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Use of random amplified polymorphic DNA (RAPD) for assessing genetic diversity of *Ocimum sanctum* (Krishna Tulsi) from different environments of Central India

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The advent of random amplified polymorphic DNA (RAPD) technique has opened up new avenues and opportunities in the diversified field of research and scientific investigations by the application of molecular markers to study the taxonomic and genetic diversity. The genetic diversity of nine accessions of *Ocimum sanctum* (Krishna Tulsi) collected from different parts of state of Madhya Pradesh has been reported in this paper using eight random amplified polymorphic DNA primers. Total of 144 bands were scored corresponding to an average of 18 bands per primer, with 137 bands showing polymorphism (95.13%) and 7 bands showing monomorphism (4.86%). All the primers individually gave more than 60% polymorphism. Jaccard similarity coefficient ranged from 0.18 to 0.48. A dendrogram constructed based on the unweighted pair group method with arithmetic mean (UPGMA) clustering method revealed two major clusters. Cluster-1 comprised of seven accessions, which was further differentiated into two subclusters and while Cluster-2 included only 2 accessions. This study revealed rich genetic diversity among *O. sanctum* accessions from Madhya Pradesh in central India, possibly allowing it to more easily adapt to environmental variations.

Key words: Polymerase chain reaction- random amplified polymorphic DNA (PCR-RAPD), genetic diversity, medicinal plant, primers, DNA extraction.

INTRODUCTION

Conservation of medicinal plants is a priority feature of environmental policies in many countries and regions. In recent years, the demand of plants for medicinal purpose is increased in the Indian market, which led to the over collection of the plants from the natural habitat. The species at one point may become extinct due to natural population of these important medicinal plant unscientific practices and destructive collection of their drug parts like bark, root, wood, stems and leaves etc. It is very important to conserve and manage the plants for future prospects. For conservation and sustainable use of plant genetic resources, accurate identification of their

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accessions using the genetic diversity study is required.

Populations in different environments may have different genetic characteristics (Duffy et al., 2009; Karimi et al., 2009). The use of molecular techniques in genetic diversity study is supported by the finding that evolutionary forces such as natural selection and genetic drift produce divergent phylogenetic branching. There are different types of molecular markers available for the assessment of genetic diversity like random amplified polymorphic DNA (RAPD), restriction fragment length polymorphism and variable number of tandem repeats etc. Amongst all, RAPDs are dominant molecular marker (Welsh and McClellan, 1990; Williams et al., 1990) with ease of methodology, simple, speed, relatively low cost and requires small amount of plant material as compared to other molecular markers (Jain et al., 1994; Becerra velasques and Gepts, 1994). RAPD molecular marker also produces numerous polymorphic, distinguishable bands (Stewart and Excoffier, 1996) which have been extensively used for DNA fingerprinting, genetic diversity and population genetic studies (Wu, 2005; Chalmers et al., 1992; Chaturvedi and Nag, 2010; Salim et al., 2010).

Ocimum sanctum (Krishna Tulsi) commonly known as holy basil belongs to family Lamiaceae, which has close to 252 genera and 6,700 species (Mabberley, 1997). *O. sanctum* has two varieties that is, black (Krishna Tulsi) and green (Rama Tulsi), with common chemical constituents (Phillip and Damodaran, 1985) and medicinal properties (Ghosh, 1995). The presence of many pharmacologically active compounds like flavonoids (orientin and vicenin), phenolics (eugenol, cirsilineol, apigenin), and anthocyanins (Mondal, 2010) in *Ocimum* provides them protection against free radical induced oxidative damage of cellular components. These are very important for their therapeutic potential and used in Ayurveda, Siddha, Greek, Roman and Unani systems of medicine (Mondal, 2010). The plant has hypoglycaemic (Giri et al.,1987), hypolipidaemic (Ravi et al.,1997), adaptogenic (Bhargava and Singh, 1981), anti-cancer (Aruna and sivaramkrishnan, 1992), radio-protective (Devi and Ganasundari, 1995), analgesic and anti-inflammatory properties (Singh et al., 1996).

The aim of the present study was to analyse the genetic diversity of sacred and medicinally important plant *O. sanctum* from different environments of central India using the RAPD molecular markers and to test the hypothesis that populations in different environments have different genetic diversity.

MATERIALS AND METHODS

Study area for sampling

The plant material for the study comprises total of 9 accessions. Geographically distinct genotypes of *O. Sanctum* (Krishna Tulsi) with an average minimum distance of 35 km were collected from various locations of Central India (Table 1 and Figure 1 a) to increase the possibility of variation. Young leaves were harvested and placed in sealable plastic bag with appropriate labels. The collected leaves were taken to the laboratory in an icebox and were used directly to isolate DNA, while excess leaf materials were stored in -80°C for future use. These sites represented different populations in different climatic and soil conditions.

Reagents and chemicals

Reagents and chemicals used include: Modified cetyl trimethylammonium bromide (CTAB) extraction buffer (2.5% CTAB), 100 mM Tris HCl (pH-8), 25 mM ethylenediaminetetraacetic acid (EDTA) (pH-8), 1.5 M NaCl, 0.2 % beta-mercaptoethanol (AR grade) (v/v), and 1% polyvinylpyrrolidone (PVP) (w/v) pure cold (-20°C) isoproponol, 70% ethanol, absolute ethanol, phenol: chloroform: isoamyl alcohol (25:24:1, v/v/v), TE buffer: 10 mM Tris HCl (pH-8.0), 0.1 mM EDTA (pH -8), enzyme: Taq DNA polymerase (Fermentas Inc.), RNase A (Fermentas Inc.), PCR Master Mix ,Tempelate DNA, RAPD Primers Bangalore Genei, TBE 5X: 54 g Tris, 27.5 g Boric acid, 20 ml 0.5 M EDTA, agarose gel , ethidium Bromide.

Genomic DNA extraction

The freshly harvested young leaves (to avoid high secondary metabolites) of *O. sanctum* were freshly collected and genomic DNA was extracted using a modified CTAB method based on the protocol of Doyle and Doyle (1987).

Qualitative and quantitative estimation of DNA

The yield and purity of DNA was determined by calculating the ratio of absorbance at 260 and 280 nm using a UV Spectrophotometer (ND-1000). DNA concentration and purity was also tested by running the samples on 0.8% agarose gel based on the intensities of band when compared with Lambda DNA marker (used to determine concentration) by visualizing under UV light, gel documentation system. The DNA was diluted to final concentration of 50 ng/µl using TE buffer and used as template DNA for RAPD analysis.

RAPD-PCR Analysis

RAPD profiles were generated using genomic DNA extracted from nine different accessions of plant *O. sanctum*, single decamer random oligonucleotide primers (Bangalore Genei, India) were used for amplification in polymerase chain reaction (PCR) following the standard protocol according to the RAPD amplification kit (Bangalore Genei). RAPD primer accessions are shown in Table 2. Each amplification reaction mixture of 50 µl volume contained about 1 µl of template DNA (50 ng), 25 µl of 2× red dye, 2 µl of primer, (Bangalore Genei Pvt. Ltd., Bangalore, India) and 22 µl de-ionised water (RNAse, DNAse free) and centrifuged briefly to mix well. The reactions without DNA were used as negative controls and without enzyme as positive control. The reactions were carried out in Gradient Automatic Thermal Cycler (PCR), (eppendorf) in the following temperature cycles: holding at 94°C for 5 min at start, followed by 8 cycles of 94°C for 45 s, 55°C for 1 min and 72°C for 1.5 min and 35 cycles of 94°C for 45 s, 55°C for 1 min and 72°C for 1 min and a final additional extension at 72°C for 10 min and a

Table 1. Different variants *of O. Sanctum* collected from the various districts of Madhya Pradesh (Central India).

S/No.	Area of the study/Districts	Population ID	Latitude	Longitude
1.	Shubham Nursery Patel Nagar, (East Bhopal) Dist. Bhopal	KT - 01	23° 16' N	77° 36' E
2	Nisarg Nursery, Purvanchal,(East Bhopal)Dist Bhopal	KT - 02	23° 16' N	77° 36' E
3	Pushpanjali Nursery, Bairagarh (West Bhopal), Distt. Bhopal	KT - 03	23° 16' N	77° 36' E
4	Hightech Nursery Ujjain, Distt. Ujjain	KT - 04	23° 09' N	75° 43' E
5	Islam Nagar Fort, (North Bhopal), Distt. Raisen	KT - 05	23° 15' N	77° 5' E
6	Vidisha City, Distt Vidisha	KT - 06	23° 32' N	77° 51' E
7	Sehore City, Distt. Sehore	KT - 07	23° 12' N	77° 00' E
8	Human Herbal Garden, MPCST office, Main Bhopal, Distt Bhopal	KT - 08	23° 16' N	77° 36' E
9	Sodalpur Village, Distt. Harda	KT - 09	21° 88' N	77° 98' E

holding temperature of 4°C. Amplified PCR-products were stored at 4°C. All the experiments were repeated thrice to ensure reproducibility.

Agarose gel electrophoresis

Amplified PCR products with molecular marker (100 bp DNA ladder) were electrophoreticaly separated in 1.2% (w/v) agarose gels and visualized through UV light and analysed using Alpha View Software (Lee,2012). All the experiments were repeated thrice to ensure reproducibility. The best gels of the replicates were used for band scoring.

RAPD data analysis and Scoring

In RAPD analysis, only the primers that displayed reproducible, scorable and clear bands were considered and determined using Alpha view software. For all accessions, bands on RAPD gels were scored as (1) when present or (0) when absent. Evaluation of fragment patterns was carried out by similarity index. The similarity index (SI) values between the RAPD profile of any two individual were calculated using the Nei genetic similarity index (Nei and Li, 1979), on the basis of the equation:

$$SI = 2Nij / (Ni + Nj)$$

Where Nij is the number of common bands shared between 2 samples i and j, Ni and Nj are the total number of DNA bands for genotypes i and j, respectively.

Using dice coefficients, a similarity matrix involving 9 accessions was generated with PAST (Paleontological statistics) software (Hammer et al., 2001). The similarity matrix data was subjected to unweighted pair group method for arithmetic average (UPGMA) cluster analysis to generate a dendrogram using average linkage procedure. The results were analysed based on the principle that a band is considered to be 'polymorphic' if it is present in some individuals and absent in others, and 'monomorphic' if present in all the individuals or accessions. Amplifications were repeated twice to confirm the results.

RESULTS AND DISCUSSION

RAPD technique has frequently been used for the detection of genetic variability in plants and successfully used in variety of taxonomic and genetic diversity studies (Rodriguez et.al., 1999; Alam et al., 2009). The patters of genetic variation help in effective conservation and management of a species. For example, the spatial structure of genetic variation can provide information for sampling strategies for *ex situ* or *in situ* conservation (Torre et al. 2008). In the conservation management of a species, knowledge of interspecies genetic variations may help to assess extinction risks such as inbreeding and evolutionary potential in a changing world (Hedrick, 2001).

PCR amplifications were tested with 10 decamer oligonucleotide random primers purchased from Bangalore Genei (RPI-1 to RPI-10) with nine different accessions of *Ocimum sanctum* obtained from different parts of Madhya Pradesh, as shown in Table 1 and Figure 1a. Of these 10 primers, the amplifications of only 8 primers (RPI-3, RPI-4, RPI-5, RPI-6, RPI-7, RPI-8, RPI-9 and RPI-10) were satisfactory, scorable and reproducible. The amplification reactions with the total of 8 primers generated 144 bands, 137 of them being polymorphic (95.13%) with molecular sizes to 12:50 ranging between 1.5 Kb and 7 bands (loci) with monomorphic being percentage mean of 4.86%, in all the accessions studied (Table 2). Primers RPI-10 (Figure 5b) produced the highest number of bands (23 bands) followed by RPI-9 (Figure 5a), RPI-7 (Figure 4a) and RPI 3 (Figure 2a) (22, 21 and 21 bands, respectively), while, primer RPI-4 (Figure 2b) produced bands of the lowest number (5 bands). Some bands were specific for some of the accessions, which may be used in the accessions discrimination. For example and in 1157, 1,131th band (RPI-9) was present in only accession KTS-04, Hitech Nursery Ujjain and accession KTS-03, Pushpanjali Nursery Bairagarh, respectively (Figure 5a). Bands less than 50 bp (RPI-10) was only present in the accession KTS-02, Nisarg Nursery, Purvanchal BHEL (Figure 5b) and band Rs. 215.38 (RPI-6) was present in only

Table 2. Polymorphism pattern demonstration by RAPD analysis in *Ocimum sanctum*.

S/No.	Primer	Accession no.	Total no. of bands	Total no. of polymorphic bands	Total no. of Monomorphic bands	Monomorphism (%)	Polymorphism (%)
1	RPI 3	AM 773310	21	20	1	4.76	65.2
2	RPI 4	AM 773769	5	4	1	20	80
3	RPI 5	AM 773770	16	16	0	0	100
4	RPI 6	AM 773771	20	19	1	5	95
5	RPI 7	AM 773312	21	19	2	9.52	90.47
6	RPI 8	AM 773773	16	15	1	6.25	93.75
7	RPI 9	AM 773315	22	22	0	0	100
8	RPI 10	AM 750045	23	22	1	4.34	95.65
Average polymorphism			144	137	7	4.86	95.13

Table 3. Jaccard's similarity coefficient among different accessions of *Ocimum sanctum* through RAPD.

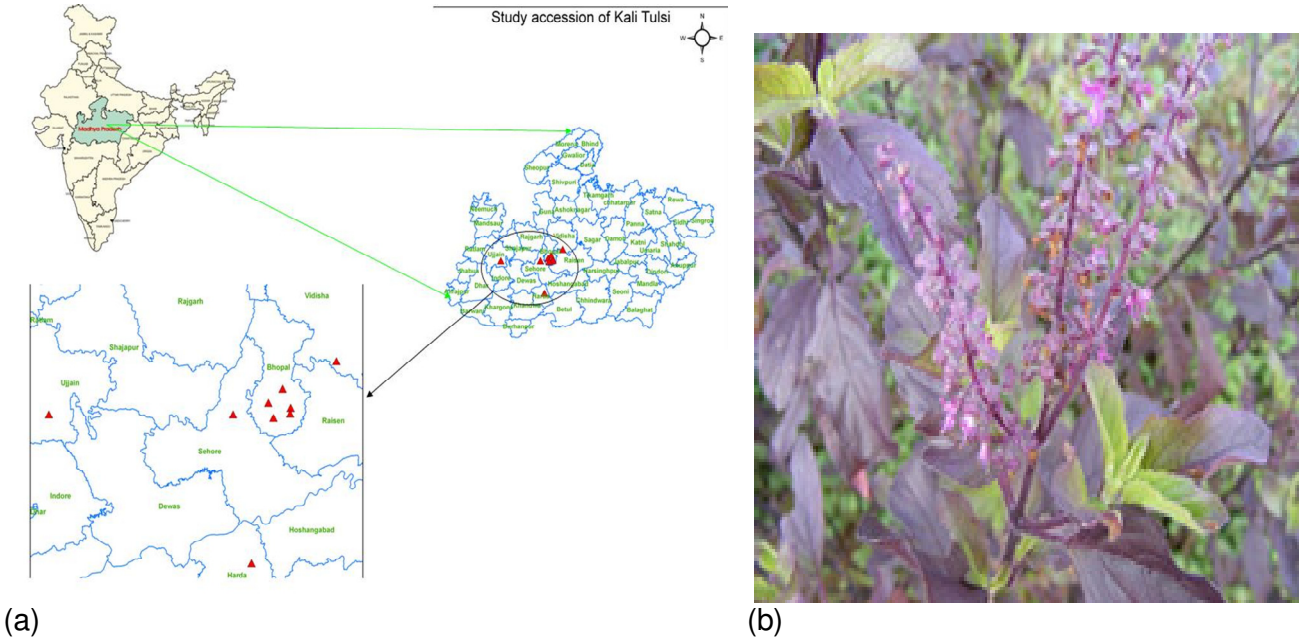
Sample ID	KTS-1	KTS-2	KTS-3	KTS-4	KTS-5	KTS-6	KTS-7	KTS-8	KTS-9
KTS-1	1								
KTS-2	0.3542	1							
KTS-3	0.2857	0.2830	1						
KTS-4	0.23438	0.2	0.4386	1					
KTS-5	0.2	0.18	0.25	0.3036	1				
KTS-6	0.26923	0.2046	0.2546	0.2407	0.3636	1			
KTS-7	0.2281	0.2128	0.3036	0.2909	0.3913	0.45	1		
KTS-8	0.2321	0.2174	0.2414	0.2281	0.3696	0.3571	0.525	1	
KYS-9	0.2909	0.2340	0.2542	0.2	0.3830	0.3721	0.3696	0.4762	1

accession KTS-01, Shubham Patel Nagar Raisen Road Nursery (Figure 3b). Bands 657.14 and the 1,325th (RPI-3) were present in only accession KTS-04, Hitech Nursery Ujjain, and 628.57 (RPI-3) was present in only accession KTS-05, Fort Bairasia Islam Nagar, Bhopal (Figure 2a). Band 775 (RPI-5) was present in the accession only KTS-02, Nisarg Nursery, Patel Nagar Raisen Road. Figure 3a shows the presence of the specific genetic, loci Oscimum sanctum distinctness of the species studied accessions. The plant with all nine accessions has produced high diversity, which indicated that the plant could survive in any adverse conditions.

There were some loci/bands, which were missing only in one or two accessions; such loci may also be of use in the *O. sanctum* accession differentiation. For example Band (between 500 to 600 bp) were present in all the primers but absent in all the accessions with RPI-7 (Figure 4a) and RPI-4 (Figure 2b) but with all other present Primers. Due to one particular band of Genomic Recombination is missing and may possibly be of use in discrimination PLANT.

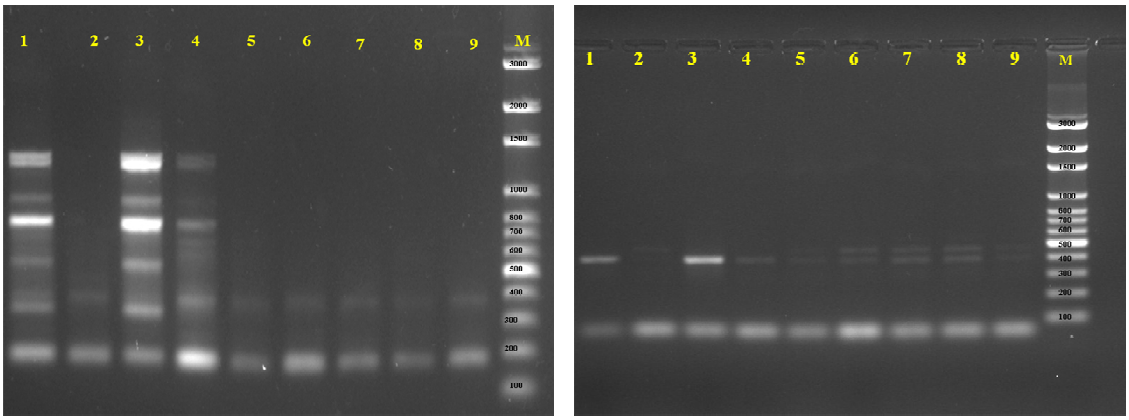
The data obtained by RAPD profile with different primers individually as well as collectively were subjected to the construction of similarity matrix using Jaccard similarity coefficient (Jaccard, 1908). Among the accessions studied, the similarity coefficient based on 8 primers ranged from 0.18 to 0.47. Accessions KTS-02, Nisarg Nursery, and KTS 05, Islamabad fort showed the lowest value of similarity index (0.18), while the highest value of similarity index (0.476) occurred between the accessions KTS-08, Human Herbal Garden, and KTS-09, Sodalpur Village, Distt. Harda (Table 3).

Cluster analysis was performed based on the Jaccard's similarity coefficient matrices calculated from RAPD data. Different clustering methods including UPGMA (unweighted paired group with arithmetic average), NJ and Bayesian tree almost produced similar results with good bootstrap and clade credibility values (Figures 6 and 7). In all accessions, three different clusters were obtained. Two accessions of Shubham Nursery (KTS-01) and Nisarga Nursery (KTS-02) showed high genetic affinity and are placed in a single cluster or clade due to less distance between the two accessions. Two accessions of Distt Sehore (KTS-07) and HHG MPCST



(a) (b)

Figure 1. Collecting sites of *O. sanctum* accessions. (B) *Ocimum sanctum.*



(a) **(b)**

Figure 2. Random amplified polymorphic DNA fragment patterns generated using RPI-3 and 4. Lane1: Shubham Nursery Patel Nagar, Lane 2: Nisarg Nursery Purvanchal BHEL, Lane 3: Pushpanjali Nursery, Bairagarh, Lane 4: Hitech Nursery Ujjain, Lane 5:Islam Nagar Fort Bairasia, Lane 6: Vidisha, Lane 7: Sehore, Lane 8: MPCST, Lane 9: Harda and M is the DNA Ladder.

(KTS-08) showed genetic similarity and form a single cluster or clade to which three other accessions of Distt Harda (KTS-09), Distt Vidisha (KTS-06), and Islam Nagar fort Raisen Road (KTS-05) are joined with some distance. Two accessions of Pushpanjali Nursery Bairagarh (KTS-03) and Hitech Nursery Distt Ujjain (KTS-04) show genetic similarity and form a single cluster or clade. All the three clusters are placed far from each other and join them with greater distance indicating their genetic difference with the others.

The geographical locality of each accession is indicated in NJ dendrogram (Figure 7), as we can see the grouping of the accession is not correlated with their geographical origin.

Genetic diversity is of great importance to the sustainability of plant populations (Wang et al., 2007). The genetic structure of plant populations reflects the interactions of many different processes such as the

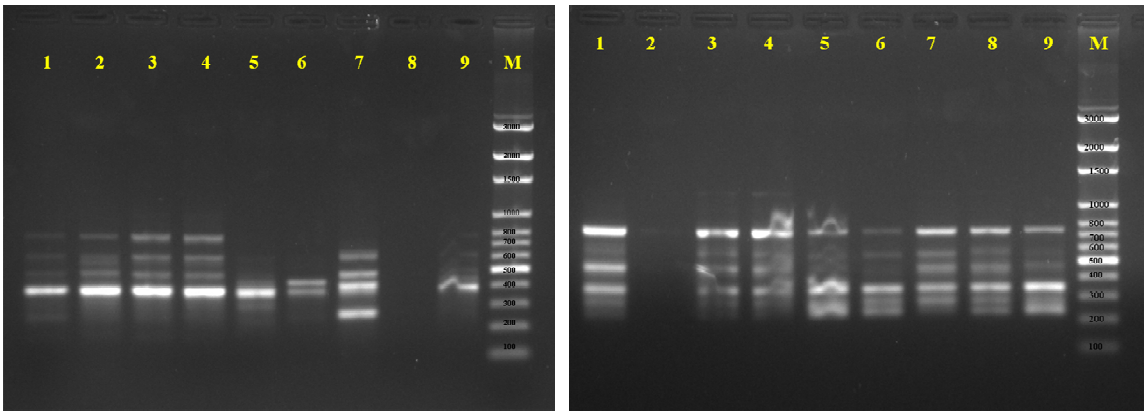


Figure 3. Random amplified polymorphic DNA fragment patterns generated using RPI-5 and 6. Lane1: Shubham Nursery Patel Nagar, Lane 2: Nisarg Nursery Purvanchal BHEL, Lane 3: Pushpanjali Nursery, Bairagarh, Lane 4: Hitech Nursery Ujjain, Lane 5:Islam Nagar Fort Bairasia, Lane 6: Vidisha, Lane 7: Sehore, Lane 8: MPCST, Lane 9: Harda and M is the DNA Ladder.

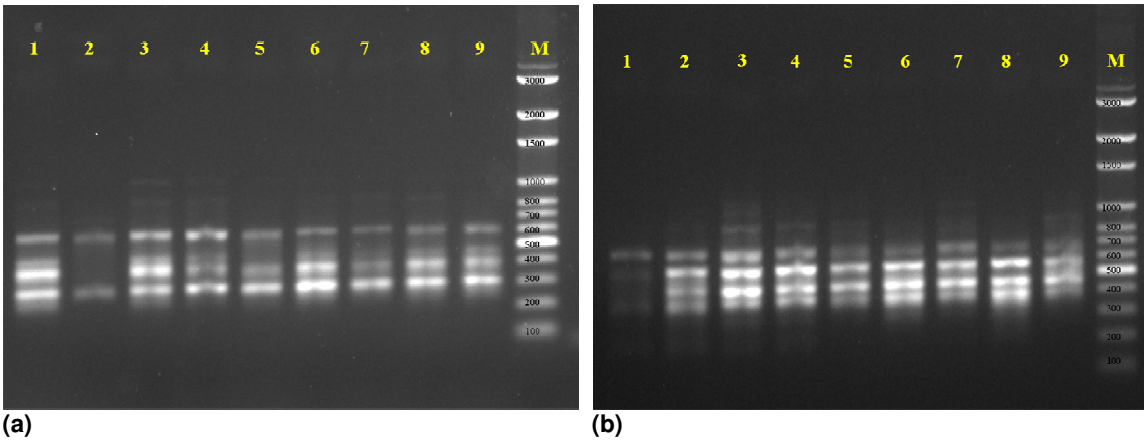


Figure 4. Random amplified polymorphic DNA fragment patterns generated using RPI-7 and 8. Lane1: Shubham Nursery Patel Nagar, Lane 2: Nisarg Nursery Purvanchal BHEL, Lane 3: Pushpanjali Nursery, Bairagarh, Lane 4: Hitech Nursery Ujjain, Lane 5:Islam Nagar Fort Bairasia, Lane 6: Vidisha, Lane 7: Sehore, Lane 8: MPCST, Lane 9: Harda and M is the DNA Ladder.

long-term evolutionary history of the species (for example, shifts in distribution, habitat fragmentation, and/or population isolation), mutation, genetic drift, mating system, gene flow, and selection (Slatkin, 1987; Schaal et al., 1998). All of these factors can lead to complex genetic structuring within populations. Based on the above results, the accessions belonging to geographically different locations had shown the high genetic diversity. High diversity indices suggest that the individuals can survive in any adverse conditions (adaptation to the environment), which is beneficial to its propagation, resources conservation, the domestication of wild species and the screen of specified locus. More genetic diversity could result from higher cross pollination and higher effective population size. Geographically isolated individuals tend to accumulate genetic variations during the course of environmental adaptations (Sarwat, 2008). Sources of polymorphism in RAPD assay may be due to deletion, addition or substitution of base within the priming site sequence (Williams et al., 1990) as shown in result.

High levels of polymorphism found in the present work showed that RAPD markers are suitable tool for genetic diversity studies. Chinese researchers have applied DNA markers extensively for characterization of botanicals from the Chinese materia medica. These markers have

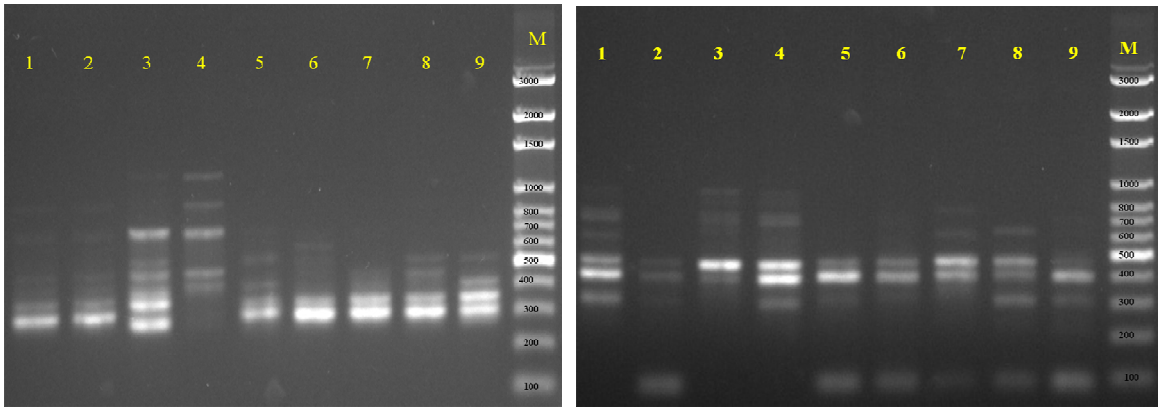


Figure 5. Random amplified polymorphic DNA fragment patterns generated using RPI-9 and 10. Lane1: Shubham Nursery Patel Nagar, Lane 2: Nisarg Nursery Purvanchal BHEL, Lane 3: Pushpanjali Nursery, Bairagarh, Lane 4: Hitech Nursery Ujjain, Lane 5:Islam Nagar Fort Bairasia, Lane 6: Vidisha, Lane 7: Sehore, Lane 8: MPCST, Lane 9: Harda and M is the DNA Ladder.

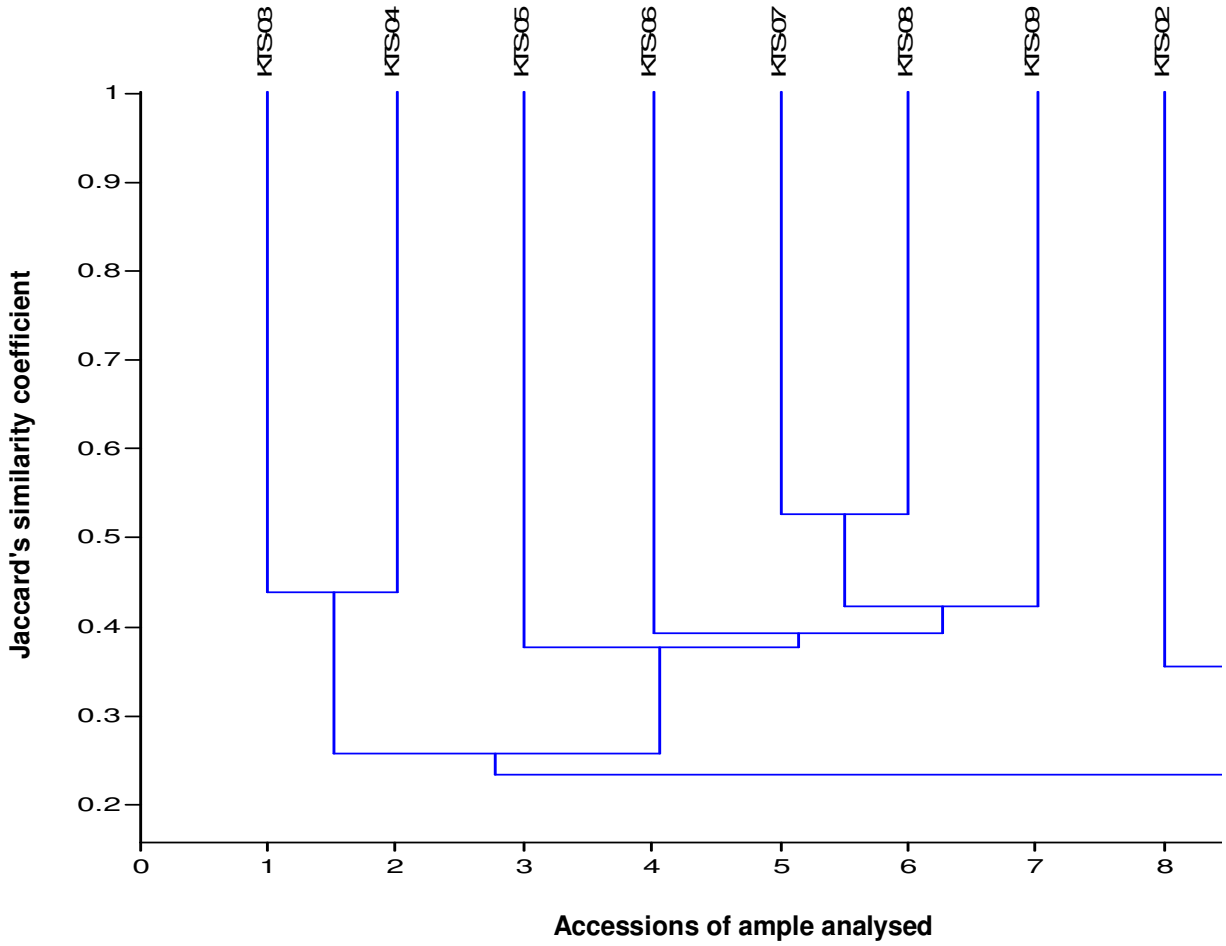


Figure 6. Paired group Jaccard coefficient matrix of *O. sanctum* accessions based on RAPD marker.

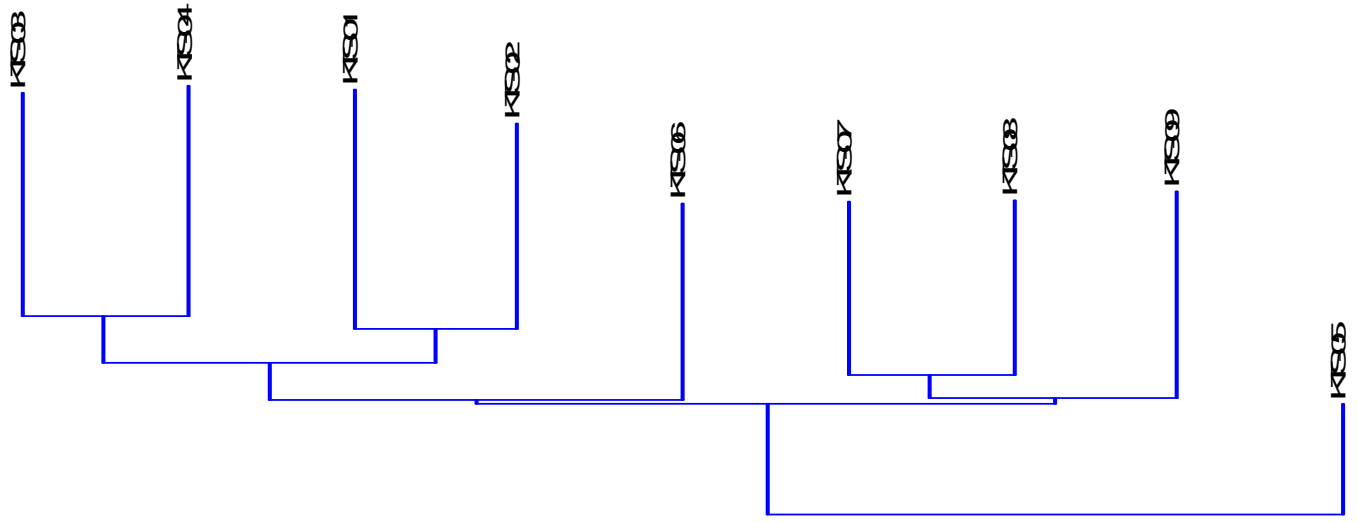


Figure 7. Euclidean matrix (neighbor joining) using similarity index (SI) of *O. sanctum* accessions based on RAPD marker.

been useful in other investigations of genetic variation among geographically distant populations *Echinaceae* spp. (Li et. al., 2003). The study was also been correlated with the other medicinal plants like black cumin. Kapila et al. (1997), Devi (2004) and Mittal et al. (2006) used morphological traits to study genetic diversity of black cummin populations. Majeed (2005) studied genetic diversity of black cummin populations based on morphological and RAPD markers. Similar results for other species from different environmental conditions were reported (Zhao et al., 2006; Lattoo et al., 2008; Kumar et al., 2009). Fingerprinting five populations of *Achillea fragrantissima* using RAPD-PCR by Morsy (2007) has also revealed similar results. This study could pave way for detailed research to understand all the aspects of this divergence.

Conclusion

The present study shows the usefulness of RAPD analysis in distinguishing *O. sanctum* species from different localities, particularly identification of the specific bands may be considered important in the identification and for conservation of species, and may lead to planning of a better management of conservation program in the country. RAPD analysis method is very simple, rapid and requires very low amount of DNA for amplification, which is advantageous for plant species, since isolation of DNA from plant is difficult due to presence of secondary metabolites.

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