

Full Length Research Paper

Diversity among 20 accession of three germplasm of the medicinal plant, *Ocimum* (*O. gratissimum*, *O. sanctum* and *O. basilicum*, Lamiaceae)

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Ocimum spp, popularly known as Tulsi, has great commercial (food and perfumery industries) and medicinal importance due to antispasmodic, stomachic, carminative, antimalarial and febrifuge properties. The aroma and flavor in *Ocimum* spp. is distinct due to the predominant aroma compound eugenol, camphor, citral etc. We used molecular techniques to assess the genetic variability and relatedness of 20 accessions of three germplasm of *Ocimum* spp. (*Ocimum gratissimum*, *Ocimum sanctum* and *Ocimum basilicum*) collected from different places of India. DNA was isolated by fixing a sample in alcohol without using liquid nitrogen. 20 accessions were analyzed through random amplified polymorphic DNA (RAPD) profiling for similarity and genetic distance, using 18 primers. The binary (1/0) data was analyzed with REEPLIT to infer the genetic distance and to construct the unweighted pair group method with arithmetic mean (UPGMA) based dendrogram. High degree of polymorphism (82.78%) was shown by RAPD markers. There was total 122 bands generated, 101 bands were polymorphic. Highest similarity was measured at approximately 0.97% and least was 0.46%. The present work showed interesting finding and proved to be a bidirectional evolution in *Ocimum* species. Therefore, RAPD markers can be used in the systematic study of wild plants and new crop. The present study would provide suitable keys for further studies.

Key words: *Ocimum*, Tulsi, genetic diversity, degree of divergence, R marker.

INTRODUCTION

Tulsi (family: Lamiaceae, *Ocimum* spp.), the holy Indian basil, is widely distributed in tropical and subtropical regions of India. The plant contains multiple bioactive substances having several health promoting and disease preventing properties (Prakash and Gupta, 2005) that are attributed to the essential oils stored in peltate glands of leaves and stems. The essential oils comprise a number of aromatic chemicals like terpenoids and phenyl propanoids. These compounds individually and in combination impart aroma and fragrance, plant-insect and

plant-pathogen interactions and are used as antioxidants in pharmaceutical industries. The red colored Tulsi also contains anthocyanin that has application as food colourants. Tulsi is a complex, variable plant. Interspecific, intraspecific hybridization and polyploidy are very common for this plant resulting in diversity with great variations (Tilwari et al., 2013). Owing to a high degree of polymorphism exhibited by the species as also abundant cross pollination, a large number of species, subspecies, varieties and strains have come into existence, which

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Table 1. Germplasm accession of *Ocimum* species.

Species	Accession No.
<i>O. sanctum</i>	OS-1
	OS-2
	OS-3
	OS-4
	OS-5
	OS-6
	OS-7
	OS-8
	OS-9
<i>O. basilicum</i>	OS-10
	OS-11
	OS-12
	OS-13
	OS-14
	OS-15
<i>O. gratissimum</i>	OS-16
	OS-17
	OS-18
	OS-19
	OS-20

make the botanical nomenclature extremely difficult (Krishna, 1981).

In view of the great diversity, the various species have been classified in broad groups, based on the geographical sources, morphological and cytological features and chemical constituents. The plants get further complicated by the presence of chemical races that do not differ morphologically, but differ in aromatic chemical constituents. Considering its potential as an aromatic, medicinal and pigmented plant and lack of molecular information about this plant, the aim of this study is to evaluate the genetic variability within the species and to determine the extent of correlation between the random amplified polymorphic DNA (RAPD) profiles. Molecular genetic tools like bar coding, random amplified polymorphism DNA are reliable method for quality control of herbal material. Finger prints obtained by RAPD can be employed for identification of herbal drug at the molecular levels. In this study, genetic variability and RAPD techniques were used for the assessment of diversity for 20 different accessions with three germplasm collections assembled from different places in India, as a prelude to crop improvement

MATERIALS AND METHODS

Collection and maintenance of varieties

Representative set of 20 accessions of three species of *Ocimum*, group 1 of *Ocimum sanctum* with 10 accessions, group 2, *Ocimum basilicum* with 6 accessions and group 3 *Ocimum gratissimum* with

4 accessions from different location of Uttar Pradesh, India (Table 1), were used for assessment of diversity. The accessions were selected randomly, which were grown to flowering stages at University of Allahabad. Three seedlings of each accession were transplanted into pots in triplicates. Greenhouse plants were irrigated to pot capacity daily and maintained at day/night temperatures of 26 to 30°C and 18 to 21°C, respectively. Taxonomic identification of each accession was conducted by Botanical Survey of India, Allahabad.

Extraction of genomic DNA

Total genomic DNA was extracted by CTAB method with some modification (Doyal and Doyal, 1990). Fresh young leaves from nursery raised plant individual genotype/accession progeny were collected in ice box. One gram leaf tissue was fixed in alcohol before cetyl trimethylammonium bromide (CTAB) DNA extraction, making liquid nitrogen unnecessary (Sharma et al., 2003). The protocol was modified slightly from the standard one as *Ocimum* species have high amount of oil and secondary metabolites (Suman et al., 1999; Khanuja et al., 1999). Alcohol was allowed to evaporate followed by grinding the tissue with mortar and pestle and then transferred into 10 ml polypropylene centrifuge tube. It contained 3 ml pre warmed (65°C) DNA buffer (2% CTAB, 20 mM ethylenediaminetetraacetic acid (EDTA), 1.4 mM NaCl, 100 mM Tris HCl pH 8.0) with 0.6 volume of β -mercaptoethanol and 2% polyvinylpyrrolidone (PVP). The tubes were shaken and incubated at 65°C for 60 min. Equal volume of chloroform: Isoamyl alcohols (24:1, v/v) have been added and tubes were shaken end to end for 10 min to make emulsion, then centrifuged at 15,000 rpm for 10 min. Supernatant have been collected and subjected to RNase treatment. Then DNA was precipitated using 0.6 volume chilled isopropanol, tubes were shaken end to end until DNA fiber appeared, centrifuged at 8000 rpm and 4°C for 10 min. Pellets were washed with washing sol (70% ethanol + 10 mM ammonium acetate), dried and then dissolved into 1.0 ml Tris EDTA (TE) buffer. Ammonium acetate 7.5 mM (0.5 vol.) has been added and kept at -20°C for 30 min, centrifuged at 8,000 rpm, 4°C for 20 min and supernatants have been collected into new tubes. DNA was precipitated using 4.5 ml absolute ethanol and pellets were dissolved in 1.0 ml TE buffer. Quantification and purity measurements of DNA have been performed by using UV spectrophotometer (Ultrospec-4000) and also analyzing the DNA on 0.8% agarose gel alongside diluted uncut lambda DNA as standard.

DNA amplification

PCR amplification was carried out by using Master Cycler gradient Thermal Cycler (Eppendorf). Amplification was carried out in 50 μ l reaction volume containing 1 \times Taq polymerase buffer (finnzyme), 1.5 mM magnesium chloride 4.5 μ l, 200 M each dNTP (Finnzyme), 20 M primer, 1 unit of the Taq DNA polymerase enzyme (Finnzyme) and 50 ng of template DNA. Thermal cycler with an initial denaturation at 94°C for 3 min, followed by 42 cycles was done. Each cycle consisted of denaturation at 94°C for 45 s, primer annealing at 42°C for 1 min, extension at 72°C for 3 min, with final extension at 72°C for 8 min. PCR products were separated on 1.5% agarose gel in 1 \times TBE buffer using ethidium bromide staining. The size of amplified fragments was determined by using size standard (3 kb DNA ladder Finnzyme). DNA fragment were visualized under UV light and photographed using VSD Image master (Pharmacia Biotech). To test the reproducibility of the RAPD markers, the reactions were repeated in duplicate.

Statistical analysis

Amplicons were scored as discrete variables, using 1 for the

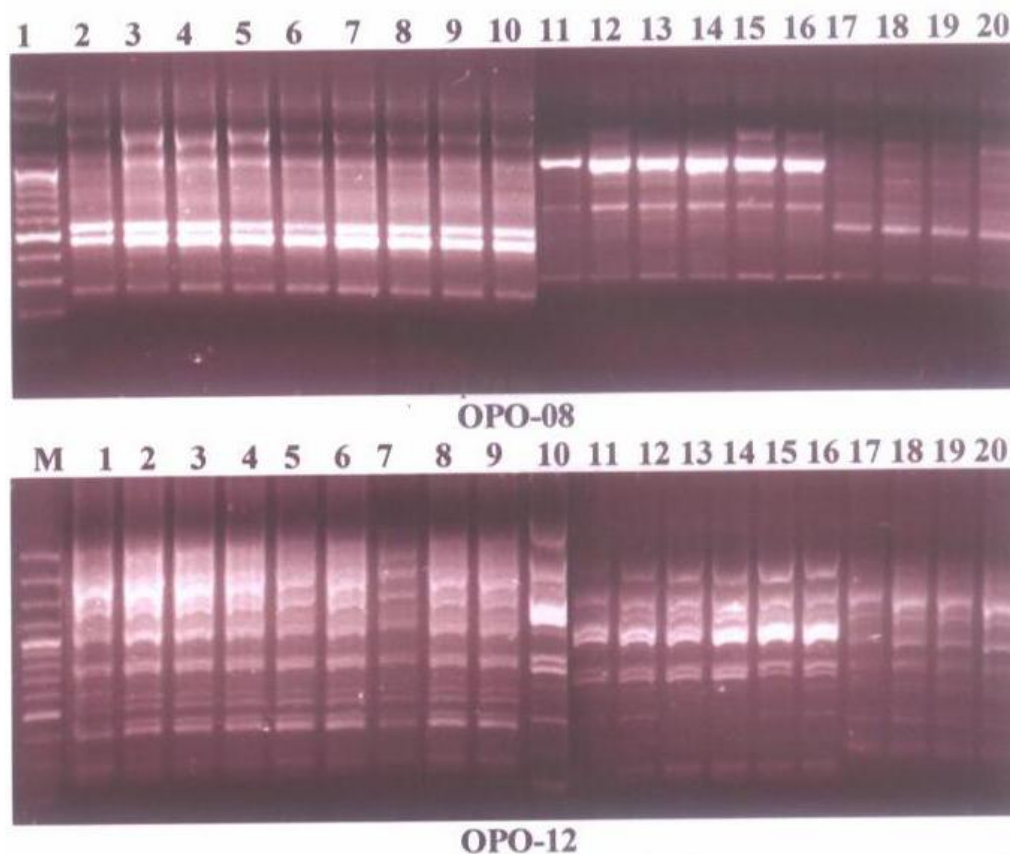


Figure 1. RAPD pattern of *Ocimum* spp.
Lane M: standard DNA marker (DNA); 1-20 represent accession from OS-1 to OS-20 by primer OPO-08 and OPO-12.

presence of bands and 0 for the absence of bands. Jaccard similarity coefficient based similarity matrix have been prepared by using the formula (Jaccard, 1908). The pattern of presence and absence of bands was submitted to NT-SYS-PC based software (SIMINT) to prepare similarity matrix. unweighted pair-group method of arithmetic average analysis (UPGMA) have been performed by SHAN which uses similarity matrix of SIMQUAL as input file. Phylogenetic tree based on similarity matrix have been viewed by using graphics (TREEPLOT).

RESULTS

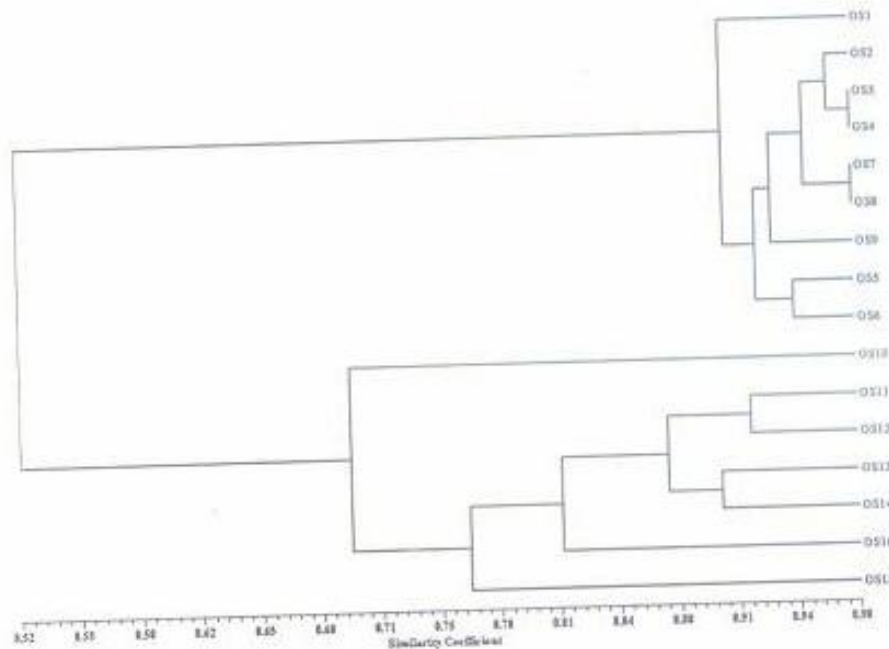
Each accession of three *Ocimum* species was considered as an individual operational taxonomic unit (OTU). Out of eighteen primers analyzed in the present study, fourteen random primers were found to produce scorable RAPD patterns and used for analysis. Two primers (OPO-17 and OPT-15) produced fuzzy and hardly repeatable bands while other two primers (OPT-10 and OPT-14) did not produce any amplification product and was thus excluded from the present study. A total 122 bands were scored from polymerase chain reaction (PCR) amplification of the genomic DNA of 20 accessions of three *Ocimum* species (Figure 1).

A high degree of polymorphism (82.78%) was shown by RAPD markers. It was observed that out of total 122 bands generated, 101 bands were polymorphic (Table 2). Average number of 9 bands were obtained per primer and amplification produced ranged in the size from 200 bp to 3.0 kb. Maximum number of 12 amplification product was obtained with the primer OPO-12, followed by 10 amplicons for Primers OPO-03 and OPT-12. Maximum number of RAPD products was obtained for Primer OPO-08 and OPT-06 (7 bands with each primer). 28 RAPD products were recorded as unique or species specific. An average of 82.78% polymorphism was shown by primers. However, 100% polymorphism was found with primers OPT-01 and OPT-06. The primer OPO-05 showed the least % of polymorphism in *Ocimum* accessions (62.50%). Though the primer OPO-08 showed a high degree of polymorphism (85.71%), its distinguishing capacity was found to be poor comparatively.

The result of RAPD analysis based genetic similarity matrix showed varying degree of genetic relatedness among *Ocimum* accessions belonging to three species. Highest similarity (0.97) was measured in the OS-4 and OS-3 and OS-7 and Os-8 accessions of *O. sanctum*. The

Table 2. Polymorphism shown by various primers.

S/No.	Primers	Oligo sequence	Total Number of Bands	Total number of polymorphic bands	Polymorphism (%)
1	OPO-02	5'ACGTAGCGT C 3'	8	6	75
2	OPO-03	5'CTG TTG CTA C 3'	10	9	90
3	OPO-04	5'AAG TCC GCT C 3'	9	8	88.8
4	OPO-05	5'CCC AGT CAC T 3'	8	5	62.50
5	OPO-08	5'CCT CCA GTG T 3'	7	6	85.71
6	OPO-12	5'CAG TGC TGT G 3'	12	8	66.6
7	OPO-13	5'GTC AGA GTC C 3'	9	6	66.6
8	OPO-14	5'AGC ATG GCT C 3'	7	6	85.71
9	OPO-18	5'CTC GCT ATC C 3'	9	8	88.88
10	OPO-01	5'GGG CCA CTC A 3'	9	9	100
11	OPO-06	5'CAA GGG CAG A 3'	7	7	100
12	OPO-12	5'GGG TGT GTA G 3'	10	9	90
13	OPO-13	5'AGG ACT GCC A 3'	8	6	75
14	OPO-20	5'GAC CAA TGC C 3'	9	8	88.88
		Total	122	101	82.78

**Figure 2.** Combined phenetic dendrogram prepared based on genetic similarities among *ocimum* species germplasm accession.

least similarity (0.46) was measured in OS-17 (accession *O. gratissimum*) and OS-10 (accession of *O. basilicum*) and OS-14 (accession of *O. basilicum*) and OS-9 (accession of *O. sanctum*). Multivariate (cluster) analysis of the genetic similarity data grouped accessions belonging to three *Ocimum* species into two major clusters (Figures 2 and 3). Cluster I (Figure 2) included all the accessions belonging to *O. gratissimum* and *O. sanctum*

(except OS-15 that is an accession of *O. sanctum* var. *tenuiflorum* and showed the closeness to second cluster). Cluster II (Figure 3) included all the accession belonging to *O. basilicum*. Maximum average genetic similarity between species was found inbetween *O. sanctum* var. *tenuiflorum* and *O. gratissimum*.

Intra-clustering between *O. sanctum* accessions and *O. basilicum* accessions showed two sub-clusters and OS-

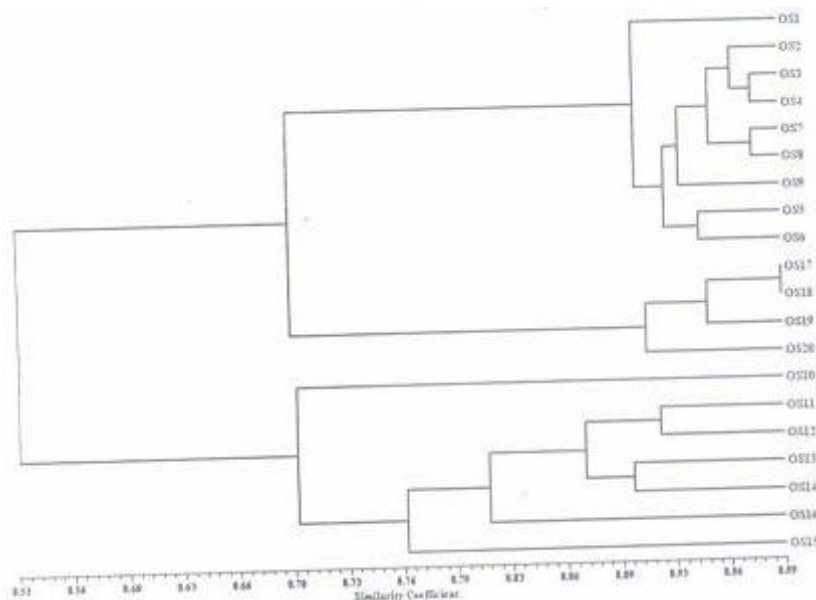


Figure 3. Interrelationship shown among the accessions *O. sanctum* and *O. basilicum*.

10 accession of *O. basilicum* showed closer genetic relatedness to *O. sanctum* cultivars (Figure 2). The combine dendrogram (Figure 3) showed nearly similar results obtained previously by other workers, who put the three *Ocimum* species into two main clusters and proving the closeness of *O. sanctum* accessions to *O. gratissimum* (Singh et al., 2004). Maximum primers used in the present study proved their capacity to distinguish different *Ocimum* species and their cultivars and hence, they are very useful in the assessment of biodiversity. It can give us intrageneric and intergeneric relationships among different species.

DISCUSSION

RAPD markers proved their advantage over morphological and chemical/biochemical (*isozyme*) markers because they detect maximum number of genetic loci (Sinde et al., 2007; Kongkiatngam et al., 1995). This is why they are applicable widely in various studies related to biodiversity assessment in laboratory. Another positive factor of RAPD is that it is less time and labor consuming, less expensive and can be easily perform (Khanuja et al., 1998). However some doubts have also been raised regarding the suitability of RAPD for diversity analysis. It is debated that most of the co-migrating RAPD bands may not be allelic or composed of similar sequences (Bowbitch et al., 1993). On the other hand, a study in some species of *Glycine max* and *Allium* have demonstrated the homology of co-migrating RAPD band (William et al., 1993). Although, investigation on genetic diversity,

interrelationship and phylogeny of *Ocimum* have been reported earlier (Khosla, 1995; Viera et al., 2001; Tilwari et al., 2013). They have also analyzed the genetic diversity of *O. gratissimum* L. at DNA level using RAPD marker. The present study also analyzes the genetic diversity at molecular level using RAPD assay. RAPD marker have been shown to detect high polymorphism then restriction fragment length polymorphism (RFLP) and amplified fragment length polymorphism (AFLP) marker (Vid et al., 2007; Sang et al., 2009; Thormann et al., 1994; Das et al., 1999).

In the present study, RAPD marker revealed high degree of polymorphism (82.78%) among the 20 accession belonging to 3 *Ocimum* species. it may be due to the possibility that most of the genetic loci is screened with RAPD markers were tone respected or of more species. *Ocimum* species were grouped in the present paper into two major clusters, one belonging to the accession of *O. basilicum*.

Previously, on the basis of morphological, cytological and oil characters, other authors have divided *Ocimum* species into two groups- '*Basilicum*' and '*Sanctum*'. *O. basilicum* and other species were included in the '*Basilicum*' group, while *O. tenuiflorum* and *O. gratissimum* were placed in the '*Sanctum*' group. The two clusters defined in the present study on the basis of similarity matrix obtained through RAPD analysis also corresponded to previously reported '*Basilicum*' and '*Sanctum*' groups, respectively. Hence, genetic divergence estimation among three *Ocimum* species under investigation suggests that phylogenetically, *O. basilicum* accessions are distantly related to other two species *O.*

O. Sanctum var. tenuiflorum and *O. gratissimum*. The result is indicative of a probably bidirectional evolutionary concept in *Ocimum* species. The study also proved that DNA can be easily extracted from plants by fixing leaves in absolute alcohol without impairing its quality for routine molecular biological work and without requirement of liquid nitrogen for DNA isolation. The study may be a preliminary step in understanding the vast area of biodiversity assessment in plant science.

Conclusion

Biodiversity assessment of *Ocimum* species on various morphological and molecular marker systems is of interest to researchers since a long time, because of its rich medicinal properties. RAPD molecular marker has been taking advantage over other techniques because it requires no prior knowledge about any particular gene in a target taxon. The present work showed interesting finding and proved a bidirectional evolution in *Ocimum* species. Therefore, RAPD markers can be used in the systematic study of wild plants and new crop and the present study would provide suitable keys for further studies.

Conflict of Interests

The author(s) have not declared any conflict of interests.

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