Detection of genetic diversity in *Ocimum* species using RAPD markers

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ABSTRACT

*Ocimum* is an important member of Lamiaceae and the genus is an important source of many essential oils and aroma chemicals in perfumery and cosmetic industries. *Ocimum* species are used as pot herbs and find diverse uses in the indigenous system of medicine of many Asian and African countries, from industrial point of view. *Ocimum* species with oil rich in camphor, citral, geraniol, linalool, linalylacetate and methyl etc., are important and can be harnessed for successful utilization in industry. There is a variation in the production of these products among different species of *Ocimum*. Therefore, precise characterization of promising species and determination of genetic variation among those are felt necessary. Until recently, ever-promising species released formally are characterized based on morphological data, organic content and yield potential. These characters differ under varying environmental condition thereby nosing problem in proper identification of species. Unlike morphological markers, cytological (chromosome number, nuclear DNA content) and molecular markers (RAPD, AFLP, ISSR etc., ) are not prone to environmental influences and accurately characterize the plants portraying the extent of genetic diversity among the taxa. Of the different markers RAPD has been widely used in the last decade in species identification programme and in accessing genetic variations among different taxa at DNA level because of its cost effectiveness and simple operation without requiring prior knowledge of species DNA sequences. RAPDs reveal similar patterns of genetic diversity when compared with other markers type, can be performed more rapidly than most other methods, and can provide vital information for the development of genetic, sampling, conservation and improvement strategies. Random amplified Random amplified polymorphic DNA (RAPD) markers were employed to study the genetic relationships between six *Ocimum* species. Of the 80 random primers screened 10 primers resulted in 73 RAPD bands of good amplification. The data structure included a total of 100 to 1000 base pair marker levels. A dendrogram was constructed using Euclidean distances by Ward’s method. Based on the number of bands all the species were grouped into three clusters and the dendrogram maximum similarity between the *Ocimum* species.

Keywords: Aquatic *Ocimum* species; RAPD; genetic diversity

INTRODUCTION

*Ocimum* is an important member of Lamiaceae and differentiation of *Ocimum* species through morphological features is inefficient and inaccurate. The genus is an important source of many essential oils and aroma chemicals in perfumery and cosmetic industries. *Ocimum* species are used as pot herbs and find diverse uses in the indigenous system of medicine of many Asian and African countries, from an industrial point of view *Ocimum* species with oil rich in camphor, citral, geraniol, linalool, linalylacetate, and methyl Charicol, eugenol, thymol, etc., are important and can be harnessed for successful utilization in the industry. The cytological report of all the taxa shows two basic chromosome number X=8 and X=12 (Morton 1962; Pushpungadan et al., 1973). It is interesting to note that the species belonging to the sanctum group have X=8.

The length of chromosomes and gross appearance of Karyotype show minor differences. The chromosomes of the *Ocimum* species can be divided into three groups based on their absolute length, which are designated as A, B and C. Group A has long chromosomes with 300µ and above B group has medium-sized chromosomes with length between 2. 0µ and 2. 5µ and C group has shorter chromosomes with a length of 1. 5µ and below. The evolution of closely related species and types of *Ocimum* species has been through structural differences and repatterning of chromosomes. There appears to be a general reduction in the total chroma-
tin length during the evolution. (Sobti and Pushpangadan 1977).

The taxonomy of all the six species along with their different species, types and races were collected from different parts of India. These were grown in the experimental garden of the Department of Biotechnology, R. K. Institute of Management and Computer Science; Bangalore, India. The details of the morphological features of all the collected species of Ocimum were studied. On the basis of morphological characters of the six species. It may be noted that the 4 species belonging to the first group that is Basilicum group are predominantly herbaceous annuale while the 5th species O. kilimandschaicum is a perrennial. In the second group that is sanctum group, O. sanctum and O. suave are biennial while others are perennial woody undershrubs. The bracts of the first group are Petiolate, flowers are more conspicuous, seed mucilaginous, where as in the second the bract are sessile, small flowers and seeds non mucilaginous. Inspite of the differences in floral characters among the Ocimum species, they are similar in the basic structure of the flower.

Therefore, precise characterization of promising species and determination of genetic variation among those are felt necessary until recently, several promising species released formally are characterized based on morphological data, organic content and yield potential. These characters differ under varying environmental condition thereby posing a problem in proper identification of species. Unlike morphological markers, cytological (chromosome numbers, nuclear DNA content) and molecular markers are not prone to environmental influences and accurately characterize the plants portraying the extent of genetic diversity among the taxa (Bennett 1987, Bennett and Smith 1991, Waugh and Powell 1992, Chalmers et al. 1994, Das et al. 1998, Rodriguez et al. 1999) of the different markers, RAPD has been widely used in the last decade in species identification programme (Schnell et al.,1995) and in accessing genetic variations among DNA level because of its cost effectiveness and simple operation without requiring prior knowledge of species DNA sequences (Williams et al.,1990, Frankel et al.,1977). RAPDs reveal similar patterns of genetic diversity when compared with other markers type and can be performed more rapidly than most other methods (Morell et al.,1995) and can provide vital information for the development of genetic sampling, conservation and improvement strategies (Waugh and Powell 1992, Chalmers et al.,1994). No report has yet been published so far either on the genetic characterization or on the extent of genetic variations existing among promising species of Ocimum using RAPD analysis.

DNA markers like restriction fragment length polymorphism (RFLP), amplified fragment length polymorphism (AFLP) and random amplified polymorphic DNA (RAPD) have been used in genetic and breeding studies in many plant species. Random amplified polymorphic DNA (RAPD) markers (Williams et al., 1990) have been used in species analysis, species identification in most plants due to technical simplicity and speed of RAPD methodology (Grits 1993). Compared to restriction fragment length polymorphism (RFLP), markers, RAPD can generate markers more rapidly but some loss of information may occur because RAPD markers are usually more dominant rather than the co-dominant as RFLP markers.

The species Ocimum basilicum L. and its chemotypes have been subjected to studies on the morphological traits and essential oil composition. The published data on basil cultivars show that they are well characterized from these points of view. DNA analysis was performed by Vieira et al., (2003) that discriminated among Ocimum spp. by RAPD investigation, even if in this study a high number of polymorphic bands was required. Only in recent times, morphological and essential oil characteristics were correlated with DNA-based molecular markers, confirming only the classification of some basil cultivars. However, from the genetic point of view, many cultivars have not been characterized yet. In fact, basil taxonomy is complex for the occurrence of inter/intraspecific hybridization and morphological similitude. Sometimes, taxonomists assigned a number of designations to the same variety (synonym) and confused different varieties as unique (homonym). Typically, cultivar identification is based on phenotypic traits, often influenced by environment, making difficult the classification. Since the aroma of each basil cultivar is predominantly determined by its genotype and depends on the main chemical components of essential oil (secondary metabolites), genetic characterization of basil cultivars represents an extremely important aspect. DNA fingerprinting allows identifying plant genotypes in an efficient manner. Randomly amplified polymorphic DNA (RAPD) analysis has emerged as a powerful technique for detecting DNA polymorphisms among cultivars or clones belonging to plant kingdom. Applications such as RAPD-based genotyping rely on in vitro amplification by polymerase chain reaction (PCR) of a target genome with thermophilic DNA polymerase. The main advantage of RAPD-PCR is the use of unique and short arbitrary primer for PCR amplification, without a priori information about the sequence of DNA template of the organism in study, indispensable in conventional PCR. This means that the DNA bands from RAPD-PCR represent anonymous DNA fragments. Besides, it is more advantageous with respect to other similar molecular marker techniques such as the more recently introduced AFLP. In fact, the RAPD - PCR technology requires reduced time and cost, and no radioactive reagents for DNA fingerprinting analysis.

The present study deals with the in situ DNA estimation and RAPD analysis of six promising species of Ocimum to identify and evaluate the extent of genetic variation existing among them.
MATERIALS AND METHODS

Leaf samples of Ocimum species were collected from the conservatory of Biotechnology Centre, Hulimavu, Department of Horticulture, Bangalore. The recently matured leaves were collected and used for DNA extraction.

The basic DNA extraction protocol was slightly modified following Porebski et al., (1997). 1g of fresh leaf tissue was grinded using liquid nitrogen and then transferred to a tube containing 20 ml of extraction buffer (3% CTAB, 100 mM Tris, 20 mM EDTA, 1. 4 M NaCl, 2% PVP and 1% B- mercaptoethanol) preheated to 65°C and maintained at this temperature for 1 hour with intermittent shaking. The centrifuge tube was brought to room temperature and 6 ml of chloroform and isoamyl alcohol (24:1) were added. The contents were mixed well by inverting the tube gently 25-30 times, and then spun at 7, 000 rpm for 15 min. The supernatant was transferred to a fresh tube and this cleanup step was repeated until a clear supernatant was obtained. Supernatant was kept overnight at 4°C to precipitate DNA by adding half a volume of 5 M NaCl and one volume of isopropanol. The DNA was pelleted by centrifuging at 10, 000 rpm for 20 min and the pellet was washed with 70% ethanol. The dried DNA pellet was resuspended in 300 µl of TE (Tris EDTA) buffer. Contaminating RNA was removed by digestion with 10 µg of Rnase for 60 min at 37°C. Proteins were removed by digestion with 25 µg of Proteinase-K. The DNA was further purified by extracting twice with an equal volume of phenol following by an equal volume of phenol:chloroform (1:1) and finally with an equal volume of chloroform. The DNA was precipitated by the addition of one volume of isopropanol and spun at 5, 000 rpm for 5 min. The final pellet was dissolved in 300 µl TE. The DNA concentration was determined using UV-Visible spectrophotometer at 260 nm and 280nm and the quality verified by gel electrophoresis on a 1% agarose gel.

DNA amplification

The basic protocol reported by Williams et al., (1990) for PCR was followed with slight modifications. A single decamer of arbitrary sequence was used in each PCR reaction. With the PCR reaction conditions optimized, informative and reproducible fingerprint profiles were carried out in 25 µl reaction mixture containing template DNA (25 ng), 10 pmol of primer ( Operon Technologies USA, Inc. ), 2. 5 mM MgCl2, 50 mM KCl, 10 mM Tris-HCl and 0. 05% Triton X-100. One unit of Taq DNA polymerase (Bangalore Genei) and 200 µM of each dNTPs (Bangalore Genei). The mixture was overlaid with one drop of mineral oil to prevent evaporation of the reaction mixture. Amplification was performed in a thermal cycler (Eppendorf, Master cycler) for 42 cycles after an initial denaturation at 94°C for 6 min. In each cycle, denaturation for 1 min at 94 °C, annealing for 1 min at 34 °C and extension for 2 min at 72 °C was programmed with a final extension step at 72 °C for 6 min after the 42 cycle.

DNA electrophoresis

Amplified DNA fragments were separated out on 1% agarose gel stained with ethidium bromide. Running buffer containing Tris-buffer, Acetic acid and EDTA (pH 8. 0) was used for electrophoresis and for preparing gels. Wells were loaded with 25 µl reaction volume and 5µl of loading buffer (Sucrose, Bromophenol blue and Xylene cyanol) together. Electrophoresis was conducted at 45 volts for 3 hours and the gel photographed under UV light using a gel dock system (Hero-lab).

DNA / Statistical analysis

Binary coding was used to score gel and each band of primer was scored of 6 species and 12 primers with 100 to 1000 base pairs marker level pairwise. Squared Euclidean distance was calculated and utilizing these distances, species were clustered following Ward’s method. The segregation of species was also assessed through principal component analysis, the statistical version 5. 0 a computer application was used to generate a dendrogram using squared Euclidean distance and Ward’s method.

RESULTS

The yield and quality of DNA extracted by following the procedure described earlier was 10-25 ng per µl for every gram of leaf sample. The DNA obtained was amplifiable and of high quality. A spectrophotometer reading of 1. 7-1. 8 (260nm/280nm) confirmed the quality of DNA. DNA isolated from 500 mg leaf tissue using 20 ml extraction buffer yielded good quality, high molecular weight DNA (above 50 kb). The quality of the DNA was also confirmed by gel electrophoresis. In this study, 80 Operon random ten-base long, single stranded primers (OPA to OPD with 20 primers in each group) were screened using the Ocimum species, which on an average gave six bands. The selected 12 primers were used for the screening of species (OPC 9-20), in which all the bands obtained were either monomorphic or polymorphic and were considered for the precise calculation of genetic diversity.

The basic protocol described elsewhere for PCR was optimized for high quality amplification and intense repeatable banding patterns. However, a reduction in the amplification of fainter bands was noticed with large changes in template DNA concentrations, while too much DNA produced a smear effect, which emphasized the importance of quantification of the DNA for clear amplification.

A representative of the PCR amplification product of six species is shown in fig. 1, which yielded sufficient polymorphisms to distinguish between species. Ward’s method of analysis with Euclidean distance gave dendrogram. Based on the number of bands all the species
were grouped into three clusters (Fig. 2). The dendrogram revealed a maximum similarity between Ocimum basilicum species. Every single species/hybrid could be identified using these selected ten primers.

Analysis of diversity based on PCR fragments amounted to saturating the genome. The analysis of six species of Ocimum suggested that diversity is moderate to high and has shown even differences.

Table 1: RAPD primers used as for amplification of Ocimum DNA

<table>
<thead>
<tr>
<th>Primer No.</th>
<th>Sequence</th>
<th>Total no. of fragments amplified.</th>
</tr>
</thead>
<tbody>
<tr>
<td>OPC-09</td>
<td>CTCACCGTCC</td>
<td>2</td>
</tr>
<tr>
<td>OPC-10</td>
<td>TGTCTGGGTG</td>
<td>3</td>
</tr>
<tr>
<td>OPC-11</td>
<td>AAAGCTGGGG</td>
<td>1</td>
</tr>
<tr>
<td>OPC-12</td>
<td>TGTCATCCCC</td>
<td>4</td>
</tr>
<tr>
<td>OPC-13</td>
<td>AAGCCTCGTC</td>
<td>9</td>
</tr>
<tr>
<td>OPC-14</td>
<td>TGGCGTCTTG</td>
<td>9</td>
</tr>
<tr>
<td>OPC-15</td>
<td>GACGGATCAG</td>
<td>7</td>
</tr>
<tr>
<td>OPC-16</td>
<td>CACACTCCAG</td>
<td>6</td>
</tr>
<tr>
<td>OPC-17</td>
<td>TTCCCCCAG</td>
<td>9</td>
</tr>
<tr>
<td>OPC-18</td>
<td>TGAGGTGGGTG</td>
<td>8</td>
</tr>
<tr>
<td>OPC-19</td>
<td>GTTGCAGAGC</td>
<td>8</td>
</tr>
<tr>
<td>OPC-20</td>
<td>ACTTCGCCAC</td>
<td>7</td>
</tr>
</tbody>
</table>

Figure 1: Gel profile of Ocimum species (O. basilicum, O. sanctu O. gratissimum, O. kilimandascharicum O. basilicum and O. sanctum) Amplified with selected primers like OPC-09 to OPC-20

Figure 2: Tree diagram for six variables Ward’s method, Euclidean distance

RAPD analysis revealed a high degree of genetic diversity among the species examined in the study, which can contribute to the crop improvement. RAPD analysis can also be used for detecting gene flow between species. Furthermore, this technique is less restricted than other molecular technique RFLP (Restricted Fragment Length Polymorphism), as no hybridization and no use of radioisotopes is required, and it is therefore more convenient for use in research centers in developing countries.

DISCUSSION

The cluster based on RAPD analysis using 10 primers depict the genetic diversity and relationship among the six species of Ocimum. The squared Euclidean distances were calculated using the data on six species with ten primers. The highest distance was observed between O. kilimandascharicum and O. sanctum and lowest distances was recorded between two species O. basilicum 1 and O. basilicum 2 and intermediate distance was observed between O. sanctum 1 and O. sanctum 2. Was in line with the results obtained in Ward’s genetic diversity calculation. This result agrees with the findings of Jude Sitaram Rao, Mohan and Annadana Seetharam 2005, Chikkaswamy et al., 2007 on Sunflower and Piper species respectively. And RAPD
molecular markers in Ocimum cultivars reported by Lal et al., 2003. The dendrogram fig: 2 showed three distinct clusters namely clusters 1, 2 and 3.

From the pattern of clustering, it was pertinent that RAPD technique was efficient in segregating species into different clusters. More significantly, the clustering had been largely successful in retaining the relationship between species as proposed by Schilling and Heiser 1981). In the light of current taxonomy at the species level, it can be seen in the clustering pattern that the series Ocimum species were clearly distinguished. The association of the species observed in the present study was similar to the pattern observed by (Sivloap and Solodenko 1998 and Khalid et al., 1999).

Similar pattern of segregation of species to that of dendrogram was found in PCA graphs. In fact, the first three principle components axes explained substantial parts of the variability (13. 84%) present in the population and hence the segregating pattern was quite relia-

The results of the present study demonstrated the utility of using RAPD markers to characterize genetic diversity among six promising species of Ocimum. Differential polymorphism was recorded in six Ocimum species showing variation in percentage of polymorphic bands from 13. 84% to 3. 07%. The observed high proportion of polymorphic loci reveals relatively high genetic variations by RAPD markers have also been reported in other species at the cultivar level (Colombo et al., 1998, Das et al., 1998, Huang et al., 2003). Wide genetic distances determined by Ward’s genetic distances revealed relatively high genetic variation among six species. The considerable polymorphism detected in the present study also illustrated the genetic diversity among Coffee species (Sera et al., 2003). The observed intraspecific differences among six Ocimum species could be ascribed to fluctuating micro and macro climatic conditions of habitat. Ward’s analysis of RAPD data also revealed that all Ocimum species belonging to the state of distant habitat in the state of Karnataka are genetically closer. The greater sensitivity of RAPDs obtained in the result of species diversity may be derived from the rapid evolution of non-coding repetitive DNA sequences detected by RAPDs. This hypothesis has been corroborated from Plomion et al., 1995.

CONCLUSION AND IMPLICATION

Thus, our results demonstrate that RAPD marker provides an effective tool for the detection and evaluation of genetic variation existing among six species of Ocimum. The species relationship revealed in the present study was in line with the current taxonomy. Based on the molecular distances revealed by RAPD, it should be possible to select the species that are near to cultivated species to introgress useful characters. In the present study, O. basil 1 and O. basil 2 were close and are being utilized for integration of useful characters.

The molecular distance between the Ocimum species seems to be associated with the extent of crossability between them i.e. as the genetic distances increases, there appears to be an associated decrease in crossability. O. basil 1 and O. basil 2 and O. sanctum were genetically near and hence crossing was successful. It would be logical to infer that the crossing was not successful even after modest efforts as O. sanctum, O. gratissimum, O. kilimandascharicum were distanced away from one another with respect to ploidy level and genetic heterogeneity. There are very few instances available where the cross compatibility of these species is indicated. The precise range of molecular distance values at which cross compatibility or incompatibility could be inferred remains a challenge and further studies would be helpful in this direction.

Base sequence variations in genome of Ocimum species under investigation determined the polymorphism revealed by DNA analysis (Alok Kalra et al., 2003 and Karp et al., 1996). In addition, DNA species does not depend from the variability of the plant growth stage and the environmental conditions. Consequently, DNA-based markers represent a classification system better than conventional methods based on agromorphological and chemical markers. In fact, numerous authors (Labra et al., 2004, Marotti et al., 1996, Morton 1962, Mullis and Faloona 1987, Fleisher 1981, Pushpagadan et al., 1975) reported that phenotypic characteristics of each Ocimum species showed great variability, being influenced by environmental factors, growth stage, age, etc. Morphological characters and essential oil composition provide only a generic classification, also they are more time-consuming and expensive than molecular techniques. DNA of Ocimum species here reported has revealed unambiguous differentiation respect to traditional investigations. A significant aspect of this study was that RAPD analysis has achieved to discriminate Ocimum species by using only few primers; our method has shown more advantages respect to other molecular markers, even recently introduced, such as AFLP. DNA-based analytical procedures may represent a routine tool to verify the identity and the quality of Ocimum species and species derived products. This would also support in the management of germplasm collections of basil for the identification of redundancy, where identical cultivars often have different names.

Our results showed that molecular tools to screen and characterize plant biodiversity provided the possibility to put order into the wide array of Ocimum species. RAPD methodology offered the possibility to test a large number of anonymous loci, and RAPD dendrogram should be used for taxonomic studies. In future, the application of RAPD-PCR to a large number of Ocimum species and accessions will be useful for selection and improvement of species/ cultivars suitable for industrial processing. In addition, the knowledge of basil chemotype diversity, revealed through this investiga-

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tion, will let an enhancement of the production of different types of essential oil depending on the flavor and fragrance requested for a specific use. Then, chemical constituents of essential oil are without doubt important from production point of view, but they are not necessarily correlated with taxonomy of the Ocimum species

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