CHAPTER 3

MOLECULAR CHARACTERISATION OF PATCHOULI GERMPLASM

3.1. Introduction

_Pogostemon cablin_ Benth., a native of Philippines is reported to be the true patchouli of commerce (Samuel 2002). However, our knowledge of patchouli oil has been vague and ambiguous for a long time since _Pogostemon cablin, P. commosum, P. hortensis, P. heynanans and P. plectranthoides_ were being cultivated for their oils, all of which were known as patchouli oil. Inferior quality oils were also reported to be extracted from _Microtoena cymosa_ and other adulterant raw materials. The prevalence of such adulterants and morphological variations in the crop caused due to climatic and edaphic factors are responsible for the taxonomic ambiguity existing in the patchouli crop. Non flowering of patchouli is another hurdle in its taxonomic characterisation. Therefore, chemotaxonomy was suggested as a solution to this problem.

But, tolerance to seasonal variation (Akhila and Thakur 1988) and impact of diurnal effects (Levinson _et al._ 1971) have been observed to influence essential oil quality in general. It is also reported that, there exist two metabolically distinct pools of terpenes in aromatic plants. One of these is sensitive to external regime while the other is relatively neutral. The relative size of these two pools may depend on the species as well as on environmental conditions (Banthorpe _et al._ 1972). Reports also suggest that natural chemical components are bio-synthesized under the influence of certain enzymes which are under genetic control (Sharma _et al._)
1988). Appropriate technological inputs can therefore modify the genetic nature of oil components on a permanent basis. Since the patchouli oil quality is dependent both on the genetic make-up of the plant as well as the environment in which it grows, selection of genotypes that favor a balance between the genetic make-up and environment can be considered.

Since the Johore variety of *P. cablin* is a genotype stable for oil quality as well as tolerant to environmental variations, it was used for large scale cultivation across the world for about three centuries. A decline in the regeneration capacity of this variety coupled with its shortened life span pose a threat to its future existence. This calls for a systematic assessment of genetic variation among the patchouli cultivars available in the country in order to expand its genetic base. An understanding of genetic relationships that exist within the patchouli germplasm, their similarities and divergences are also expected to evolve from this study.

The present study focuses at locating distinctive RAPD markers in the patchouli germplasm that will help to distinguish one patchouli ecotype from the other and segregate adulterants from original patchouli raw material.
3.2. Materials and Methods

3.2.1. DNA Extraction

3.2.1.1. Materials

a. **Instruments, plastic ware, glassware and others**

Refrigerated micro-centrifuge, -20°C deep freezer, 4°C freezer, refrigerator, freeze drier, 1.5 ml autoclaved eppendorf tubes, 250 ml conical flask, autoclaved mortar and pestle (one each for every sample), micropipettes (2-20µl, 20-200µl, 200-1000µl), autoclaved micro-tips, water bath (at 60°C) and liquid nitrogen were used for the DNA extraction procedure.

b. **Reagents**

2x CTAB buffer, chloroform: isoamyl alcohol (24:1), ribonuclease, 75% ethyl alcohol, isopropanol, TE buffer (10:1) were the reagents used for the DNA extraction procedure.

3.2.1.2. Method (Doyle and Doyle 1990)

i. Five to 7.5ml of CTAB isolation buffer [2% hexadecyltrimethyl ammonium bromide (CTAB: Sigma), 1.4M NaCl, 20mMEDTA, 100mM Tris-HCl, pH-8.0] was preheated in a flask to 60°C in a water bath. 0.3% 2-mercaptoethanol was added to this.
ii. One gram leaf tissue each from the seven patchouli accessions were weighed and frozen overnight in liquid nitrogen and ground to a fine powder using a pre-chilled mortar and pestle.

iii. The pre-heated (60°C) CTAB isolation buffer was added to the ground tissue. The sample was then incubated for 45 min. in a 50ml oak ridge polypropylene centrifuge tube with occasional gentle swirling.

iv. The sample was extracted once with chloroform: isoamylalcohol (24:1) by mixing gently but thoroughly. This produced two phases, an upper aqueous phase which contained DNA and a lower chloroform phase that contained some degraded proteins, lipids and many secondary compounds. The interface between these two phases contained most of the junk cell debris and many degraded proteins.

v. The phases were then concentrated by spinning the sample at 6000x g for 10 min. at room temperature. The aqueous phase was removed with a wide bore pipette and transferred to a clean polypropylene centrifuge tube.

vi. Twenty to 25µl (10 mg/ml) RNase was added and mixed several times by inversion of the tube. The sample was then incubated at 37°C for one hour.

vii. Equal quantity of chloroform : isoamylalcohol (24:1) was added and mixed for 10 min. by inversion. The sample was centrifuged at 6000x g for 10 min. at room temperature.
viii. The supernatant was removed carefully into a fresh tube. Cold isopropanol (0.6 volume was added to this and the tubes were gently swirled. DNA was spooled out using the pipette tip and transferred to 1-2ml of wash buffer (75% EtOH).

ix. This was kept in a freezer (-20°C) for 30 min. and given a spin for 10 min. at 10,000x g (4°C)

### 3.2.2. Polymerase Chain Reaction (PCR)

#### 3.2.2.1. Materials

**a. Instruments**

DNA Thermocycler (Eppendorf), microfuge, auto pipettes, UV transilluminator, submarine electrophoresis unit with power supply, -20°C deep freezer, vortex machine, refrigerator, laminar air flow and a gel documentation system equipped with a CCD camera was used.

**b. Reagents**

Template DNA, 10x PCR buffer, dNTP mix, ethidium bromide, Taq DNA polymerase, MgCl₂, sterile water and primers (Table 7) were the reagents used for the PCR mix.

#### 3.2.2.2. Method (Abraham et al. 2005)

i. The buffer, MgCl₂, dNTP’s and primer were tapped, given a short spin and placed in ice.
ii. The master mix components were added into a labeled and autoclaved eppendorf tube in the order mentioned in Table 8. Taq polymerase was added last in the mix, just before adding the DNA. The mix was tapped, vortexed and given a short spin and placed back in ice.

iii. The mix was aliquoted into PCR micro tubes at the rate of 20µl per tube. Five µl each of the respective DNA template was added to the aliquoted master mix to get a final volume of 25µl. The lids of the eppendorf tubes were tightly closed.

iv. The PCR tubes containing the master mix and the sample DNA were given a short spin and placed in the PCR machine. The PCR reaction was performed by switching on the PCR machine and selecting the programme for RAPD (Table 9).

v. At the end of the PCR programme run, the tubes were taken out and 2.5µl of 10x loading dye was added and mixed well by giving a spin for 2 to 5 seconds and stored at 4ºC till electrophoresis.

3.2.3. Visualization of the amplified product

a. Preparation of Agarose gel

i. The gel casting tray was cleaned with distilled water and dried before fixing the side blocks. The comb was placed at the appropriate end using uniform gaps between the comb tips and casting tray so that the tip of the comb is 0.5 to 1.0 mm above the plate surface. The casting tray was then leveled prior to pouring agarose.
Table 7

List of primers that generated unambiguous RAPD bands

<table>
<thead>
<tr>
<th>S.No.</th>
<th>Primers used</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>OPJ 20</td>
</tr>
<tr>
<td>2</td>
<td>OPR 19</td>
</tr>
<tr>
<td>3</td>
<td>OPJ 19</td>
</tr>
<tr>
<td>4</td>
<td>OPAN 10</td>
</tr>
<tr>
<td>5</td>
<td>OPAN 20</td>
</tr>
<tr>
<td>6</td>
<td>OPE 01</td>
</tr>
<tr>
<td>7</td>
<td>OPE 18</td>
</tr>
<tr>
<td>8</td>
<td>OPG 03</td>
</tr>
<tr>
<td>9</td>
<td>OPG 10</td>
</tr>
<tr>
<td>10</td>
<td>OPH 03</td>
</tr>
<tr>
<td>11</td>
<td>OPI 06</td>
</tr>
<tr>
<td>12</td>
<td>OPAK 05</td>
</tr>
<tr>
<td>13</td>
<td>OPAM 11</td>
</tr>
<tr>
<td>14</td>
<td>OPQ 18</td>
</tr>
<tr>
<td>15</td>
<td>OPR 01</td>
</tr>
<tr>
<td>16</td>
<td>OPR 15</td>
</tr>
<tr>
<td>17</td>
<td>OPS 11</td>
</tr>
<tr>
<td>18</td>
<td>OPC 05</td>
</tr>
</tbody>
</table>

Table 8

Composition of the master mix components for PCR (for 80 samples)

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Components</th>
<th>Stock conc.</th>
<th>Required conc.</th>
<th>One reaction (µl)</th>
<th>Master mix for 82 reactions (µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Autoclaved water</td>
<td>-</td>
<td>-</td>
<td>12.86</td>
<td>1054.52</td>
</tr>
<tr>
<td>2</td>
<td>PCR Buffer</td>
<td>10x</td>
<td>1x</td>
<td>2.5</td>
<td>205.0</td>
</tr>
<tr>
<td>3</td>
<td>MgCl₂</td>
<td>25mM</td>
<td>2mM</td>
<td>2.0</td>
<td>164.0</td>
</tr>
<tr>
<td>4</td>
<td>dNTP mix</td>
<td>10mM</td>
<td>200µM</td>
<td>0.5</td>
<td>41.0</td>
</tr>
<tr>
<td>5</td>
<td>Primer</td>
<td>10 picomoles/µl</td>
<td>20 picomoles/µl</td>
<td>2.0</td>
<td>164.0</td>
</tr>
<tr>
<td>6</td>
<td>Taq polymerase</td>
<td>5 units/µl</td>
<td>0.7 units/reaction</td>
<td>0.14</td>
<td>11.48</td>
</tr>
<tr>
<td></td>
<td><strong>Sub Total</strong></td>
<td></td>
<td></td>
<td><strong>20</strong></td>
<td><strong>1640.0</strong></td>
</tr>
<tr>
<td>7</td>
<td>DNA Template</td>
<td>10ng/µl</td>
<td>50ng</td>
<td>5.0</td>
<td></td>
</tr>
<tr>
<td></td>
<td><strong>Total</strong></td>
<td></td>
<td></td>
<td><strong>25.0</strong></td>
<td></td>
</tr>
</tbody>
</table>
ii. Sufficient quantity of 1x TAE buffer was prepared to fill the electrophoresis tank as well as to prepare the gel. A 2% w/v agarose mix was prepared using 1x TAE buffer in an autoclaved conical flask.

iii. The contents were boiled over flame with stirring for 1-2 min. till all the agarose powder dissolved giving a clear solution in the flask. The solution was cooled at 55 - 60ºC and 10mg/ml (stock) ethidium bromide solution added to the molten agarose [0.05µl/ml (v/v)] and mixed well.

iv. The molten agarose was slowly and carefully poured into the casting tray and allowed to solidify for 45 to 60 min. in room temperature.

b. Gel Electrophoresis

i. 1x TAE buffer was poured into the gel tank so that its level stood 5mm above the gel. The solidified gel with the gel casting tray and comb was placed into the gel tank containing buffer.

ii. The comb was then pulled out carefully, taking care that the sample wells were located towards the negative electrode. Samples were loaded in wells by placing the pipette tips under the buffer just above the well taking care not to damage the walls and bottom of the well.

iii. The first and last wells were loaded with 1µg of molecular weight marker (EcoR1 + Hind III digest) and the seven wells between the two markers were loaded with 1µg DNA sample each of KER 1, KAR 1, CHE 1, MEG 1, DEL 1, KAR 2 and KER 2 from left to right (Figs. 3, 4 and 5) of the gel.
iv. The lid of the gel tank was closed and the electrical leads were attached to the power pack. Appropriate voltage (5V/cm of distance between the two electrodes) was then applied till the time the bromophenol blue migrated an appropriate distance through the gel.

v. The electric current was turned off, the leads removed and the gel incubated in sufficient volume of ethidium bromide (1µg/ml) in distilled water for 30 to 45 min. in the dark.

vi. The ethidium bromide solution was poured out as per the prescribed norms of the lab and rinsed with distilled water. The gel was observed in ultra-violet light and photographed using the gel documentation system.

3.2.4. Data Scoring and Analysis

Polymorphic bands were scored as 1 (present) and 0 (absent). A band was considered polymorphic if it was present in at least one accession and absent in others. Only clearly scorable bands were considered. Fragment scoring was performed manually with the help of Adobe Photoshop Software (Adobe® photoshop®7.0). The data matrix for the seven patchouli accessions and eighteen primer combinations used, was used to calculate pair wise genetic similarity based on Jaccards co-efficient (GSj). The resulting similarity matrix (Table 10) was subjected to clustering using the Unweighted Pair Group Method using Arithmetic means (UPGMA) algorithm and principal co-ordinate analysis with the help of computer programme NTSYS pc version 2.1 (Rolf 2000).
**Table 9**

**PCR Programme for RAPD**

<table>
<thead>
<tr>
<th>Steps</th>
<th>Process</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Denaturation at 94°C</td>
<td>3 – 4 min.</td>
</tr>
<tr>
<td>2</td>
<td>Denaturation at 94°C</td>
<td>30 sec.</td>
</tr>
<tr>
<td>3</td>
<td>Annealing at 36°C</td>
<td>1 min.</td>
</tr>
<tr>
<td>4</td>
<td>Polymerization at 72°C</td>
<td>2 min.</td>
</tr>
<tr>
<td>5</td>
<td>Repeat steps 2 to 4 forty times</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>Extended polymerization at 72°C</td>
<td>10 min.</td>
</tr>
<tr>
<td>7</td>
<td>Hold at 4°C</td>
<td>Infinitely</td>
</tr>
</tbody>
</table>

**Table 10**

**Matrix of pairwise genetic similarity between seven patchouli accessions based on Jaccard’s co-efficient**

- **KER 1** 1.000
- **KAR 1** 0.950 1.000
- **CHE 1** 0.672 0.691 1.000
- **MEG 1** 0.645 0.662 0.685 1.000
- **DEL 1** 0.513 0.532 0.614 0.658 1.000
- **KAR 2** 0.579 0.597 0.710 0.747 0.658 1.000
- **KER 2** 0.145 0.152 0.181 0.171 0.233 0.211 1.000
3.3. Results

Twenty five primers were tested initially for their ability to generate amplification products. Of the twenty primers that produced amplification products, eighteen were chosen for their ability to generate unambiguously scorable RAPD bands. A total of 129 bands were scored with an average of seven bands scored per primer. The number of scorable bands generated by a single primer ranged from four in OPAN 10 to thirteen in OPS 11. Portions of gels showing typical amplification products are shown in Figs. 3, 4 and 5. The sizes of RAPD products developed on the agarose gel ranged from 1.375 Kilo base pairs to 25 Kilo base pairs.

Monomorphic bands of 5.14 kilo base pairs were generated by the primers OPJ-20, OPE-01, OPR-19 (Fig.3), OPS-11 and OPC-05 (Fig.5) whereas that of 2.16 Kilo base pairs were generated by the primer OPS 11 (Fig. 5) across all the seven accessions.

Polymorphic bands were generated in KER 2 by the primers OPAN 20, OPE 01, OPE 18, OPG 03, OPG 10, OPH 03, OPI 06, OPAK 05, OPQ 18, OPR 01, OPR 15 and OPC 05.

Monomorphic bands were generated by the primers OPAN 20 (Fig.3), OPE 18, OPG 03, OPG 10, OPH 03, OPI 06, OPAK 05 (Fig. 4) and OPQ 18 (Fig. 5) in the cultivars KER 1, KAR 1, CHE 1, MEG 1, DEL 1 and KAR 2. However, in KER 1 and KAR 1 monomorphic bands were generated by the primers OPJ 20, OPR 19, OPE 01 (Fig.3), OPG 03, OPG 10 (Fig. 4), OPS 11 and OPC 05 (Fig.5).

While the primer OPJ 20 generated similar bands of 3.53 Kb size in MEG1 and KAR 2 (Fig. 3), the primer OPAK 05 was observed to generate polymorphism in the
patchouli cultivar MEG 1 (Fig. 4). Polymorphism was also observed in CHE 1, MEG 1 and DEL 1 generated by the primers OPS 11 (Fig. 5), OPG 10 and OPG 03 (Fig. 4).

A cluster analysis of the RAPD profiles (Fig. 6) show that KER 1, KAR 1, CHE 1, MEG 1, DEL 1, KAR 2 and KER 2 are observed to form three clusters. One cluster consisting of KER 1 and KAR 1, the second consisting of CHE 1, MEG 1, DEL 1 and KAR 2 and the third constituting the lone accession KER 2 which is an outlier. The patchouli accession KER 2 is observed to exhibit a genetic similarity of only 25% with the other two clusters.

The individuals of the first cluster i.e. KER 1 and KAR 1 exhibited 97% genetic closeness between each other whereas the second cluster showed a 60% genetic closeness to the former cluster (Fig. 6). The patchouli accessions DEL 1 showed a 65% similarity with the cluster consisting of CHE 1, MEG 1 and KAR 2. The accession CHE 1 has a similarity index of 0.70 with the more recently evolved accessions MEG 1 and KAR 2 that are 75% genetically similar to each other.

The polymorphism generated by the primer OPJ-20 in MEG 1 and KAR 2 (Fig. 3), OPS-11 in CHE 1 (Fig. 5), OPG-10 in MEG 1 and OPG-03 in DEL 1 (Fig. 4) undoubtedly support the taxonomic relationship obtained from the cladogram that CHE 1, MEG 1, DEL 1 and KAR 2 belong to a common genetic stock and hence can function as components of the secondary gene pool.

However, since the patchouli accession KER 2 exhibits only a genetic similarity of 25% with the others it may be considered an outlier.
Fig. 3 RAPD profiles of seven patchouli accessions using primers OPJ 20, OPR 19, OPJ 19, OPAN 10, OPAN 20 and OPE 01
Lanes (M) Marker, (1) KER 1, (2) KAR 1, (3) CHE 1, (4) MEG 1, (5) DEL 1, (6) KAR 2, (7) KER 2
Fig. 4 RAPD profiles of seven patchouli accessions using primers OPE 18, OPG 03, OPG 10, OPH 03, OPI 06 and OPAK 05
Lanes (M) Marker, (1) KER 1, (2) KAR 1, (3) CHE 1, (4) MEG 1, (5) DEL 1, (6) KAR 2, (7) KER 2
Fig. 5 RAPD profiles of seven patchouli accessions using primers OPAM 11, OPQ 18, OPR 01, OPR 15, OPS 11 and OPC 05
Lanes (M) Marker; (1) KER 1, (2) KAR 1, (3) CHE 1, (4) MEG 1, (5) DEL 1, (6) KAR 2, (7) KER 2
Fig. 6 Cladogram based on the RAPD profile analysis of seven patchouli accessions
3.4 Discussion

Random Amplified Polymorphic DNA (RAPD) characterization studies of the seven patchouli accessions show that the primers OPJ 20, OPE 01, OPR 19 (Fig. 3), OPS 11 and OPC 05 (Fig. 5) generated monomorphic bands across all the seven patchouli accessions. This helps in proving their common ancestry. The bands can therefore be considered genus specific (Zhang et al. 1996).

The polymorphism generated by the primers OPAN 20, OPE 01, OPE 18, OPG 03, OPG 10, OPH 03, OPI 06, OPAK 05, OPQ 18, OPR 01, OPR 15 and OPC 05 in the KER 2 accession (Figs. 3, 4 and 5), enabled the segregation of KER 2 from the other accessions. The polymorphism also supports the result of the cluster analysis that KER 2 is an outlier and exhibits a similarity index of less than 0.25 with the other members of the germplasm (Fig. 6). In view of this, KER 2 was excluded from the field evaluation. In the present study, RAPD has evolved as a valuable tool to help rule out taxonomic ambiguity in the patchouli germplasm. The same methodology may be suggested to segregate adulterants (Wolf et al. 1999) from original patchouli raw material.

The other patchouli accessions excluding KER 2 were observed to possess a common DNA fingerprint pattern (Figs. 3, 4 and 5). This helped in the identification of taxa and determination of phylogenetic relationships and intra specific diversity at a molecular genetic level. The ability of this method to distinguish between taxa has useful implications in botanical analysis. The monomorphic bands formed by the primers OPAN-20 (Fig. 3), OPE-18, OPG-03, OPG-10, OPH-03, OPI-06, OPAK-05 (Fig. 4)
and OPQ-18 (Fig.6) present in KER 1, KAR 1, CHE 1, MEG 1, DEL 1 and KAR 2 indicate that they are species specific markers (Li et al. 1999; Sharma and Jana 2002; Simioniuc et al. 2002).

The lone polymorphic band formed by the primer OPAK-05 in MEG 1 can be considered as a marker band (Zeng et al. 2002) of the patchouli accession MEG 1. The polymorphism generated by the primer OPJ-20 in MEG 1 and KAR 2 (Fig.3), OPS-11 in CHE 1 (Fig.5), OPG-10 in MEG 1 and OPG-03 in DEL 1 (Fig.4) undoubtedly support the taxonomic relationship obtained from the cladogram that CHE 1, MEG 1, DEL 1 and KAR 2 belong to a common genetic stock and hence can function as components of the secondary gene pool (Jain et al. 1994 and Cao et al. 2006).

In contrast to the preliminary characterisation studies, it was observed that KER 1 and KAR 1 shared a common molecular pattern. The bands formed by primers OPJ 20, OPR 19, OPE 01, OPG 03, OPG 10, OPS 11 and OPC 05 clearly indicate a similarity between the KER 1 and KAR 1 accessions. The banding pattern complemented the cladogram results establishing a 97% similarity between KER 1 and KAR 1. Therefore, KER 1 can serve as a dependable substitute for KAR 1 and function as a member of the primary gene pool (Kapteyn et al. 2002).

The similarities in the DNA fingerprints of KER 1 and KAR 1 accessions of patchouli strongly suggest a possibility of their common ancestry (Figs. 3, 4 and 5). The results indicate that KER 1 and KAR 1 could have belonged to the same genetic stock that got separated owing to an isolation or barrier at some point of time during their evolution. The cluster consisting of CHE 1, MEG 1, DEL 1 and KAR 2 exhibit a genetic
closeness of 60% with the first cluster. Therefore, these accessions can make up the secondary gene pool of patchouli and serve as substitutes of KAR 1 and KER 1.
BIBLIOGRAPHY


Rolf, F. J. 2000. NTSYSpc Numerical taxonomy and multivariate analysis system, Exeter software, New York, USA.


* Original not seen