Chapter 6

Inter- and intra-geographical group relationship amongst neem provenances
INTER- AND INTRA-GEOGRAPHICAL GROUP RELATIONSHIP AMONGST NEEM PROVENANCES

6.1 INTRODUCTION

The RAPD profiles have generally been carried out for studies on determination of genetic variability within germplasm, between wild and cultivated plants and at inter- and intra-species level within finite populations in case of several plants. Thus, RAPD analysis was employed for assessing the genetic diversity in coffee and it was found that differences in morphology and geographical origin of the genotype was reflected in similarities in the RAPD patterns (Orozco-Castillo et al., 1996). On the other hand the study in case of Eucalyptus genotypes with RAPD markers revealed the power of the technique in resolving ambiguities in sampling and genotype identification (Keil and Griffin, 1994). In case of Arachis germplasm, Lanham et al. (1992) have determined the extent of genetic variability for exploitation in the groundnut improvement programme. In a similar type of study, Virk et al. (1995) have analysed the germplasm collection of rice accessions by RAPD methods. This study has enabled a reliable and rapid method of classifying rice accessions which were new or unknown. RAPD technology has also been chosen as a means of generating markers to investigate the gene introgression in plants obtained after sexual hybridization as in case of crosses of Brassica sp (Lifol et al., 1997). In this way several other plants for various applications have been studied using RAPD technique (reviewed in Ranade and Sane, 1995). RAPD technology, despite the above-mentioned and other reports of applications, has problems of reproducibility of profiles, since these are produced by primer annealing
invariably under low stringency conditions. The primary result of this low annealing stringency is generation of faint bands interspersed with prominent bands. These faint bands are generally not scored during data analysis. This is in fact a major drawback with RAPD technology. In order to alleviate these problems, several workers have employed polyacrylamide gels for the resolution of RAPD profiles and silver staining of these gels to reveal the profiles. Silver staining reportedly increases the sensitivity of detection by two, to five folds (Caetano-Anolles et al., 1991, 1992; Huff and Bara, 1993; Huff et al., 1993, 1994; Novy et al., 1994). This has resulted in a reduction in misscoring of bands, especially in situations involving epistatic interactions between primer sites (Heun and Helentjaris, 1993). This technique has been successfully used in identification of intra-cultivar genetic heterogeneity in Cranberry (Novy and Vorsa, 1995).

This chapter describes the augmentation of the potential of RAPD studies for a more comprehensive analysis of genetic variability in case of neem. A small sub-set of provenances representing individuals of same as well as different geographical areas was selected for the study with the expectation that a better estimate of genetic variation could be arrived at using the higher resolution silver staining technique.

6.2 MATERIALS AND METHODS

6.2.1 Plant material

The plant material was selected from amongst the genotypes as listed in Table 4.1 of Chapter 4.
Table 6.1 The provenances selected for study were grouped in four subsets A,B,C,D.

<table>
<thead>
<tr>
<th>Subset</th>
<th>S. No.</th>
<th>Sample No. as in Table 4.1</th>
<th>Provenances (Country)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>1</td>
<td>1</td>
<td>Lucknow (India)</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>5</td>
<td>Lucknow (India)</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>8</td>
<td>Lucknow (India)</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>35</td>
<td>Kanpur 27 (India)</td>
</tr>
<tr>
<td>B</td>
<td>5</td>
<td>36</td>
<td>Kanpur 28 (India)</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>37</td>
<td>Kanpur 46 (India)</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>38</td>
<td>Kanpur 48 (India)</td>
</tr>
<tr>
<td>C</td>
<td>8</td>
<td>74</td>
<td>Pakistan 1 (Pakistan)</td>
</tr>
<tr>
<td></td>
<td>9</td>
<td>75</td>
<td>Pakistan 2 (Pakistan)</td>
</tr>
<tr>
<td>D</td>
<td>10</td>
<td>76</td>
<td>Poi Tao (Thailand)</td>
</tr>
<tr>
<td></td>
<td>11</td>
<td>77</td>
<td>Uthai Thani (Thailand)</td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>78</td>
<td>Ban Noung Hai (Thailand)</td>
</tr>
<tr>
<td></td>
<td>13</td>
<td>79</td>
<td>Khao Luang (Thailand)</td>
</tr>
<tr>
<td></td>
<td>14</td>
<td>80</td>
<td>Ban Huey Sai (Thailand)</td>
</tr>
</tbody>
</table>

6.2.2 DNA isolation

The genomic DNA was isolated from neem leaves by the method of de Kochko and Hamon (1990) and its quality and quantity was analysed as described in Chapter 3.

6.2.3 RAPD reaction

The reaction mixture and PCR conditions were the same as those described in Chapter 4.
6.2.4 Primers used

Primers OP-T05, OP-T06, OP-T08, OP-T10, OP-T12, OP-F02, OP-F03, OP-F04, OP-U10, OP-U15, OP-U17, OP-U18, OP-U19, and OP-U20, were selected from the Operon kits for the RAPD reactions, since these had generated informative profiles when used for the genetic diversity studies as described in Chapter 4. The primer sequences are given in Annexure 1, at the end of the thesis.

6.2.5 Agarose gel electrophoresis

After RAPD-PCR was completed, the amplification products were electrophoresed in 1% agarose gel as already detailed in Chapter 4. The profiles were revealed by ethidium bromide staining of the gels.

6.2.6 Polyacrylamide gel electrophoresis

RAPD produces a range of bands which vary widely in intensity making them difficult to visualize using conventional agarose gel electrophoresis procedures. RAPD profiles resolved in polyacrylamide gel electrophoresis and stained by silver nitrate allowed more detailed analysis of complex fingerprints.

Procedure: For a 10 ml polyacrylamide gel of strength 8% (w/v), the following composition was used:

- bis:acrylamide mix (29:1), 30% (w/v) 2.66ml
- TBE Buffer, pH 8.0, 10X stock 1ml
- Sterile water 5.27ml
- APS (ammonium persulphate) 10%(w/v), freshly made 70μl

147
TEMED (N,N,N',N'-tetramethylethylenediamine) 3.5μl

The mixture was poured in the sealed mould of glass plates, the appropriate slot former was inserted and the entire assembly left aside for 30 minutes to allow polymerization of the acrylamide gel. Loading dye was mixed with the sample and loaded on the gel. Electrophoresis was carried out till the bromophenol blue marker dye had migrated to 3/4th distance from the well along the length of the gel.

**6.2.7 Ethidium Bromide staining of agarose gel**

The RAPD-PCR profiles obtained were resolved on 1% agarose gel. The gel was stained with ethidium bromide (0.5μg/ml concentration) for 15-20 min. The excess stain was de-stained in de-ionized water with gentle shaking for 30 min, prior to visualization and archiving using Nighthawk gel documentation system (pdi Inc., USA).

**6.2.8 Silver staining of polyacrylamide gel**

The PCR profiles obtained were resolved on polyacrylamide gels, and this enabled us to detect more number of bands. A fast and highly sensitive silver staining method as given by Caetano and Gresshoff (1994), was used with a few modifications. This procedure reportedly which allowed an accurate detection of nucleic acid, down to 1pg DNA/mm² band cross section with minimum background staining.

**Procedure:** The silver staining of polyacrylamide gel basically consisted of 6 steps, which are given in Table 6.2
Table 6.2: Steps involved in silver staining of polyacrylamide gels

<table>
<thead>
<tr>
<th>S. No</th>
<th>Steps</th>
<th>Reagent</th>
<th>Time required</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Fixation</td>
<td>Fixer solution (acetic acid, 7.5% v/v)</td>
<td>10 min</td>
</tr>
<tr>
<td>2.</td>
<td>Wash (3X)</td>
<td>Deionized water</td>
<td>2 min each</td>
</tr>
<tr>
<td>3.</td>
<td>Silver impregnation</td>
<td>Silver Solution (AgNO₃ 1.5g/l, formaldehyde, 0.056% v/v)</td>
<td>30 min</td>
</tr>
<tr>
<td>4.</td>
<td>Rinse</td>
<td>Deionized water</td>
<td>5 sec</td>
</tr>
<tr>
<td>5.</td>
<td>Image development</td>
<td>Developer (Na₂CO₃ 30g/l, HCHO, 0.056% v/v, 200μg/l sodium thiosulphate)</td>
<td>5-10 min</td>
</tr>
<tr>
<td>6.</td>
<td>Stop</td>
<td>Fixer Solution (acetic acid, 7.5% v/v, chilled at 4°C)</td>
<td>5 min</td>
</tr>
</tbody>
</table>

All the chemicals used were of analytical grade and solutions were prepared in deionized water. The fixer solution could be stored at room temperature, but the stop solution should be stored at 4°C. The silver nitrate/formaldehyde and developer solutions were prepared fresh every time. After optimum staining the polyacrylamide gels were dried between cellophane sheets and were photographed and scanned using gel documentation system, (pdi Inc.).

6.2.9 Data analysis

The bands in both staining techniques were scored for presence and absence and a data matrix was made as described earlier in chapter 4. Computer analysis of the data was also carried out as described in Chapter 4.
6.3 RESULTS

6.3.1 RAPD products resolved on Agarose gel after staining with ethidium bromide

A total of 108 scorable RAPD bands varying in size between 180bp-3000bp were generated with 14 primers. Some of the DNA markers were shared among all the genotypes, and were described as invariable or monomorphic, as in case of primer OP-F02 and OP-F03, as shown in Figure 6.1 and 6.2(b). The monomorphic band in these figures is indicated by solid arrow. These two invariable markers may have been amplified from highly conserved regions of the neem genome, whereas others were unique to a particular group, as in case of primer OP-AH03 (Fig 6.2 a), OP-F03 (Figure 6.2 b) and OP-U20 (Figure 6.2 c). These markers are indicated in the figure by an open arrow and are specific to the group of provenances form Thailand. The other typical profiles obtained with primer OP-U10, OP-U15 and OP-T08 are shown in Figure 6.3(a), 6.3(b) and 6.3(c) respectively.

6.3.2 RAPD products resolved on polyacrylamide gel after silver staining

Optimization of silver staining for RAPD profiles on polyacrylamide gel: The silver staining procedure of nucleic acids is based on a photochemically derived silver stain (Goldman and Merril, 1982), originally designed for the staining of protein. The detection of nucleic acids was optimized using a double digested HindIII/EcoRI λ DNA resolved by polyacrylamide gel electrophoresis, using the modified protocol of Ceatano-Anolles and Gresshoff, (1994). Image development was in the
1.1. RAPD profiles of neem DNAs with OP-F02 primer. The amplification products from DNAs of 14 neem provenances were analyzed on 1% agarose gels. The lanes marked 1-14 contain samples as listed in Table 6.1. DNA molecular weight markers (λ DNA double digested with EcoRI and HindIII) are indicated as lane M.

The solid arrow indicates a monomorphic band present in all provenances.
Figure 6.1
The solid arrow shows monomorphic and open arrow shows the Thailand provenance group.
Fig. 6.3 RAPD profiles of neem DNAs using 3 primers, namely, (a) OP-U10, (b) OP-U15 and (c) OP-T08. The amplification products from DNAs of 14 neem provenances were analysed on 1% agarose gels. The lanes marked 1-14 contain samples as listed in Table 6.1. DNA molecular weight markers (λ DNA double digested with EcoRI and HindIII) are indicated as lane M.
Figure 6.3
presence of sodium thiosulphate, as suggested by Blum et al. (1987) and using higher concentration of formaldehyde. This procedure was highly sensitive and prevents background staining and is also without any loss in contrast. It uses less silver and no oxidizing pretreatment is required. This protocol has fewer steps and stains complex mixtures of DNA resolved in non-denatured polyacrylamide gels.

The silver nitrate concentration was taken as 1g/l and 1.5ml/l of formaldehyde 37% (w/v) in silver nitrate was added freshly. The high concentration of formaldehyde in silver nitrate solution improved both sensitivity and contrast. While band intensity increased with time, a minimum silver nitrate impregnation time of 30 min gave optimal sensitivity. Reduction of silver by formaldehyde was concentration dependent. Originally Merril et al. (1981), had used 2g/l of silver. An increase amount of formaldehyde beyond 1.5ml/l though increased sensitivity, but also increased background staining, while reducing development time (Bassam et al., 1991). Dark brown-black bands were produced as opposed to the orange light brown colours obtained at lower concentrations. Optimal band intensity was attained in 5-10 min development time.

The reaction was performed at lower temperature, 8-12°C in the presence of 1mg/l sodium thiosulphate. Thiosulphate dissolves insoluble silver salts by complex formation, removing silver ions from the gel surface which in turn decreases non specific staining background (Caetano Anolles et al., 1991). Image development in the presence of different thiosulphate concentrations was investigated. The general effect was a reduction of background staining with no apparent change in development time. A concentration of 1.5mg/l sodium thiosulphate was sufficient to reduce nonspecific background
staining without noticeably affecting DNA image development. Higher concentrations have no noticeable benefit and can reduce the sensitivity of DNA detection. The presence of thiosulphate eliminated the formation of dark precipitate in the gel and developer solution. This optimized staining procedure was used for the analysis of complex DNA banding patterns.

**RAPD products resolved on polyacrylamide gel after silver staining**: The complex DNA banding patterns resulting from the amplification of genomic DNA by single short primer of arbitrary sequence consisted of a range of bands, which varied widely in intensity when visualized using conventional ethidium bromide staining of agarose gels. The more sensitive method of silver staining of polyacrylamide gel was therefore used for a more efficient detection of polymorphic bands. In the present study, a total of 91 scorable RAPD markers varying in size between 120bp-3200bp were generated with seven primers. The primers used for silver staining studies were OP-T06, OP-F02, OP-F03, OP-F04, OP-U18, OP-U19 and OP-U20. The profile of amplification products from different neem provenances resolved by electrophoresis in 8% polyacrylamide gel are shown in Fig 6.4 and 6.5. The improved silver staining method revealed more bands in the RAPD profiles relative to that after ethidium bromide staining.

**6.4 DISCUSSION**

**6.4.1 Optimization of silver staining technique**

Silver staining procedure provides sensitive and reproducible results. The method used for staining has been optimized for polyacrylamide gel. Originally, silver staining was used effectively in the detection of small amounts of nucleic acids.
double digested with EcoRI and HindIII) as indicated as lane M.

Lanes marked 1-14 contain samples as listed in Table 6.1. DNA molecular weight marker (λ DNA from DNAs of 14 necn provenances were analyzed on 8% polyacrylamide after silver staining. The amplification products

Fig. 6.4 RAPD profiles of necn DNAs using primers OP-F04(α) and OP-T06β. The amplification products
Fig. 6.4 (contd.) RAPD profiles of neem DNAs using primer OP-U18(c) and OP-U19(d) The amplification products from DNAs of 14 neem provenances were analyzed on 8% polyacrylamide silver stained gel. The lanes marked 1-14 contain samples as listed in Table 6.1. DNA molecular weight marker (λ DNA double digested with EcoRI and HindIII) is indicated as lane M.
Fig.6.5 RAPD profiles of neem DNAs with 4 primers, namely, (a) OP-F04, (b) OP-U20, (c) OP-F02 and (d) OP-F03. The amplification products from DNAs of 9 different neem provenances were resolved on 8% polyacrylamide gels and revealed after silver staining. The lanes 1-9 contain DNA from provenances Lucknow, Kanpur 27, Pakistan1, Pakistan2, Poi Tao, Uthai Thani, Ban Noug Hai, Khao Luang and Ban Huey Sai respectively. DNA molecular weight marker (λ DNA double digest with EcoRI and HindIII) is indicated as lane M.
However, more demanding application, such as analysis of complex DNA profiles generated in DNA amplification fingerprinting was reported by Caetano-Anolles et al. (1991, 1992 and 1993).

The procedure used for silver staining gave minimum background, had fewer steps, took less time and produced the least number of staining artifacts. Several staining parameters alter the effectiveness of silver staining. These are discussed below.

**Nucleic acid fixation**: Fixation step prevents the diffusion of separated nucleic acid molecules within the gel matrix and helps to remove and neutralize unwanted chemicals like silver and buffer. Fixation was found to be very important for sensitivity. Omitting or limiting fixation to very short exposures resulted in poor image development. In turn, longer exposures resulted in band fading (Result not shown). A minimum of 5 minute immersion in 7.5% acetic acid maintained the limit of detection of DNA fragments of various sizes.

**Gel Washing**: This step removes the acid, trace substances and remnants of soluble gel components that may have been carried over from the fixation step, and that can interfere with staining. A minimum of 3 washes of 2 min each in deionized water were found to be adequate.

**Silver impregnation**: The presence of formaldehyde in the silver solution improves sensitivity and contrast. Formaldehyde reduces silver at a very low rate but enough to produce initial nucleation sites around the staining substrate. These sites favor the rapid build up of silver deposits during the development step. Optimal staining was achieved after 30 minutes.
**Post impregnation Wash**: The remnant silver nitrate which can cause brown precipitates was removed at this step. A longer rinse greater than 10 seconds resulted in fading band pattern.

**Image development**: The development requires an abrupt change in pH which inevitably causes the formation of insoluble silver salts. These precipitates attach to the gel surface and decrease image contrast by increasing background staining. Decreasing the concentration of silver on the surface of the gel by prior washing avoids silver precipitation and also decreases sensitivity (Wray *et al*., 1981). This was optimized at a concentration of 1.5 gm/l silver nitrate and 5 second wash after impregnation. However, silver ion complex reacts, with sodium-thiosulfate, decreases the free silver ion concentration, reduces the kinetics of reduction, and thus increases the redox potential in the surrounding matrix and minimize background staining. A concentration of 200μg/l sodium thiosulphate effectively reduced nonspecific background staining. This concentration of sodium thiosulphate produced noticeable improvement in image development. Formaldehyde concentration of 0.054% (v/v) provided on optimal stain. Lower concentrations have the general effect of reducing sensitivity. In contrast, higher formaldehyde concentrations increase sensitivity but also background staining. Higher concentration also considerably reduced the development time, producing dark brown bands; while lower formaldehyde concentration below 0.056% (v/v) produced light brown colour bands. The temperature of development solution is crucial. Typically, it was kept between 8-10°C. If temperature was above 10°C, the image developed quickly and browning of the gel surface was the usual effect.
6.4.2 RAPD analysis

Many genetic properties are still unknown in a majority of tree species and it is, therefore, important that the gene pool be preserved and the level of intra-population diversity and inter-population differentiation evaluated. The estimation of genetic features in populations and at the individual level has been strongly influenced by polymerase chain reaction (PCR). Genetic variation for identification of cultivars and validation of genetic relationship between and within tree species have been made using RAPD markers, as in case of *Mangifera indica*, mahoganies, *Rubus*, *Malus*, *Eucalyptus*, *Picea abies* and *Theobroma cacao* (Dunemann et al., 1994; Chalmers et al., 1994; Graham and McNicol, 1995; Schnell et al., 1995; Scheepers et al., 1997; Whitkus et al., 1998).

Neem tree is believed to be monotypic in India, since only one species is recognized, *Azadirachta indica*. However, Thailand neem is reported as a different species, *Azadirachta siamensis*. RAPD-PCR based technique was applied to a small subset, consisting of provenances of different geographical regions. In this study comparisons could be made both between and within genotype subsets.

The total number of markers and proportions of unique and invariable markers are valuable parameters in determining intra-specific variability and genetic relationship. It appears that a sufficient number of markers can be generated using a few selected primers, to enable comparison within a small sample size. Same set of primer was used for silver staining of RAPD products. When RAPD products were separated on the polyacrylamide gel additional bands which could not be resolved on agarose gel could be distinguished. Table 6.3 summarizes...
the results obtained with the two electrophoresis and staining methods.

Table 6.3: RAPD results obtained with the two electrophoresis and staining methodologies:

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Agarose gel + Ethidium bromide staining</th>
<th>Polyacrylamide gel + Silver staining</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total number of primers used</td>
<td>14</td>
<td>7</td>
</tr>
<tr>
<td>Total number of bands detected</td>
<td>108</td>
<td>91</td>
</tr>
<tr>
<td>Average number of loci/primer</td>
<td>7.7</td>
<td>13</td>
</tr>
<tr>
<td>Range in genetic similarity</td>
<td>0.2-0.75</td>
<td>0.2-0.8</td>
</tr>
</tbody>
</table>

It was assumed that the amplification products of differing electrophoretic mobilities were non-allelic and products with same mobilities to be allelic. This has been previously demonstrated for different species of *Glycine* and *Allium* (Wilkie *et al.*, 1993., Williams *et al.*, 1993). Indirect but very significant, supporting evidence of allelism is derived from the conformity of taxonomic classifications based on RAPD data which may be widely accepted and based on more conventional approaches involving morphology, cytology and enzyme electrophoresis.

Cluster analysis method (UPGMA) was used to generate a dendrogram. The analysis was carried out using Nei and Lei (1979) index. The genetic similarity among the genotypes studied was relatively high (0.8) suggesting narrow genetic base. The dendrogram obtained in case of silver stained profiles readily separated the 14 provenances into two major groups (X and Y), which being further divided into two minor sub-groups.
X1 and X2; Y1 and Y2 respectively (Figure 6.6, with shaded box). X1 group formed cluster of genotypes belonging to one provenance Lucknow, which was closely related to another sub-cluster X2 consisting of accessions from another provenance Kanpur of close geographic location. Another major group formed sub-clusters, Y1 of genotypes belonging to Pakistan provenance and Y2 belonging to genotypes of Thailand provenance. The Thailand samples belong to another species A. siamensis, but shows close similarity with A. indica (Pakistan provenance). The data grouped the samples according to their geographical origin. Matching the clustering results of these samples with their collection sites, it was clear that geographical distributions of most samples in each of these groups were well defined. Such type of results have been reported by Liu (1997), where genetic variation in geographical distribution of Stylosanthes scabra has been revealed by RAPD analysis. Provenance relationships in Norway spruce using RAPD pattern has been reported by Scheepers et al. (1997), where 42 RAPD bands, separated the nine provenances into two major groups. RAPD markers have been used for differentiation and estimation of genetic diversity among different populations of T. dicoccoides originating primarily from Northern Israel (Fahima et al., 1999). These result shows that RAPD technique is a useful and powerful tool for population analysis.

The grouping pattern was identical among genotypes for data sets as reported in Chapter 4. Although, specific values of these parameters varied with number of sample size. With this technique sufficient number of markers can be generated by using selected primers and applying silver staining technique. Similar type of study has been done in case of Cranberry cultivars where genetic heterogeneity has been studied using silver stain RAPD (Novy and Vorsa, 1995). This enabled
comparison with small sample size. This implies that the RAPD method can be used to discriminate among species even when few primers are used in combination with polyacrylamide gel and silver staining technique. When the similar set of study was made by resolving the RAPD products on agarose gel and stained with ethidium bromide, a loose clustering was obtained as shown in the dendrogram (Fig. 6.6, shown with an open box). The subgroups were not so well defined according to their geographic origin. Agarose gel resolution is not so sensitive and gives few markers. Hence, more number of genotypes and primer combinations need to be studied for analysing relationship in case of ethidium bromide stained agarose gel. This method appears to be an effective approach in resolving genetic variations, fingerprinting species and grouping germplasm into geographical races. This is in contrast with morphological and other molecular methods that do not show this depth of resolution.

6.5 REFERENCES


