CHAPTER 4
ASSESSMENT OF GENETIC DIVERSITY IN WALSURA TRIFOLIATA USING RAPD

Introduction

Population is the principal and fundamental unit of evolution of a species. It is defined as a group of individuals of a particular species grow together in a community (Odum, 1971). To understand the population biology of a species, it is necessary to study the population ecology and population genetics. Population genetics is the analysis of genetic variation within and between the populations. The molecular basis of the essential biological phenomena in plants should be understood for the effective conservation, management and efficient utilization of plant genetic resources. The assessment of genetic diversity within and between populations is routinely performed at the molecular level using various laboratory based techniques such as allozyme or DNA analysis, which measure the levels of variation directly (Mondini et al., 2009).

Genotypic variation which exhibits differences in the genetic makeup of individuals is the prevalence of evolution. The level of variation and its influence on environment attributes significantly to genetic diversity. Genetic diversity can therefore be defined as any variation in the nucleotides, genes, chromosomes or whole genomes of the plant. Natural populations in general have high level of genetic variation (Hubby and Lewontin 1966).

Genetic diversity is generally influenced by various factors like mutation, genetic drift, gene flow and natural selection. Genetic variability in a population can be assessed through the number of polymorphic genes in the population, the number
of alleles for each polymorphic gene and the proportion of heterozygous loci per individual. Information on genetic diversity is also valued for the management of germplasm and for evolving conservation strategies. Molecular markers are the best tools for determining genetic relationships (Chyi et al., 1992).

**Molecular markers**

Genetic diversity of an organism can be measured and quantified by three types of markers namely

i) Phenotypic marker (difference in morphological features)

ii) Biochemical markers (difference in protein and metabolites) and

iii) Molecular markers (difference in nucleotide sequence)

Phenotypic and biochemical markers are relatively less efficient and are highly sensitive to environmental and ontological changes. Molecular markers are polymorphic proteins or DNA sequences that can be used as indicators of genome-wide variation. They play a major role in assessing patterns of genetic variation within and among species (Alphey, 2005; Pramod et al., 2009). They also give extremely useful information for the analysis of population structure, level of gene flow, phylogenetic relationships, pattern of historical biogeography, analysis of parentage and genetic variation and relatedness within and between population and species (UNEP, 1995). The characteristic features of molecular markers are: polymorphic in nature, distributed throughout the genome, very simple, cost effective, high reproducibility, more reliability, co-dominance and not influenced by environmental cues.

There are many molecular markers available and are being developed. They include Restriction Fragment Length Polymorphism (RFLP) (Bostein et al., 1980), Random Amplified Polymorphic DNA (RAPD) (Williams et al., 1990), Arbitrarily

**Randomly Amplified Polymorphic DNA (RAPD)**

RAPD is a molecular marker which is less expensive and is a rapid method to determine the genetic diversity, variation and relatedness of plants and animals species. It was developed by William et al. (1990) and Welsh and McClelland (1990) independently by using the different size of oligo-nucleotide sequences for the amplification of DNA randomly in the genome. RAPD is a dominant molecular marker because it is only based on the presence and absence of loci (Rafalski et al., 1998). It requires only relatively small quantity of DNA and need not have any prior knowledge on the sequence of template DNA. The major disadvantage of RAPD molecular marker is less reproducibility and it is highly sensitive to laboratory changes (IAEA, 2002). This limitation can be overcome by converting the RAPD data into Sequence Characterized Amplified Region (SCAR) molecular marker based on their DNA sequence (Paran and Michelmore, 1993).

This method has been successfully used for the identification of hybrid origin, QTL identification, molecular systematics and estimation of genetic diversity, variation and relatedness in *Hevea brasiliensis* (Varghese et al., 1997),
Changium smyrnioides (Fu et al., 2003), Jatropha (Ram et al., 2007), Catheranthus roseus (Shaw et al., 2009), Chilli (Makari et al., 2009), Pinus roxburghii (Ghildiyal et al., 2009) Jatropha curcas (Ikbal et al., 2010), Azima tetracantha (Hepsibha et al., 2010), Andrographis paniculata (Wijarat et al., 2011), Withania somnifera (Dharmar and Britto, 2011), Cyamopsis tetragonoloba (Manivannan and Anandakumar, 2013), and Semecarpus kurzii (Das and Mandal, 2013). The present study analysed the genetic variation within and between the populations of Walsura trifoliata using five random primers by the Randomly Amplified Polymorphic DNA (RAPD) for the first time.

Materials and Methods

Collection site

For this study samples of Walsura trifoliata were collected from three different localities one from the coastal forest and two from the Eastern Ghats. From each location samples were collected from three accessions. Thus, a total of 9 accessions were collected for analyzing genetic diversity of Walsura trifoliata. The accessions and place name is given in table 4.1. Fresh and young leaf samples were collected to isolate genomic DNA. Molecular study was conducted at Pondicherry Centre for Biological Sciences, Pondicherry -5.
Table 4.1: Collection of 9 accessions *Walsura trifoliata* from different sites.

<table>
<thead>
<tr>
<th>S.No.</th>
<th>Accessions</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>WKP0101</td>
<td>Puthupet</td>
</tr>
<tr>
<td>2</td>
<td>WKP0102</td>
<td>Puthupet</td>
</tr>
<tr>
<td>3</td>
<td>WKP0103</td>
<td>Puthupet</td>
</tr>
<tr>
<td>4</td>
<td>WKB0104</td>
<td>Kambakkam</td>
</tr>
<tr>
<td>5</td>
<td>WKB0105</td>
<td>Kambakkam</td>
</tr>
<tr>
<td>6</td>
<td>WKB0106</td>
<td>Kambakkam</td>
</tr>
<tr>
<td>7</td>
<td>WNP0107</td>
<td>Nagallapuram</td>
</tr>
<tr>
<td>8</td>
<td>WNP0108</td>
<td>Nagallapuram</td>
</tr>
<tr>
<td>9</td>
<td>WNP0109</td>
<td>Nagallapuram</td>
</tr>
</tbody>
</table>

**Genomic DNA isolation**

The new young leaves from all the accessions were collected in zip-Lock cover containing silica beads for genomic DNA isolation. The collected leaves were washed properly with double distilled water and kept in -20 °C for storage and DNA isolation in future.

**Genomic DNA isolation Procedure**

Genomic DNA was isolated from tender young leaves by the standard CTAB (Cetyl trimethyl ammonium bromide) method (Doyle and Doyle 1990) with slight modifications as follows.

1. Fresh 1 g of leaf sample was homogenized properly by adding 1 ml of pre-warmed (60 °C) CTAB extraction buffer with sterilized mortar and pestle.
2. The homogenized mixture was transferred into 1.5 ml centrifuge tube and incubated at 60 °C in water bath for 1 hour with intermittent swirling for every 15 minutes.

3. The mixture was emulsified with an equal volume of Chloroform : Isoamyl alcohol (24:1) by gentle inversions.

4. The content was centrifuged at 10000 rpm for 10 min at room temperature and supernatant were taken out carefully and transferred into new sterile 1.5 ml centrifuge tube.

5. Equal volume of ice cold isopropanol was added with the supernatant and mixed gently. The content was incubated at -20 °C for at least 20 minutes.

6. The precipitated DNA was spooled out using disposable pipette tip and washed twice with 70% ethanol by centrifuging 10000 rpm for 5 minutes.

7. The pellet was dried in room temperature and dissolved in 1 ml of TE buffer.

8. Stored at -20 °C for further analysis.

**Composition of DNA extraction buffer**

- Tris hydrochloride (pH 8.0) (100 mM)
- Na-EDTA salt (pH 8.0) (20 mM)
- Sodium chloride (NaCl) (1.4 M)
- CTAB 2% (w/v)
- Polyvinylpyrrolidone (PVP) 0.25 g
- β - mercaptoethanol (0.2%)
Quantification of the extracted gDNA

Agarose gel (0.8%) was prepared in 1X TBE buffer. To each well, 5 µl of genomic DNA sample and 1 µl of 6X loading dye were loaded carefully with standard DNA ladder marker DNA (Invitrogen). The electrophoresis was carried out in Biorad 200 V power pack at a constant volume of 55 volts for 1 hour till the dye moved to a few centimeters from the wells. The gel was visualized under UV light and quantified manually and photographed.

PCR Amplification

Random Amplified Polymorphic DNA (RAPD) analysis

The RAPD analysis was performed following the methodology of Williams et al., (1990). Each amplification of 25 µL reaction volume contained 2.5 µL of 10X assay buffer (100 mM Tris-Cl, pH 9.0, 500 mM KCl, 15 mM MgCl$_2$ and 0.1% gelatin), 200 mM of each dNTPs (dATP, dTTP, dCTP and dGTP) (Eurofins, Germany) 15 ng primer, 0.5 unit of Taq DNA polymerase (Sangon, Shanghai, China) and 24 ng of DNA template. The amplification reaction was carried out using a Agilent cycler 2200 (Germany). Amplification was performed in three steps. In the first, the sample was maintained at 94 ºC for 5 minutes for complete denaturation of the template DNA. The second step consisted of 42 cycles, each cycle with three temperature regimes, i.e. at 92 ºC for 1 min for denaturation of template DNA, at 37 ºC for 1 min. for primer annealing and at 72 ºC for 2 min. for primer extension followed by complete polymerization at 72 ºC for 7 min. The soaking temperature was 4 ºC. After completion of the PCR, 2.5 µl of 6X loading dye (MBI Fermantas, Lithuania) was added to the amplified product, which was deep-frozen at –20 ºC for future use.
Agarose Gel Electrophoresis

The amplicons were separated in 1.5 % agarose gel for RAPD. Electrophoresis was performed at a constant voltage at 60 V for 3 hours. The amplicons were visualized under UV light and photographed. The gel was also documented by Gel Doc 2000 (Bio-Rad, USA) for scoring the bands. The amplicon size was determined by comparison with the ladder (Gene Ruler 100 bp ladder plus). The entire process was repeated at least twice to ensure reproducibility.

Data scoring

The data were scored as ‘1’ for band presence and ‘0’ for absence for each primer genotype combination for RAPD analysis. All bands (monomorphic and polymorphic) were considered to avoid under/over estimation of the genetic similarity (Gherardi et al. 1998). The data were used as discrete variables in a binary matrix.

Statistical data analysis

Jaccard’s similarity coefficient (Jaccard, 1908) was measured as well as a phylogram based on similarity coefficients generated by the unweighted pair group method using arithmetic means (UPGMA) (Sneath and Sokal, 1973) and SAHN clustering. The most informative primers were detected by comparing all primers with those of the pooled data using Mantel Z statistics (Mantel 1967). Most informative primers were used for a diagrammatic representation of the banding pattern of 9 individual specimens of Walsura trifoliata. The entire analysis was performed using the statistical package NTSYS pc 2.02e (Rohlf, 2000). The resolving power of the RAPD primer was calculated according to the method of Prevost and Wilkinson (1999). Resolving power (RP) was calculated as RP=SIb (Ib= Band informativeness) =1-[2 ´(0.5-P)], where P is the proportion of the 9 plant
specimens containing the band. The RAPD Primer Index (RPI) was calculated based on the Polymorphic index. This index (PIC) was calculated as $\text{PIC} = 1 - \sum P_i^2$; $P_i$ is the band frequency of the $i^{th}$ allele (Smith et al., 1997). In case of RAPDs, the PIC was considered to be $1 - p^2 - q^2$, where $p$ is band frequency and $q$ is no band frequency (Ghislain et al., 1999). The PIC value was then used to calculate the RAPD primer index (RPI). RPI is the sum of the PIC of all markers amplified by the same primer.

**Results**

Out of the 30 RAPD primers screened for polymorphism survey in pooled DNA of 9 accessions of *Walsura trifoliata* collections, 24 primers were not amplified while 6 primers (OPA-01, OPA-02, OPA-03, OPA-04, OPA-05, and OPA-07) amplified and showed polymorphic bands (Table 3.2). The reason for the non-amplifications of the 24 primers could not be explained. Probably the sample DNA did not have any binding site for the primers. The amplification pattern is shown in Fig 3.1a to Fig 3.1f and the details of the RAPD analyses in table 4.2.

PCR analysis of 9 accessions of *Walsura trifoliata* with 5 polymorphic random markers generated a total of 470 scorable bands. In accessions containing 9 accessions, the highest number of bands was obtained with primer OPA-04 (104) followed by OPA-02 (101) while minimum number of band was generated with OPA-07 (58). Maximum number of bands per marker per accessions was obtained from OPA-04 (11.6) followed by OPA-02 (11.2) and minimum number of bands recorded in OPA-07 (6.4). Among the amplified RAPD primers, OPA-04 shows more bands (8.67) produced across the genotypes, while the less number of bands were generated by OPA-07 (2.76). The amplicon size from all the five studied primers was ranged from 300 to 4500 bp. The polymorphic information content (PIC) ranged from 0.33 to 0.12 with average of 0.20.
Table 4.2: DNA marker and amplified products used to generate RAPD marker in *Walsura trifoliata* accessions.

<table>
<thead>
<tr>
<th>S. No</th>
<th>RAPD Marker</th>
<th>Marker Sequence (5' to 3')</th>
<th>PIC value</th>
<th>Range of amplicons in individual genotypes</th>
<th>Range of amplification size (bp)</th>
<th>Total No. of bands</th>
<th>Average No. of bands per genotypes</th>
<th>Average No. of bands across genotypes</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>OPA-01</td>
<td>CAGGCCCT TC</td>
<td>0.16</td>
<td>1-8</td>
<td>350-3800</td>
<td>65</td>
<td>7.2</td>
<td>3.85</td>
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<tr>
<td>2</td>
<td>OPA-02</td>
<td>TGCCGAGCTG</td>
<td>0.30</td>
<td>1-9</td>
<td>300-4500</td>
<td>101</td>
<td>11.2</td>
<td>7.77</td>
</tr>
<tr>
<td>3</td>
<td>OPA-03</td>
<td>AGTCAGCC AC</td>
<td>0.18</td>
<td>1-9</td>
<td>400-4500</td>
<td>77</td>
<td>8.6</td>
<td>4.53</td>
</tr>
<tr>
<td>4</td>
<td>OPA-04</td>
<td>AATCGGGCTG</td>
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<td>7-9</td>
<td>300-4500</td>
<td>104</td>
<td>11.6</td>
<td>8.67</td>
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<tr>
<td>5</td>
<td>OPA-05</td>
<td>AGGGGTCTTG</td>
<td>0.13</td>
<td>1-6</td>
<td>350-3750</td>
<td>65</td>
<td>7.2</td>
<td>2.95</td>
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<tr>
<td>6</td>
<td>OPA-07</td>
<td>GAAACGGGTG</td>
<td>0.12</td>
<td>1-5</td>
<td>300-4250</td>
<td>58</td>
<td>6.4</td>
<td>2.76</td>
</tr>
</tbody>
</table>
Figure 4.1A to 1D: RAPD banding pattern in 9 accessions of *Walsura trifoliata* L. with different random primers.

1- Standard DNA Marker, 2-4 Samples collected from Puthupet, 5-7 Samples collected from Kambakkam, 8 – 10 Samples collected from Nagallapuram

Fig 4.2 Dendrogram analysis of RAPD profile for 9 accessions of *Walsura trifoliata*
Discussion

Morphological characters in plants may be affected by environmental conditions, and a species grown in different environmental conditions may be different and thus the use of morphologically characters for classification may result in some mistakes. Efficiency of a molecular marker technique depends upon the amount of polymorphism it can detect among the set of accessions under investigation. These techniques extensively used in many crops like rice (Ravi et al., 2003) and common bean (Marotti et al., 2006). In the present study Walsura trifoliata was screened using 30 primers for polymorphism survey and 24 primers could not be amplified. The similar non-amplification of decamer primers was reported by Hosaka et al., 1984, Cisneros and Quiros (1995) and Mattagajasingh et al. (2006), in different plant species. Reproducible results were obtained using specific primer and DNA template combination. RAPD analysis was also used to identify accepted relationship between genomes of Musa and Brassica (Demeke et al., 1992) and other medicinal plants like Oroxyllum indicum (Jayaram and Prasad, 2008), Vitex rotundifolia (Yuan et al., 2008), Withania coagulans (Gilani et al., 2009), Colues sp (Muthusamy et al., 2011), Withania somnifera (Dharmar and Britto, 2011) and Semecarpus kurzii (Das and Mandal, 2013). Huang et al., (2008) assessed interspecific and inter population variation in three species of Ceriops and recorded low genetic diversity at population level. Pawar et al., (2013) assessed the genetic variation between Xylocarpus spp and found that the species are closely related with minor variation.

In the present study RAPD cluster analysis (Fig 4.2) dendrogram showed distinguished relationship and grouped into six major clusters. Cluster I consists of 2 accessions, of these accessions WNP0107 and WNP0108 from Nagallapuram, Andhra Pradesh showed similarity of 81%. Cluster II consists of 2 accessions, of
these accessions WKB0105 and WKB0104 from Kambakkam, Tamilnadu showed 78% similarity among the accessions. Cluster III consists of 2 accessions, of which WKP0103 and WKP0102 from Puthupet, Tamilnadu showed 85% similarity. Cluster IV consists of one accession, of which WKP0101 from Puthupet, Tamilnadu showed 74% similarity with all accessions and 11% intra cluster divergence with WKP0102 and WKP0103 of same accessions. Cluster V consists of one accession, of which WKB0106 from Kambakkam, Tamilnadu showed 70% similarity with WKP0101, WKP0102 and WKP0103 and 14% Intra cluster divergence with WKB0104, WKB0105. Cluster VI consists of one accession, of which WNP0109 from Nagallapuram, Andhra Pradesh showed 62% similarity with Kambakkam and Puthupet accessions and 8% divergence with within the accessions. The slight variation may due to its genotypic variation, isolated distribution and adaption to dissimilar edaphic and environmental factors.