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DNA fingerprinting of *Plumbago zeylanica* L. using ISSR Markers

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Inter-simple sequence repeat markers (ISSR) were used to estimate genetic diversity of *Plumbago zeylanica* L. a medicinal plant which was collected from five accessions of Southern Western Ghats. Of the 12 primers screened, 6 produced highly polymorphic DNA fragments. The total number of amplified DNA fragments was 35. The overall observed and effective number of alleles, over all genetic diversity and percentage of polymorphic loci were calculated using POPGENE software. Nei's overall genetic diversity or heterozygosity was 0.2194. The genetic identity between the population ranged from 0.5714 to 0.8571 and the genetic distance ranged from 0.2231 to 0.5596. The overall observed and effective number of alleles was about 1.5429 and 1.3847 respectively and overall percentage of polymorphism of this species was 54.29. The population (p_5) exhibited high percentage (93 %) of polymorphism which was considered to be a superior genotypes, which can be conserved.

Plumbago zeylanica / DNA

***P**lumbago zeylanica* L. is an important medicinal herb belonging to the family Plumbaginaceae. Local name is Vellai Sidhirai Mulam. The plant is used for the treatment of dyspepsia, piles, diarrhoea and skin diseases. The tuberous roots are the main source of plumbagin (2-methyl-5-hydroxy-1, 4-naphtoquinone), which is used to formulate the anticancer drug (Krishnaswamy and Purushothaman, 1980).

Genetic diversity is at the lowest hierarchy, without genetic diversity, a population cannot evolve and adapt to environmental changes (Templeton, 1993). Genetic diversity refers to any variation in the nucleotides, genes, chromosomes or whole genomes of organisms (Andayani et al. 2001).

The extensive development of molecular techniques for genetic analysis in the past decade has led to the increase the knowledge of plant genetic diversity. The molecular markers best suited for detecting genetic diversity should be relatively easy and inexpensive to use and should evolve rapidly enough to be variable within populations. Inter-simple sequence repeat (ISSR) markers which have been used to investigate the genetic diversity of natural populations (Zietkiewicz et al.1994).

Characterization of the genetic diversity and examination of the genetic relationship among *Plumbago zeylanica* are important for the sustainable conservation and increased use of plant genetic resources. Hence, in the present study, ISSR markers were employed to study the extent of genetic diversity among five populations of *Plumbago zeylanica* in the Southern Western Ghats.

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Habit of *P. zeylanica*

Material and Methods

Plant materials

The plant samples were collected from different geographical locations in the Western Ghats of India such as Marunthuvalmalai, Courtallum, Achankoil, Maruthamalai hills and Anchetty. The samples were randomly collected from each population. The voucher specimens are deposited in the St. Xavier's College Herbarium (XCH), Palayamkottai, Tamil Nadu, India.

Genomic DNA isolation and purification

Tender unfolded leaf samples were collected from five different locations and stored at -70° C for DNA extraction. The total genomic DNA was extracted from the samples using the modified CTAB method (Doyle and Doyle, 1987) and purified according to standard method followed by Sambrook and Russel (2000). Concentration of the purified genomic DNA in each case was adjusted to 10 ng/ µl in different aliquots and stored at -70° C for PCR amplification.

PCR amplification for ISSR fingerprinting

Six ISSR primers were used for PCR amplification of the genomic DNA of *Plumbago zeylanica* (Williams et al. 1990). PCR reactions were carried out in a final volume of 25 µl, which contained 2.5 µl 10 X taq polymerase buffer, 4.0 µl of deoxyribonucletides (dNTPs), 0.5 µl $MgCl_2$, 0.1 µl of taq DNA polymerase, 2.5 µl of deca oligonucleotide primer, 3.0 µl of tamplate DNA and 12.4 µl of sterile dis.H_20.

The reaction mixture was subjected to programmed PCR-amplification in a Perkin-Elmer Gene Amp PCR system (Model, 2400). Amplification process included, initial denaturation of DNA at 95ºC for 5 minutes, denaturation at 94ºC for 30 seconds, annealing temperature varied from 34-39ºC for each primer for 1 minute and extension at 72ºC for 2 minutes. This was repeated for thirty five cycles and final extension at 72ºC for 5 minutes followed by storage at 4ºC till electrophoresed.

The amplification products were resolved by electrophoresis on 1.5 % agarose gel containing ethidium bromide along with 1 Kb ladder DNA as a standard molecular weight size marker. The gels were visualized under UV transilluminator and image was captured using gel documentation system Alpha Imager 1200.

Data analysis

Amplification profiles of five genotypes were compared with each other. Genetic similarity matrix among populations of each plant samples was calculated using the standard coefficient method (Nei and Li, 1979). The dendrogram was constructed using the UPGMA (Unweighed Pair Group Method with Arithmetic Average) (Sneath and Sokal, 1973) algorithm in SHAN clustering module of NTSYS-pc software version 1.5 (Rohlf, 1989). The genetic diversity within and between populations according to Nei's formula (Nei, 1973) was calculated using POPGENE package version 1.31software (Yeh et al. 1999).

Results and Discussion

The genetic diversity and the relationships between five populations of *Plumbago zeylanica* L. was analyzed with six ISSR random primers (10-mer) which produced resolved amplified fragments that varied from 5 to 7 with different primers. The differences in fragment size with different primers used for ISSR assay suggested that each of the primer amplified a different set of loci in the genome and a total of 35 ISSR fragments were observed. The size of the ISSR fragments varied from 0.1 to 1.0 kbp (Plate 1).

The mean genetic heterozygosity or diversity (*H*) ranged from 0.2023 to 0.2978. The P_3 population was found to be the least diverse (0.2023), P_3 population displayed the high level of variability (0.2978) and the P_2 population revealed intermediate diversity (0.2671). Nei's overall genetic diversity or heterozygosity was 0.2194. The genetic identity between the population ranged from 0.5714 to 0.8571 and the genetic distance ranged from 0.2231 to 0.5596 Table 3. The overall observed and

effective number of alleles was about 1.5429 and 1.3847 respectively and overall percentage of polymorphism of this species was 54.29. The highest Shannon Information Index (*I*) in the population P_3 was 0.42 (Table 1).

Table- 1: Analysis of polymorphism obtained with ISSR primers in different accessions of *P. zeylanica*

Accession	*Na*	*Ne*	*H*	*I*	NPL	% of polymorphism
P_1	1.5524	1.3241	0.2800	0.3772	35	82.58
P_2	1.5783	1.3340	0.2671	0.3827	37	91.73
P_3	1.5290	1.5867	0.2023	0.3241	32	89.48
P_4	1.5592	1.3752	0.2972	0.4779	29	84.86
P_5	1.5293	1.3726	0.2978	0.2245	45	93.49

Na – Observed number of alleles;*Ne* – Effective number of alleles; *H* – Gene diversity; *I* – Shannon Information Index; NPL – Number of Polymorphic Loci

Table- 2: Genetic and gene diversity within and between the populations of *P. zeylanica* for ISSR markers

S.No	Primers	Sequence 5'-3'	NPF	H_T	*Hs*	G_{ST}	*Nm*	BF
1.	HBIO809	(AG)8G	5	0.4440	0.3295	0.3324	1.8334	0.7457
2	HBIO812	(GA)8T	5	0.3232	0.2344	0.3806	1.8438	0.6568
3	HBIO816	(CA)8T	7	0.3488	0.2240	0.3417	1.7341	0.7459
4	HBIO834	(AG)8T	7	0.4099	0.3391	0.4390	1.5300	0.7453
5	HBIO835	(AG)8C	5	0.2949	0.3232	0.3225	1.5340	0.7457
6	HBIO840	(GA)8T	6	0.3616	0.2424	0.3341	0.9401	0.7859

HT - Total diversity; *Hs* – Gene diversity within populations; *GST* - Genetic differentiation; *Nm* – Gene flow; NPF- Number of polymorphic fragments; BF- Band frequency

Table -3: Nei's unbiased measures of Genetic distance and Genetic identity for *P. zeylanica* L.

Pop ID	P 1	P2	P3	P4	P5
P1	****	0.5271	0.6823	0.6234	0.7715
P2	0.3113	****	0.6874	0.7742	0.8214
P3	0.3452	0.3420	****	0.57.14	0.8571
P4	0.4846	0.4821	0.5432	****	0.6524
P5	0.5672	0.5347	0.2331	0.5596	****

Nei's genetic identity (above diagonal) and genetic distance (below diagonal)
P1-Marunthuvalmalai, P2-Courtallum, P3-Achankoil, P4-Maruthamalai hills and P5 Anchetty

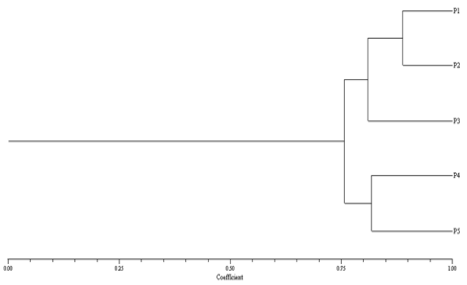

Fig.1:Dendrogram showing genetic relationship among the five different accessions of *P. zeylanica* based on Nei & Li coefficient

The average gene diversity within the population (*Hs*) was 0.2821, the highest (*Hs*) was 0.3391 and the lowest (*Hs*) was 0.2240. The total diversity (H_T) ranged from 0.2949 to 0.4440 and the average was 0.3604. The mean level of genetic differentiation (G_{ST}) between the populations was 0.3583 and the G_{ST} ranged from 0.3225 to 0.4390. The band frequency ranged from 0.6568 to 0.7859 and average was 0.7375. The estimated gene flow from one population to the other generation (*Nm*) was 1.8249, while the lowest was 0.9401 and the highest was 1.8438 between the populations of *P. zeylanica*. The highest percentage of polymorphism was 93.49 in the accession P_5 and the lowest percentage of polymorphism was 82.58 in the accession P_1 (Table 2).

The present study revealed a relatively high level of genetic diversity in *P. zeylanica* based

on ISSR markers. Using UPGMA algorithm a dendrogram was constructed to infer phylogenetic relationships between the five populations (Fig. 1). A Jaccard's matrix was used to produce a dendrogram based on SI, which showed distinct separation of the five accessions into two major groups having a similarity of 76%. Among the two clusters, the accessions belonging to the upper cluster were collected from P_1, P_2 and P_3 which had 80 % similarity, while accessions belonging to the lower cluster were from P_4 and P_5 and both the clusters showed 76 % similarity. The Lower cluster again divided into two sub clusters from P_4 and P_5 and both the sub clusters had 83 % similarity. The upper cluster was grouped into two major sub clusters collected from P_1, P_2 and P_3, which had a similarity of 80 %.

The genetic diversity of *P. zeylanica* collected from 226 populations (Debbe et al. 2009) using ISSR markers was done earlier. ISSR were used to estimate genetic diversity within and among 10 populations of *Rhodiola chrysanthemifolia* (Tao Xia, 2007). 116 discernible DNA fragments were generated of which 104 (89.7%) were polymorphic, indicating substantial genetic diversity at the species level. The percentage of polymorphic bands ranged from 21.97% to 48.8. They suggested that the main factor responsible for the high level of differentiation among populations is probably the historical, geographical and genetic isolation of populations in a harsh mountainous environment.

In the present study results were obtained using ISSR markers in *P. zeylanica*. 6 primers were used and produced band varying from 5 to 7 and a total of 35 polymorphic bands were observed. Overall genetic diversity or heterozygosity was 0.2194 and percentage of polymorphic loci was 54.29.

As pointed out by Wolff et al. (1994) and Apostol et al. (1996) large scale characterization of plant species in varying geoclimatic conditions can be performed using various parameters such as seed morphometric traits, molecular markers and isozymes, However, environmental factors as well as the developmental stage of the plants influence such traits. Various environmental factors play a very important role in genetic diversity of an organism.

Understanding of a level and partitioning genetic variability within the species would provide an important input to determine the appropriate management strategies (Rashmi et al. 2004). According to Jayaram and Prasad (2008) and Chengxin et al. (2003) the study of distributive patterns of genetic variation would provide baseline data for conservation and collection strategies of the species. Artyukova et al. (2004) reported that the mean genetic heterozygosity may be helpful in estimating gene diversity of the populations of rare and endangered species. The results suggest that ISSR markers are useful in distinguishing the populations of chosen medicinal plants according to geographical origin and confirm the importance of genetic studies for designing germplasm conservation strategies.

Conclusion

Analysis of ISSR data can be used to detect genetic differentiation of *P. zeylanica*. Thus in the present study the population which exhibited high percentage of polymorphism was considered to be the superior genotypes. Development of molecular technique makes it easy to analyze genetic characteristics of a specific species in varying environmental conditions The P_5 accession collected from Anchetty had the highest percentage of polymorphism. Such superior genotypes of these medicinal plants could be collected and conserved through *ex-situ* and *in-situ* conservation.

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