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ACKNOWLEDGEMENTS. We thank Prof. B. N. Goswami, Director, Indian Institute of Tropical Meteorology, Pune for encouragement and providing the necessary facilities. We also thank the NOAA website for providing tree ring parameters data and CRU for providing climatic data.

Received 22 October 2013; revised accepted 9 April 2014

Efficacy of two dominant marker systems, ISSR and TE-AFLP for assessment of genetic diversity in biodiesel species *Pongamia pinnata*

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The extent of genetic diversity was assessed in 12 *Pongamia* accessions from different regions of Delhi and surrounding areas using two dominant markers, namely ISSR and three endonuclease AFLP (TE-AFLP). Five ISSR primers and two TE-AFLP primer combinations generated a total of 12 and 48 polymorphic bands respectively. The Jaccard's dissimilarity coefficient ranged from 0 to 0.90 for ISSR and from 0 to 0.67 for TE-AFLP markers. The polymorphic information content of both markers was equal. However, TE-AFLP had much higher values of marker index and resolving power compared to those obtained for ISSR markers. This study demonstrates the usefulness of dominant markers like ISSR and TE-AFLP for assessment of genetic diversity in *Pongamia* for which microsatellites markers are still not available. However, high multiplex ratio, easy scorability and other high band attributes of TE-AFLP markers make them more suitable compared to ISSR for genetic diversity analysis.

Keywords: Dominant markers, genetic diversity, *Pongamia* accessions.

PONGAMIA pinnata L. Pierre (locally known as karanja), a member of the family Fabaceae, is a non-edible oil-producing tree which has been recognized as a major biodiesel species in India[1]. The species is indigenous to India and Southeast Asia, from where it has spread to other parts of the world. In urban areas it is a common avenue tree primarily grown for shade and aesthetic value due to its brilliantly coloured flowers and shiny leaves. Its seed oil content is about 32–42%, and can be converted into biodiesel which is at par with that of *Jatropha curcas*[2]. In the past few years *Pongamia* has attracted interest of several investors, including many from the United States as a biodiesel crop. However, availability of any improved and characterized planting stock has been the major bottleneck in harnessing the biofuel potential of this plant. A large proportion of trees do not flower at all and commercially attractive levels of fruiting are observed in only a small fraction of total trees[3]. There is a large phenotypic diversity in this species, thus providing an opportunity for genetic improvement[2]. More recently, initiatives have been taken towards identification of superior genotypes and their characterization.

Assessment of genetic diversity is a prerequisite for efficient conservation and utilization of genetic resources. During the past two decades, several high-throughput PCR-based technologies such as randomly amplified polymorphic DNA (RAPD), inter-simple sequence repeats (ISSR) and amplified fragment length polymorphisms (AFLP) have been developed to assay genetic polymorphism at the DNA level. Among these, RAPD[4], ISSR[5] and more recently, AFLP[6] have been increasingly used for detailed genetic analysis. All these technologies are accessible and they quickly provide large number of polymorphic markers with universal reagents and assay protocols without prior genetic information of the concerned species. However, due to their dominant behaviour, ISSR and AFLP markers have less information per locus than co-dominant markers. A number of studies have been conducted on *Pongamia* using dominant markers such as RAPD, ISSR, AFLP and TE-AFLP[3,7–10]. As there are not many microsatellite markers reported for *Pongamia*, the present study was aimed to assess the efficacy of two dominant markers in a set of 12 *Pongamia* accessions for analysis of genetic diversity.

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Table 1. *Pongamia pinnata* accessions used in this study

Accession ID	Location	GBH* (cm)	100 seed weight (g)	Oil content (%)
P001M	Central Delhi	82	115	37.8
P001/5	Progeny of P001M	NA	NA	NA
P003	Central Delhi	102	132	37.93
P019M	Central Delhi	69	115	33.53
P019/10	Progeny of P019M	NA	NA	NA
P026	Central Delhi	53	No fruiting	NA
P053	South Delhi	85	91	31.85
P077	South Delhi	37	113	30.6
P110	South Delhi	150	127	34.4
P132	Ghaziabad, Uttar Pradesh	62	No fruiting	NA
P175	South Delhi	72	114	33.77
P183	Ghaziabad, Uttar Pradesh	137	No fruiting	NA
Mean		81.60	115.29	34.27
SD		31.71	13.00	2.77
Range		37–150	91–132	30.6–37.93

*GBH, Girth at breast height.

Table 2. ISSR and TE-AFLP primer sequences used in this study

ISSR		TE-AFLP	
UBC812	5′GAGAGAGAGAGAGAGA-A3′	*Eco*RI primer	
UBC814	5′CTCTCTCTCTCTCTCTCT-A3′	E-AG	5′GACTGCGTACCAATTC-AG3′
UBC818	5′CACACACACACACACA-G3′	*Pst*I primer	
UBC848	5′CACACACACACACACA-R*G3′	P-G	5′GACTGCGTACATGCAG-G3′
UBC836	5′AGAAGAAGA AGAAGA-GY*A3′	P-C	5′GACTGCGTACATGCAG-C3′

*R, Purine; Y, Pyrimidine.

Pongamia accessions were collected from different locations in the National Capital Region (NCR) of Delhi and each tree was marked with Global Positioning System (GPS) for future reference. Data on 100 seed weight were taken by weighing randomly sampled 100 seeds from each accession (Table 1). Oil content was estimated by solvent extraction method using *n*-hexane as solvent in a Soxhlet apparatus (SOCS PLUS, Pelican Equipments, Chennai) following the protocol described by Kaushik *et al.*[2]. Total genomic DNA was extracted from lyophilized leaves following a CTAB-based procedure[3,11]. Initially, 20 UBC-ISSR and 6 TE-AFLP primers were screened for their amplification and degree of polymorphism and finally five ISSR and two TE AFLP primer combinations were chosen for genetic diversity analysis.

The typical PCR mix for ISSR contained 50 ng genomic DNA in 1x reaction buffer, 1.5 mM $MgCl_2$, 10 pmol primer and 1 U *Taq* DNA polymerase (Bio tools) in 20 µl reaction volume. PCR amplification conditions were as follows: 94°C for 5 min followed by 30 cycles of 94°C (30 sec)/42°C (30 sec)/72°C (60 sec) and a final extension at 72°C for 10 min. The amplification products were resolved on 1.5% agarose gels.

The protocol for TE-AFLP was based on van der Wurff *et al.*[12] with the modification that in the present study, *Eco*RI, *Pst*I and *Mse*I were used instead of *Bam*HI, *Xba*I, and *Rsa*I that were used in the original protocol. Genomic DNA (250 ng) was digested using 5 units of *Eco*RI and *Pst*I and 2.5 units of *Mse*I followed by enzyme inactivation at 70°C for 10 min. *Eco*RI and *Pst*I adapters were ligated to the digested DNA using 10 pmol of each adaptor and 1 unit of T4 DNA ligase at 20°C for 2 h. The ligation mix was diluted to 1 : 10 in TE buffer (10 mM Tris, 0.1 mM EDTA). Preamplification of ligation products was accomplished using *Eco*RI and *Pst*I adapter-specific primers without any selective nucleotide. This enables the use of the same preamplification library for selective amplification using *Eco*RI and *Pst*I primers with any selective nucleotide(s). Selective amplification was done using γ32P-ATP labelled *Eco*RI primers with two selective nucleotides in combination with unlabelled *Pst*I primers with one selective nucleotide. The primer sequences used for selective amplification are given in Table 2. The PCR profiles for selective amplification were: ten cycles of 30 sec at 94°C, 30 sec at 70°C, and 60 sec at 72°C followed by 30 cycles of 94°C for 30 sec, 60°C for 30 sec, and 72°C for 60 sec. A final extension of 5 min at 72°C was given to allow completion of elongation of the products. All PCR reactions were performed in a Gene Amp PCR 9700 Thermal Cycler. The samples were size fractionated on 6% polyacrylamide gels using Sequigen GT (Bio-Rad, Hercules, USA) under denaturing conditions. The fragments were detected by autoradiography.

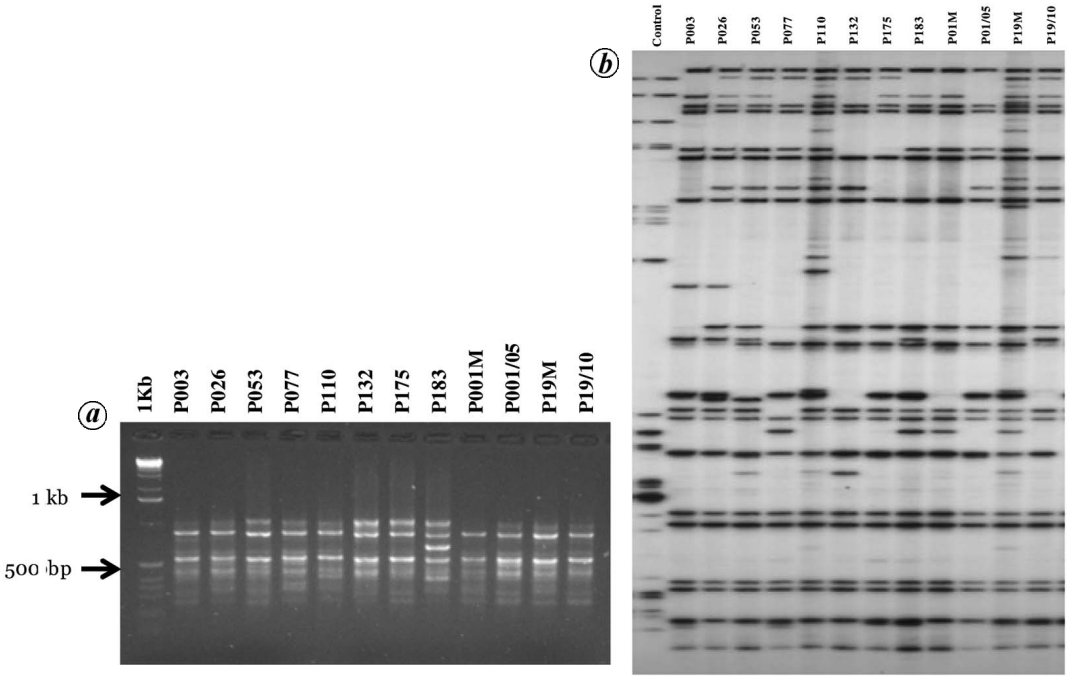

Figure 1. ***a***, Representative ISSR profile using primer UBC848. ***b***, Representative TE-AFLP profiles using primer combination E-AG × P-G of 12 *Pongamia pinnata* accessions.

The amplified fragments were scored manually for their presence (denoted as '1') or absence (denoted as '0') for each primer combination. The Jaccard's dissimilarity matrix was subjected to unweighted pair group method of arithmetic averages clustering in order to construct the phonetic dendrogram using DARwin software (version5.0.157)[13]. The reliability and robustness of the phenograms were tested by bootstrap analysis for 1000 bootstraps for computing probabilities in terms of percentage for each node of the tree using the DARWin software.

Genotyping data from ISSR and TE-AFLP were used for assessing the discriminatory power of the respective assays by evaluating three parameters, namely polymorphism information content (PIC), marker index (MI) and resolving power (RP).

Twelve *Pongamia* accessions were analysed using five ISSR primers and two TE-AFLP primer combinations which generated a total of 12 and 48 polymorphic bands respectively. The representative gel profiles obtained with the two methods are shown in Figure 1. In general, profiles obtained with TE-AFLP were clearer and had a larger number of bands than those obtained with ISSR.

The banding attributes obtained with both the methods are summarized in Table 3. A total of 95 bands were detected using two TE-AFLP primer combinations, whereas five ISSR primers detected only 23 bands. This shows that TE-AFLP has high multiplex ratio per primer. The number of polymorphic bands detected with ISSR and TE-AFLP was 12 (52.2%) and 48 (50.5%) respectively. The average per cent polymorphism and average PIC values for both the markers were almost same. However, all other banding attributes such as scorability, effective multiplex ratio (EMR), RP and MI were markedly higher in TE-AFLP than ISSR (Table 3).

The Jaccard's dissimilarity coefficient ranged from 0 to 0.90 for ISSR and from 0 to 0.67 for TE-AFLP. The dendrogram obtained using ISSR and TE-AFLP data is shown in Figure 2 *a* and *b* respectively. The overall topology of majority of accessions was similar in both dendrograms, with few exceptions. In ISSR dendrogram, the mother trees P001M and P019M clustered together with their respective progenies, but in TE-AFLP dendrogram the mother accession, P001M and its progeny grouped together in cluster I, while the mother accession P019M and its progeny grouped in cluster II. Three major clusters were formed with both the datasets (Figure 2). The four accessions namely P053, P077, P110 and P175 were grouped in the same cluster, i.e. cluster III in both ISSR and TE-AFLP dendrograms. On the contrary, accessions P003, P026, P132 and P183 are grouped in different clustering patterns in both marker systems. Bootstrap values obtained with TE-AFLP data were marginally better than those obtained with ISSR data (Figure 2 *a* and *b*).

In recent years, studies have been conducted to assess molecular diversity in *P. pinnata* using molecular markers such as RAPD[8], ISSR[7,8], AFLP[3,8–10] and TE-AFLP[3]. Both TE-AFLP and AFLP indicated a high level of genetic diversity of *P. pinnata* collected from different locations of NCR, Delhi[3] while ISSR indicated narrow

Table 3. Comparative band attributes of ISSR and TE-AFLP in *P. pinnata*

Marker system	ISSR					TE-AFLP	
Primer combination	UBC812	UBC814	UBC818	UBC848	UBC836	E-AG × P-C	E-AG × P-G
Total bands	4	3	6	7	3	56	39
Polymorphic bands	2	2	2	5	1	30	18
Polymorphism (%)	50	66.7	33.3	71.4	33.3	53.6	46.2
PIC	0.47	0.38	0.22	0.29	0.28	0.33	0.34
Marker index	0.94	0.75	0.43	1.47	0.28	9.78	6.1
Resolving power	2.33	2	0.5	4.33	1.67	14	9.5
Total bands	23					95	
Polymorphic bands	12					48	
Average polymorphism (%)	52.2					50.5	
EMR	2.4					24	
PIC	0.33					0.33	
MI	0.78					7.94	
RP	2.16					11.75	

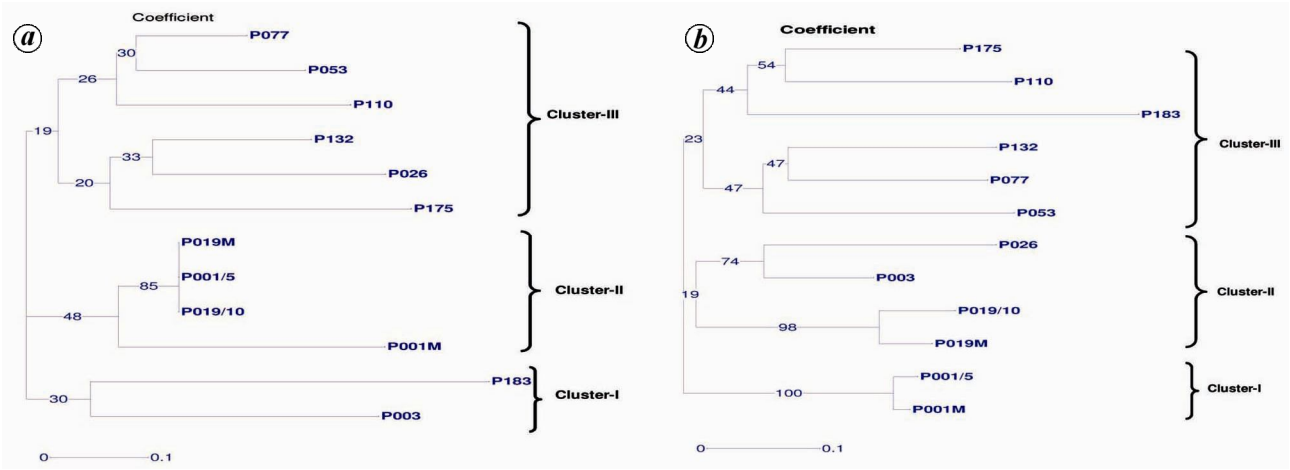

Figure 2. ***a***, Dendrogram showing neighbour joining clustering of accessions using ISSR markers data. ***b***, Dendrogram showing neighbour joining clustering of accessions using TE-AFLP markers data.

genetic diversity within the trees from several regions of Odisha[7]. AFLP detected higher levels of genetic diversity (100%) in natural populations of *P. pinnata*, whereas RAPD and ISSR showed lesser genetic diversity (approx 10%)[8].

Due to their high genetic diversity, the accessions from NCR of Delhi provide greater scope for selection of candidate plus trees with respect to functional diversity for oil, seed yield and biofuel properties. It is, therefore, important to characterize accessions from this region for genetic diversity.

The high level of genetic diversity observed in this study within the limited number of accessions with both the marker systems is consistent with the fact that the plants used in the study were selected from diverse locations. However, high multiplexing ratio, easy scorability and other high band attributes of TE-AFLP markers make them more suitable over ISSR for genetic diversity analysis. The grouping of P001 and P019 with their respective progenies by TE-AFLP indicates their higher accuracy of characterization. Thus, both the methods were found to be efficient in distinguishing the genotypes and elucidating their genetic relatedness. However, TE-AFLP may be preferred over ISSR when a large number of accessions need to be genotyped with greater accuracy.

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ACKNOWLEDGEMENTS. We thank the Department of Science and Technology, New Delhi for providing funds. S.S.S. thanks the Council of Scientific and Industrial Research, New Delhi for providing fellowship.

Received 12 November 2013; revised accepted 16 April 2014