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POTENTIAL RAPD MARKERS FOR POPULATION STUDIES IN TREE LEGUMES

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Abstract

RAPDs were quite efficient in bringing out the diversity at DNA level among non-edible legumes viz., *Acacia nilotica, Adenanthera pavonina, Prosopis juliflora, Pithecolobium dulce, Clitoria ternatea* and *Pongamia pinnata*. The RAPD primer index reveals the information content of the RAPD primer *per se*. Of the 82 primers tested, OPE 8, OPI 6, OPL 2, OPL 16, OPI 18, OPI 13, OPI 14, OPP 1, OPE 20 and OPI 4 with comparatively higher primer index were more informative and can be used for further DNA finger printing and population studies in tree legumes. CTAB protocol was found to be superior in isolating genomic DNA of good quality. The 260/280 ratios varied between 1.70 and 2.09. Though the genomic DNA isolated by potassium acetate method was found to be intact in 0.8% agarose gel, the yield was significantly lower than the modified CTAB method.

Introduction

Tree legumes play a vital role in many agro forestry systems by providing high quality fodder, nutrient rich mulch, fuel wood, timber and ecosystem stability through microenvironment amelioration (Gutteridge & Shelton, 1993). Assessment of genetic diversity using DNA based molecular markers is one of the key aspects in crop improvement and germplasm conservation (Cooke, 1995). AFLP, RFLP, RAPD and SSR are the commonly used molecular markers in genetic diversity analysis. AFLP and RFLP employ complicated methodologies besides the need for large quantity of DNA (Kochert, 1994; Vos *et al.*, 1995). Among the PCR based molecular markers, RAPD offers several advantages such as higher frequency of polymorphism, technical simplicity, rapidity, requirement of few nanograms of DNA (1000 times less than is needed for RFLP reactions) and unlike SSR, RAPD does not require prior sequence information (Grattapaglia *et al.,* 1992). Therefore RAPD was used in the DNA finger printing of tree legumes for which, no sequence information are available.

A prerequisite for exploiting the advantage of the molecular marker technology relies on the ability to extract genomic DNA of good quality suitable for polymerase chain reaction (Chakraborti *et al*., 2006). Extraction of good quality DNA from tree species is more difficult due to co-precipitation of impurities such as terpenes, polyphenolics (Aganga & Tshnwane, 2003) and highly viscous polysaccharides, which are often abundant in the foliage of perennials (Do & Adams, 1991; Shepherd *et al*., 2002). As a consequence, the DNA becomes unsuitable for PCR and many tree species require more complex extraction methods than do annual plants (Scott & Playford, 1996). Hence, a simple and efficient protocol is highly solicited for RAPD based genetic diversity analysis in tree legumes. The present study was aimed to develop a protocol for extraction of DNA from *Acacia nilotica, Adenanthera pavonina, Prosopis juliflora, Pithecolobium dulce, Pongamia pinnata* and *Clitoria ternatea*, and also to identify potential polymorphic RAPD primers for further inter and intra population diversity studies.

Materials and Methods

DNA Isolation: Genomic DNA was isolated from 5 tree legumes viz., *A.nilotica, A.pavonina, P.juliflora, P.dulce, P.pinnata* and a herb *C.ternatea* by adopting CTAB procedure (Doyle & Doyle, 1987) with necessary modifications. Tender leaves (2.0g) were ground to fine powder in liquid nitrogen and the nucleic acids were extracted using 10 ml of preheated (65°C) CTAB buffer (100mM trizma® base-pH 8.0; 20mM EDTA-pH 8.0; 1.4M NaCl) containing 1.0% poly vinyl pyrrolidone10000 and 0.2% 2-mercapto ethanol under incubation at 65°C for an hr with occasional mixing. Equal volume of chloroform: iso amyl alcohol (24:1) was added to the samples, mixed gently for 10min., on gel rocker and centrifuged at 3000 rpm for 20 min., (Eppendorf Centrifuge 5810R). The upper aqueous phase was collected and the DNA was precipitated by adding equal volume of isopropanol (stored at -20°C). The DNA spool was hooked out and washed twice with 70% ethanol to remove the traces of CTAB and chloroform. The pellet was dried in laminar flow and resuspended in 500µl of TE (10mM tris and 1mM EDTA) buffer.

DNA Purification: The RNA contamination was eliminated by treating the samples with 5µl of RNaseA (10000 ppm) at 37°C for 1hr. The samples were then incubated at 60°C for an hr after treating with 5µl of proteinase K (20000 ppm), 50µl of 3M Sodium acetate (pH 5.2) and 5µl of SDS (25%). Saturated phenol (500µl) was added and the upper phase was collected carefully without disturbing the middle layer after centrifugation at 10000 rpm for 20 min., (Heraeus Sepatech, Biofuge 15R). The DNA was further purified using chloroform: isoamyl alcohol (24:1) then precipitated by two volumes of 95% chilled ethanol. The DNA pellet was washed twice with 70% ethanol, dried and resuspended in 150µl of TE buffer.

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DNA Quantification: The isolated DNA was quantified using NanoDrop1000® and also in DyNA Quant-200 Fluorimeter (Hoefer, Pharmacia Biotech, USA) with Calf Thymus DNA as standard. The crude DNA was diluted using sterile water in to a final concentration of 30ng μl^{-1}. The quality of the DNA was checked by 260/280 ratio using NanoDrop1000® and further confirmed in 0.8% w/v agarose (Sigma Aldrich Chemicals, Bangalore) gel electrophoresis.

PCR amplification: PCR was performed with 82 RAPD primers in 15 µl reaction volume containing 60 ng genomic DNA, 0.2 µ*M* primer (Sigma-Aldrich, India), 0.2 m*M* each of dNTPs, (Fermentas Life Sciences, Canada), 2 m*M* $MgCl_2$, 1× *Taq* buffer (GeNei, India) and 0.5U *Taq* DNA polymerase (GeNei, India). The amplification was carried out in PTC-100™ thermal cycler (MJ Research, USA) programmed for 44 cycles of denaturation at 94ºC for 1 min, annealing at 37°C for 1 min and extension at 72°C for 1 min preceded and followed by initial denaturation at 94°C for 4 min., and final extension at 72°C for 5 min. Amplified products were electrophoresed in 2% w/v agarose gels (GeNei, India) stained with Ethidium bromide. The reproducibility of the DNA profiles was tested by repeating the PCR amplifications twice with each primer.

Diversity analysis of PCR amplified products: Clearly resolved, unambiguous RAPD markers were scored as presence (1) or absence (0), and the data was obtained in the form of a rectangular matrix. The data matrix was then used to deduce genetic similarity index (Nei & Li, 1979) using NTSYS version 2.0 (Rohlf, 1990). Cluster analysis was carried out based on genetic distance using UPGMA (unweighted pair group method using arithmetic averages) (Sneath & Sokal, 1973). To characterize the capacity of each primer to detect polymorphic loci Polymorphism Information Content (PIC) was calculated as PIC = 1- p^2 - q^2, where p is band frequency and q is no band frequency (Ghislain *et al*., 1999). The higher the PIC value, the more informative RAPD marker is. The PIC values for the RAPD markers generated by the same primer were cumulated and named as RAPD Primer Index (RPI) (Raina *et al.,* 2001).

Results and Discussion

Chemotypic heterogeneity among species may not allow optimal DNA yield with a single isolation protocol. Thus, even closely related species may require different DNA extraction protocols (Loomis, 1974; Weishing *et al*., 1995). However, enough quantity of good quality genomic DNA was consistently obtained with the protocol described in this paper. The quantity of DNA obtained by adopting the modified CTAB procedure was 694, 1142, 1503, 1907, 2776 and 2378 ng μl^{-1} for *A. nilotica, A. pavonina, P. juliflora, P. dulce, P. pinnata* and *C. ternatea* respectively as against 200, 240, 160, 1110, 735 and 730 ng μl^{-1} by employing the potassium acetate method (Dellaporta *et al*., 1983). The quality of the DNA was also found to be superior as the 260/280 ratio varied between 1.70 and 2.09. Though the genomic DNA isolated by potassium acetate method was found to be intact in 0.8% agarose gel, the yield was significantly lower than the modified CTAB method (Table 1).

Table 1. Quantity and quality of DNA obtained by adopting modified CTAB and potassium acetate method

S. No.	Legume species	DNA Yield µg g^{-1} of leaf tissue	
		CTAB (260/280)	Potassium acetate method
1.	*Acacia nilotica*	52.10 (2.05)	15.00
2.	*Adenanthera pavonina*	85.65 (1.70)	18.00
3.	*Prosopis juliflora*	112.73 (1.74)	12.00
4.	*Pithecolobium dulce*	143.03 (2.09)	83.25
5.	*Pongamia pinnata*	208.20 (2.00)	55.13
6.	*Clitoria ternatea*	178.35 (2.06)	54.75

All the 82 RAPD primers employed in the present investigation exhibited 100% polymorphism with an average of 13 marker loci per primer. It indicates the ability of RAPD to reveal genetic diversity among the tree legumes investigated. Number of RAPD loci generated was the highest for OPE 8 (25) and the lowest for OPM 7 and OPM 8 (7 each) (Table 2). Many investigators have generated reliable and consistent banding patterns with RAPDs (Chalmers *et al*., 1992; Waugh & Powell, 1992; Lerceteau *et al*., 1997; Clerc *et al*., 1998), though the reproducibility of RAPD markers was questioned in several studies (Weeden *et al.,* 1992; Jones *et al.,* 1997).

Similarity indices derived from the polymorphic data revealed the extent of relatedness among the tree legumes. Comparatively higher similarity was observed between *P. pinnata* and *A. pavonina* whereas, *C. ternatea* and *A. nilotica* were only 50.15% similar at DNA level (Table 3). The legume species investigated were grouped into two major clusters. *A. nilotica* alone formed a separate cluster and all other species clustered together in another major cluster (Fig. 1). In the present investigation, the genetic distance among the tree legumes was considerably higher and the RAPDs were quite efficient in bringing out this diversity at DNA level. One of the immediate uses of this study is to identify the primers, which are likely to be efficient in revealing genetic diversity in tree legumes. Short-listing such primers will be highly useful for further molecular analysis of vast tree legume germplasms. The RAPD primer index reveals the information content of the RAPD primer *per se*. Of the 82 primers, OPE 8 (8.50), OPI 6 (7.67), OPL 2 (7.11), OPL 16 (7.44), OPI 18 (6.61), OPI 13 (6.50), OPI 14 (6.44), OPP 1 (6.44), OPE 20 (6.22) and OPI 4 (6.00) with comparatively higher primer index (Table 2) were more informative and can be used for further DNA finger printing and population studies in tree legumes.

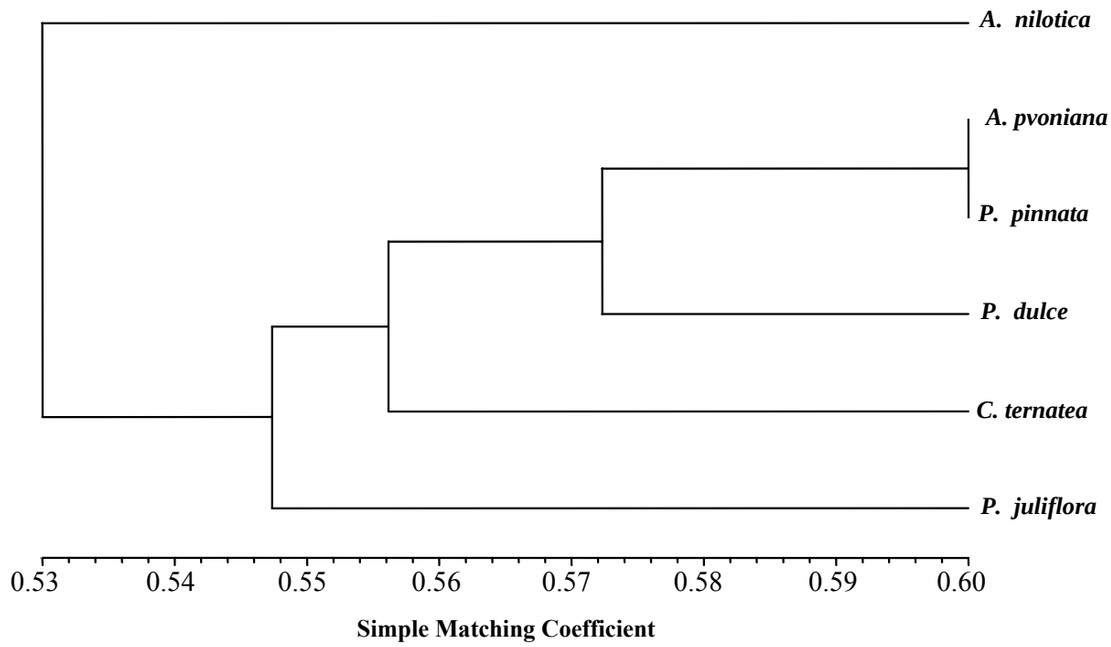

Fig. 1. Dendrogram showing the genetic relatedness among the tree legumes.

Table 2. Details of markers generated among the legume species and RAPD Primer Index

S. No.	Random Primer	Polymorphic markers	RPI	S. No.	Random primer	Polymorphic markers	RPI
1.	OPE7	10	3.17	42	OPP3	14	5.06
2.	OPE8	25	8.50	43	OPP6	15	4.67
3.	OPE9	14	5.11	44	OPP7	14	4.28
4.	OPE12	13	4.61	45	OPP8	12	4.56
5.	OPE13	13	4.94	46	OPN2	13	4.56
6.	OPE14	8	3.22	47	OPN3	10	3.11
7.	OPE15	10	3.94	48	OPN4	10	3.44
8.	OPE16	13	3.94	49	OPN5	11	4.06
9.	OPE19	13	4.50	50	OPN6	11	3.94
10.	OPE20	17	6.22	51	OPN7	17	5.28
11.	OPI1	14	5.39	52	OPN8	13	4.28
12.	OPI2	13	4.33	53	OPN9	12	4.17
13.	OPI4	16	6.00	54	OPN10	13	4.33
14.	OPI5	14	4.94	55	OPN11	8	3.17
15.	OPI6	20	7.67	56	OPN12	9	2.89
16.	OPI7	16	5.67	57	OPN13	15	4.83
17.	OPI9	13	4.67	58	OPN14	15	4.67
18.	OPI11	9	2.56	59	OPN15	12	4.11
19.	OPI12	9	3.83	60	OPN16	10	3.44
20.	OPI13	17	6.50	61	OPN17	11	3.56
21.	OPI14	18	6.44	62	OPN19	9	3.00
22.	OPI15	13	4.11	63	OPN20	11	3.56

Table 2. (Cont'd.).

S. No.	Random Primer	Polymorphic markers	RPI	S. No.	Random primer	Polymorphic markers	RPI
23.	OPI16	14	4.72	64	OPM7	7	2.44
24.	OPI18	16	6.61	65	OPM8	7	1.94
25.	OPI19	13	4.17	66	OPM9	11	3.72
26.	OPL1	14	4.78	67	OPM11	13	3.78
27.	OPL2	19	7.11	68	OPM12	13	5.17
28.	OPL3	12	4.61	69	OPM13	15	5.00
29.	OPL4	13	5.06	70	OPM14	14	4.39
30.	OPL6	11	3.78	71	OPM15	11	4.00
31.	OPL7	10	3.83	72	OPM16	12	4.00
32.	OPL9	8	2.39	73	OPM17	14	4.44
33.	OPL12	15	5.67	74	OPK3	9	2.83
34.	OPL10	14	4.61	75	OPK4	15	4.56
35.	OPL11	15	5.61	76	OPK6	11	3.89
36.	OPL13	14	5.28	77	OPK9	15	4.78
37.	OPL16	19	7.44	78	OPK13	15	5.00
38.	OPL17	13	4.89	79	OPK14	13	4.61
39.	OPL18	15	5.94	80	OPK17	9	3.50
40.	OPP1	17	6.44	81	OPA3	10	3.50
41.	OPP2	12	4.89	82	OPA15	14	4.61

Table 3. Similarity index values for the tree legumes generated by simple matching coefficient

	A.nilotica	***A.pavonina***	***C.ternatia***	***P.juliflora***	***P.dulce***	***P.pinnata***
A. nilotica	1.0000					
A. pavonina	0.5476	1.0000				
C. ternatia	0.5015	0.5711	1.0000			
P. juliflora	0.5308	0.5560	0.5454	1.0000		
P. dulce	0.5453	0.5684	0.5380	0.5534	1.0000	
P. pinnata	0.5392	0.5987	0.5633	0.5422	0.5770	1.0000

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