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Standardization of DNA Extraction and Optimization of RAPD-PCR Conditions in *Garcinia indica*

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Abstract: Genetic analysis of plant relies on high yields of pure DNA samples. DNA isolation is difficult in woody plants because of presence of polysaccharides, tannins, alkaloids, polyphenols and other secondary metabolites that interfere during isolation. Here we report for the first time a fast, reliable and less expensive method of genomic DNA isolation from leaves of *Garcinia indica*. The modified CTAB protocol includes addition of Polyvinylpyrrolidone (PVP) separately in each tube and precipitation with 5M NaCl along with chilled ethanol, which increased the solubility of polysaccharides. Without use of RNase or Proteinase K, were able to isolate pure and sufficient amount of DNA, which proved to be amenable to RAPD analysis. We also optimized RAPD-PCR conditions such as annealing temp, amount of DNA and Taq polymerase etc. A preliminary study of variation within *G. indica* species was carried out with nine plants with twenty decamers. Out of which six primers showed polymorphism while three had monomorphic banding pattern. In continuation with these promising results, efforts are underway to screen more plants with different geographical locations and with more number of primers to study genetic diversity.

Key words: Endemic plant, morphological variation, DNA isolation, polyphenols and secondary metabolites, RAPD conditions

INTRODUCTION

Garcinia indica (family- Clusiaceae) is a tree endemic to tropical rain forests of Western Ghats of India. Fruits are rich source of Hydroxycitric Acid, an important biologically active plant metabolite used as an anti-obesity and anti-cholesterol drug (Sullivan *et al*., 1977). Fat extracted from the seeds is used in cosmetics as emollient. Plants are cross-pollinated in nature and are polygamodioecious. Generally plants are propagated through seeds. The seed raised progeny exhibit lot of variations in fruit shape and size, thickness of fruit rind, leaf morphology and flowering pattern. Generally a tree bears drooping branches, which gives slender or conical shapes to the tree. But branching habits like erect or horizontal were also noticed in some plants. The trees with horizontal branches occupy larger canopy and gives typical conical or dome shaped appearance to plant. The leaf shape of these plants also varies from oblong elliptical to ovate (Sahasrabudhe and Deodhar, 2009). Variation is also found in shape of fruits i.e., round with beak, round flat, apple shaped, pear shaped etc. Extent of genetic diversity within the species limits the production of elite stock for improving the quality and productivity. Same genotypes when raised under different ecoclimatic conditions give rise to many significantly different phenotypic features conversely many genotypes; phenotypically look alike although they are endowed by unique genetic features. Molecular markers provide the best estimates of genetic diversity that are independent of effect of various environmental factors. RAPD is the most widely used technique as it requires low quantities of DNA, it is a fast and easy assay and it does not require any sequence data for primer design (Ercisli *et al*., 2007; Koc *et al*., 2009). RAPD technique is successfully used in determining genetic diversity in various plants from Western Ghats such as *Dendrocalamus strictus and Bambusa bambos* (Biradar *et al*., 2005) ground parrot (Chan *et al*., 2008) and *Humboldtia brunosis* (Borges *et al*., 2010) along with *Urginea indica* (Sathyanarayana *et al*., 2008), *Gmelina arborea* (Shankar *et al*., 2009), Sweet Sorghum (Kacahapur *et al*., 2009), *Allium stracheyi* (Ranjan *et al*., 2010). But up till now no attempts have been made to apply the molecular techniques to screen the intraspecific variability in *G. indica*. There are reports with other species of

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Garcinia viz., *G. cambogia*, *G. mangostana* (Sando *et al*., 2004). *G. indica* being a tree species is a difficult plant for DNA isolation due to its high polyphenolic content that may interfere with the quantity and quality of DNA. We report here the modified CTAB protocol derived from the method that was originally developed for other plants (Doyle and Doyle, 1987; Lodhi *et al*., 1994; Sathyanarayana *et al*., 2008). Modifications were made to yield sufficient and pure DNA (Staden *et al*., 2008). The protocol optimizes the various conditions for RAPD such as annealing temperature, DNA concentration and the amount of Taq polymerase (Padmalatha and Prasad, 2006).

MATERIALS AND METHODS

Plant material: This study was carried out from March 2007 to Dec. 2008. Young leaves of *G. indica* were collected just prior to extraction from V.G.Vaze college campus and medicinal and aromatic plant Garden of S.H. Kelkar Company.

Reagents and chemicals: Extraction Buffer contains 20 mM EDTA (disodium) pH 8, 100 mM Tris (adjust the pH to 8 using concentrated HCl), 1.4 M NaCl, 2% CTAB, 0.2% β-mercaptoethanol (add just before the use).

- 5 M NaCl
- Chloroform: isoamylalchohol (24:1)
- Polyvinylpyrrolidone (PVP) (MW 40,000)
- Chilled ethanol
- 0.1X TE buffer

DNA isolation: DNA was extracted by CTAB method (Lodhi *et al*., 1994) with few modifications, (1) Young pale green leaves of *G.indica* were collected just before the extraction. (2) Three grams of leaf sample was grounded in liquid nitrogen in pre- chilled mortar and pestle. (3) To this 9 mL of extraction buffer was added and the tissue was homogenized thoroughly. (4) The reaction mixture was transferred to the test tubes and to each tube 50 mg of PVP was added separately and incubated in water bath at 60°C for 1 h with in between shaking. (5) All the tubes were allowed to cool. (6) Equal amount of chloroform: isoamylalcohol (24:1) was added and mixed well by inversion. Mixture was centrifuged at 10,000 rpm for 25 min. (7) Aqueous layer was collected and equal amount of chloroform: isoamylalcohol (24:1) was added and mixed well slowly. (8) Mixture was centrifuged at 10,000 rpm for 15 min at room temperature. (9) Aqueous layer was collected and the above two steps were repeated twice. (10) Supernatant was collected in a clean glass tube and 0.5 volumes of 5 M NaCl was added, followed by 2 volumes of chilled ethanol. (11) DNA was allowed to precipitate as threads. (12) DNA was hooked out in a microcentrifuge tube and 1.5 mL of 80% ethanol was added. (13) Spin the tubes at 5000 rpm for 5 min at 4°C. Supernatant was thrown and the pellet was allowed to dry overnight. (14) Next day pellet was dissolved completely in 0.1X TE buffer and stored at -20°C until used.

Quantification and purity of DNA: Quantification of genomic DNA was done by visualizing DNA on 0.8% Agarose gel in 1X TAE buffer containing Ethidium bromide (10 μg mL^{-1}) in a range of 1, 2, 3, 4 and 5 μL in separate lanes. The concentration of extracted DNA was also estimated by visual comparison of the band with 100 bp marker DNA. The purity and concentration of the extracted DNA was also checked by measuring absorbance on Varian Cary 50 UV spectrophotometer at 260 and 280 nm. Purity was analyzed by absorbance ratios i.e., 260/280 nm.

PCR amplification: PCR amplification was carried out with random decamer primers obtained from Operon technologies Inc., CA, USA. Amplification was performed in a 25 μL reaction volume and contained 40 ng of DNA template, 2.5 μL 1X assay buffer, 1 μL of dNTPs mixture, 2 μL of primer and 0.4 U of Taq DNA polymerase containing 20 mM Tris HCl (pH 8.0), 100 mM KCl, 0.1 M EDTA, 1 M DTT, 0.5% Tween-20 and 50% glycerol. Taq polymerase dNTPs and 1X assay buffer were purchased from Bangalore Genei, India. Reaction mixture was overlaid with 15 μL of Mineral oil to prevent evaporation. PCR reactions were maintained for initial denaturation at 94°C for 4 min, followed by 40 cycles of 1 min at 94°C (denaturation), 1 min at 50°C (annealing) and 2 min at 72°C (extension). The final extension for 7 min at 72°C and hold temperature was maintained at 8°C. After amplification, PCR products were stored at 4°C till electrophoresis. PCR products were mixed with 2.5 μL of 10X loading dye (0.25% bromophenol blue, 0.25% xylene cyanol and 40% sucrose, w/v) and electrophoresis was carried on 1.6% Agarose gel containing ethidium bromide (10 μg mL^{-1}) in 1X TAE buffer at 100 v for 2 h. A 100 bp DNA ladder was used as a standard molecular weight marker.

RESULTS AND DISCUSSION

DNA extraction for woody plants is difficult due to the presence of contaminants such as polyphenols, polysaccharides and other secondary metabolites. The problems encountered include degradation of DNA due to endonucleases, co-isolation of highly viscous polysaccharides. Polyphenols, which are powerful

oxidizing agents, can reduce the yield and purity of extracted DNA (Khanuja *et al*., 1999; Puchooa, 2004). Experiments with CTAB method of extraction yielded very less DNA. Hence we made few modifications in extraction procedure. (1) Proper choice of leaf material was very important for the DNA extraction. *G. indica* is characterized by red pigments in young leaves. Such leaves yielded very poor DNA. Fully expanded young leaves devoid of red colorization were selected for DNA extraction. Yield was also poor when mature leaves were used for the extraction. (2) We made few modifications in the extraction buffer, NaCl concentration was changed from 2 to 1.4 M. Use of 0.2% β-mercaptoethanol instead of 2%. The key step of our protocol was addition of PVP (50 mg $tube^{-1}$) separately at the time of extraction had a considerable effect on neutralization of polyphenols and prevented oxidation of secondary metabolites Incubation period was also standardized. For that purpose we tried 30 min 1 h, 90 min and 2 h. It was found that incubation at 60°C for 1 h gave good yield of DNA. After addition of chloroform: isoamylalcohol (24:1), we standardized the centrifugation period. Centrifugation at 10,000 rpm for 25 min was required to separate the aqueous layer. This process was repeated two times. The purity of genomic DNA was dependent on the number of washes. Three times washes combined with a short-run centrifugation was sufficient for DNA purification and removal of endogenous nucleases or other proteins. (3) As CTAB is soluble in ethanol, residual amounts are removed in the subsequent wash. Use of chilled ethanol for DNA precipitation improved the yield. The addition of 5M NaCl and ethanol helped in precipitation of DNA. It is also reported that the addition of high concentration of NaCl increased the solubility of polysaccharides in ethanol, effectively decreasing co-precipitation of the polysaccharides and DNA (Fang *et al*., 1992). We could isolate good quality of DNA without any RNA and protein contamination (depicted with arrow in Fig. 1). The freer the DNA is from contaminants, the easier it is to resuspend the pellet.

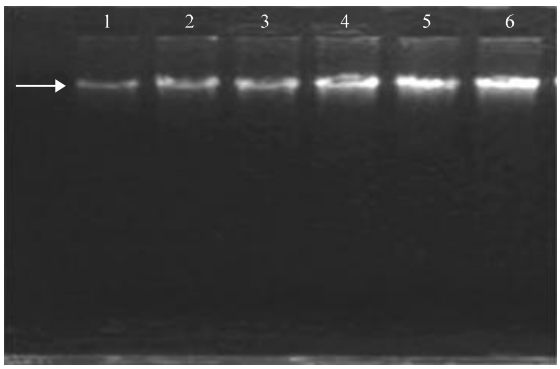

Fig. 1: Genomic DNA of *G.indica* genotypes resolved on 0.8% agarose gel

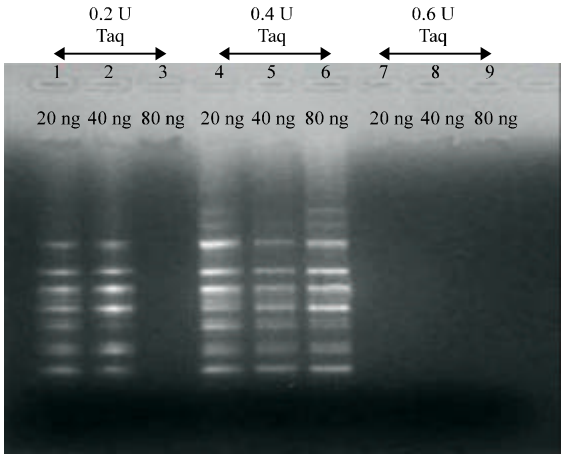

Fig. 2: Effect of variation in amount of Taq polymerase and DNA template on PCR amplification

Table 1: Optimization of PCR parameters

PCR parameter	Tested range	Optimum concentration
DNA concentration	20, 40 and 80 ng	40 ng
Taq polymerase	0.2, 0.4 and 0.6 U	0.4 U
Annealing temperature	35, 40, 45 and 50°C	50°C

RAPD analysis: To get consistent, reproducible and sharp amplicons, we optimized the RAPD reaction parameters such as amount of DNA, amount of Taq polymerase and annealing temperatures which are depicted in Table 1. Figure 2 depicts the effect of various concentrations of Taq polymerase and amount of DNA template on PCR amplification. In Fig. 2, lane 1 to 3 depicts 0.2 U Taq polymerase along with 20, 40 and 80 ng of DNA. Lane 4 to 6 uses 0.4 U Taq polymerase with 20, 40 and 80 ng of DNA. Lane 7 to 9 uses 0.6 U Taq polymerase with 20, 40 and 80 ng of DNA. When 0.2 U Taq polymerase was used, amplification was observed in case of 20 and 40 ng of DNA but bands were sheared. At 0.2 U Taq and 80 ng of DNA, there was no amplification (Fig. 2, lane 1 to 3). When 0.4 U Taq was used good amplification was observed at all DNA concentrations viz., 20, 40 and 80 ng of DNA (Fig. 2, lane 4, 5 and 6), but repeated results were achieved with 40 ng of DNA template. In case of 0.6 U Taq polymerase, all the three DNA concentrations i.e. 20, 40 and 80 ng, no amplification was observed (Fig. 2, lane 7, 8 and 9). Hence for further experiments 40 ng of DNA and 0.4 U Taq polymerase was used. Similarly various annealing temperatures checked for RAPD analysis were 36, 40, 45 and 50°C (Fig. 3). At 40 and 36°C no

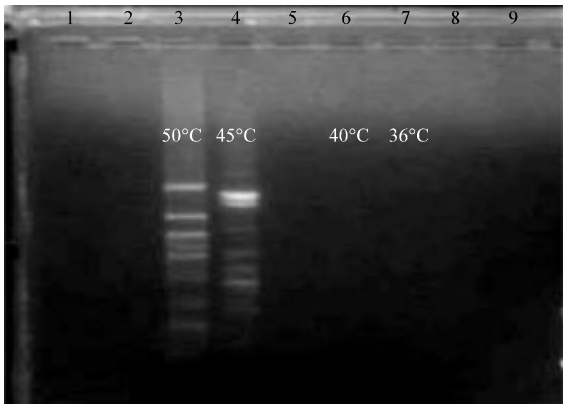

Fig. 3: Represents RAPD-PCR pattern obtained at various annealing temperatures

amplification was achieved (Fig. 3, lane 6 and 7). Amplification was achieved at 50 and 45°C (Fig. 3, lane 3 and 4) but the number of sharp and reproducible bands was more at 50°C (Fig. 3, lane 3) so 50°C was chosen. In general, RAPD reactions are carried out at low annealing temperature 34 to 38°C. But there are reports where temperature as high as 50°C is used (Fernandez, 2002). For *G. mangostana* annealing temperature required was 41°C (Sompong, 2004).

To make the preliminary study of genetic diversity within *G. indica* species, 9 plants were randomly chosen from the premises of Kelkar College and medicinal and aromatic plant garden of S.H.Kelkar Company, Mulund, Mumbai. Primers used for RAPD analysis were from Operon Technologies (KIT D). In all we screened 20 primers. Out o f 20 primers used for initial screening, 8 primers did not give any amplification. Remaining 12 primers were used for further analysis. The oligonucleotide sequences of selected primers and number of polymorphic bands produced by each primer were given in the Table 2.Out of selected 12 primers only 6 primers produced polymorphic bands. Total numbers of polymorphic bands were 28. The size of amplicons was in the range of 1200 to 250 bp. The highest number of polymorphic bands (11) was obtained with OPD-13 while the lowest number (2) was obtained with primer OPD-05. Different primers showed variation in their ability to detect polymorphism. The percentage polymorphism was in the range of 13 to 37.5%. Out of 12 RAPD primers used for present analysis primers OPD-05, OPD-08, OPD-18 and OPD-20 showed more or less similar banding pattern. Among this OPD-18 gave almost similar banding pattern in all genotypes. In Fig. 4a, (M- Molecular marker, Lane 1: G1, Lane 2: G2, Lane 3: G3, Lane 4: G4, Lane 5: G5, Lane 6: G6, Lane 7: G7, Lane 8: G8 and Lane 9: G9). This primer had

Table 2: Nucleotide sequences that detected polymorphism and the number of polymorphic bands generated by each primer

Primer	Sequence	Polymorphic bands
OPD-02	GGACCCAACC	4
OPD-05	TGATCGGACA	2
OPD-07	TTGGCACGGG	4
OPD-08	GTGTGCCCCA	3
OPD-11	AGCGCCATTG	4
OPD-13	GGGGTGACGA	11

produced amplicons of very low molecular weight ranging from 600 to 250 bp. Genotype G9 failed to amplify with this primer. This primer showed monomorphic banding pattern. Figure 4b shows agarose gel electrophoresis of 9 genotypes obtained using OPD-11. With this primer no amplification was obtained for genotypes 8 and 9. This primer showed more or less similar banding pattern with one or two polymorphic bands. The amplified products were in the range of 1100 and 400 bp. A common band of 800 bp is present in all genotypes. Genotypes G1 (Lane 1), G5 (Lane 5), G6 (Lane 6) and G7 (Lane 7) have showed a band of 1000 bp which is absent in rest of the genotypes. Genotype G4 just above 1000 bp showed a unique band just which is absent in all other genotypes (depicted with arrow). Figure 4c represents amplification pattern of primer OPD-13. Among all the tested primers, this produced a unique banding pattern among all nine genotypes. Amplicons were in the range of 1000 to 300 bp. This primer had produced eleven polymorphic bands (depicted with arrow). Genotypes G1 (Lane 1), G6 (Lane 6) and G9 (Lane 9) showed almost similar banding pattern. Genotype G4 (Lane 4) produced only two bands. Genotypes G7 (Lane 7) and G8 (Lane 8) though produced more or less similar banding pattern with other genotypes but failed to produce amplicons of lower molecular weight. Among the 12 primers, selected only primer OPD-12 and OPD-13 gave amplification to genotype G9 (Fig. 4c). The G9 has ovate leaves instead of normal elliptical leaves and horizontal branching pattern. Such plants are observed more in number in Sindhudurga region. The remaining primers did not produce any amplification for this genotype. RAPD had been used to study genetic diversity within *Jatropha curcas*. Selected 10 decamers produced 60% polymorphism proving that germplasm had been collected from different locations (Subryamanyam *et al*., 2010). In case of *Azima tetracantha*, RAPD markers revealed wide variation reflecting high amount of genetic diversity (Hepsibha *et al*., 2010). Erturk *et al*. (2009) reported successful use of RAPD markers to detect polymorphism and genetic relationship among *Prunus spinosa*. Similar type of work was carried out to study phylogenetic relationship in endangered plant *Allium stracheyi* (Lodhi *et al*., 1994). A simple and efficient. RAPD had been successfully employed to study genetic diversity in

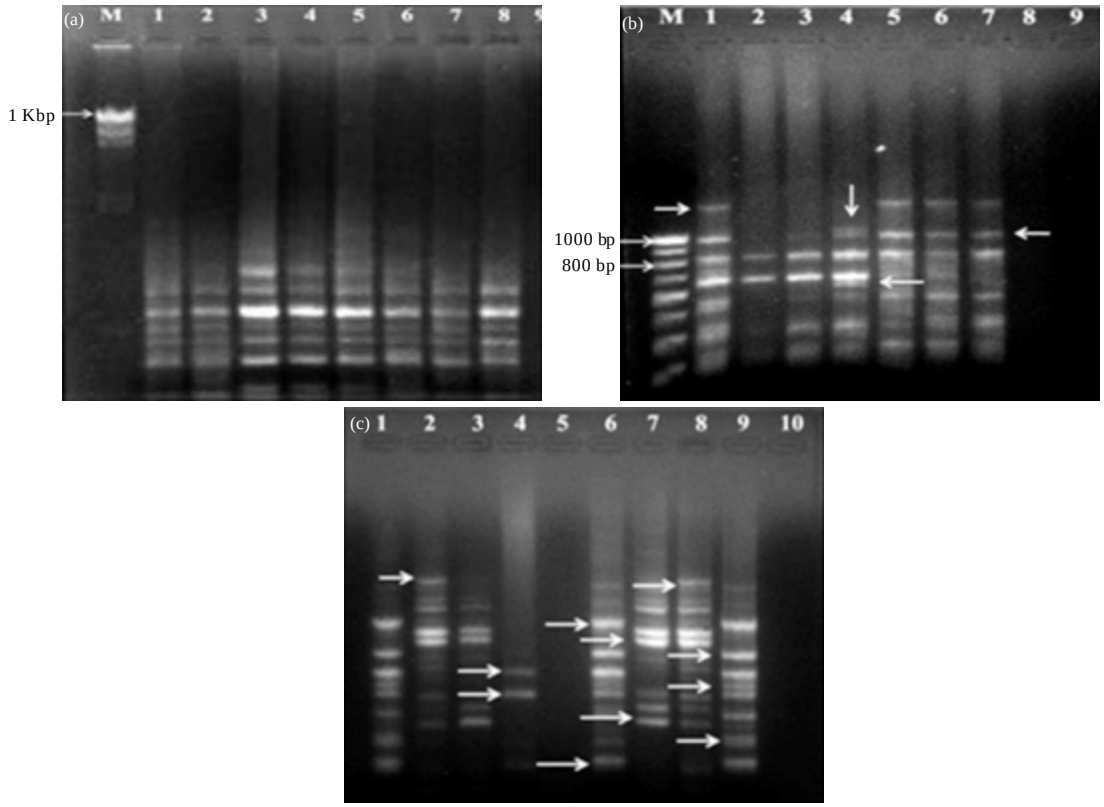

Fig. 4: RAPD pattern of nine *G indica* genotypes produced with primer (a) OPD-18, (b) OPD-11 and (c) OPD-13

other *Garcinia* species. Sando *et al*. (2004) conducted a preliminary survey using randomly amplified DNA fingerprinting to examine level of genetic diversity in *Garcinia mangostana*. Among 37 accessions examined, 26 (70%) of accessions showed no marked variations. Over 530 loci screened, 8 accessions (22%) showed very low level of variations. The remaining 3 accessions showed marked variations and these *Mangostana* groups were 63 to 70% dissimilar to other *Garcinia* species. Sompong (2004) used RAPD technique to check genetic similarities in micropropagated system. They obtained somaclones as well as nodular callus lines from *mangostana* leaf. Eight decamers could successfully be used to amplify DNA from the samples and showed no polymorphism in somaclones. Thus we can say that our preliminary results are in support with other *Garcinia* species where there was morphological variation seen at species level but very less amount of polymorphism revealed by RAPD.

CONCLUSION

This was the preliminary study carried out to assess amount of genetic variation within *G. indica*. However this data what we got is not sufficient to come to any conclusion. There are large number of plants with ovate leaves and horizontal branching patterns found in various localities of Sindhudurga district of Maharashtra state, India. The efforts are underway to locate such plants as well as large number of plants in various populations of Western Ghats of India and screen the genetic diversity within and among the populations.

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