

Study of Relationships among Twelve *Phyllanthus* Species with the Use of Molecular Markers

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Abstract: The present investigation was undertaken to describe the relationships among twelve species of *Phyllanthus* collected in India by help of molecular markers. In total, 259 marker loci were assessed, out of which 249 were polymorphic revealing 96.13% polymorphism. Nei's similarity index varied from 0.35 to 0.76 for RAPD (Random Amplified Polymorphic DNA) and from 0.31 to 0.76 for ISSR marker systems. Cluster analysis by the unweighted pair group method (UPGMA) of Dice coefficient of similarity generated dendrogram with more or less similar topology for both the analyses that offered a better explanation for diversity and affinities between the species. The phylogenetic tree obtained from both RAPD and ISSR (Inter Simple Sequence Repeat) markers has divided the 12 species into two groups: group I consisting of only one species *Phyllanthus angustifolius* (Sw.) Sw and group II with the rest of 11 species. Basically, these results were in compliance with notable morphological characterization. The present study revealed high variation among the species of *Phyllanthus* and will help to identify different *Phyllanthus* species.

Keywords: genetic variation; ISSR; medicinal plant; RAPD

The genus *Phyllanthus* belonging to family Euphorbiaceae is an important group of medicinal plants used for various purposes. In *Phyllanthus emblica* L., syn: *Emblica officinalis* Gaertn., the fruit is used for diverse applications in health-care, food and cosmetic industry. It has been well studied for immunomodulatory, anticancer, antioxidant and antiulcer activities (DNYANESHWAR *et al.* 2006). *Phyllanthus amarus* (*Phyllanthus niruri* L.) is an important folk remedy used in the treatment of a variety of ailments (GEETHA *et al.* 2003). In India, it is predominantly used as a cure for liver disorders (NADKARNI 1976; KRITIKAR & BASU 1993). The aqueous extract from *Phyllanthus amarus* has been reported to inhibit DNA polymerase of hepatitis-B and woodchuck

hepatitis virus. Therefore proper identification of genotype remains important for protection of both the public health and industry. Chemo-profiling and morphological evaluation are routinely used for the identification of genotype. Chemical complexity and lack of therapeutic markers are some of the limitations associated with the identification of genotype. Molecular markers have provided a powerful new tool for breeders to search for new sources of variation and to investigate genetic factors controlling quantitatively inherited traits. The molecular approach for the identification of plant varieties/genotypes seems to be more effective than traditional morphological markers because it allows direct access to the hereditary material and makes it possible to understand the relation-

ships between individuals (WILLIAMS *et al.* 1990; PATERSON *et al.* 1991). Genetic polymorphism in medicinal plants has been widely studied, which helps in distinguishing plants at inter- and/or intra-specific level. The most important role of conservation is to preserve the genetic variation and evolutionary process in viable populations of ecologically and commercially viable varieties/genotypes in order to prevent potential extinction. PCR-based molecular markers are widely used in many plant species for identification, phylogenetic analyses, population studies and genetic linkage mapping (WILLIAMS *et al.* 1990). Both RAPD (Random Amplified Polymorphic DNA) and ISSR (Inter Simple Sequence Repeat) markers, based on PCR (Polymerase Chain Reaction) techniques, have proved to be a reliable, easy to generate, inexpensive and versatile set of markers that rely on repeatable amplification of DNA sequence using single primers. The RAPD and ISSR markers can be used in the study of the genetic variability of species or natural populations and in the identification of genotypes (WILDE *et al.* 1992; KOLLER *et al.* 1993; LASHERMES *et al.* 1993; WILKIE *et al.* 1993; WOLFF & PETERS-VAN RUN 1993; PHARMAWATI *et al.* 2004; MOHAPATRA & ROUT 2005; BARIK *et al.* 2006). In this communication, we report the feasibility of PCR-based DNA (RAPD and ISSR) markers for identification and phylogenetic study of different *Phyllanthus* species.

MATERIALS AND METHODS

Plant materials

Twelve species of *Phyllanthus* were collected from natural forest of Orissa, India and used for molecular analyses. These species are *Phyllanthus nivosus* (L.), *Phyllanthus flaternus* (Linn.), *Phyllanthus reticulatus* Poir., *Phyllanthus acidus* (Linn.), *Phyllanthus nivosus* Varigata (Linn.), *Phyllanthus* spp. Acc No. 1, *Phyllanthus rotundifolius* (Klein. & Willd), *Phyllanthus angustifolius* (Sw.) Sw, *Phyllanthus emblica* (Linn), *Phyllanthus uninaria* L., *Phyllanthus virgatus* G.Forst., *Phyllanthus amarus* Schum & Th. These species were identified on the basis of morphological characteristics and comparison with voucher specimens recorded in the central herbarium of Botanical Survey of India.

DNA isolation and quantification

DNA was extracted from fresh leaves using the cetyl-trimethyl ammonium bromide (CTAB) method (DOYLE & DOYLE 1990). Approximately, 20 mg of fresh leaves was ground to powder in liquid nitrogen using a mortar and pestle. The ground powder was transferred to a 50 ml falcon tube with 10 ml of CTAB buffer [2% (w/v) CTAB, 1.4M NaCl, 20mM EDTA, 100mM Tris (tris (hydroxymethyl) aminomethane)-HCl, pH 8.0, and 0.2% (v/v) β -mercaptoethanol]. The homogenate was incubated at 60°C for 2 h, extracted with an equal volume of chloroform/isoamyl alcohol (24:1 v/v) and centrifuged at 10 000 \times g for 20 min. DNA was precipitated from the aqueous phase by mixing with an equal volume of isopropanol. After centrifugation at 10 000 \times g for 10 min, the DNA pellet was washed with 70% (v/v) ethanol, air-dried and resuspended in TE (Tris-EDTA buffer) (10mM Tris-HCl, pH 8.0, and 0.1mM EDTA) buffer. DNA quantifications were performed by visualizing under UV light, after electrophoresis on 0.8% (w/v) agarose gel at 50 V for 45 min and compared with a known amount of lambda DNA marker (MBI, Fermentas, Richlands B.C., Old). The resuspended DNA was then diluted in TE buffer to 5 μ g/ μ l concentration for use in polymerase chain reaction (PCR).

Primer screening

Thirty decamer primers, corresponding to kits A, D, and N from Operon Technologies (Alameda, California, USA) and twenty synthesized ISSR primers (M/S Bangalore Genei, Bangalore, India) were initially screened using one species of *Phyllanthus*, i.e. *Phyllanthus virgatus*, to determine the suitability of each primer for the study. Primers were selected for further analysis based on their ability to detect distinct, clearly resolved and polymorphic amplified products within the species. To ensure reproducibility, the primers generating no, weak, or complex patterns were discarded.

RAPD and ISSR assay

Polymerase chain reactions (PCR) with single primer were carried out in a final volume of 25 μ l

Table 1. Total number of amplified fragments and the number of polymorphic fragments generated by PCR using selected RAPD primers

Primer	Primer sequence	Total No. of bands	No. of polymorphic bands	Polymorphism percentage	No. of unique bands	Band range (kbp)
OPA-01	5'-TGCCGAGCTG-3'	24	24	100	3	0.4–2.1
OPA-04	5'-AATCGGGCTG-3'	18	18	100	2	0.25–2.4
OPA-10	5'-GTGATCGCAG-3'	20	20	100	3	0.3–2.3
OPD-02	5'-GGACCCAACC-3'	8	7	87.4	2	0.5–1.8
OPD-11	5'-AGCGCCATTG-3'	12	10	83.3	1	0.3–2.3
OPD-18	5'-GAGAGCCAAC-3'	15	15	100	1	0.2–2.1
OPD-20	5'-ACCCGGTCAC-3'	11	11	100	0	0.3–2.2
OPN-06	5'-GAGACGCACA-3'	20	20	100	3	0.3–2.5
OPN-15	5'-GGTGAGGTCA-3'	14	11	78.5	2	0.4–2.4
OPN-16	5'-AAGCGACCTG-3'	15	14	93	2	0.2–3.0
Total		157	150	95.5	11	0.2–3.0

containing 20 ng template DNA, 100 μ M of each deoxyribonucleotide triphosphate, 20 ng of decanucleotide primer (M/S Operon Technology), 1.5mM MgCl₂, 1X Taq buffer [10mM Tris-HCl (pH 9.0), 50mM KCl, 0.001% gelatin], and 0.5 U Taq DNA polymerase (M/S Bangalore Genei, India). Amplification was performed in a PTC-100 thermal cycler (M.J. Research Inc., Watertown, MA, USA) programmed for a preliminary 2 min denaturation step at 94°C, followed by 40 cycles of denaturation at 94°C for 20 s, annealing at required temperature for 30 s and extension at 72°C

for 1 min, finally at 72°C for 10 min for amplification. Amplification products were separated alongside a molecular weight marker (1.0 kb plus ladder, M/S Bangalore Genei) by 1% and 1.5% (W/V) agarose gel for RAPD and ISSR, respectively. Electrophoresis was done in 1X TAE (Tris acetate EDTA) buffer, stained with ethidium bromide and visualized under UV light. Gel photographs were scanned through a Gel Documentation System (Gel Doc. 2000, BioRad, California, USA) and the amplification product sizes were evaluated using the software Quantity one (BioRad, USA).

Table 2. Total number of amplified fragments and the number of polymorphic fragments generated by PCR using selected ISSR Primers

Primer	Primer sequence	Total no. of bands	No. of polymorphic bands	Polymorphism percentage	No. of unique bands	Band range (kbp)
IG-01	5'AGGGCTGAGGAGGGC-3'	12	12	100	1	0.5–1.6
IG-03	5'GAGGGTGGAGGATCT-3'	08	08	100	1	0.5–1.6
IG-10	3'-(AG) ₈ T-5'	12	12	100	0	0.3–1.8
IG-11	3'-(AG) ₈ C-5'	12	12	100	1	0.3–1.6
IG-13	3'-(AC) ₈ G-5'	11	11	100	1	0.4–2.2
IG-14	3'-(GA) ₈ A-5'	18	17	94.4	2	0.3–2.5
IG-15	3'-(GA) ₈ T-5'	15	14	93.33	0	0.4–2.0
IG-23	3'-(GA) ₈ C-5'	14	13	92.85	1	0.3–2.1
Total		102	99	97.05	7	0.3–2.5

Data analysis

Data were recorded as presence (1) or absence (0) of band products from the photographic examination. Each amplification fragment was named by the source of the primer, the kit letter or number, the primer number and its approximate size in base pairs. Bands with similar mobility to those detected in the negative control, if any, were not scored. A pair-wise matrix of distances between landraces was determined for the RAPD and ISSR data using the Dice formula (NEI & LI 1979) in the Free Tree programme (PAVLICEK *et al.* 1999). The average of similarity matrices was used to generate a tree by UPGMA (unweighted pair-group method arithmetic average) using NTSYS- PC version 2.1 (ROHLF 2000).

RESULTS AND DISCUSSION

The present study offers an optimization of primer screening for the evaluation of genetic relationships among twelve *Phyllanthus* species collected in India. DNA extraction of *Phyllanthus* proved difficult due to the presence of secondary metabolites and essential oil content. A modified

CTAB method by Doyle and Doyle proved to be fruitful. The modified method included a higher concentration of CTAB (4%), EDTA (50mM) and 1% 2-mercaptoethanol. Importantly purification by choloform: isoamyl alcohol (24:1) was performed twice. Significant quantities of DNA were always successfully extracted by this modified method that varied from 200 to 1000 ng in different *Phyllanthus* species. The reproducibility of both RAPD and ISSR primer amplification was detected by performing separate runs of PCR with DNA extraction from different preparations. No significant differences were observed in different experiments although occasional variation in the intensities of individual bands was detected. Bands with the same mobility were considered as identical fragments receiving equal values regardless of their staining ability. When multiple bands in a region were difficult to resolve, data on that region were not included in the analysis. As a result ten RAPD and eight ISSR primers were selected out of thirty RAPD and twenty ISSR primers screened, as they generated clear and scorable bands with considerable polymorphism.

Using ten RAPD primers, 157 bands were produced with an average of ~ 16 bands per primer out of which 150 were polymorphic revealing 95.54%

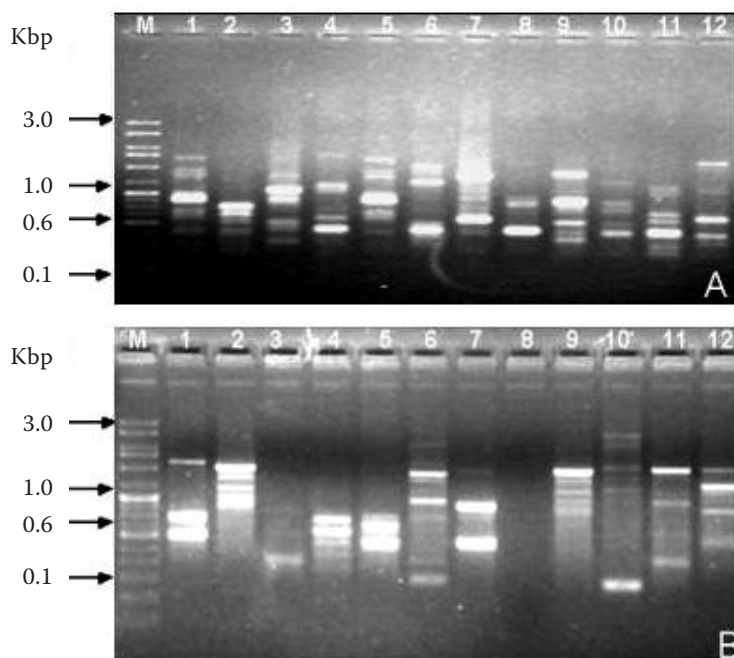


Figure 1. RAPD banding patterns of twelve *Phyllanthus* species generated by the primers OPA-01 (A) and OPD-18 (B); M – molecular weight ladder (kb). 1 – *Phyllanthus nivosus*, 2 – *Phyllanthus flaternus*, 3 – *Phyllanthus reticulatus*, 4 – *Phyllanthus acidus*, 5 – *Phyllanthus nivosus* Varigata, 6 – *Phyllanthus* spp. Acc No. 1, 7 – *Phyllanthus rotundifolius*, 8 – *Phyllanthus angustifolius*, 9 – *Phyllanthus emblica*, 10 – *Phyllanthus uninaria*, 11 – *Phyllanthus virgatus*, 12 – *Phyllanthus amarus*

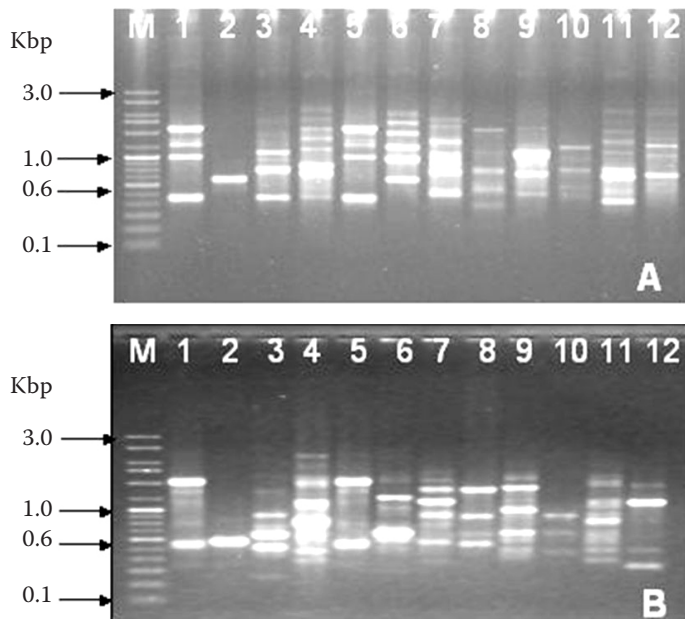


Figure 2. ISSR banding patterns in 12 *Phyllanthus* species obtained from PCR amplification by ISSR primers IG-10(A) and IG-14(B); M indicates DNA size marker; 1 – *Phyllanthus nivosus*, 2 – *Phyllanthus flaternus*, 3 – *Phyllanthus reticulatus*, 4 – *Phyllanthus acidus*, 5 – *Phyllanthus nivosus* Varigata, 6 – *Phyllanthus* spp. Acc No. 1, 7 – *Phyllanthus rotundifolius*, 8 – *Phyllanthus angustifolius*, 9 – *Phyllanthus emblica*, 10 – *Phyllanthus uninaria*, 11 – *Phyllanthus virgatus*, 12 – *Phyllanthus amarus*

polymorphism. The size of the RAPD fragments ranged from 0.2 to 3.0 kilo base pairs (Table 1). The banding profile by RAPD primers OPA-01 and OPD-18 is shown in Figure 1. The primer OPA-01 amplified a maximum of 24 fragments whereas OPD-02 produced the lowest number of amplified bands (08). Similarly, 102 amplified ISSR products were scored across 12 species of *Phyllanthus* by eight selected custom synthesized ISSR primers with 97.05% polymorphism. The average number of amplification products per ISSR primer was ~13. The size of ISSR amplified fragments varied from 0.3–2.5 kilo base pair

(Table 2). The banding pattern by ISSR primers IG-10 and IG-14 is presented in Figure 2. The genetic variation through RAPD and ISSR markers has been highlighted in a number of medicinal plants (BAI *et al.* 1997; ROUT *et al.* 1998; PAL & RAYCHAUDHURI 2003; ROUT 2006). The results show that both marker systems are efficient enough to distinguish 12 species of *Phyllanthus* and to reveal molecular relationship among them. The resolution of ISSR markers (97.08%) is higher in comparison with RAPD markers (95.54%). The similarity value ranged from 0.35 to 0.76 in the case of RAPD and from 0.31 to 0.76 for ISSR.

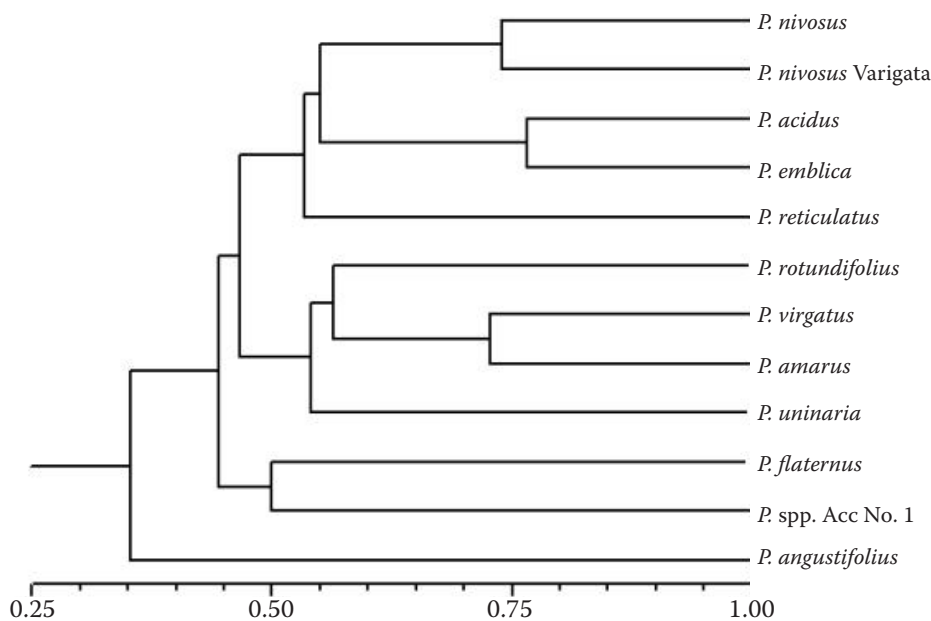


Figure 3. Dendrogram showing the cluster analysis of 12 *Phyllanthus* species using RAPD markers

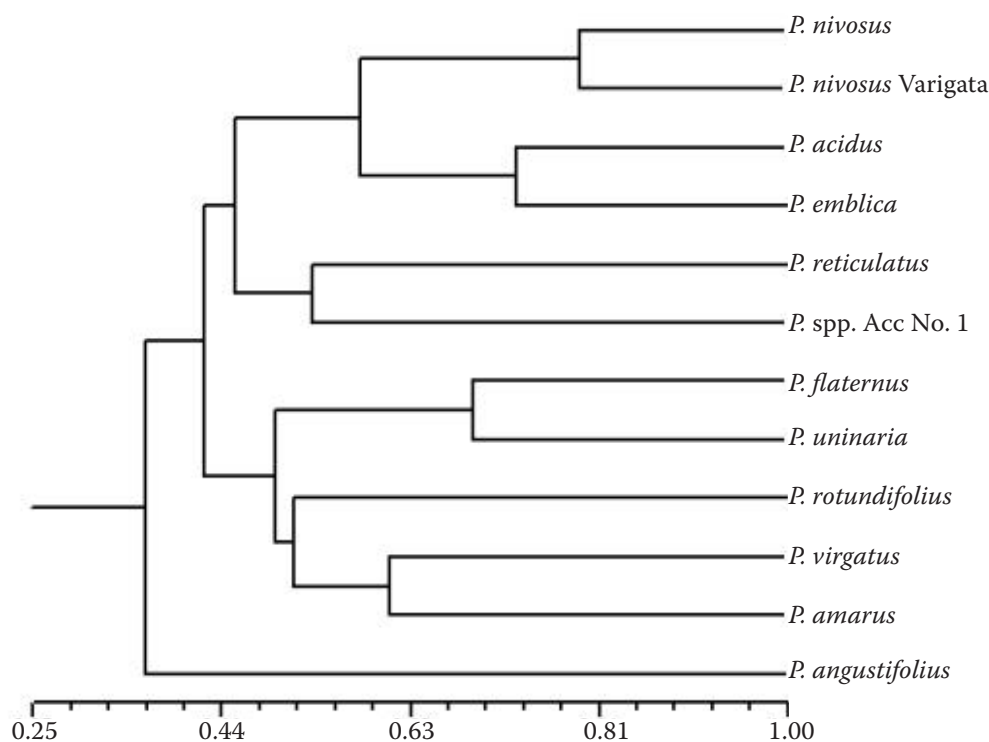


Figure 4. Dendrogram showing the cluster analysis of 12 *Phyllanthus* species using ISSR markers

The similarity matrix obtained in the present study was used to construct a dendrogram by the UPGMA method for both RAPD and ISSR data (Figures 3 and 4). The dendrograms generated by both approaches (RAPD and ISSR) were in broad agreement with each other and also with accepted taxonomy; two major groups were obtained and most of the related species were found to be grouped together. *Phyllanthus angustifolius* morphologically distinct from the rest 11 species was grouped isolated in group I by both molecular approaches. At the molecular level *Phyllanthus angustifolius* had six unique RAPD bands and five unique ISSR bands. The remaining eleven species positioned in group II are differentiated into two clads by both marker systems. The first clad contains six species (*Phyllanthus* spp. Acc No. 1, *Phyllanthus reticulatus*, *Phyllanthus nivosus*, *Phyllanthus nivosus* varigata, *Phyllanthus acidus*, *Phyllanthus emblica*) and the other clad comprises five species (*Phyllanthus flatarnus*, *Phyllanthus uninaria*, *Phyllanthus rotundifolius*, *Phyllanthus virgatus* and *Phyllanthus amarus*). *Phyllanthus acidus* and *Phyllanthus emblica* as well as *Phyllanthus nivosus* and *Phyllanthus nivosus* varigata were grouped together by both approaches, whereas

Phyllanthus spp. Acc No. 1 and *Phyllanthus reticulatus* formed a single cluster with the use of ISSR and two separate clusters by RAPD approach. *Phyllanthus amarus* and *Phyllanthus virgatus* in clad II were always grouped together by both approaches. The differences in the number of individuals estimated by RAPD markers in this study are similar to the results obtained by RAJASEGER *et al.* (1997) in RAPD studies of *Ixora coccinea* and *Ixora javanica*. These authors also found that the taxa-specific RAPD and ISSR bands could be utilized for identification.

It can be concluded that RAPD and ISSR markers may be a more useful tool for the identification of *Phyllanthus* species than morphological characters. The present findings can help the identification of different *Phyllanthus* species and genetic variation between the species. The genetic variability in a gene pool is normally considered as being the major resource available to breeders for improvement programmes.

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