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Phylogenic Study of Twelve Species of *Phyllanthus* Originated from India through Molecular Markers for Conservation

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ABSTRACT

The objective of the study was to characterize the germplasm for identification and phylogeny study for conservation. Identification and characterization of germplasm is an important link between the conservation and utilization of plant genetic resources. The present investigation was undertaken to draw the phylogenetic relationship between twelve species from India belonging to genus Phyllanthus with the 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 varies from* 0.23 *to* 0.76 *for RAPD and* 0.26 *to* 0.81 *for ISSR marker systems. Cluster analysis by unweighted pair group method* (*UPGMA*) *of Dice coefficient of similarity generated dendogram with more or less similar topology for both the analysis that gave a better reflection of diversity and affinities between the species. The phylogenetic tree obtained from both RAPD and ISSR marker has divided the* 12 *species in two groups: group* I *consisting of only one species Phyllanthus angustifolius and the group* II *with the rest* 11 *species. This molecular result is comparable to notable morphological characteristics. The present study revealed the distant variation within the species of Phyllanthus. This investigation will help for identification and conservation of Phyllanthus species.*

Keywords*: *Genetic Variation*, *ISSR*, *Medicinal Plant*, *RAPD

1. Introduction

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 healthcare, food and cosmetic industry. It has been well studied for immunomodulatory, anticancer, antioxidant and antiulcer activities [1]. *Phyllanthus amarus* is an important folk remedy used in the treatment of a variety of ailments [2]. In India, it is predominantly used as a cure for liver disorders [3,4]. The aqueous extract from *Phyllanthus amarus* has been reported to inhibit DNA polymerase of Hepatitis-B and woodchuck hepatitis virus. Proper identification of genotype, therefore, remains important for protection of both the public health and industry. Chemo profiling and morphological evaluation are routinely used for 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 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 relationships between individuals [5,6]. Genetic polymorphism in medicinal plants has been widely studied which helps in distinguishing plants at inter- and / or intra-species 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 analysis, population studies and genetic linkage mapping [5]. Both RAPD and ISSR marker, based on PCR techniques have proven to be a reliable, easy to generate, inexpensive and versatile set of marker that rely on repeatable amplification of DNA se-

quence 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 [7-14]. In this communication, we report the feasibility of PCR-based DNA (RAPD and ISSR) marker for phylogeny study and identification for conservation of *Phyllanthus* species.

2. Materials and Methods

2.1. Plant Materials

Twelve species of *Phyllanthus* were collected from natural forest of Orissa, India and used for molecular analysis.

2.2. DNA Isolation and Quantification

DNA was extracted from fresh leaves by using the Cetyltrimethyl ammonium bromide (CTAB) method [15]. 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, 1.4 M NaCl, 20 mM EDTA, 100 mM 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 x *g* for 20 min. DNA was precipitated from the aqueous phase by mixing with an equal volume of isopropanol. After centrifugation at 10,000 x *g* for 10 min, the DNA pellet was washed with 70% (v/v) ethanol, air-dried and resuspended in TE (10 mM Tris-HCl, pH 8.0, and 0.1 mM 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 µg/µl concentration for use in polymerase chain reaction (PCR).

2.3. Primer Screening

Thirty decamer primers, corresponding to kits A, D, and N from Operon Technologies (Alameda, California, USA) and twenty synthesized ISSR primer (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.

2.4. RAPD and ISSR Assay

Polymerase chain reactions (PCR) with single primer were carried out in a final volume of 25 µl containing 20 ng template DNA, 100 µM of each deoxyribonucleotide triphosphate, 20 ng of decanucleotide primer (M/S Operon Technology), 1.5 mM $MgCl_2$, 1X Taq buffer [10 mM Tris-HCl (pH 9.0), 50 mM 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).

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 distance between landraces was determined for the RAPD and ISSR data using Dice formula [16] in the program Free Tree [17]. The average of similarity matrices was used to generate a tree by UPGMA (unweighted pair-group method arithmetic average) using the program Tree view.

3. Results and Discussion

The present study offers an optimization of primer screening for evaluation of genetic relationship between twelve *Phyllanthus* species collected from Indian origin. DNA extraction of *Phyllanthus* proved difficult due to presence of secondary metabolites and essential oil content. A modified CTAB method by Doyle and Doyle proved to be fruitful. The modified method included 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 1000ng in different *Phyllanthus* species. The reproducibility of both RAPD and ISSR primer amplification were detected by performing separate runs of PCR with DNA extraction from different preparation. No significant differences were observed in different experiments although occasional variation in the intensities of individual bands was detected. Bands with 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 of that region were not included for 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% polymorphism. The size of the RAPD fragments ranged from 0.2 to 2.4 Kilo base pairs (**Table 1**). The banding profile by RAPD primer OPA-01 and OPD-18 has been shown in the **Figure 1**. The primer OPA-01 amplified a maximum of 24 fragments whereas OPD-02 produced least 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 primer IG-10 and IG-14 are presented in **Figure 2**. The genetic variation through RAPD and ISSR markers has been highlighted in a number of medicinal plants [18-21]. The result shows that both the marker systems are efficient enough to distinguish 12 species of *Phyllanthus* and in revealing molecular relationship among them. The resolution of ISSR markers (97.08%) is high in comparison to RAPD markers (95.54%). The similarity matrix of RAPD and ISSR data after multivariant analysis using Nei and Li's coefficient has been presented in **Tables 3** & **4** respectively. The similarity value ranged from 0.23 to 0.76 in case of RAPD and from 0.26 to 0.81 for ISSR. The similarity matrix obtained in the present study was used to construct a dendrogram with the UPGMA method by both RAPD and ISSR data (**Figures 3** & **4**). The dendograms generated by both the approaches (RAPD and ISSR) were with 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 angustifolia*, morphologically distinct from the rest 11 species had been grouped isolated in group-I by both the molecular approaches. At the molecular level *Phyllanthus angustifolia* is having six unique RAPD bands and five unique ISSR bands.

The remaining eleven species positioned in group II are differentiated into two clad by both the marker system. The first clad having six species (*Phyllanthus spp* "Acc No-1", *Phyllanthus reticulus*, *Phyllanthus nivosus*, *Phyllanthus nivosus* "varigata", *Phyllanthus acidus*, *Phyllanthus emblica*) and other clad having five species (*Phyllanthus flatarnus*, *Phyllanthus urinaria*, *Phyllanthus rotundifolius*, *Phyllanthus virgatus* and *Phyllanthus amarus*). *Phyllanthus acidus* and *Phyllanthus emblica* as well as *Phyllanthus nivosus* and *Phyllanthus nivosus* "varigata" are grouped together by both the approaches, where

Table 1. Total number of amplified fragments and 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

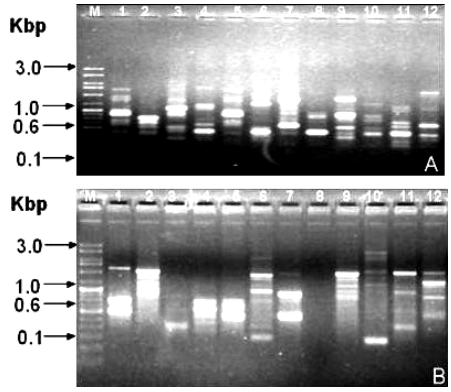

Figure 1. RAPD banding patterns of twelve species of *Phyllanthus* generated by the primers OPA- 01 (A) and OPD-18 (B) M – Molecular weight ladder (kb). 1-*Phyllanthus nivosus*, 2-*Phyllanthus flaternus*, 3-*Phyllanthus reticulus*, 4-*Phyllanthus acidus*, 5-*Phyllanthus nivosus* "Varigata", 6-*Phyllanthus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

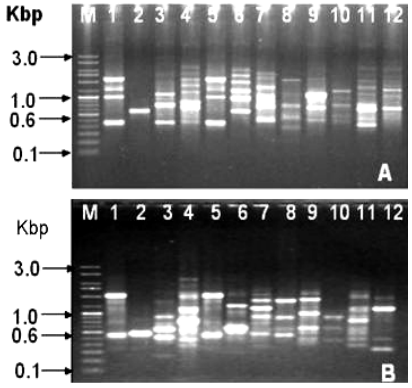

Figure 2. ISSR banding pattern in 12 species of *Phyllanthus* obtained from PCR amplification by ISSR primer IG-10(A) and IG-14(B). M indicates DNA size marker; 1-*Phyllanthus nivosus*, 2-*Phyllanthus flaternus*, 3-*Phyllanthus reticulus*, 4-*Phyllanthus acidus*, 5-*Phyllanthus nivosus* "Varigata", 6-*Phyllanthus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

Table 2. Total number of amplified fragments and 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'	8	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$8A-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

Table 3. Similarity matrix of 12 species of *Phyllanthus* generated by RAPD markers.

	P1	**P2**	**P3**	**P4**	**P5**	**P6**	**P7**	**P8**	**P9**	**P10**	**P11**	**P12**
P1	1.00											
P2	0.31	1.00										
P3	0.53	0.40	1.00									
P4	0.57	0.43	0.50	1.00								
P5	0.74	0.25	0.52	0.54	1.00							
P6	0.45	0.49	0.51	0.48	0.50	1.00						
P7	0.55	0.54	0.43	0.52	0.44	0.41	1.00					
P8	0.41	0.23	0.41	0.33	0.39	0.29	0.36	1.00				
P9	0.58	0.50	0.56	0.76	0.50	0.56	0.56	0.40	1.00			
P10	0.38	0.37	0.43	0.46	0.38	0.46	0.54	0.35	0.45	1.00		
P11	0.45	0.54	0.42	0.59	0.46	0.51	0.63	0.35	0.58	0.51	1.00	
P12	0.41	0.41	0.35	0.47	0.45	0.44	0.52	0.36	0.45	0.55	0.72	1.00

P1-*Phyllanthus nivosus*, P2-*Phyllanthus flaternus*, P3-*Phyllanthus reticulus*, P4-*Phyllanthus acidus*, P5-*Phyllanthus nivosus* "Varigata", P6-*Phyllanthus spp* "Àcc No.1", P7-*Phyllanthus rotundifolius*, P8-*Phyllanthus angustifolius*, P9-*Phyllanthus emblica*, P10-*Phyllanthus uninaria*, P11-*Phyllanthus virgatus*, P12-*Phyllanthus amarus.*

Table 4. Similarity matrix of 12 species of *Phyllanthus* generated by ISSR markers.

	P1	P2	P3	P4	P5	P6	P7	P8	P9	P10	P11	P12
P1	1.00											
P2	0.32	1.00										
P3	0.37	0.29	1.00									
P4	0.56	0.50	0.42	1.00								
P5	0.81	0.36	0.32	0.64	1.00							
P6	0.46	0.50	0.53	0.57	0.43	1.00						
P7	0.47	0.41	0.42	0.39	0.55	0.46	1.00					
P8	0.29	0.44	0.26	0.44	0.36	0.33	0.32	1.00				
P9	0.57	0.50	0.48	0.72	0.55	0.52	0.47	0.38	1.00			
P10	0.36	0.69	0.29	0.54	0.48	0.46	0.57	0.47	0.43	1.00		
P11	0.43	0.48	0.28	0.45	0.53	0.41	0.57	0.37	0.41	0.57	1.00	
P12	0.44	0.46	0.27	0.38	0.56	0.32	0.49	0.33	0.42	0.52	0.64	1.00

P1-*Phyllanthus nivosus*, P2-*Phyllanthus flaternus*, P3-*Phyllanthus reticulus*, P4-*Phyllanthus acidus*, P5-*Phyllanthus nivosus* "Varigata", P6-*Phyllanthus spp* "Àcc No.1", P7-*Phyllanthus rotundifolius*, P8-*Phyllanthus angustifolius*, P9-*Phyllanthus emblica*, P10-*Phyllanthus uninaria*, P11-*Phyllanthus virgatus*, P12-*Phyllanthus amarus*.

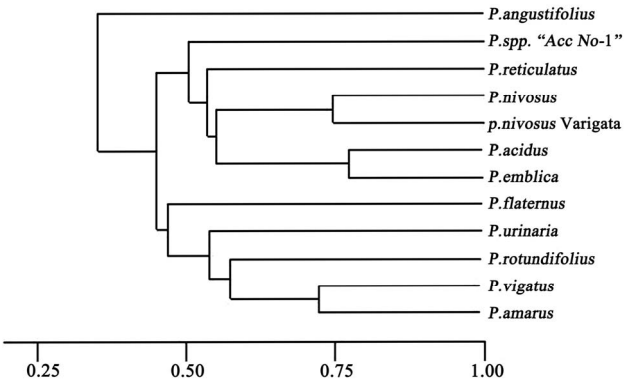

Figure 3. Dendogram showing the cluster analysis of 12 species of *Phyllanthus* using RAPD markers. 1-*Phyllanthus nivosus*, 2-*Phyllanthus flaternus*, 3-*Phyllanthus reticulus*, 4-*Phyllanthus acidus*, 5-*Phyllanthus nivosus* "Varigata", 6-*Phyllanthus spp* "Àcc No.1", 7-*Phyllanthus rotundifolius*, 8-*Phyllanthus angustifolius*, 9-*Phyllanthus emblica*, 10-*Phyllanthus uninaria*, 11-*Phyllanthus virgatus*, 12-*Phyllanthus amarus*.

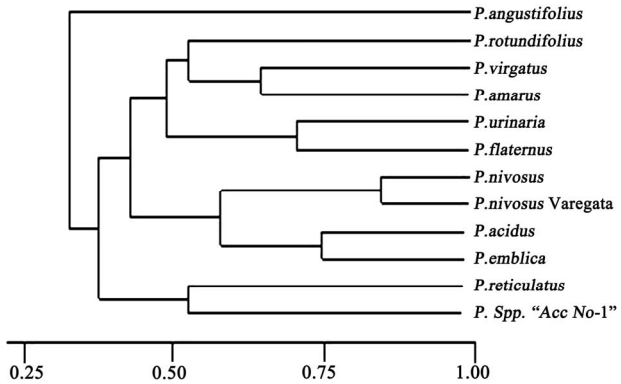

Figure 4. Dendrogram showing the cluster analysis of 12 species of *Phyllanthus* using ISSR markers. 1-*Phyllanthus nivosus*, 2-*Phyllanthus flaternus*, 3-*Phyllanthus reticulus*, 4-*Phyllanthus acidus*, 5-*Phyllanthus nivosus* "Varigata", 6-*Phyllanthus spp* "Àcc No.1", 7-*Phyllanthus rotundifolius*, 8-*Phyllanthus angustifolius*, 9-*Phyllanthus emblica*, 10-*Phyllanthus uninaria*, 11-*Phyllanthus virgatus*, 12-*Phyllanthus amarus*.

as *Phyllanthus spp* "Acc No-1" and *Phyllanthus reticulus* forming single cluster in case of ISSR are grouped separately in RAPD approach. *Phyllanthus amarus and Phyllanthus virgatus* in clad II is always grouped together in both the approaches. The differences in number of individuals estimated by RAPD markers in this study are similar to the result obtained by Rajaseger *et al*. [22] in RAPD studies of the *Ixora coccinea* and *Ixora. javanica*. They also found that the taxa-specific RAPD and ISSR bands could be utilized to define the identification.

The present findings include the identification and genetic variation within twelve species of *Phyllanthus*. The dendogram shows the distant variation within the species. The genetic relation through RAPD and ISSR markers provides a reliable method for identification of species than morphological characters. This investigation as an understanding of the level and partitioning of genetic variation within the species would provide an important input into determining efficient management strategies. The genetic variability in a gene pool is normally considered as being the major resource available to breeders for improvement program.

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