Identification of Species-Diagnostic Inter Simple Sequence Repeat Markers for Ten *Phyllanthus* Species

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Phyllanthus has been widely used in traditional medicine as an antipyretic, a diuretic, and to treat liver diseases and viral infections. Correct genotype identification of medicinal plant material remains important for the botanical drug industry. Limitations of chemical and morphological approaches for authentication have generated the need for newer methods in quality control of botanicals. In the present study, attempts were made to identify species-diagnostic markers for ten *Phyllanthus* species using the inter simple sequence repeat-polymerase chain reaction (ISSR-PCR) fingerprinting method. PCR amplification using seven ISSR primers resulted in significant polymorphism among the populations from different species. *P. angustifolius* and *P. urinaria* showed monomorphic frequency of maximum (63.88%) and minimum (20.64%), respectively. Seventeen species-diagnostic markers were identified for seven species (*P. acidus*, *P. emblica*, *P. fraternus*, *P. urinaria*, *P. rotundifolius*, *P. amarus*, and *P. angustifolius*) while no marker was detected for *P. reticulatus*, *P. nivosus*, and *P. virgulatus*. A maximum of six species-diagnostic markers were identified for *P. acidus* and a minimum of only one of 755 bp was available for *P. amarus*. Among the seventeen markers, nine were present in all individuals of particular species. The species-specific differences in fragment numbers and sizes could be used as diagnostic markers to distinguish the *Phyllanthus* species quickly.

Key words: ISSR, *Phyllanthus*, Species-Diagnostic Markers

Introduction

The genus *Phyllanthus* L. (Phyllanthaceae, formerly Euphorbiaceae) consists of about 800 species of trees, shrubs, and annual or biennial herbs distributed throughout the tropical and subtropical regions of both hemispheres (Govaerts *et al.*, 2000). *Phyllanthus* has been used in Ayurvedic medicine for over 2000 years and has an enormous number of traditional uses in many countries (Unander *et al.*, 1990). *Phyllanthus emblica* L. fruit is used for diverse applications in healthcare, food and cosmetics industry. It has been well studied for immunomodulatory, anticancer, antioxidant, and antiulcer activities (Dnyaneshwar *et al.*, 2006). In Ayurvedic Pharmacopoeia and Indian Herbal Pharmacopoeia, it forms a main ingredient of various multi-component formulations. *P. amarus* has been extensively used in pharmacological research due to its anti-HBV (hepatitis B virus, Liu *et al.*, 2001), anti-HIV (Notka *et al.*, 2004), antimutagenic (Raphael *et al.*, 2002), anti-inflammatory (Raphael and Kuttan, 2003), and antioxidant (Harikumar and Kuttan, 2004) properties. Recent pharmacological research has been focused on the inhibitory effects of *P. amarus* and related species on HBV, which has been found to be associated with chronic liver disease and primary liver cancer.

Unfortunately, a great deal of confusion exists among scientists regarding plant identification, which makes evaluation of published information difficult. *P. amarus* is considered by some authors as a variety of *P. niruri*, while in several reports one name is indicated to be synonymous of the other. *P. amarus*, *P. reticulatus*, *P. fraternus*, *P. urinaria*, *P. virgulatus*, and *P. rotundifolius* are all annual herbs, growing to a similar height (60–70 cm) with a similar pattern of branching, leaf size and phylotaxy, flowers and fruits. Microscopic inspection of the number of sepals and fruit surface is needed for correct identification of plants (Chantaranothai, 2007). Moreover, these plant species commonly grow together in

the same open habitat and wastelands, and, sometimes, identical species have different names or different species have the same name often lead to confusion in cultivation. Therefore, there exists the possibility of collecting the wrong plant species for medical uses. However, ethnomedical uses and some aspects of pharmacological activities among these species are different (Bunyaprapasara and Chokechaichareon, 2000).

Selection of elite plants for clonal propagation and seed orchard has been an important attempt carried out by breeders for tree improvement. Therefore, precise identification and classification of commercial lines of different *Phyllanthus* species are important for protection of both the public health and industry. Although phenotypic analysis is a traditional method for identification of medicinal plants, morphological characteristics are often unreliable or inconclusive, mainly due to the large influence exerted by environmental factors. Chemoprofiling evaluation is also routinely used for identification of medicinally important genotypes but chemical complexity and lack of therapeutic markers are some of the limitations associated with the identification of genotypes. So morphological criteria and chemotaxonomy are not preferred as suitable markers to verify the identity of each species. Recently, species-diagnostic markers are common among sytematicists and germplasm managers for identification, validation, and their conservation. Plant breeders extensively use the DNA-based species-specific markers for pharmacognostic characterization of medicinal plants and herbal medicine for the purpose of quality control and standardization (Joshi *et al*., 2004). They also prevent the introgression of closer or distant taxa, which may lead to certain negative consequences like loss of genetic diversity, genetic assimilation, and out-breeding depression on population viability. Hence, the aim of the present study was to identify molecular genetic differences that could be used as species-specific diagnostic markers for the proper identification and quantification of *Phyllanthus* species.

Table I. *Phyllanthus* populations used for the development of species-diagnostic markers.

No.	Species	No. of individuals
1	*Phyllanthus reticulatus*	15
2	*Phyllanthus nivosus*	15
3	*Phyllanthus acidus*	20
4	*Phyllanthus emblica*	20
5	*Phyllanthus fraternus*	12
6	*Phyllanthus urinaria*	10
7	*Phyllanthus rotundifolius*	10
8	*Phyllanthus virgulatus*	10
9	*Phyllanthus amarus*	15
10	*Phyllanthus angustifolius*	5

Material and Methods

Plant material

We examined 132 genotypes belonging to ten *Phyllanthus* species (*P. reticulatus*, *P. nivosus*, *P. acidus*, *P. emblica*, *P. fraternus*, *P. urinaria*, *P. rotundifolius*, *P. virgulatus*, *P. amarus*, and *P. angustifolius*) for the development of species-diagnostic DNA markers (Table I).

DNA isolation and quantification

DNA was extracted from fresh leaves using the cetyl-trimethyl ammonium bromide (CTAB) method (Doyle and Doyle, 1987). Approx. 200 mg of fresh leaves were ground to a powder in liquid nitrogen using a mortar and pestle. The 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 9838 × *g* for 20 min. DNA was precipitated from the aqueous phase by mixing it with an equal volume of isopropanol. After centrifugation at 9838 × *g* for 10 min, the resultant 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 comparing with a known amount of lambda DNA marker (MBI, Fermentas, Richlands B.C., QLD, Australia). The resuspended DNA was then diluted in TE buffer to 5 μg/μl concentration for use in polymerase chain reaction (PCR).

Primer screening

Twenty synthesized inter simple sequence repeat (ISSR) primers (M/S Bangalore Genei, Bangalore, India) were initially screened 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.

ISSR assay

PCRs with a 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 oligonucleotides synthesized primer (M/S Bangalore Genei, Bangalore, India), 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). Amplification was performed in a PTC-100 thermal cycler (MJ 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, extension at 72 °C for 1 min, and finally amplification at 72 °C for 10 min. Amplification products were separated alongside a molecular weight marker (1.0 kb plus ladder, M/S Bangalore Genei) by 1.5% (w/v) agarose gel. Electrophoresis was done in 1X TAE (Tris acetate/EDTA) buffer. The gel was prestained with ethidium bromide and visualized under UV light. Gel photographs were scanned through a Gel Documentation System (Gel Doc. 2000, BioRad, Herculus, CA, USA), and the amplification product sizes were evaluated using the software Quantity one (BioRad).

Data analysis

During data analysis, only reproducible polymorphic bands in amplification reactions were considered as present. Each band was treated as a separate putative locus, and scored as present (1) or absent (0) in each genotype. The binary data developed by manual scoring of the ISSR fingerprints were used for further population genetic analyses. The numbers of monomorphic and polymorphic bands were derived from the binary data, and their percentages were calculated. The presence of an ISSR fragment in a particular species population and its absence in all other species was designated as species-diagnostic marker. Bands with a frequency above 80% in a particular species and a frequency below 20% in the other species were considered as potential species-specific marker as described by Gili *et al.* (2004).

Table II. ISSR primers used for determination of species-diagnostic markers in *Phyllanthus* species.

Primer code	Primer sequence
Non-anchored	
IG-01	5'-AGGGCTGGAGGAGGGC-3'
IG-02	5'-AGAGGTGGGCAGGTGG-3'
IG-03	5'-GAGGGTGGAGGATCT-3'
5'-Anchored	
IG-10	3'-$(AG)_8$T-5'
IG-11	3'-$(AG)_8$C-5'
IG-12	3'-$(AC)_8$T-5'
IG-13	3'-$(AC)_8$G-5'
IG-14	3'-$(GA)_8$8A-5'
IG-15	3'-$(GA)_8$T-5'
IG-23	3'-$(GA)_8$C-5'
3'-Anchored	
IG-18	3'-ACC $(GT)_6$-5'

Results

Among the twenty primers tested, only eleven of them produced unambiguous DNA fragments. All individuals of the ten *Phyllanthus* species extensively amplified using these eleven ISSR primers (Table II) and produced 780 fragments ranging from 175 bp to 1930 bp. The minimum size fragment of 175 bp was amplified by the primer IG-14 in *P. rotundifolius* and the maximum size fragment of 1930 bp was amplified in *P. acidus* by primer IG-23. The total number of loci ranged from 55 (*P. nivosus*) to 119 (*P. acidus*). When individual species were considered, a maximum of 54 monomorphic bands were recorded in *P. rotundifolius* but the highest percentage of monomorphism (63.88%) was calculated in the populations of *P. angustifolius.* Similarly, a minimum number of 18 monomorphic bands with the lowest ISSR monomorphism percentage (20.64%) was recorded in *P. urinaria* (Table III).

This study revealed seventeen species-diagnostic markers for seven species (*P. acidus*, *P. emblica*, *P. fraternus*, *P. urinaria*, *P. rotundifolius*, *P. amarus*, and *P. angustifolius*), out of ten species (Table IV). However, ISSR primers failed to detect even a single species-diagnostic marker for *P. reticulatus*, *P. nivosus*, and *P. virgulatus*. The ISSR profile of ten *Phyllanthus* species amplified with primer IG-14 showed species-diagnostic markers for *P. acidus* (1850 bp), *P. fraternus* (1115 bp), and *P. rotundifolius* (175 bp) (Fig. 1). Similarly, two species-diagnostic markers for *P. acidus* (at 1930 bp and 1615 bp), and one each for *P. urinaria*

Table III. Level of polymorphism among different *Phyllanthus* species.

Species	Total no. of bands	No. of bands		Percentage	
		Monomorphic	Polymorphic	Monomorphic	Polymorphic
P. reticulatus	64	28	36	43.75	56.25
P. nivosus	55	39	16	41.81	29.08
P. acidus	119	34	85	28.57	71.43
P. emblica	83	36	47	43.37	56.63
P. fraternus	76	32	44	42.10	57.90
P. urinaria	87	18	69	20.64	79.36
P. rotundifolius	94	54	28	57.40	34.15
P. virgulatus	62	26	36	41.93	58.07
P. amarus	68	30	38	44.11	55.89
P. angustifolius	72	46	26	63.88	59.73

Table IV. Species-specific markers identified in different *Phyllanthus* species.

Species	Primer (bp)	Same species		Different species	
		No. of individuals	Presence (%)	No. of individuals	Presence (%)
P. amarus	IG-12 (755)	15	86.6	117	0
P. acidus	IG-10 (1850)	20	100	112	0
P. acidus	IG-11 (1615)	20	100	112	0
P. acidus	IG-12 (1175)	20	100	112	0
P. acidus	IG-15 (1055)	20	90	112	1.76
P. acidus	IG-16 (260)	20	80	112	12.5
P. acidus	IG-23 (1930)	20	100	112	0
P. emblica	IG-11 (1275)	20	100	112	0
P. emblica	IG-15 (1180)	20	100	112	4.4
P. fraternus	IG-10 (1115)	12	100	120	0
P. fraternus	IG-12 (780)	12	83.3	120	0
P. urinaria	IG-23 (1500)	10	90	122	6.55
P. urinaria	IG-10 (350)	10	100	122	0
P. rotundifolius	IG-14 (175)	10	100	122	0
P. rotundifolius	IG-11 (1750)	10	86.6	122	3.2
P. angustifolius	IG-12 (380)	5	80	127	1.5
P. angustifolius	IG-15 (1580)	5	100	127	0

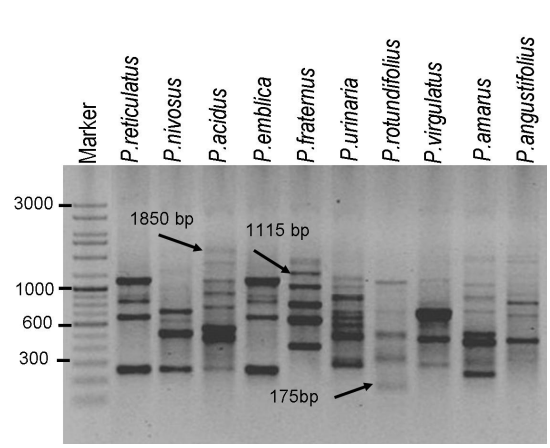

Fig. 1. ISSR banding profile showing species-specific markers generated by the primer IG-14 in ten *Phyllanthus* species.

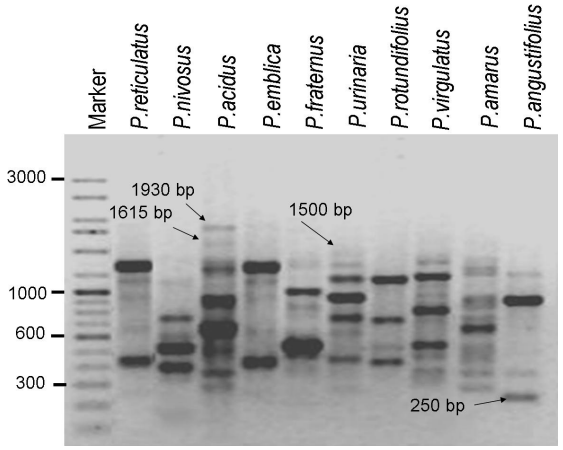

Fig. 2. ISSR banding profile showing species-specific markers generated by the primer IG-23 in ten *Phyllanthus* species.

(at 1500 bp) and *P. angustifolius* (at 250 bp) were found in the ISSR profile amplified by primer IG-23 (Fig. 2). A maximum of six species-diagnostic markers were identified for *P. acidus* and a minimum of only one was available for *P. amarus* at 755 bp by primer IG-12.

Among the diagnostic markers identified, ten markers belonging to six different species, *i.e. P. acidus* (4), *P. emblica* (2), *P. fraternus* (1), *P. urinaria* (1), *P. rotundifolius* (1), and *P. angustifolius* (1), had a frequency of 100% (Table IV). IG-12 generated the only marker for *P. amarus* and was present in 86.6% of the individuals and absent in the other population. *P. acidus* had a maximum of four diagnostic markers [IG-10 (1850), IG-11 (1615), IG-12 (1175), IG-23 (1930)] which were absent in all other populations. Two diagnostic markers of *P. emblica* [IG-11 (1275), IG-15 (1180)] had 100% frequency but the latter fragment was present in individuals of other species at 4.4% frequency. The diagnostic marker identified for *P. fraternus* [IG-10 (1115)], *P. urinaria* [IG-10 (350)], *P. rotundifolius* [IG-14 (175)], and *P. angustifolius* [IG-15 (1580)] were present in every individual of the particular species (100%) and absent in other species. All other markers showed a more than 20% occurrence in individuals of other species and were excluded.

Discussion

DNA-based molecular markers have proved their utility in fields like taxonomy, physiology, embryology, and genetics. As the science of plant genetics progressed, researchers have tried to explore these molecular marker techniques for their applications in commercially important plants, such as food crops, and horticultural plants, and recently in pharmacognostic characterization of herbal medicine. DNA-based techniques have been widely used for authentication of medicinally important plant species. This is especially useful in case of those that are frequently substituted or adulterated with other species or varieties that are morphologically and/or phytochemically indistinguishable. Due to the advancements in the DNA fingerprinting techniques, species-specific markers are widely used in the field of molecular taxonomy, particularly in animals (Gili *et al.*, 2004; Maier *et al.*, 2001). Isolation of specific diagnostic markers for particular taxa could be of great use in proper identification of these taxa. However, only a few reports on species-specific markers in plants are available (Scheef *et al.*, 2003; Kochieva, 1999; Kochieva *et al.*, 2004; Balasaravanan *et al.*, 2006; Theerakulpisut et al., 2008; Feng et al., 2010).

Molecular marker analysis has been widely used for differentiation of a large number of medicinal plant species from their close relatives or adulterants (Shaw and But, 1995; Cheng *et al.*, 1997, 2000; Kochieva, 1999; Sasikumar *et al.*, 2004). Scheef *et al.* (2003) identified two RAPD-based species-specific markers for two *Agrostis* species (*A. capillaries* – colonial type and *A. palustris* – creeping type) and subsequently developed (SCAR) markers, which were tested in 17 cultivars belonging to four species. In another study, Kochieva *et al.* (2004) revealed both species- and cultivar-specific markers in the genus *Syringa*. Balasaravanan *et al*. (2006) identified eight species-specific diagnostic markers for six species of *Eucylaptus*. Feng *et al*. (2010) studied the internal transcribed spacer (ITS) regions of *Angelica sinensis* and seven other *Angelica* species used as adulterants for authentication of *A. sinensis*. Theerakulpisut *et al.* (2008) developed specific SCAR markers that could differentiate *P. amarus*, *P. debilis*, and *P. urinaria* from several closely related species, some of which are commonly used as food or traditional medicine, such as *P. acidus*, *P. emblica*, *P. chamaepeuce*, *P. mirabilis*, *P. myrtifolius*, *P. pulcher*, and *P. androgynus*. However, ISSR being a highly informative and reproducible marker has not been employed for identification of *Phyllanthus* species.

In the present study, we focused on the identification of species-specific ISSR markers for ten commercially and medicinally important *Phyllanthus* species. Species-diagnostic ISSR markers were identified in *P. acidus*, *P. emblica*, *P. fraternus*, *P. urinaria*, *P. rotundifolius*, *P. amarus*, and *P. angustifolius* while no diagnostic markers were observed in *P. reticulatus*, *P. nivosus*, and *P. virgulatus*. A similar problem was experienced while developing species-specific markers using RAPDs (Scheef *et al.*, 2003) and ISSRs (Balasaravanan *et al.*, 2006). This could be due to the occurrence of high divergence among the particular species populations.

It is inferred that more primers should be used in future to develop species-diagnostic markers for these species. The developed species-diagnostic markers can be used for the precise and rapid identification of *Phyllanthus* species. This is

also extremely important to accelerate breeding programs and for protection of breeder's rights and quality control. Sequencing of these markers and developing PCR-based primers from the sequencing data will be used as ready reckoners to satisfy the above requirements.

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