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Development of ITS based SCAR Markers for Some Medicinally Important Species of *Phyllanthus*

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Abstract: Internal Transcribed Spacer (ITS) 1 and 2 were sequenced for five species of the genus *Phyllanthus* namely *P. emblica*, *P. reticulatus*, *P. amarus*, *P. fraternus* and *P. urinaria*, with a view to identify accurately herbaceous species. All the five species used in the study are medicinally important Affinities among these species were deduced through the help of phylograms generated from their ITS sequences. It was found that internal transcribed spacer 1 showed more potential than internal transcribed spacer 2 in assessing their relationship at molecular level. SCAR markers based on ITS 1 sequences of *P. urinaria*, *P. amarus* and *P. emblica* were designed. All three markers successfully distinguished these species from other closely related species. This is an important tool for identifying plant parts used in ayurvedic medicine.

Key words: *Phyllanthus emblica*, *P. reticulatus*, *P. amarus*, *P. fraternus*, *P. urinaria*, molecular identification

INTRODUCTION

Euphorbiaceae is a heterogeneous family represented by genera that are medicinally important. Of these, the genus *Phyllanthus* includes many economically important species containing various phytochemicals. The plants belonging to this genus have developed various life forms ranging from herbs, shrubs to tall canopy trees (Balakrishnan and Chakrabarty, 2007).

Five species namely *Phyllanthus emblica*, *P. reticulatus*, *P. amarus*, *P. fraternus* and *P. urinaria* were selected for the present investigation with a view to develop an authentic identification marker for the selected species in general and the herbaceous species in particular, using ITS sequences. Several medicinal properties have been already reported for all the five species. Fruits of *P. emblica* are a rich source of vitamin C; these are mainly used for their hepatoprotective and antioxidant activities (Bhattacharya *et al*., 1999; Jose and Kuttan, 2000). *Phyllanthus reticulatus* is a shrub and is claimed to have antidiabetic activity .The phytochemical screening of the residues revealed the presence of terpenoids, glycosides, proteins, carbohydrates and absence of alkaloids and steroids (Kumar *et al*., 2007). *P. amarus*, a herb is also important and well known for uses against hepatitis infection. It is also used for curing stomach ailments like dyspepsia, diarrhoea, dysentery and urinogenital problems (Jain *et al*., 2003). *Phyllanthus fraternus*, a closely related herb of *P. amarus* also exhibits hepatoprotective properties (Padma and Setty, 1999). Aqueous extract of *P. fraternus* against allyl alcohol induced oxidative stress in the liver of rats showed positive effects (Sailaja and Setty, 2006). Antioxidative and cardio protective effects of *P. urinaria* L. on Doxorubicin induced cardio toxicity have also been demonstrated (Chularajmontri *et al*., 2005).

Molecular marker based on DNA analysis (Farooq and Azam, 2002; Tanee *et al*., 2009) is a modern approach based on which proper assessment of closely related plant species could be determined. Increasing popularity of herbal medicines has projected molecular marker technology as a powerful weapon for quick identification of plants with suitable medicinal properties. India is a rich source of medicinal plants and market value of all these therapeutically important plant species is increasing day by day (Joshi *et al*., 2004). So keeping in mind the growing value of medicinal plants, it is essential to generate molecular markers that will help to authenticate plant species of medicinal importance.

Internal transcribed spacer regions are sequences located in eukaryotic rRNA genes between the 18S and 5.8S rRNA coding regions (ITS1) and between the 5.8S and 25S (26S) rRNA coding regions (ITS2). Studies of restriction site variation in rDNA in populations of animals and plants have shown that while coding regions are conserved, these spacer regions are variable (Gerbi,

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1985). These spacer sequences have a high evolutionary rate and are present in all known nuclear rRNA genes of eucaryotes (Jorgensen *et al*., 1987; White *et al*., 1991; Xu *et al*., 2006). They are useful for phylogenetic analysis among related species or among population within a species because these sequences evolve more rapidly than coding regions (Baldwin, 1993; Soltis and Kuzoff, 1993; Suh *et al*., 1993; Bayer *et al*., 1996). Development of primers from variable regions of ITS1 and ITS2 is another strategy that has been applied for species identification in bacteria (Chiu *et al*., 2005), fungi (Martin and Rygiewicz, 2005) and plants (Chiou *et al*., 2007). There are some reports on different *Phyllanthus* species from China (Lee *et al*., 2006) and Thailand (Chiou *et al*., 2007). In one of these papers primers designed based on ITS2 sequences proved to be suitable for a broad application in the authentication of herbal materials (Chiou *et al*., 2007). Here from our sequenced data we also tried to develop some species-specific PCR primers with which it will be possible to differentiate some of these species quickly and accurately and could be used as SCAR markers.

Herbaceous species of *Phyllanthus*, especially *P. amarus*, *P. urinaria* and *P. fraternus* are very similar in phenotypic characters and usually grow in the same habitat. Since the content of phytochemicals are variable in these species, their medicinal uses are also different. Therefore, there exist the potential risks of collecting wrong plants for medicinal uses. Again uses of dried fruits and leaves of *P. emblica* in local markets are rampant and it needs quality checking in terms of raw material. So the aim of the present investigation is to throw light on ITS sequences of *Phyllanthus* species based on which genetic relationships between species can be determined. Besides this we designed PCR primers based on ITS1 sequences for *P. amarus*, *P. urinaria* and *P. emblica* to be used as SCAR markers.

MATERIALS AND METHODS

This study was conducted from 2005 to 2009.

Plant leaves of all the five species were collected from different districts of West Bengal, India (Table 1). The tissues were brought to the laboratory and stored in -70°C until further analysis. Genomic DNA was isolated from these leaves within seven days of collection.

DNA extraction, ITS amplification and sequencing: Total genomic DNA was isolated from leaf materials following the DNA isolation method of Joshi *et al*. (2004). Leaf samples were collected and stored at -70°C until further use. These were ground to powder using liquid nitrogen, the powder collected from 1 g of frozen tissue was dissolved in 6 mL of buffer with the following composition-100 mM Tris-HCl (pH 8), 20 mM EDTA (pH 8), 1.5M NaCl, Cetyl Trimethyl Ammonium Bromide (CTAB) (2% w/v), β-mercaptoethanol (0.3% v/v) (added to buffer just before use), 100 mg PVP per gram of tissue (added in the mortar while crushing the tissue). The suspension was incubated at 65°C for 30 min with intermittent pH monitoring. 1:1 chloroform: isoamylalcohol (24:1) was added to it after cooling at room temperature. It was then centrifuged at 665 g for 10 min at 25°C and the supernatant was pipetted out and again centrifuged with equal volume of chloroform-isoamylacohol (24:1) mixture at the same speed. After recovering the clear supernatant 1/10th volume of 3M sodium acetate and twice the volume of chilled ethanol at 4°C were mixed with it to centrifuge at 665 g for 10 min to precipitate DNA. The pellet was washed with 70% ethanol (v/v) and dissolved in autoclaved triple distilled water. RNA contaminations were removed by adding RNase A at a concentration of 250 μg per ml of DNA solution and incubating for 30 min at 37°C. After incubation all the samples were extracted with equal volume of chloroform by centrifuging at 665 g at room temperature. The supernatant was collected and mixed with equal volume of isopropyl alcohol and 1/10th volume of 3 M ammonium acetate and centrifuged at 665 g at 4°C to precipitate the DNA from the solution. DNA pellets were then washed with 70% ethanol, dried and dissolved in triple distilled water. The purity of the genomic DNA was checked by calculating the ratio Absorbance 260/Absorbance 280, a ratio of 1.8 confirmed high quality genomic DNA.

Table 1: Morphological characters and collection sites of five *Phyllanthus* species

Name of species	Collection site	Habit
Phyllanthus emblica	North 24 parganas, Burdwan, South 24 parganas, Nadia	Very tall tree. Normally reaching a height of 60 ft. (18 m)
Phyllanthus reticulatus	North 24 parganas, Burdwan	*Phyllanthus reticulatus* is a large straggling or climbing shrub growing from 8 to 10 ft in height (Kritikar and Basu, 2003)
Phyllanthus amarus	North 24 parganas, Burdwan	Erect annual herb, 10-90 cm long, leaves oblong or sometimes oblong obovate with obtuse or rounded or occasionally cuneate base (Chaudhary and Rao, 2002)
Phyllanthus fraternus	North 24 parganas, Kolkata	Erect annual herb. 10-60 cm in heights, leaves elliptic-oblong with obtuse apex (Chaudhary and Rao, 2002)
Phyllanthus urinaria	North 24 parganas, Burdwan, Kolkata, South 24 parganas	Erect or sometimes procumbent annual herb. 10-70 cm long leaves generally hispidulous along margins, oblique at base, distinctly mucronate at apex (Chaudhary and Rao, 2002)

Table 2: Several features of ITS 1 and 2 regions of different *Phyllanthus* species

Species name	Accession sites	Accession No. (ITS 1)	Average size of ITS1 bp	Average GC% of ITS1	Accession No. (ITS 2)	Average size of ITS2 bp	Average GC% of ITS2
Phyllanthus emblica	North 24 parganas Burdwan	EU626399	218	52.29%	EU626400	212	53.92%
	South 24 parganas	EU876843			EU876843		
	Nadia.	FJ 391969			FJ 391970		
		FJ 391971			FJ 391972		
Phyllanthus reticulatus	North 24 parganas Burdwan	EU309042	223	57.53%	EU309043	217	66.33%
		EU876844			EU876845		
Phyllanthus amarus	North 24 parganas Burdwan	EU429327	222	56.88%	EU429328	206	52.73%
		EU876848			EU876849		
Phyllanthus fraternus	North 24 parganas Kolkata	EU429329	220	58.18%	EU429330	211	52.15%
		EU876846			EU876847		
Phyllanthus urinaria	North 24 parganas	EU626401	220	49.09%	EU626402	203	47.71%
	Burdwan	EU876850			EU876851		
	Kolkata	FJ391973			FJ391974		
	South 24 parganas	FJ391975			FJ391976		

ITS1 and ITS2 regions were separately amplified using primers (5'ACGAATTCATGGTCCGGTGAAGTGT TCG3') and (5'TAGAATTCCCCGGTTCGCTCGCCGT TAC 3') (Sun *et al*., 1994) and (5'GCTGCGTTCTTCA TCGATGC3') and (5'GCATCGATGAAGAAC GCAG C3') (White *et al*., 1990). The amplification parameters were 2 min at 94°C followed by 35 cycles of 1 min denaturing at 94°C, 30 sec of annealing at 48°C, 1 min extension at 72°C ending with 30 min final extension at 72°C. A slightly higher than 400 bp fragments were obtained for each ITS1 and ITS2. The amplified products were then cleaned using a PCR clean up kit (Bangalore Genei, India) for complete removal of dNTPS and primer dimers. The concentration of PCR product was determined and the amount needed for ligation for the specific PCR fragment was calculated by the conversion table subjected to ligation in a plasmid vector pTZ57R/T and transformed into a strain of *E. coli* XL1-Blue. All the steps of ligation and transformation were performed following manufactures protocol (InsTA clone PCR Cloning Kit, Fermentas). Positive clones were selected by blue-white screening procedure. All these clones were further scrutinized using a colony PCR step using the bacterial colonies as DNA templates and performing the same cycle as before. Colonies, which produced the desired fragment, selected, cultured overnight and subjected to plasmid DNA extraction. Plasmid DNA was sequenced using universal primers. All the sequences were edited by comparing them with the existing sequences in the NCBI database with the help of Clustal W programme (EMBL-EBI). Completed sequences were submitted to GENBANK database (Table 2). ITS1 and ITS2 sequences were separately aligned to construct respective phylograms (with bootstrap values and Phylip as tree type option) using online GeneBee ClustalW1.83 services.

All the ITS 1 sequences from different accessions of *P. amarus*, *P. urinaria* and *P. emblica* were put into Primer 3.0 software (version 0.4.0) to obtain species specific Primers. Three pairs of species-specific primers were obtained as follows:

PA (F) 5'CCGCGAACAATTTTATCCAC3' T_M-60.32
PA (R) 5 TTGAAAGGAGCAACGCCTAT3' T_M-59.85

PU (F) 5'GGCCTCGTGTGTGATCCTAT3' T_M-59.96
PU (R) 5'GAGATATCCGTTGCCGAGAG3' T_M-59.80

PE (F) 5'TTTAGTCACTGCGGATGGTG3' T_M-59.72
PE (R) 5'GAGATATCCGTTGCCGAGAG3' T_M-59.80

With the first two primer pair [PA (F/R) and PU (F/R)] we have amplified the DNA samples of all the three herbaceous species in study. The PCR amplification cycle was, 2 min hot start at 94°C, denaturation at 94°C for 1 min, annealing at 52°C for 30 sec, amplification at 72°C for 1 min, a final extension of 5 min at 72°C. Total 35 cycles were performed. Annealing temperatures were adjusted for different species (52°C for *P. urinaria* and 60°C for *P. amarus*) to obtain better results. With the third primer pair [PE (F/R)] specific for *P. emblica* dried fruit samples as well as leaf samples were examined using the above-mentioned cycle.

RESULTS

The ITS region was sequenced from 14 different accessions consisting of 5 different species locally growing in West Bengal, India .All the five species were identified by traditional morphological method (Table 1). Two different sets of forward and reverse primers (sequences provided in materials and method) were used to amplify ITS1 and ITS2 separately. All the four primers were designed using the conserved flanking sequences of rRNA genes around ITS1 and ITS2 so that full-length ITS1 and ITS2 regions could be obtained along with these conserved sequences. A slightly higher than 400 bp

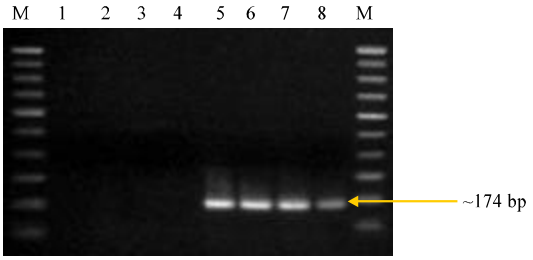

Fig. 1: DNA amplification profile using PU [F and R] primers. Lanes M-100 bp DNA ladder, 1-*P. emblica*, 2- *P. reticulatus*, 3- *P. amarus*, 4-*P. fraternus*, 5-8 *P. urinaria*

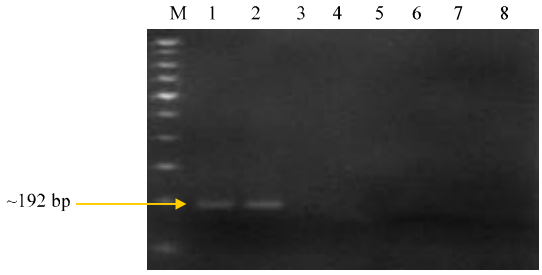

Fig. 2: DNA amplification profile using PA [F and R] primers. Lanes M-100 bp DNA ladder, 1,2-*P. amarus*; 3,4-*P. fraternus*; 5, 6- *P. urinaria*; 7- *P. emblica*; 8- *P. reticulatus*

fragment was obtained for each ITS1 and ITS2 consisting of varying length of ITS1 and ITS2 sequences for different species shown in Table 2. It was clear from the data that the average length of ITS1 is 220 bases whereas length of ITS2 is variable. Besides this one or two samples of most of the samples showed that these two regions were rich in GC content. The GC% was found to be ranged from 47-58% in case of ITS1 and from 47-66% in case of ITS2.In most of the accessions the GC% was constant, but some of the accession showed higher GC%. Two phylograms were constructed depending upon sequences of the region 18S-ITS1-5.8S and 5.8S-ITS2-26S.

Primer pair designed for *P. urinaria* [PU (F/R)] showed one clear and distinct band of ~174 bp (Fig. 1), but there were no such band for other species samples. In case of *P. amarus* specific primers [PA (F/R)] the desired band at ~192 bp was present in all *P. amarus* samples but that was obtained by increasing the annealing temperature to 60°C (Fig. 2) to reduce the effect of cross-reaction. Again when *P. emblica* leaf and dried fruit DNA samples were subjected to PCR amplification they produced the desired fragment of ~212 bp (Fig. 3).

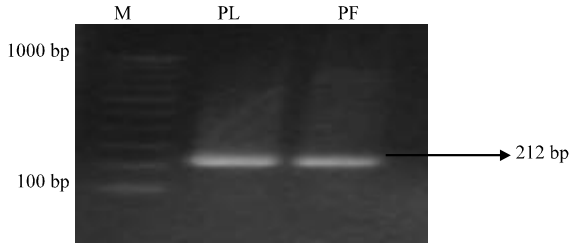

Fig. 3: DNA amplification profile using PE [F and R] primers. Lanes M-100 bp DNA ladder, PL-*Phyllanthus emblica* DNA from leaves. PF-*Phyllanthus emblica* DNA from fruits

DISCUSSION

In recent years ITS based phylogenetic studies immensely contributed in the understanding of ancestral relationships. In the genus *Phyllanthus* there were some studies based on these sequences done in China (Lee *et al*., 2006) and other countries (Kathriarachchi *et al*., 2006). India is a large country and several species of this genus grow here and used for medicinal purpose. For proper use of these species it is very important to know their proper identity. This investigation is such an attempt to develop ITS marker for proper assessment of relationship among five species of the genus *Phyllanthus*.

Phylogram generated from 18S-ITS1-5.8S (Fig. 4) showed that different species formed separate clades and it also showed that *P. amarus* and *P. fraternus* are two very close species. Phylogram generated from 5.8S-ITS2-26S region also showed close proximities for herbaceous species but in a complex manner. The position of *P. reticulatus* in both the phylograms was constant, i.e., it was positioned very far from all the herbaceous species. We also compared the ITS1 and ITS 2 sequences of these two species with the sequences of *P. niruri* from NCBI databank and found that there is high dissimilarity between them, which again proves that *P. amarus* and *P. fraternus* are two different species from *P. niruri*. Therefore, this type of clustering technique could be easily used for unknown samples so that their genetic identity could be known.

Several other special features of ITS1 and ITS2 regions were observed in this study. Alignment of ITS1 and ITS2 sequences separately revealed conserved stretches in both the regions. Different authors from time to time have thrown light on conserved stretches and their functional role in processing rRNA. Two conserved regions one in ITS1 (AACCCCGGCGCGGAAAGCGCCA AGGAA) (Fig. 5) and another in ITS2 (CGTGGCATTCGG TGGTTGAA) were found in our study. Liu and Schardl

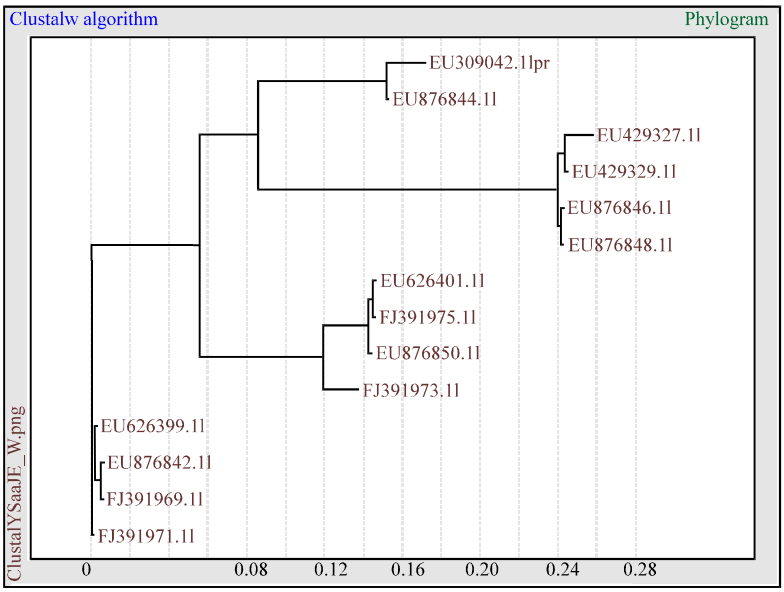

Fig. 4: Phylogram generated from 18S-ITS1-5.8S sequences

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EU309042 GCCACTTTAACAAACCCCGGCGCGGAGAGCGCCAAGGAAAATGAACAGAAAAGTGAGAGC 107
EU876844 GCCACTTTAACAAACCCCGGCGCGGAAAGCGCCAAGGAAAATGAACAGAAAAGTGAGAGC 167
EU626399 GCCACATAAACCAACCCCGGCGCGGAATGCGCCAAGGAAAACGAATCTAAAAGAGAGAAC 163
FJ391971  GCCACATAAACCAACCCCGGCGCGGAATGCGCCAAGGAAAACGAATCTAAAAGAGAGAAC 164
FJ391969  GCCACATAAACCAACCCCGGCGCGGAATGCGCCAAGGAAAACGAATCTAAAAGAGAGAAC 164
EU876842 GCCACATAAACCAACCCCGGCGCGGAATGCGCCAAGGAAAACGAATCTAAAAGAGAGAAC 174
EU626401 GCCGTGTAAACCAACCCCGGCGCGGAATGCGCCAAGGAAAACAAACATAAAAGCGAGAAC 168
FJ391975  GCCGTGTAAACCAACCCCGGCGCGGAATGCGCCAAGGAAAACAAACATAAAAGCGAGAAC 168
EU876850 GCCGTGTAAACCAACCCCGGCGCGGAATGCGCCAAGGAAAACAAACATAAAAGCGAGAAC 157
FJ391973  GCCATGTAAACCAACCCCGGCGCGGAATGCGCCAAGGAAAACAAACAAAAAAGCGAGAAC 165
EU429327 GCCACG-AAACAAACCCCGGCGCGGAAAGCGCCAAGGAACACGAACGTATAAGCGAGAAC 162
EU876846 GCCACG-AAACAAACCCCGGCGCGGAAAGCGCCAAGGAACACGAACGTATAAGCGAGAAC 163
EU429329 GCCACG-AAACAAACCCCGGCGCGGAAAGCGCCAAGGAACACGAACGTATAAGCGAGAAC 166
EU876848 GCCACG-AAACAAACCCCGGCGCGGAAAGCGCCAAGGAACACGAACGTATAAGCGAGAAC 164
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Fig. 5: Conserved stretches in ITS 1 region

(1994) found a conserved motif within ITS 1 in many flowering plants, which they found absent in other eukaryotic kingdom and species, this short stretch (AAGGAA) was also found to be present in our ITS1 sequences. It was suggested that the sequence served as a critical recognition element for r RNA processing. It was proposed that it forms a hairpin structure during the r RNA processing and probable hairpin structures are also available in literature for Rice (*Oryza sativa*) and *Arabidopsis thaliana*. Following those structures here we have also drawn a probable hairpin structure (Fig. 6), which again strongly support this hypothesis by Liu and Schardl (1994).

Results obtained from amplification studies based on species specific PCR primers designed from ITS1 sequences of *P. urinaria*, *P. amarus* and *P. emblica*

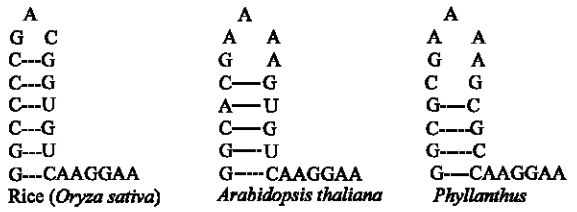

Fig. 6: Probable hairpin structure for *Phyllanthus* along with rice and *Arabidopsis*

showed that while primers from *P. urinaria* (Fig. 1) samples clearly distinguished it from other closely related herbs at one chance, *P. amarus* specific primer cross reacted with other samples which upon elevation of annealing temperature gave clear and distinct band and the cross reacting band also got diluted (Fig. 2). For the *P. emblica* leaf and fruit samples the *P. emblica*-specific primer gave the expected band at ~212 bp (Fig. 3) that showed the accuracy of the method to distinguish *P. emblica* samples. Bands obtained from all these amplification procedures were very bright and clear which can be attributed to the presence of multiple copies of these spacer regions in the genome. Genomic DNA required for this type of amplification procedure is also very low (~5 ng) which is another positive aspect of this kind of study. So we found that in comparison to SCAR marker development (usually developed from RAPD (Dnyaneshwar *et al*., 2006; Neeraj *et al*., 2008; Theerakulpisut *et al*., 2008) or AFLP markers) ITS specific marker generation is much easier and also highly reproducible.

Lee *et al*. (2006) in their investigation assessed phylogenetic relationships among 18 *Phyllanthus* species based on nrITS sequences along with chloroplast ATPB and RBCL sequences. Cladistic analysis indicated that the genus is paraphyletic and strongly demarcates between the closely related species *P. niruri* and *P. amarus*. Same authors also developed a multiplex PCR assay to differentiate three medicinally important species *P. amarus*, *P. niruri* and *P. urinaria*. In the present investigation SCAR markers based on ITS1 sequences have been developed for the species *P. amarus*, *P. urinaria* and *P. emblica* to identify them from other species of the genus *Phyllanthus* growing in India.

In another report (Kathriarachchi *et al*., 2006) nrITS and plastid matK DNA sequence data for 95 species of tribe *Phyllantheae* including representatives of all subgenera of *Phyllanthus* were analysed. According to them these results are generally concordant with each other, although some species are placed differently in the plastid and ITS trees, indicating that hybridization paralogy is involved. In conclusion, it could be stated that the data on length and sequence of ITS1 and ITS2 determined in the present report could be used successfully for the assessment of genetic relationships at the interspecific level in the genus *Phyllanthus* in India. In this study ITS1 was found to be more informative than ITS2. Apart from that we have developed highly sensitive and specific PCR primers for detection of three therapeutically important species of the genus *Phyllanthus* which could be treated as SCAR markers. These primers may be widely useful in identification of plant parts of all these species used extensively in Ayurvedic medicines.

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