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SCAR Markers for Correct Identification of *Phyllanthus amarus*, *P. fraternus*, *P. debilis* and *P. urinaria* used in Scientific Investigations and Dry Leaf Bulk Herb Trade

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- *Phyllanthus urinaria*
- SCAR marker

Abstract

The trade in *Phyllanthus* material as bulk herb is rampant and mainly involves herbaceous species such as *Phyllanthus amarus, P. fraternus*, P. *debilis* and *P. urinaria*. These species are very important in herbal medicines and have varied activities. In India these species grow sympatrically and there are chances of deliberate or ignorant adulteration of crude drugs, lowering the efficiency of the medication for its intended purpose. Secondly, incorrect identification may also lead to erroneous reports on activities/molecules. To overcome this problem in crude drug (dry leaf power) and compliment morphological identification in live plant, we have developed SCAR markers for all four species. In each species, we selected one fragment as being monomorphic between accessions but differing in size between species. These species-specific fragments were selected, cloned and sequenced. Based on the sequences, primer pairs were designed and amplification conditions standardized. SCAR markers were isolated from population DNA amplification profiles and validated by sequencing. The species-specific SCAR primers could retrieve the same size and sequence of fragments as in the RAPD profile. These fragments are 1150 bp, 317 bp, 980 bp and 550 bp in size for *P. amarus, P. fraternus, P. debilis* and *P. urinaria*, respectively. Additional fragments in *P. debilis* and *P. urinaria* indicate different alleles. The retrieval of same size and sequence of species-specific unique SCAR markers from the respective accessions (mixed DNA sample of same accessions) indicates the usefulness to study natural hybridization between the species in addition to adulteration.

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Introduction

Phyllanthus (Euphorbiaceae) is one of the largest and most diversified genera, having 833 species [1] and is commonly found in the moist humid tropics throughout the world. The plant is very important medicinally and has been used in traditional medicine by Europeans, Chinese and Indians since ancient times, mainly for treating jaundice. In addition, it is also believed to possess anticancerous, anti-HIV, and anti-inflammatory properties. More specifically, the herbaceous species *P. amarus* Schum. and Thonn, *P. fraternus* Webster and *P. debilis* Klein ex Willd have been reported to be extensively used for jaundice and *P. urinaria* L for urinary tract diseases. Modern research with *Phyllanthus* focuses on its potential for fighting viruses, specifically the hepatitis B virus [2] as well as the malaria parasite [3].

Despite extensive historical use of a group of species comprised of *P. amarus, P. fraternus, P. debilis* and even *P. urinaria* (**Fig. 1**), confusion still exists concerning proper identification and nomenclature of the species commonly known as "Bhuianavala" or "Bhumyamalaki" in the Indian literature and Ayurveda. Earlier research grouped all of these species, especially *P. amarus, P. fraternus* and *P. debilis,* under the single species name *P. niruri* which was later described as `niruri complex'. The species *P. niruri* is an American species and not at all found in India. Webster (1957) [4] and Mitra and Jain (1985) [5] showed that *P. niruri* of Hooker is actually represented by the three species described above. Thus, confusion in identification of these herbaceous species is largely due to the use of common vernacular names for all species, their similarity in gross morphology, a close proximity in the growth

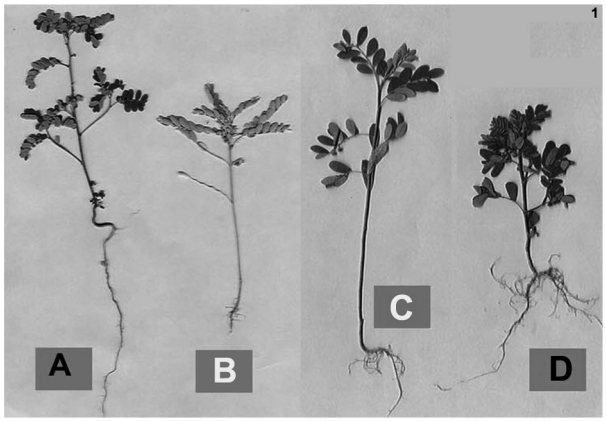

Fig. 1 Scanned picture of *Phyllanthus* species analyzed in this investigation showing morphological similarity. These species grow sympatrically in India and hence there are chances of deliberate or ignorant adulteration of crude drugs (**A**: *P. amarus*, **B**: *P. fraternus*, **C**: *P. debilis* and **D**: *P. urinaria*.).

habitat and the range of diverse morphological features. This misidentification leads to deliberate or ignorant adulteration of crude drugs leading to a decreased or deleterious effects of the drug when administered. The present study was targeted to develop molecular markers that would help in the unambiguous identification of the above species. The marker can also be used in quality check of dry herbs samples exported from India.

For this purpose the SCAR (sequence characterized amplified region) markers were chosen. SCAR DNA analysis was developed to produce more specific and reproducible results that are less sensitive to changes in reaction conditions [6], [7], [8], [9]. SCAR markers amplify distinct single fragments whose sizes are the same as those of the RAPD clones [7], [10]. The presence or absence of the fragment indicates variation in sequence and low sensitivity to reaction conditions facilitating their use for marker-assisted selection and fingerprinting. These markers have already been successfully derived from RAPD fragments in *Lactuca* [7] and *Triticum* [9]. Genotype-specific SCAR markers for identification purposes were developed by Vidal et al. [11]. SCAR primer pairs were designed to identify morphologically similar colonial and creeping bentgrass (*Agrostis*) species [12]. The SCAR marker identified was found to be useful for identification of *P. emblica* (tree species of *Phyllanthus*) in its commercial samples and in Triphala churna, a multi-component Ayurvedic formulation [13]. Species-specific molecular markers for *Bambusa balcooa* and *B. tulda* have been developed to allow proper identification for avoiding unintentional adulteration that affects the quality and quantity of paper pulp production [14]. A SCAR-based indirect selection method for a dominant blast-resistance gene in rice was reported to facilitate marker-assisted selection in rice breeding programs [15]. RAPD and SCAR markers were used for purity testing of F1 hybrid seed in chili pepper (*Capsicum annuum*) [16] and early identification of cytoplasmic male sterility [17]. SCAR markers have also been used for dispersal and phylogeography studies [18].

In the present study, we examined the accessions from *P. amarus, P. fraternus*, *P. debilis* and *P. urinaria* for similarity and differences in the amplified fragment profiles generated through RAPD primers and utilized the uniqueness of the fragments to generate SCAR markers with the objective of correct identification of herbaceous *Phyllanthus* species of the "niruri complex". This will not only help in determining the composition of dry leaf samples exported as bulk herbal drug but will also aid in identifying correct species for use in research related to activity prospection.

Materials and Methods

Plant material

The plant material of each of the four species (*P. amarus, P. debilis, P. fraternus* and *P. urinaria*) was collected from different geographical locations throughout the country. Twenty plants were collected for each accession. From this collection, 10 accessions were randomly selected for each species after proper identification according to Chaudhary and Rao [1], in order to assess the similarity and differences in the profiles. The identification was based on the keys described for the species and by comparison with the herbarium specimen in the National Gene Bank for Medicinal and Aromatic Plants (sponsored by Department of Biotechnology, Government of India) maintained at CIMAP, Lucknow. A different set of 25 randomly selected accessions was taken from the germplasm in the genebank for validation of SCAR primers specific to respective species. The seeds and voucher specimens from these accessions were submitted to the Gene Bank. The accession numbers and places of collections are provided as Supporting Information.

DNA isolation and PCR amplification

Total DNA was extracted from pooled accessions (20 plant samples from each accession, approximately 3 g of leaf tissue) as described by Khanuja et al. [19]. The concentration of the DNA was checked both by taking OD at 260/280 nm and running on a 0.8% agarose gel along with standard λ DNA. All accessions of the four species were then analyzed through RAPD using 40 random decamer oligonuleotides (20 each of MAP and OPO kits) according to the protocol described by Shasany et al. [20]. The sequence information and the source of the random primers are available in the same report. Amplification reactions were repeated thrice and the fragments appearing consistently were scored for the presence and absence of bands in the RAPD profiles. In each species, we selected the primers generating monomorphic fragments between the accessions but differing in size between species for further analysis. Then the pooled DNA of all the accessions of same species was amplified with the selected primers.

Cloning and sequencing of amplified fragments

Specific fragments obtained from PCR amplification profiles with selected primers were eluted from agarose gels (QIAquick gel extraction kit following manufacturer's instructions; QIAGEN GmbH), cloned in pBluescript II SK (+), transformed into *Escherichia coli* DH5α for blue white selection on X-gal/IPTG plate and screened for the DNA fragments of the desired sizes in the recombinant clones. The recombinant plasmids were then isolated for sequencing using the Qiagen plasmid purification kit (QIAGEN GmbH) on an ABI 377 automated DNA Sequencer (Applied Biosystems) and sequences were analyzed through the Sequence analysis software v3.3.

Primer design, synthesis and optimization of amplification conditions

Forward and reverse oligonucleotide primers were designed for *Phyllanthus* sp. (*P. amarus*, *P. fraternus*, *P. debilis* and *P. urinaria*)

on the basis of end sequences of unique fragments using the software Primer Premier 4 v 2.0 (Premier Biosoft). The sequence characterized amplified region (SCAR) primer sequences comprised the original 8 – 9 bases of the RAPD primer, followed by approximately 18 bases in the DNA sequence at the 3′ region and an A or T at the 5′ end. The primers were synthesized on an Automated DNA/RNA Synthesizer ABI 392 (Applied Biosystems) and resulting oligonucleotides were purified manually using the manufacturer's guidelines. The purified primer was lyophilized, dissolved in sterile water and the concentration was determined by measuring the OD at 260/280 nm and also by running on a 0.8 % gel. The primer samples were diluted to 20 ng/mL and used for amplification. Equal amounts of DNA samples were mixed from accessions of each plant species separately and amplified with the SCAR primers specific to respective species. The conditions for amplification were standardized for each of the primer pairs taking into consideration the T_m values of the primers. The T_m values and GC content of the primers are mentioned in ◉ **Table 3**.

Validation of the SCAR markers

DNA samples of the 4 species in this investigation were analyzed with the specific primer pairs defined for each species; other species supposed as adulterants were also included to examine the amplification profiles. PCR products were run on 1.2 % agarose gels containing 0.5 μg/mL ethidium bromide. The presence or absence of fragments was visually scored and compared with other species. The specific amplified fragments implicated as species-specific (generated by SCAR primers) were eluted from the agarose gel and end sequenced.

Results

The four species of the niruri complex (*P. amarus, P. fraternus, P. debilis* and *P. urinaria*) were analyzed using 40 decanucleotide primers. Of these, two primers (MAP9 : 5′ CGGGATCCGC 3′ and MAP10 : 5′ GCGAATTCCG 3′) were observed to show polymorphism and exhibited DNA fragments specific to the four species (*P. amarus, P. fraternus, P. debilis* and *P. urinaria*) (◉ **Table 1**). One species-specific fragment was identified for each of the four species amplified either through MAP 09 and MAP10 primers (◉ **Fig. 2**, ◉ **Table 2**). The fragment specific for *P. amarus* was 1150 bp long and the one specific for *P. fraternus* was 317 bp long as amplified with the MAP 10 primer. A fragment of 980 bp, identified to be specific for *P. debilis*, and one of 550 bp specific for *P. urinaria* were amplified with the MAP 09 primer.
These two primers carry restriction enzyme sites, *Bam* HI (GGATCC in MAP 09) and *Eco* RI (GAATTC in MAP 10). The DNA samples for all accessions of the same species were pooled and amplified with these primers. From the profiles generated by the amplification of pooled DNA, specific fragments were cloned

Molecular weight (bp)	*P. amarus*	*P. fraternus*	*P. debilis*	*P. urinaria*
MAP 09 with Bam HI site				
1 150	*			*
1 050	*			
980			**	
600	*	*		
550				**
MAP 10 with Eco RI site				
1 225	*	*		
1 150	**			
990	*			*
975		*	*	
850	*		*	
625		*		*
580	*			*
520	*			
430				*
317		**		

** Indicate fragments taken for cloning and development of SCAR marker.

Table 1 Presence of the fragments in the profiles of different species when amplified with MAP 09 and 10 primers

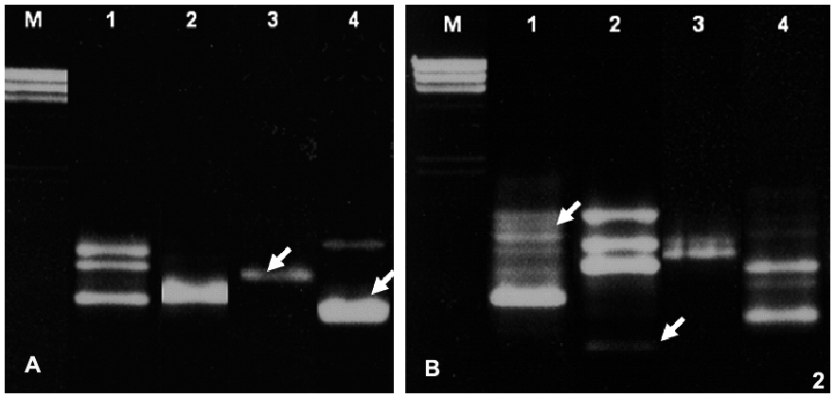

Fig. 2 Amplified profiles of *Phyllanthus* with MAP 09 (**A**) and MAP 10 (**B**) primers from pooled DNA of 10 accessions of each species. The arrows in lane 3 and 4 of A indicate the unique fragments from *P. debilis* (980 bp) and *P. urinaria* (550 bp) respectively. Similarly, the arrows in lane 1 and 2 of B indicate the unique fragments from P. *amarus* (1150 bp) and *P. fraternus* (317 bp, light fragment) respectively. M: DNA Marker λ Hind III digest; Lanes 1 to 4: *P. amarus*, *P. fraternus*, *P. debilis*, *P. urinaria*.

in pBluescript II SK+ vector using these restriction sites. The sequences of the cloned fragments were obtained by sequencing from both ends using T3 (reverse primer) and T7 (forward primer) primers and submitted to the GenBank (NCBI) (**Table 2**). These sequences were used to design forward and reverse primer pairs for each of the four species (**Table 3**). Each primer was a 29-mer carrying 8 bases of the original primer (including 6 bases of the restriction site), 20 additional bases down-stream similar to the end sequences of the fragments and an extra T or A at the 5'end. The sequences were also analyzed for homology through BLAST search in the public database NCBI. BLAST analysis of the SCAR marker sequences of *P. amarus, P. fraternus* and *P. urinaria* showed no significant homology (E value = 3.1 – 0.013) with any of the sequences in the existing online database, whereas the SCAR marker sequence of *P. debilis* exhibited significant homology with the chloroplast sequence of several plants in the database.

Amplification conditions were standardized for all primer pairs and were used to amplify the genome of all four *Phyllanthus* species separately, to retrieve species-specific fragments (**Table 4**). The species-specific primer pairs generated specific amplification products for each of the respective species when amplified, along with other species as control, using the optimized conditions. The *P.amarus*-specific primer pair generated a single fragment of 1150 bp with DNA sample of *P. amarus* and no amplification product with other species amplified simultaneously thus validating specificity (**Fig. 3A**). The *P. fraternus* specific primer pair generated a fragment of about 317 bp with the DNA sample of *P. fraternus* and a fragment of low intensity of about 500 bp with the DNA sample of *P. urinaria* and no fragments

Table 2 Specific unique fragments generated by the primes and genebank accession numbers of end sequences

Species	Species-specific SCAR marker and fragment size in bp	primer amplifying marker	Restriction enzyme in the primer	Genebank accession numbers (number of bases from the end)	
				From 5′end	From 3′end
P. amarus	GRB/EA$_{1150}$	MAP 10	*Eco* RI	EI367000 (248 bases)	EI367027 (252 bases)
P. fraternus	GRB/EF$_{317}$	MAP 10	*Eco* RI	EI367008 (247 bases)	EI367035 (248 bases)
P. debilis	GRB/BD$_{980}$	MAP 09	*Bam* HI	EI366953 (246 bases)	EI366979 (247 bases)
P. urinaria	GRB/BU$_{550}$	MAP 09	*Bam* HI	EI366960 (246 bases)	EI366986 (248 bases)

Table 3 Species-specific primers synthesized to generate SCAR markers

S. No.	Species	Primer sequences	Tm (°C)	% GC content
1	*P. amarus*	Forward primer: 5′A**GAATTCCG**TATCTTCGTATACGTCATGA 3′	55.8	34.5
		Reverse primer: 5′A**GAATTCCG**TTCAAGCACAGCGGAAGAAG 3′	61.4	48.3
2	*P. fraternus*	Forward primer: 5′A**GAATTCCG**TGTTCTCGTTGAGCAAGGAT 3′	60.0	44.8
		Reverse primer: 5′T**GAATTCCG**ATAGCCCAAAACGCAAAACA 3′	58.6	41.4
3	*P. debilis*	Forward primer 5′T**GGATCCGC**ATAGAAATTCAGGAACTAGG 3′	60.0	44.8
		Reverse primer 5′T**GGATCCGC**GGACAACCAATGAGGGACGG 3′	67.1	62.1
4	*P. urinaria*	Forward primer 5′T**GGATCCGC**AAAGTGAGAAAATACATATC 3′	57.2	37.9
		Reverse primer 5′T**GGATCCGC**TAGCAAGAAATTATAGCACA 3′	58.6	41.4

Table 4 Optimized conditions for amplification of SCAR markers with primer pairs designed for *Phyllanthus* spp

Name of the Species	Step I		Step II						No. of cycles	Step III	
	Initial Denaturation		Denaturation		Annealing		Extension			Final Extension	
	Temp (°C)	Time (min)	Temp (°C)	Time (min)	Temp (°C)	Time (min)	Temp (°C)	Time (min)		Temp (°C)	Time (min)
P. amarus	94	5	94	1	65	1	72	2	40	72	5
P. fraternus	94	5	94	1	55	1	72	1	40	72	5
P. debilis	94	5	94	1	60	1	72	1.5	40	72	5
P. urinaria	94	5	94	1	55	1	72	1	40	72	5

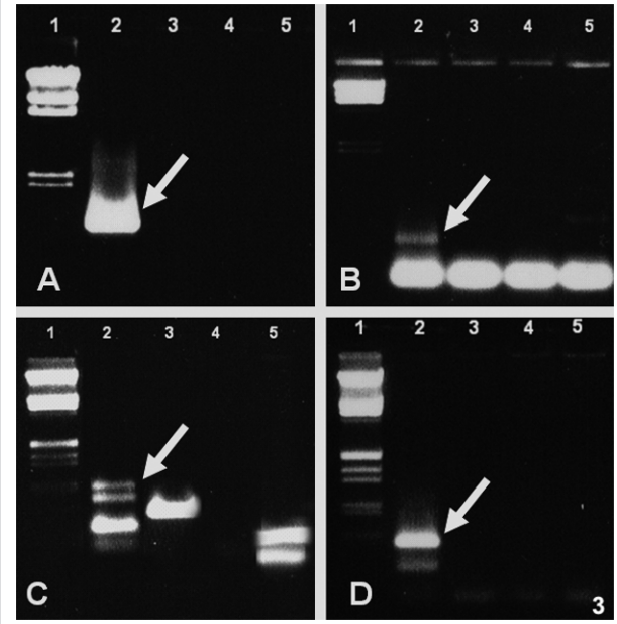

Fig. 3 Species-specific SCAR markers in *Phyllanthus.* The arrows indicate species-specific fragments retrieved as SCAR markers after amplification from different species. **A** *Phyllanthus amarus*-specific SCAR marker. Lane 1: DNA Marker λ Hind III digest; Lane 2: DNA fragment generated through *P. amarus*-specific SCAR primer pair (indicated with arrow, 1150 bp); Lane 3 to 5: Amplification of *P. fraternus*, *P debilis* and *P. urinaria* with *P. amarus*-specific primer pair. **B** *Phyllanthus fraternus*-specific SCAR marker. Lane 1: DNA Marker λ Hind III digest; Lane 2: DNA fragment generated through *P. fraternus*-specific SCAR primer pair (indicated with arrow, 317 bp); Lane 3 to 5: Amplification of *P. amarus*, *P debilis* and *P. urinaria* with *P. fraternus*-specific primer pair. **C** *Phyllanthus debilis* specific SCAR marker. Lane 1: DNA Marker λ Hind III + EcoRI digest; Lane 2: DNA fragment generated through *P. debilis*-specific SCAR primer pair (indicated with arrow, 980 bp); Lane 3 to 5: Amplification of *P. amarus*, *P fraternus* and *P. urinaria* with *P. debilis* specific primer pair. **D** *Phyllanthus urinaria*-specific SCAR marker. Lane 1: DNA Marker λ Hind III + EcoRI digest; Lane 2: DNA fragment generated through *P. urinaria*-specific SCAR primer pair (indicated with arrow, 550 bp); Lane 3 to 5: Amplification of *P. amarus*, *P. fraternus* and *P. debilis* with *P. urinaria* specific primer pair.

with the DNA samples of the other species used as control (**Fig. 3B**). The primer pair specific to *P. debilis* generated a fragment of 980 bp with the DNA sample of *P. debilis. P. debilis* DNA also produced two shorter fragments of 564 bp and 125 bp. This primer pair also generated a fragment of 125 bp in *P. urinaria* in addition to *P. debilis,* whereas a heavy fragment was obtained with *P. amarus* DNA (about 500 bp) (**Fig. 3C**). The primer pair specific to *P. urinaria* amplified two fragments of about 550 bp and 200 bp with the DNA sample of *P. urinaria* and no fragments with the DNA samples of the other species (**Fig. 3D**).

Discussion

As most of the species of the subsection *Swartziani* in the section *Phyllanthus* grow sympatrically in India, morphological identification is obviously difficult [21]. The confusion connected to the name *P. niruri* is due to Linneaus's inclusion of synonyms which actually belong to different species [22]. Concerning *P. niruri*, the broad concept adopted by Linneaus has led subsequent botanists to place at least a dozen different herbaceous species of *Phyllanthus* under this one name. According to Webster [22], true *P. niruri* is native and restricted to the "New World" and all the "Old World" records of *P. niruri* must be referred to as *P. amarus*. Webster [4] and Mitra and Jain [5] showed that *P. niruri* L. of Hooker is actually represented by 3 different species – *P. amarus* Schum. and Thonn., *P. fraternus* Webster and *P. debilis* Klein ex Willd. These species were therefore said to collectively form the "niruri complex". *P. urinaria*, although belonging to a different section, grows sympatrically to the other 3 species of the "niruri complex". *P. fraternus* represents one of the less common species of the complex. *P. amarus* is recorded in few publications as a distinct species but none of them indicated *P. amarus* as the most common representative of the *P. niruri* complex [5]. This plant is used for traditional and herbal remedies all over the world. Also, the crude drug (dried leaves) is exported from India. Hence, attempts were made to differentiate them through species specific molecular markers – SCARs (sequence characterized amplified regions) conclusively to ascertain the presence of species in the crude drug.

The monomorphic fragment common to all accessions but unique to the species concerned was selected to develop the marker. Simultaneously, the fragment was ensured to be polymorphic compared to other species. This was ascertained through intra-specific RAPD analysis for all four species with the primers (10 random accessions from each species). Species-specific primer pairs were synthesized for all species-specific fragments, taking into account their end sequences, for further amplification towards marker development. The properties of the sequences of the fragments were also taken into consideration while selecting the RAPD marker to be converted into a SCAR marker.

A comparison of RAPD and SCAR markers showed some differences between the profiles, with RAPD showing a dominant single band while SCAR had two (*P. urinaria*) or three bands (*P. debilis*). In an individual PCR experiment, the SCAR is amplified from both parental chromosomes so that if the amplified regions differ in length between the two copies (e.g., because of the presence of repetitive DNA in one of them) heterozygosity may be manifested by the SCAR marker and thus it behaves as codominant. By amplifying alleles in the basic population, it is possible to convert a dominant RAPD locus into a co-dominant SCAR marker [7]. Kim et al. [23] and Lee et al. [24] while working on SCAR markers in *Pyrus* sps reported dominant single fragments in RAPD while SCAR has one or two fragments leading to the conclusion that the fragments amplified with specific primers used in the investigation may be located in different alleles. Accordingly, the size difference in the amplified fragments could be used as identification marker for cultivars. These studies indicate that the fragments amplified by *P. urinaria* and *P. debilis* specific SCAR primer pairs in the present study may be from different alleles. While SCARs share the advantages of STSs (sequence tagged sites) [25], they are distinct from the latter in two aspects. SCARs are primarily defined genetically. Therefore, they can be used not only as physical landmarks in the genome but also as genetic markers. In addition, SCARs may contain repetitive DNA sequences within the amplified fragment as they are analyzed by PCR only; their uniqueness is determined by the sequence and spacing of the primer sequences, rather than by hybridization.

The amplification products obtained by SCAR primers were analyzed through BLAST [24] to generate further information on the amplified products. Thus, SCAR primers can also provide useful

information related to the sequence organization at a RAPD locus [26] and may help in molecular characterization. In the present analysis, the SCAR marker sequences of *P. amarus, P. fraternus* and *P. urinaria* showed no significant homology (E value = 3.1 – 0.013) with any of the sequences of the existing online database. So, all these sequences can be regarded as unique or unknown sequences, whereas the SCAR marker sequence of *P. debilis* exhibited significant homology with the chloroplast sequence of several plants in the database. All specific fragments retrieved from the gel showed similar sequences to the initially isolated RAPD fragments indicating that the primers designed are able to amplify the same loci from different species.

The trade of *Phyllanthus* material as bulk herb is widespread but as the species demonstrates differential activities incorrect identification of the species will lead to ineffective use. To address this concern, we sought a reproducible marker for differentiating the species for correct identification. This will compliment the morphological analysis used to determine the identity of intact plants before being taken for further research as well as permit detection of the species in bulk herbs where intentional or unintentional mixing occurs. The retrieval of the SCAR markers with same sizes and sequences from the random mixtures of accessions and the uniqueness in sequences indicates that the marker can also be used to study the occurrence of natural hybridization leading to morphological intermediates and ultimately evolution of different species.

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