The herbs of genus Phyllanthus (Phyllanthaceae) are well known for their medicinal properties and contribution to indigenous remedies (Calixto et al., 1998). The green medicines produced from these herbs are used in many parts of the world. In India, they also form an important component of Ayurveda (an Indian System of Medicine). Three of the commonly used Phyllanthus herbs in preparation of herbal medicines are P. amarus, P. fraternus and P. urinaria. P. amarus has several phytocomponents that render the plant with hepatoprotective, anticancer, antifungal, antioxidant, anti-inflammatory, anti-aging, antiamnestic and antiviral properties (reviewed by Patel et al., 2011). The plant extract of P. fraternus is used for treating biliary and urinary problems, bacterial and viral infections, hepatitis, common cold, venereal diseases, diabetes, dyspepsia, malaria and dysentery (Nishiura et al., 2004); P. urinaria contains phytochemicals which possess anti-inflammatory, antioxidant, anti-aging, antiviral, anti-angiogenic, anticancer and analgesic properties (Yang et al., 2007; Fang et al., 2008; Tang et al., 2010; Huang et al., 2011). Although all three species are used in preparation of herbal medicines, these herbs differ in their pharmaceutical characteristics (Jain et al., 2008), use of molecular tools (in laboratories where the samples are further processed) along with morpho-taxonomic analysis can confirm correct identification of herbs. Also, before carrying out scientific investigations and clinical studies, the correct identification of the species is essential. Nuclear markers have been used to distinguish some tree, shrub or herb species of Phyllanthus (Bandhopadhyay and Raychaudhuri, 2010; Jain et al., 2008; Manissoon et al., 2010; Li et al., 2013). The only study where chloroplast DNA (cpDNA) has been used for identification, is the analysis of sequence variations of psbA-trnH region in some species of Phyllanthus (Srirama et al., 2010). To the best of our knowledge, there are no other studies where cpDNA markers have been used for identification of medicinal herbs of Phyllanthus sp. The present investigation is the first study where, universal primers of cpDNA have been used for PCR–RFLP technique (Polymerase Chain Reaction–Restriction Fragment Length Polymorphism) technique, to analyze polymorphisms in six chloroplast genome regions (approx. 16.4 kb) of P. amarus, P. fraternus and P. urinaria and consequently distinguish them.

1. Introduction

The herbs of genus Phyllanthus (Phyllanthaceae) are well known for their medicinal properties and contribution to indigenous remedies (Calixto et al., 1998). The green medicines produced from these herbs are used in many parts of the world. In India, they also form an important component of Ayurveda (an Indian System of Medicine). Three of the commonly used Phyllanthus herbs in preparation of herbal medicines are P. amarus, P. fraternus and P. urinaria. P. amarus has several phytocomponents that render the plant with hepatoprotective, anticancer, antifungal, antioxidant, anti-inflammatory, anti-aging, antiamnestic and antiviral properties (reviewed by Patel et al., 2011). The plant extract of P. fraternus is used for treating biliary and urinary problems, bacterial and viral infections, hepatitis, common cold, venereal diseases, diabetes, dyspepsia, malaria and dysentery (Nishiura et al., 2004); P. urinaria contains phytochemicals which possess anti-inflammatory, antioxidant, anti-aging, antiviral, anti-angiogenic, anticancer and analgesic properties (Yang et al., 2007; Fang et al., 2008; Tang et al., 2010; Huang et al., 2011). Although all three species are used in preparation of herbal medicines, these herbs differ in their pharmaceutical activities (Srirama et al., 2010). This implies that any mixing of species during collection, can result in herbal preparations with lower efficacy or even adverse effects (Wieniawski, 2001).

P. amarus, P. fraternus and P. urinaria are similar in their morphology and plant habit. In many parts of the Asian continent, they normally grow together and are collected by local laborers, who are unable to distinguish them morphologically; thus resulting in undesirable admixtures (Rao, 2011; Srirama et al., 2010). However, since these herbs collected for raw drug trade, retain some of their original morphological characteristics (Jain et al., 2008), use of molecular tools (in laboratories where the samples are further processed) along with morpho-taxonomic analysis can confirm correct identification of herbs. Also, before carrying out scientific investigations and clinical studies, the correct identification of the species is essential.

Nuclear markers have been used to distinguish some tree, shrub or herb species of Phyllanthus (Bandhopadhyay and Raychaudhuri, 2010; Jain et al., 2008; Manissoon et al., 2010; Li et al., 2013). The only study where chloroplast DNA (cpDNA) has been used for identification, is the analysis of sequence variations of psbA-trnH region in some species of Phyllanthus (Srirama et al., 2010). To the best of our knowledge, there are no other studies where cpDNA markers have been used for identification of medicinal herbs of Phyllanthus sp. The present investigation is the first study where, universal primers of cpDNA have been used for PCR–RFLP technique (Polymerase Chain Reaction–Restriction Fragment Length Polymorphism) technique, to analyze polymorphisms in six chloroplast genome regions (approx. 16.4 kb) of P. amarus, P. fraternus and P. urinaria and consequently distinguish them.

2. Materials and methods

2.1. Plant material

Leaf material of P. amarus, P. fraternus and P. urinaria were sampled from eight different locations of Northern India (Table S1). The three
species were morphologically identified according to Chaudhary and Rao (2002). Fresh leaves of five individuals of each species from each collection site were frozen and stored at −80 °C till DNA extraction.

2.2. DNA extraction, amplification and digestion

Leaves of each species were used for total genomic DNA extraction (Torres et al., 1993), subsequently quantified and working dilutions of 5 ng/μL were made.

Six pairs of universal cpDNA primers (CD, DT, FV, K1K2, TF, VL; described in Dumolin-Lapegue et al., 1997) were used for PCR amplification. The cpDNA primers are universal as their sequences are conserved over a wide range of plant taxa and therefore can be used to amplify cpDNA regions from a large variety of plants (Dumolin-Lapegue et al., 1997). The amplification was carried out in 30 μL of reaction mixture containing 0.2 μM of each primer, 200 μM of each of the four dNTPs, 2 mM MgCl₂, 1.0 U of Taq DNA polymerase in 1X buffer and 15 ng of genomic DNA. The PCR was set with an initial cycle of 4 min at 94 °C, followed by 30 cycles of 45 s at 94 °C, 45 s at 50–54 °C, 2.0 min–4 min and 30 s at 72 °C, and finally 10 min extension at 72 °C (Table S2). PCR products were run on 1.2% agarose gel in 1X TBE buffer, along with 1 kilobase (kb) ladder as molecular size marker. Amplified products (10 μl per reaction) were digested with the restriction enzymes, Hinfl (2 U/reaction) and TaqI (5 U/reaction). The digestion mixture (20 μl) was incubated for 18 h. Restriction products were separated on 2.4% agarose gels, run at 3 V/cm for 3 h with 100-base pair (bp) and 50 bp ladders, as molecular size markers. All the gels were stained with ethidium bromide, photographed and documented using Gel Doc XR+ (BioRad) with Image Lab™ software.

3. Results

Six regions of chloroplast genome of each species (P. amarus, P. fraternus and P. urinaria) were amplified using six pairs of universal cpDNA primers (CD, DT, FV, K1K2, TF and VL). The size of the amplified fragments with the primers DT, FV, K1K2 and VL were approximately 1900 bp, 3700 bp, 2800 bp and 3900 bp respectively, in the three species (Fig. 1A). Amplification with primer pair CD resulted in a 2000 bp fragment in P. amarus and P. fraternus and 2400 bp in P. urinaria and TF amplified 1750 bp fragment in P. amarus and P. fraternus and 1700 bp in P. urinaria (Fig. 1A). The total size of amplified cpDNA regions analyzed was 16.4 kb (approx). PCR–RFLP technique with the 12 combinations (i.e. 6 primer pairs × 2 restriction enzymes), resulted in distinct restriction patterns in P. amarus, P. fraternus and P. urinaria (Fig. 1B, S1, S2). However, fragments obtained with TF-TaqI could not be clearly resolved and therefore not included in the analyses. All combinations in the three Phyllanthus species resulted in fragments below 1.0 kb except FV-Hinfl and VL-Hinfl which showed fragments of approximately 1.4 kb and 2.0 kb respectively, in addition to many smaller (i.e. <1 kb) fragments.

Fig. 1. PCR amplification (A) and restriction patterns of chloroplast DNA regions (B) of Phyllanthus amarus, P. fraternus, P. urinaria. A: PCR fragments obtained for six chloroplast DNA regions in P. urinaria (Pu), P. amarus (Pa), and P. fraternus (Pf). Lanes: 1 to 3: VL region; 4 to 6: FV region; 7 to 9: TF region; 10 to 12: DT region; 13 to 15: CD region; 16 to 18: K1K2 region. M: 1 kb-Ladder molecular size marker; arrow indicates 3 kb band. B: Restriction patterns of six chloroplast DNA regions in P. urinaria (Pu), P. amarus (Pa), and P. fraternus (Pf). Lanes: 1 to 3: VL-Hinfl combination; 4 to 6: FV-Hinfl combination; 7 to 9: TF-Hinfl combination; 10 to 12: DT-Hinfl combination; 13 to 15: CD-Hinfl combination; 16 to 18: K1K2-Hinfl combination. M: 100 bp-Ladder (left) and 50 bp-Ladder (right) molecular size markers; arrows indicate 500 bp band.
All fragments between 1 kb and 100 bp were scored for analysis of polymorphisms. Of the total scorable bands, 69% of bands were polymorphic. Of the 44 polymorphic fragments, 16 fragments showed distinct fragment size for each of the three Phyllanthus species (Table 1).

4. Discussion

PCR–RFLP using universal primers of cpDNA is a fast, easy to perform and reproducible technique (Mondini et al., 2009). In the present investigation, PCR–RFLP of six cpDNA regions revealed distinct restriction patterns in the three Phyllanthus species with indistinguishable morphological features. Considering the individual polymorphic fragments in each primer pair-restriction enzyme combination (listed in Table 1), except the polymorphic fragments of K1K2-Hinfl and K1K2-TaqI, all other combinations produced one or more polymorphic fragments that could distinguish all three Phyllanthus species simultaneously. Maximum number (six) of polymorphic fragments was observed in the combinations DT-Hinfl and TF-Hinfl and therefore, one can suggest that any one of these combinations may be tried for distinguishing Ph. amarus, P. fraternus and Ph. urinaria. For further confirmation, besides these, one may choose to consider other polymorphic fragments which produce clear, resolvable and unambiguous distinctions between the three species, eg CD-(Hinfl)I produces 490 bp in Ph. amarus, 575 bp in Ph. fraternus and 700 bp in Ph. urinaria; and (FV-Hinfl)I shows 680 bp in Ph. amarus, 440 + 240 bp in Ph. fraternus and no corresponding fragment in Ph. urinaria.

For confirming identification of Ph. amarus, Ph. debilis, Ph. fraternus and Ph. urinaria, Jain et al. (2008) developed species-specific SCAR markers. In this study, each species (i.e. Ph. amarus, Ph. fraternus and Ph. urinaria) required a different SCAR/primer pair sequence for confirming the identification of that species. However, PCR–RFLP with any one of the primer pair-restriction enzyme combinations (used in the present study) can distinguish/confirn the identity of all three species in the same experiment. For identification of some Phyllanthus herbs from Southern India, sequencing of psbA-trnH segment of cpDNA has proved to be successful (Srimala et al., 2010). Our study encompasses a larger genome size (approx. 16.4 kb) and therefore instead of sequencing, we have taken advantage of indels and single nucleotide polymorphisms at the restriction sites of six cpDNA regions, to show distinct restriction patterns for Ph. amarus, Ph. fraternus and Ph. urinaria.

To conclude, we believe that present study, using PCR–RFLP of cpDNA regions with the mentioned primer pair-restriction enzyme combinations, will be beneficial at the base level i.e. correct identification of Ph. amarus, Ph. fraternus and Ph. urinaria before using them for ethnomedical research, clinical investigations, raw drug trade and consequently for manufacture of herbal drugs. The study also adds to the knowledge of application of molecular tools available for analysis/identification of Phyllanthus sp. and can be experimented for use in other medicinal/economically important plants.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.sajb.2013.09.011.

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