# Assessing species admixtures in raw drug trade of *Phyllanthus,* a hepato-protective plant using molecular tools

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## ARTICLE INFO

## ABSTRACT

*Ethnopharmacological relevance: Phyllanthus* (Euphorbiaceae) species are well known for their hepato-protective activity and are used in several ethno-medicines in indigenous health care systems in India.
*Aim of the study:* To assess species admixtures in raw drug trade of *Phyllanthus* using morphological and DNA barcoding tools.
*Materials and methods:* Samples of *Phyllanthus* used in raw drug trade were obtained from 25 shops in southern India. Species admixtures in the samples were assessed by identifying species using morpho-taxonomic keys. These identities were further validated by developing species specific DNA barcode signatures using the chloroplast DNA region, *psbA-trnH*. DNA from the market samples were extracted and amplified using the forward (*psbA*F – GTTATGCATGAACGTAATGCTC) and reverse primer (*trnH*R – CGCGCATGGTGGATTCACAAATC). The amplified products were sequenced at Chromous Biotech India, Bangalore. The sequences were manually edited using Chromas Lite. Species identities were established by constructing a neighbor-joining tree using MEGA V 4.0.
*Results:* Morphological analysis of market samples revealed six different species of *Phyllanthus* in the trade samples. Seventy-six percent of the market samples contained *Phyllanthus amarus* as the predominant species (>95%) and thus were devoid of admixtures. The remaining 24% of the shops had five different species of *Phyllanthus* namely *Phyllanthus debilis, Phyllanthus fraternus, Phyllanthus urinaria, Phyllanthus maderaspatensis,* and *Phyllanthus kozhikodianus.* All identities, except those for *Phyllanthus fraternus*, were further confirmed by the species specific DNA barcode using chloroplast region *psbA-trnH*.
*Conclusion:* Our results show that market samples of *Phyllanthus* sold in southern India contain at least six different species, though among them, *Phyllanthus amarus* is predominant. DNA barcode, *psbA-trnH* region of the chloroplast can effectively discriminate *Phyllanthus* species and hence can be used to resolve species admixtures in the raw drug trade of *Phyllanthus*.

## 1. Introduction

A common problem with raw drug trade has been the admixtures with morphologically allied and geographically co-occurring species (Bisset, 1984; Khatoon et al., 2006; Mitra and Kannan, 2007; Nair et al., 1983; Sunita, 1992; Ved and Goraya, 2008). In India and other south Asian countries, over 80% of the medicinal plants for raw drug trade are predominantly collected from the wild, by local farmers or collectors who often rely only on their experience in identifying the species being collected (Vinay, 1996; Menon, 2003). Services of specialists like taxonomists are rarely availed for authentication (Anon., 2002). Thus, it is not uncommon to find admixtures of related/allied species and infrequently also of other unrelated genera. Among the reasons attributed for species admixtures are the apparent confusion in vernacular names between indigenous systems of medicine and local dialects, non-availability of authentic plant, similarity in morphological features, etc. (Mitra and Kannan, 2007). The possibility of admixtures is

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particularly high when the species in question co-occurs with morphologically similar species. Frequently, admixtures could also be deliberate due to adulteration (Mitra and Kannan, 2007). The consequences of species admixtures can range from reducing the efficacy of the drug to lowering the trade value (Wieniawski, 2001), besides threatening the safety of herbal medicines (Song et al., 2009).

In India, *Phyllanthus* (Euphorbiaceae) constitutes one of the most important groups of species traded as raw herbal drug (Ved and Goraya, 2008). These species are also exported in powder form for the extraction of a number of phytochemicals or for use in the preparation of traditional formulations in the treatment of liver disorders (Kamble et al., 2008). The raw herbal trade samples of *Phyllanthus* comprise 3–5 different species (Khatoon et al., 2006). Most samples contain *Phyllanthus amarus* (Keezharnelli) along with *Phyllanthus fraternus* and *Phyllanthus maderaspatensis* (Khatoon et al., 2006; Ved and Goraya, 2008). The annual volume of *Phyllanthus* trade in India is about 2000–5000 meteric tonnes (Ved and Goraya, 2008). All most all of this is sourced from the wild or natural populations of the species (Kuipers, 2003; Ved and Goraya, 2008). However, both due to a high level of morphological similarity among the co-occurring species as well as taxonomic controversies plaguing the group (Chaudhary and Rao, 2002; Ganeshaiah et al., 1998), raw drug samples often contain species admixtures (Dymock, 1883; Dymock et al., 1893; Kirtikar and Basu, 1975; Nadkarni, 1954; van Rhede, 1690). Species admixtures may have significant implications on the quality and efficacy of the eventual phytomedicine made from these mixtures (Song et al., 2009). Khatoon et al. (2006) showed that the three species of *Phyllanthus* (*Phyllanthus amarus*, *Phyllanthus fraternus* and *Phyllanthus maderaspatensis*) that are often mixed together have significantly different phytochemistry. Only one of the three species, *Phyllanthus amarus*, was found to contain phyllanthin and hypophyllanthin, the two major compounds believed to be responsible for the hepatoprotective activity (Calixto et al., 1998).

Considering the adverse consequences such species admixtures may have on the eventual drug efficacy, it is imperative that the admixtures are avoided in raw herbal trade and where existing, methods be developed to identify the admixtures. In recent years, efforts have been made to accurately identify medicinal plants used in raw drug trade to ensure the purity, quality and safety of drugs (Jayasinghe et al., 2009). Besides conventional methods including examination of wood anatomy and morpho-taxonomical keys, several-DNA-based methods have been developed for the identification of medicinal plants (Sucher and Carles, 2008). For example, a rapid detection method based on DNA sequences has been developed for identifying three *Bupleurum* species, *Bupleurum kaoi* Liu Chao et Chuang, *Bupleurum falcatum* L. and *Bupleurum chinense* DC., in the processed herbal material using *ITS* regions (Lin et al., 2008). A sequence-specific oligonucleotide probe (SSOP) array has been developed using the sequence differences between these three species for identification (Lin et al., 2008). Misra et al. (2006) developed an AFLP based detection of adulterants in crude drug preparations of the safed musli (*Chlorophytum*) complex. Jain et al. (2008) developed SCAR markers to identify three species of *Phyllanthus* used in dry leaf bulk herb trade (Jain et al., 2008). With the advent of DNA barcode tools, attempts are being made to use several candidate barcode regions to identify species. For example, the chloroplast *psbA-trnH* spacer region has been used to identify *Ephedra* species in dietary supplements (Techen et al., 2006).

In this study, we assess the extent of species admixtures in *Phyllanthus* raw drug trade in South India using both morpho-taxonomical keys and the chloroplast *psbA-trnH* spacer region. We discuss the implications of the results for raw drug trade.

## 2. Materials and methods

### 2.1. Plant materials

The genus *Phyllanthus* L. (Euphorbiaceae) is traditionally known for its medicinal value especially against liver disorders (Ved and Goraya, 2008). *Phyllanthus amarus*, a predominant species occurring in South India, has been shown to suppress the growth and replication of Hepatitis B virus (Venkateswaran et al., 1987; Thyagarajan et al., 1988; Yeh et al., 1993; Jayaram and Thyagarajan, 1996; Lee et al., 1996; Paranjape, 2001). Several species such as *Phyllanthus amarus*, *Phyllanthus fraternus* and *Phyllanthus debilis* have been reported to be extensively used for curing jaundice; *Phyllanthus urinaria* has been recommended for curing urinary tract diseases (Jain et al., 2008). Phyllanthin and hypophyllanthin, both present in *Phyllanthus amarus* have been shown to protect hepatocytes against carbon tetrachloride ($CCl_4$) and galactosamine induced cytotoxicity in rats (Syamsundar et al., 1985). In South India, about 22 herbaceous *Phyllanthus* species are distributed, mostly in moist areas, wastelands and in agricultural land. All of these species share a common vernacular name, Keezhanelli (in Tamil) and Kirunelli (in Kannada) and thus in the perception of the local collectors, these species are used interchangeably (Ved and Goraya, 2008).

### 2.2. Sampling

#### 2.2.1. Authenticated species samples

Sixteen species of *Phyllanthus* were collected from different parts of South India (Table 1). These species were authenticated using the morphological characters (Webster, 1955, 1957) by the Botanical Survey of India, Kolkata, the premier national organization for taxonomic identification and classification of plant species. For each of the species, herbarium specimens were prepared and deposited at the Herbaria of the School of Ecology and Conservation, University of Agricultural Sciences, Bangalore and at the Central National Herbarium (CAL), Botanical Survey of India, Kolkata. These samples hereafter referred to as "reference" material were used to develop species specific DNA barcode signatures. These DNA signatures were used in validating the species identities obtained from the market samples.

#### 2.2.2. Raw drug trade samples

Based on an inventory of shops and traders obtained from the FRLHT, Bangalore (http://frlht.org.in/), we short listed 25 shops in the three states of southern India viz. Karnataka, Tamil Nadu and Kerala (Fig. 1). In all these shops, *Phyllanthus* is sold under a common vernacular name, Keezhanelli or Kirunelli. About 100 g of the herb samples was purchased from each of the shops. All samples were fresh and retained most of the original features of the plants including the leaves and fruits. The total number of plants in each of the samples was counted. Based on morpho-taxonomical traits, each of the plants was then identified to the species level by two taxonomists independently. In more than 95% of the samples, the identities matched. In cases, where the identities did not match, further inspection of the material was conducted and the species identities were arrived. The percentage of each of the species in a sample was then computed.

### 2.3. DNA analysis

In the absence of a universal plant DNA barcode (unlike that in animal systems), a number of candidate gene regions have been suggested to be used as barcodes for plants, most of them located in the chloroplast genome coding regions [*accD*, *matK*, *ndhJ*, *rpoB2*, *rpoC1*, and *ycf5*, Chase et al., 2007; Lahaye et al., 2008; *rbcL*, *trnH-*

**Table 1**
List of *Phyllanthus* species occurring in South India along with their voucher number, collection locations, and Gen Bank Accession number.

| Sl no. | Species name | Voucher no. | Location | Gen Bank Accession No. |
|---|---|---|---|---|
| 1 | *Phyllanthus kozhikodianus* Sivar. & Manilal | SK124A, C,D | Kerala Forest Research Institute, Peechi, Kerala | GQ409804-6 |
| | | Ku1_c8[a] | Kulasegharam, Tamil Nadu | GU598570 |
| | | Ku1_c9[a] | Kulasegharam, Tamil Nadu | GU598571 |
| | | Ku1_c11[a] | Kulasegharam, Tamil Nadu | GU598572 |
| | | Ku1_c10[a] | Kulasegharam, Tamil Nadu | GU598578 |
| 2 | *Phyllanthus rheedi* Wight | SK117A-C | Neliyampathy, Palghat, Kerala | GQ409807-9 |
| 3 | *Phyllanthus debilis* Klein ex Willd. | SK105A | Nesari, Kolhapur, Maharashtra | GQ409810 |
| | | SK119 | Alleppey, Kerala | GQ409811 |
| | | SK122B | Ernakulam, Kerala | GQ409812 |
| | | Ku1_a1[a] | Kulasegharam, Tamil Nadu | GU598567 |
| | | Kul_c3[a] | Kulasegharam, Tamil Nadu | GU598568 |
| 4 | *Phyllanthus urinaria* L. | SK114A-C | Neliyampathy, Palghat, Kerala | GQ409813-15 |
| | | Tr1_1[a] | Trivandrum, Kerala | GU598573 |
| | | Tr1_2[a] | Trivandrum, Kerala | GU598574 |
| 5 | *Phyllanthus amarus* Schumach. | SK101A-C | Bangalore, Karnataka | GQ409816-18 |
| | | SK104A-B | Pune, Maharashtra | GQ409819-20 |
| | | M1_b1[a] | Madurai, Tamil Nadu | GU598561 |
| | | C2_1[a] | Chennai, Tamil Nadu | GU598562 |
| | | Ko1_3[a] | Kollam, Kerala | GU598563 |
| | | M3_a1[a] | Madurai, Tamil Nadu | GU598564 |
| | | B3_a1[a] | Bangalore, Karnataka | GU598565 |
| | | Th1_4[a] | Thrissur, Kerala | GU598577 |
| 6 | *Phyllanthus tenellus* Roxb. | SK116A-C | Bangalore, Karnataka | GQ409821-23 |
| 7 | *Phyllanthus reticulatus* Poir. | SK 226A, C | Bangalore, Karnataka | GU598539-40 |
| 8 | *Phyllanthus lawii* J.Graham | SK 265A, SK506 A | Coorg, Karnataka | GU598556-57 |
| 9 | *Phyllanthus rotundifolius* Klein ex Willd. | SK414A-C | Kanyakumari, Tamil Nadu | GU598548-50 |
| 10 | *Phyllanthus maderaspatensis* L. | SK112-A-C | Chennai, Tamil Nadu | GU598536-38 |
| | | S1_a3[a] | Shivagangai, Tamil Nadu | GU598575 |
| | | S1_b3[a] | Shivagangai, Tamil Nadu | GU598576 |
| 11 | *Phyllanthus missionis* Hook.f. | SK484A, B; SK 547A | Courtallum, Tamil Nadu | GU598553-55 |
| 12 | *Phyllanthus emblica* L. | SK227B | Bangalore, Karnataka | GU598547 |
| 13 | *Phyllanthus indofischeri* Bennet | SK541A-C | BRT Hills, Karnataka | GU598558-60 |
| 14 | *Phyllanthus talbotii* Sedgw. | SK554A, B | Moem, Goa | GU598551-52 |
| 15 | *Phyllanthus acidus* (L.) Skeels | SK225A-C | Bangalore, Karnataka | GU598541-43 |
| 16 | *Phyllanthus polyphyllus* Willd. | SK115A-C | Bangalore, Karnataka | GU598544-46 |
| 17 | *Phyllanthus fraternus* Webster | V4_a1[a] | Virudhunagar, Tamil Nadu | GU598566 |
| | | V4_c1[a] | Virudhunagar, Tamil Nadu | GU598569 |

[a] The trade samples of *Phyllanthus* species obtained from the market.

*psbA*, Kress and Erickson, 2008]. The region *psbA-trnH* proposed by Kress et al. (2005) and Shaw et al. (2005) is one of the variable non-coding regions of the plastid genome in angiosperms. More recently, the Consortium of Barcode of Life (CBOL) proposed the use of *rbcL+matK* and *psbA-trnH* as the standard plant barcode for land plants after the scrutiny of different chloroplast regions (CBOL, 2009).

For the purpose of this study, we shortlisted three chloroplast regions namely *matK, trnE-trnF* and *psbA-trnH* and evaluated the utility of these markers in distinguishing multiple individuals of three taxonomically authenticated species (*Phyllanthus amarus, Phyllanthus tenellus* and *Phyllanthus polyphyllus*). The percent inter- and intra-specific divergences of these species were then computed. Among the three regions, *psbA-trnH* showed a significant difference between the inter- and intra-specific divergences and thus clearly distinguished the three species of *Phyllanthus* (Table 2). For the purpose of this study, we therefore selected *psbA-trnH* as the barcode to assess the species admixtures.

*2.3.1. Genomic DNA extraction, amplification and sequencing*

DNA from the reference samples ($n = 16$ species with multiple individuals in each species) were extracted using leaves following the cTAB method (Doyle and Doyle, 1987). Briefly, 100 mg of the tissue was ground to fine powder using liquid nitrogen, in a sterile mortar and pestle. The ground tissue was extracted in 1 ml of extraction buffer (100 mM Tris–HCl, pH 8.0; 20 mM $Na_2$ EDTA; 2% (w/v) CTAB; 1.4 M NaCl) along with 10 mg of PVPP and 1% β-mercaptoethanol. The entire contents were thoroughly mixed and transferred to a centrifuge tube and incubated at 65 °C for 1 h with intermittent shaking. After incubation, it was cooled to room temperature and 0.5 ml of chloroform:isoamyl alcohol (24:1 v/v) was added and mixed gently by inverting the tubes until it formed an emulsion. The mixture was centrifuged at 12,000 rpm for 10 min and the clear aqueous phase was transferred to a new sterile tube. Centrifugation was repeated twice by adding chloroform:isoamyl alcohol (24:1 v/v) amounting to 1/7th volume of the supernatant. Finally, double the volume of chilled isopropanol was added to the supernatant. It was then subjected to centrifugation at 12,000 rpm for 10 min at room temperature. The supernatant was discarded and the pellet was washed twice with 70% ethanol and centrifuged at 12,000 rpm for 5 min. The supernatant was then drained and the pellet was dried in an oven at 37 °C. After drying, the pellet was re-suspended in 100 μl of TE buffer (10 mM Tris–HCl, pH 8.0; 1 mM $Na_2$ EDTA; 1.0 M NaCl). The RNA was removed by incubating the re-suspended pellet in RNase at 37 °C for 30 min.

The genomic DNA thus obtained was quantified using a nano-photometer and by visual inspection on 0.8% agarose gel. Working concentration of genomic DNA was prepared by diluting the stock solution to a concentration of 20 ng/μl. The components of the amplification reaction were optimized and a typical 25 μl of PCR reaction mixture comprised of 2.5 μl PCR buffer at 1× (supplied with 10× concentration) with 15 mM $MgCl_2$; 1 μl primer (5 pmol); 2.5 μl of dNTP's from 1 mM stock; 0.5 u/25 μl reaction mixture of Taq-polymerase (Sigma) (stock 3 u/μl); 2 μl template DNA with the concentration of 20 ng/μl and volume made up to 25 μl with sterile water. The thermocycler program for PCR for *psbA-trnH* (*psbA*F – GTTATGCATGAACGTAATGCTC; *trnH*R – CGCGCATGGTGGATTCA-CAAATC; Kress et al., 2005) was set for 1 min at 94 °C, followed by

**Table 2**
Percent inter- and intra-specific divergences of three *Phyllanthus* species (*Phyllanthus amarus*, *Phyllanthus tenellus* and *Phyllanthus polyphyllus*) using three chloroplast regions.

| | Sequence divergence | | |
| --- | --- | --- | --- |
| | Mean percent intra-specific divergence ± SD | Mean percent inter-specific divergence ± SD | $p$ |
| *matK* | 33.9 ± 0.3018 | 39.6 ± 0.2474 | 0.286 |
| *psbA-trnH* | 2.056 ± 0.011 | 10.715 ± 0.161 | 0.005 |
| *trnE-trnF* | 59.244 ± 0214 | 63.378 ± 0.16 | 0.271 |

40 cycles of 30 s at 94 °C, 40 s annealing at 53 °C and 40 s extension at 72 °C and a final extension cycle of 5 min at 72 °C. The amplified products were sequenced at Chromous Biotech India, Bangalore. The sequences were manually edited using Chromas Lite. A neighbor-joining tree was constructed using these sequences in MEGA V 4.0 (Tamura et al., 2007).

### 2.3.2. Validation of trade samples

For validating the identities of the trade samples, we randomly selected 18 individuals from 10 shops for which the species identity was arrived at through morpho-taxonomic identification (Table 3). Genomic DNA was extracted, amplified against chloroplast region *psbA-trnH* and sequenced as described above. The sequences thus generated were analyzed along with those of the "reference" species. A neighbor-joining tree was constructed using both the raw drug trade samples and the authenticated samples in MEGA V 4.0.

Finally the resolution of the species identities using *psbA-trnH* sequences was calculated by dividing the entire phylogenetic tree into equal longitudinal segments. The number of clades that



**Fig. 1.** Map showing the collection sites of *Phyllanthus* raw drug trade samples in South India. The names corresponding to the codes are presented in Table 4.

**Table 3**
*Phyllanthus* trade samples that were used for molecular analysis.

| Shops/species | Phyllanthus amarus | Phyllanthus debilis | Phyllanthus urinaria | Phyllanthus fraternus | Phyllanthus kozhikodianus | Phyllanthus maderaspatensis |
|---|---|---|---|---|---|---|
| Bangalore 3 | B3-a1 | | | | | |
| Thrissur 1 | Th1-4 | | | | | |
| Kollam 1 | Ko1-3 | | | | | |
| Trivandrum 1 | | | Tr1-1, Tr1-2 | | | |
| Kulasegharam 1 | | Ku1-c3, Ku1-a1 | | | Ku1-c8, Ku1-c9, Ku1-c10, Ku1-c11 | |
| Madurai 1 | M1-b1 | | | | | |
| Madurai 3 | M3-a1 | | | | | |
| Virudhunagar 4 | | | | V4-a1, V4-c1 | | |
| Shivagangai 1 | | | | | | S1-a3, S1-b3 |
| Chennai 2 | C2-1 | | | | | |

The codes indicate the shops from where the material was obtained. The numbers after the hyphen indicate sample number of plants obtained from the shop.

**Table 4**
Percentage of different *Phyllanthus* species found in raw drug trade calculated using morpho-taxonomical characters ("*n*" refers to the total number of individuals in the sample).

| Shops | Species | | | | | | |
|---|---|---|---|---|---|---|---|
| | Codes | Phyllanthus amarus | Phyllanthus debilis | Phyllanthus urinaria | Phyllanthus fraternus | Phyllanthus kozhikodianus | Phyllanthus maderaspatensis |
| Bangalore 1 ($n=87$) | B1 | 100 | 0 | 0 | 0 | 0 | 0 |
| Bangalore 2 ($n=73$) | B2 | 100 | 0 | 0 | 0 | 0 | 0 |
| Bangalore 3 ($n=50$) | B3 | 100 | 0 | 0 | 0 | 0 | 0 |
| Thrissur ($n=34$) | Th1 | 100 | 0 | 0 | 0 | 0 | 0 |
| Kollam 1 ($n=33$) | Ko1 | 100 | 0 | 0 | 0 | 0 | 0 |
| Kollam 2 ($n=27$) | Ko2 | 100 | 0 | 0 | 0 | 0 | 0 |
| Trivandrum 1 ($n=61$) | Tr1 | 0 | 0 | 100 | 0 | 0 | 0 |
| Trivandrum 2 ($n=80$) | Tr2 | 100 | 0 | 0 | 0 | 0 | 0 |
| Trivandrum 3 ($n=19$) | Tr3 | 100 | 0 | 0 | 0 | 0 | 0 |
| Kulasegharam ($n=87$) | Ku1 | 8.05 | 85.05 | 2.3 | 0 | 4.6 | 0 |
| Nagercoil ($n=56$) | N1 | 42.85 | 0 | 0 | 0 | 0 | 57.15 |
| Madurai 1($n=120$) | M1 | 100 | 0 | 0 | 0 | 0 | 0 |
| Madurai 2 ($n=81$) | M2 | 100 | 0 | 0 | 0 | 0 | 0 |
| Madurai 3 ($n=104$) | M3 | 100 | 0 | 0 | 0 | 0 | 0 |
| Virudhunagar 1 ($n=28$) | V1 | 100 | 0 | 0 | 0 | 0 | 0 |
| Virudhunagar 2 ($n=54$) | V2 | 100 | 0 | 0 | 0 | 0 | 0 |
| Virudhunagar 3 ($n=58$) | V3 | 5.17 | 0 | 0 | 94.83 | 0 | 0 |
| Virudhunagar 4 ($n=115$) | V4 | 0 | 0 | 0 | 100 | 0 | 0 |
| Shivagangai, ($n=160$) | S1 | 1.8 | 0 | 0 | 0 | 0 | 98.2 |
| Rajapalayam ($n=66$) | R1 | 100 | 0 | 0 | 0 | 0 | 0 |
| Kayattar ($n=60$) | Ka1 | 98.3 | 0 | 0 | 0 | 0 | 1.7 |
| Chennai 1 ($n=63$) | C1 | 100 | 0 | 0 | 0 | 0 | 0 |
| Chennai 2 ($n=25$) | C2 | 100 | 0 | 0 | 0 | 0 | 0 |
| Chennai 3($n=50$) | C3 | 100 | 0 | 0 | 0 | 0 | 0 |
| Chennai 4 ($n=83$) | C4 | 100 | 0 | 0 | 0 | 0 | 0 |

resolved in these segments (in the increasing order from left to right) was identified and converted to percent resolution of different species of *Phyllanthus*.

## 3. Results and discussion

Morphological analysis indicated six different species of *Phyllanthus* in the trade samples (Table 4). Seventy-six percent of the shops ($n=19$) contained *Phyllanthus amarus* as the predominant species (>95%) and thus were devoid of admixtures. The remaining 24% ($n=6$) of the shops had five different species of *Phyllanthus*, namely *Phyllanthus debilis, Phyllanthus fraternus, Phyllanthus urinaria, Phyllanthus maderaspatensis, Phyllanthus kozhikodianus*. However here again, the shops had these species in nearly pure form with little admixtures (Table 4). With the lone exception of the shop at Kulashegharam, in which four species (*Phyllanthus amarus, Phyllanthus debilis, Phyllanthus urinaria, Phyllanthus kozhikodianus*) were recovered, two species were recovered from four shops; the rest of the shops ($n=20$) had only one species. Interestingly, in a couple of regions, neighboring shops had entirely different species. For example, while Trivandrum 2 and Trivandrum 3 had *Phyl-lanthus amarus*, a neighboring shop Trivadrum 1 had *Phyllanthus urinaria*. Similarly, while Virudhunagar 1 and Virudhunagar 2 had *Phyllanthus amarus*, the neighboring shops, Virudhunagar 3 and Virudhunagar 4 had *Phyllanthus fraternus*. Assuming that the material stocked in the shops is normally obtained from a single source or from collectors of a specific region, the above results appear perplexing. Enquiries with the traders indicated that the supplies are made usually by wholesale agents who in turn obtain them from sub-agents and collectors. It is likely that the collectors source the material from a large area and thus inadvertently collect more than one species. The predominance of *Phyllanthus amarus* in over 75% of the shop samples reflects the underlying distribution of the species in peninsular India. *Phyllanthus amarus* is widely distributed in India compared to other species such as *Phyllanthus debilis, Phyllanthus urinaria, Phyllanthus fraternus* and *Phyllanthus kozhikodianus* that are more restricted in their distribution.

The *psbA-trnH* region effectively discriminated all of the 16 reference species of *Phyllanthus*. Using these species specific DNA barcode signatures, we further validated the trade samples. The bar code separated all the trade samples into clear clades cor-
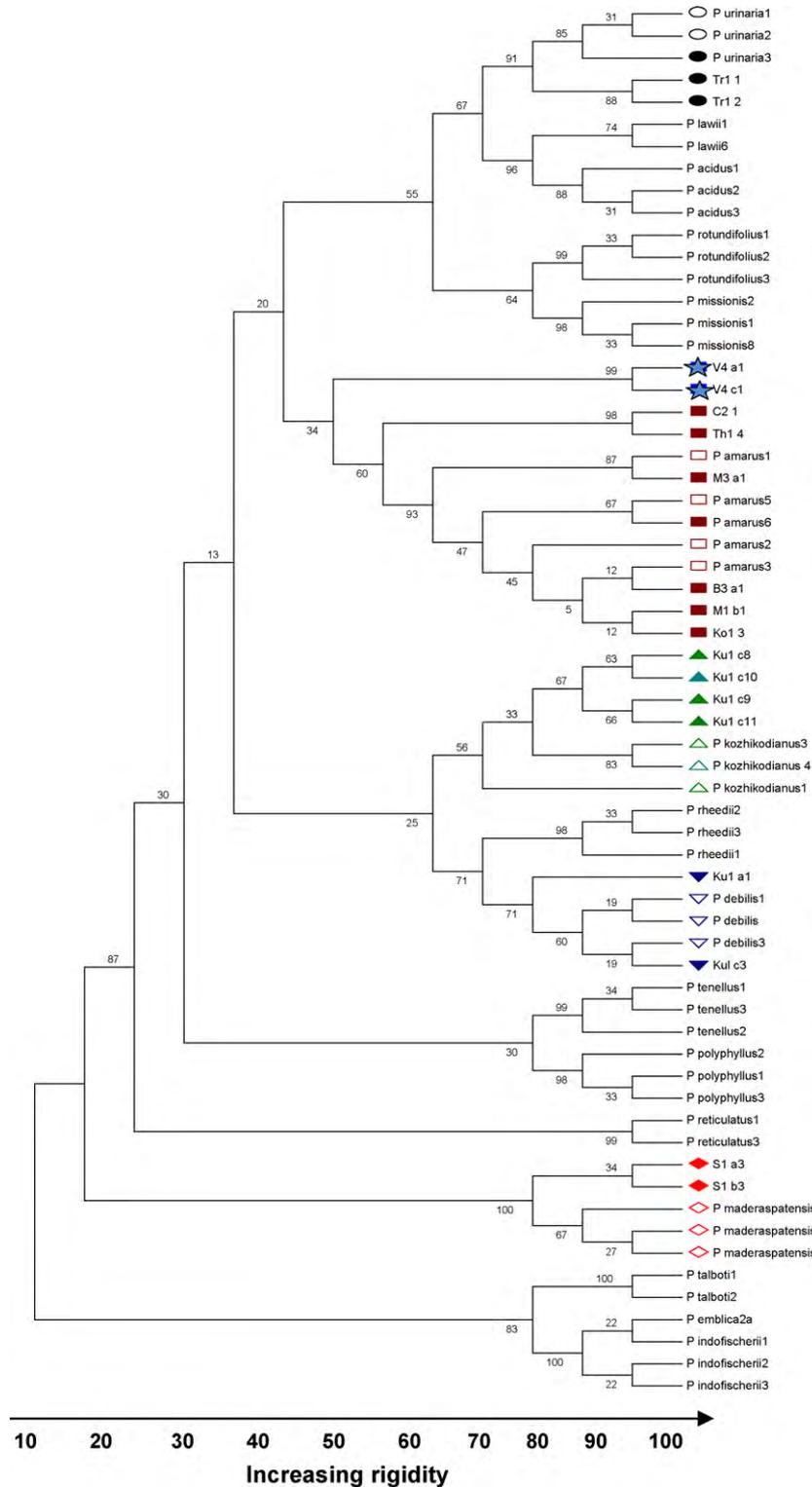
**Fig. 2.** Neighbor-joining tree of reference *Phyllanthus* species and raw drug trade samples (solid symbols) using *psbA-trnH*. Open symbols refer to the reference species corresponding to the species identified in trade. Codes for the trade samples are as presented in Table 3. The numbers at the nodes refer to the bootstrap values.

responding to the respective reference species. The phylogenetic tree shows a clear clustering of individuals of different species obtained from trade samples with those of the reference species (Fig. 2). Thus of the 18 individuals, 16 could be categorized into five distinct species (*Phyllanthus amarus* Schumach., *Phyllanthus kozhikodianus* Sivar. and Manilal, *Phyllanthus urinaria* L., *Phyllanthus tenellus* Roxb., *Phyllanthus maderaspatensis* L.). In all of these 16

representative samples, the DNA barcode based species assignation matched that deduced through morpho-taxonomic analysis. V4-a1 and V4-c1 that were taxonomically identified as *Phyllanthus fraternus* could not be verified because of lack of authenticated sample of this species. These results demonstrate that *Phyllanthus* species can be effectively discriminated using molecular data. Molecular analysis can effectively discriminate different species in trade samples

to an extent of ~80% even when the species taxonomic identity is not known.

Notwithstanding the lack of a global consensus on a universal bar code for plants and the current imbroglio on the nature of the barcode, several workers have repeatedly shown that the chloroplastic region, *psbA-trnH* spacer sequences might be one of the more preferred candidates for species identification (Kress et al., 2005; Shaw et al., 2005). In a comparative study of seven loci, Pennisi (2007) found that the discriminatory power of *psbA-trnH* was the highest (69%). More recently, Song et al. (2009) successfully demonstrated the utility of this region in discriminating medicinal plants of the family Polygonaceae. In our own study, we found that *psbA-trnH* can successfully discriminate 16 of the *Phyllanthus* species analyzed so far and can be used to discriminate species in trade. Thus while the search for an universal barcode(s) for plants is still on, we argue that candidate regions like *psbA-trnH* can be used for identification and authentication of specific taxa, as demonstrated in this study. The major challenge of using the DNA-based identification and authentication of plants would be to deal with dry plant samples and their constituent parts that are normally encountered in raw drug trade. A further challenge would be to use this technique in identifying species admixtures that are in tissue powder form. Under these circumstances, rarely is conventional taxonomical diagnosis possible. Success of DNA-based authentication in dry samples, be it in intact plants or tissue powder forms would lie in standardizing DNA extraction protocols as well as in obtaining good amplification. We are currently exploring to extend the technique for identifying species admixtures in dry powder form as well.

In summary, and contrary to the general perception, our results show surprisingly little admixtures of *Phyllanthus* species in raw drug trade. This is interesting considering that many of the species are morphologically similar in appearance, co-occur, share a common vernacular name, and in certain cases are riddled with taxonomic problems.

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## References

Anon., 2002. Demand study for selected medicinal plants. A Report Prepared for the Ministry of Health and Family Welfare, Govt. of India, Department of Indian Systems of Medicine and Homeopathy and World Health Organization, vol. 1. Centre for Research, Planning and Action, New Delhi.

Bisset, W.G., 1984. Herbal Drugs and Phytopharmaceuticals. CRC Press, London.

Calixto, J.B., Santos, A.R.S., Filho, V.C., Yunes, R.A., 1998. A review of the plants of the genus *Phyllanthus*: their chemistry, pharmacology, and therapeutic potential. Medical Research Reviews 18, 225–258.

CBOL, 2009. A DNA barcode for land plants. Proceedings of the National Academy of Sciences USA 106, 12794–12797.

Chase, M.W., Cowan, R.S., Hollingsworth, P.M., Van den Berg, C., Madriñán, S., Petersen, G., Seberg, O., Jørgsensen, T., Cameron, K.M., Carine, M., Pedersen, N., Hedderson, T.A.J., Conrad, F., Salazar, G.A., Richardson, J.E., Hollingsworth, M.L., Barraclough, T.G., Kelly, L., Wilkinson, M., 2007. A proposal for a standardised protocol to barcode all land plants. Taxon 56, 295–299.

Chaudhary, L.B., Rao, R.R., 2002. Taxonomic study of herbaceous species of *Phyllanthus* L. (Euphorbiaceae) in India. Phytotaxonomy 2, 143–162.

Doyle, J.J., Doyle, J.L., 1987. A rapid DNA isolation procedure for small quantities of fresh leaf tissue. Phytochemistry Bulletin 19, 11–15.

Dymock, W., 1883. The Vegetable Materia Medica of Western India. Education Society's Press, Bombay.

Dymock, W., Warden, C.J.H., Hooper, D., 1893. *Pharmacographia indica*: A History of the Principal Drugs of the Vegetable Origin. Kegsm Paul, Trench, Trubner & Co., Ltd., London, pp. 261–265.

Ganeshaiah, K.N., Ganesan, R., Uma Shaanker, R., Meera, C., 1998. *Phyllanthus niruri*: A Taxonomic Hurdle or Hurdled by Taxonomists. Amurth, August, 3–8.

Jain, N., Shasany, A.K., Singh, S., Khanuja, S.P.S., Kumar, S., 2008. 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. Planta Medica 74, 296–301.

Jayaram, S., Thyagarajan, S.P., 1996. Inhibition of HBs Ag secretion from Alexander cell line by *Phyllanthus amarus*. Indian Journal of Pathology and Microbiology 39, 211–215.

Jayasinghe, R., Niu, L.H., Coram, T.E., Kong, S., Kaganovitch, J., Xue, C.C.L., Li, C.G., Pang, E.C.K., 2009. Effectiveness of an innovative prototype subtracted diversity array (SDA) for fingerprinting plant species of medicinal importance. Planta Medica 75, 1180–1185.

Kamble, M.B., Dumbre, R.K., Rangari, V.D., 2008. Hepatoprotective studies of herbal formulations. International Journal of Green Pharmacy 2, 147–151.

Khatoon, S., Rai, V., Rawat, A.K.S., Mehrotra, S., 2006. Comparative pharmacognostic studies of three *Phyllanthus* species. Journal of Ethnopharmacology 104, 79–86.

Kirtikar, K.R., Basu, B.D., 1975. Indian Medicinal Plants. Bishen Singh Mahendra Pal Singh, New Connaught Place, Dehrahun.

Kress, J.W., Erickson, D.L., 2008. DNA barcodes: genes, genomics, and bioinformatics. Proceedings of the National Academy of Sciences USA 105, 2761–2762.

Kress, J.W., Wurdack, J.K., Zimmer, A.E., Weigt, A.L., Janzen, H.D., 2005. Use of DNA barcodes to identify flowering plants. Proceedings of the National Academy of Sciences USA 102, 8369–8374.

Kuipers, S.E., 2003. Trade in medicinal plants. In: Bodeker, G., Bhat, K.K.S., Burley, J., Vantomme, P. (Eds.), Medicinal Plants for Forest Conservation and Health Care, FAO, Rome, pp. 45–59.

Lahaye, R., van der Bank, M., Bogarin, D., Warner, J., Pupulin, F., Gigot, G., Maurin, O., Duthoit, S., Barraclough, T.G., Savolainen, V., 2008. DNA barcoding the floras of biodiversity hotspots. Proceedings of the National Academy of Sciences USA 105, 2923–2928.

Lee, C.D., Ott, M., Thyagarajan, S.P., Shafritz, D.A., Burk, R.D., Gupta, S., 1996. *Phyllanthus amarus* down-regulates hepatitis B virus mRNA transcription and replication. European Journal of Clinical Investigation 26, 1069–1076.

Lin, W.Y., Chen, L.R., Lin, T.Y., 2008. Rapid authentication of *Bupuleurum* species using an array of immobilized sequence-specific oligonucleotide probes. Planta Medica 74, 464–469.

Menon, P., 2003. Conservation and Consumption – A Study on the Crude Drug Trade in Threatened Medicinal Plants in Thiruvananthapuram District, Kerala. KRPLLD, CDS, Trivandrum.

Misra, A., Shasany, A.K., Shukla, A.K., Sundaresan, V., Jain, S.P., Bagchi, G.D., Singh, J., Khanuja, S.P.S., 2006. AFLP-based detection of adulterants in crude drug preparations of the 'safed musli' complex. Natural Product Communications 2, 93–97.

Mitra, S.K., Kannan, R., 2007. A Note on unintentional adulterations in ayurvedic herbs. Ethnobotanical Leaflets 11, 11–15.

Nadkarni, A.K., 1954. Dr. K.M. Nadkarni's Indian Materia Medica. Popular Book Depot, Bombay.

Nair, V.K., Yoganarasimhan, K.R., Murthy, K., Shantha, T.R., 1983. Studies on some South Indian market samples of ayurvedic drugs II. Ancient Science of Life 3, 60–66.

Paranjape, P., 2001. Indian Medicinal Plants: Forgotten Healers. Chaukhamba Sanskrit Pratisthan, Delhi, pp. 48–49.

Pennisi, E., 2007. Taxonomy. Wanted: a barcode for plants. Science 318, 190–191.

Shaw, J., Lickey, E.B., Beck, J.T., Farmer, S.B., Liu, W.S., Miller, J., Siripun, K.C., Winder, C.T., Schilling, E.E., Small, R.L., 2005. The tortoise and the hare. II. Relative utility of 21 noncoding chloroplast DNA sequences for phylogenetic analysis. American Journal of Botany 92, 142–166.

Song, J., Yao, H., Li, Y., Li, X., Lin, Y., Liu, C., Han, J., Xie, C., Chen, S., 2009. Authentication of the family polygonaceae in Chinese pharmacopoeia by DNA barcoding technique. Journal of Ethnopharmacology 124, 434–439.

Sucher, N.J., Carles, M.C., 2008. Genome-based approaches to the authentification of medicinal plants. Planta Medica 74, 603–623.

Sunita, G., 1992. Substitute and Adulterant Plants. Periodical Experts Book Agency, New Delhi.

Syamsundar, K.V., Singh, B., Thakur, R.S., Hussain, A., Kiso, Y., Hikino, H., 1985. Antihepatotoxic principles of *Phyllanthus niruri* herb. Journal of Ethnopharmacology 14, 41–44.

Tamura, K., Dudley, J., Nei, M., Kumar, S., 2007. MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. Molecular Biology and Evolution 24, 1596–1599.

Techen, N., Khan, I.A., Pan, Z., Scheffer, B.E., 2006. The use of polymerase chain reaction (PCR) for the identification of *Ephedra* DNA in dietary supplements. Planta Medica 72, 241–247.

Thyagarajan, S.P., Subramanian, S., Thirunalasundari, T., Venkateswaran, P.S., Blumberg, B.S., 1988. Preliminary study: the effect of *Phyllanthus amarus* on chronic carriers of hepatitis B virus. The Lancet 11, 764–766.

van Rhede, A., 1690. Horti Muluhorici Purs De&m de Herhis er Diversis Illurum Specienus, vol. 10. Someren, Amsterdam, pp. 29–31.

Ved, D.K., Goraya, G.S., 2008. Demand and Supply of Medicinal Plants in India. FRLHT.

Venkateswaran, P.S., Millman, I., Blumberg, B.S., 1987. Effects of an extract from *Phyllanthus niruri* on hepatitis B and woodchuck hepatitis viruses: in vitro and in vivo studies. Proceedings of the National Academy of Sciences USA 84, 274–278.

Vinay, T., 1996. Camp Workshop: Plants Under Threat – New List Forged' Medicinal Plant Conservation, vol. 2. Newsletter of the IUCN Species Survival Commission. Bundesamt für Naturschutz, Bonn, Germany.

Webster, G.L., 1955. Studies of the Euphorbiaceae, phyllanthoideae I. Taxonomic Notes on the West Indian Species of *Phyllanthus*, 176. Contributions from the Gray Herbarium of Harvard University, Cambridge, MA, pp. 45–63.

Webster, G.L., 1957. A monographic study of the West Indian species of *Phyllanthus*. Journal of the Arnold Arboretum 38, 295–373.

Wieniawski, W., 2001. Risk assessment as an element of drug control. WHO Drug Information 15, 7–11.

Yeh, S.F., Hong, C.Y., Huang, Y.L., Liu, T.Y., Choo, K.B., Chou, C.K., 1993. Effect of an extract from *Phyllanthus amarus* on hepatitis B surface antigen gene expression in human hepatoma cells. Antiviral Research 20, 185–192.