DNA FINGERPRINTING OF SELECTED THAI *PHYLLANTHUS* SPECIES BY RAPD ANALYSIS

Juthatip Manissorn[1,2], Nijsiri Ruangrungsi[2], Thatree Phadungcharoen[2], and Suchada Sukrong[1,2,*]

[1]CU-Drugs and Health Products Innovation and Promotion Center, Faculty of Pharmaceutical Sciences, Chulalongkorn University, Bangkok 10330, Thailand [2]Department of Pharmacognosy and Pharmaceutical Botany, Faculty of Pharmaceutical Sciences, Chulalongkorn University, Bangkok 10330, Thailand

ABSTRACT: Random amplified polymorphic DNA (RAPD) markers were used to discriminate and construct the genetic relationship between twelve *Phyllanthus* species (Euphorbiaceae) existing in Thailand. They are *P. amarus* Schum. & Thonn., *P. urinaria* L., *P. emblica* L., *P. taxodiifolius* Beille in Lecomte, *P. collinsiae* Craib, *P. acidus* (L.) Skeels, *P. pulcher* Wall. Ex Müll.Arg., *P. debilis* Klein ex Willd., *P. virgatus* G.Forst., *P. acutissimus* Miq., *P. ankorensis* Beille in Lecomte, and *P. reticulatus* Poir. in Lam. *Phyllanthus* species have been widely used as fruits, medicinal as well as ornamental plants. The medicinal effects of *Phyllanthus* are diverse including antipyretic, analgesic, anti-inflammatory, antihepatotoxic and antiviral activity. Nine out of eighty RAPD primers were screened for DNA polymorphism. Primer OPS-01, OPS-03, OPS-07, OPS-08, OPS-12, OPS-19, OPD-02, OPD-04, and OPD-07 produced unique DNA fingerprints that individuated the twelve *Phyllanthus* species. RAPD bands were scored and grouped by distance analysis using Dice similarity coefficient by Nei and Li. A dendrogram was generated by using an unweighted pair group method with arithmetic average (UPGMA) cluster analyses. Twelve *Phyllanthus* species are divided into 4 major groups. The RAPD result shows highly DNA polymorphism that was confirmed by 50% of Dice similarity index. Obtained fingerprints indicate the efficiency of RAPD marker for discrimination *Phyllanthus*.

Keywords: *Phyllanthus*, Euphorbiaceae, RAPD, DNA fingerprinting, DNA polymorphism

INTRODUCTION: The Genus *Phyllanthus* belongs to the Family Euphorbiaceae. It consists of 800 species growing in tropical and subtropical areas[1]. Several *Phyllanthus* species have been used for medicinal applications in many countries such as India, China, Mexico, America, Australia and Thailand[2-4]. Twelve *Phyllanthus* species, *P. amarus* Schum. & Thonn., *P. urinaria* L., *P. emblica* L., *P. taxodiifolius* Beille in Lecomte, *P. collinsiae* Craib, *P. acidus* (L.) Skeels, *P. pulcher* Wall. Ex Müll.Arg., *P. debilis* Klein ex Willd., *P. virgatus* G.Forst., *P. acutissimus* Miq., *P. ankorensis* Beille in Lecomte, and *P. reticulatus* Poir. in Lam. have been widely used in Thai folk medicine. The medicinal activities of *Phyllanthus* species are antipyretic, analgesic, antiinflammatory, anti-hepatotoxic and antiviral [5-8]. Fruits of the two well-known species, *P. acidus* L. and *P. emblica* L., contains high contents of vitamin C and have been used for relief of coughing[9]. *P. amarus* is an important herbal medicine due to its effective antiviral activities especially toward the hepatitis B virus[10-12]. The herbaceous species, *P. amarus*, *P. urinaria*, *P. debilis*, and *P. virgatus* are similar in plant habit and plant morphology. However, their pharmacological activities are different. In Thailand, *P. amarus* and *P. urinaria* are misidentified and sold in local markets under the same name.

DNA-based techniques are widely used to identify important economic plants and medicinal plants[13-18]. These techniques have been found to be useful and accurate for determination of genetic variation in plants[19]. Random amplified polymorphic DNA (RAPD) is a robust and simple assay and one of the most commonly used DNA techniques for primary assay of genomic DNA[20-22]. One of the most important applications of RAPD is the identification of plant species with no prior sequence data or clarification of the difference between species[23]. Therefore, the objectives of this study were to discriminate between twelve important *Phyllanthus* species in Thailand based on the RAPD technique. A dendrogram was also constructed to show species relationships.

*To whom correspondence should be addressed.
E-mail: suchada.su@chula.ac.th, Tel. +66 2218 8364

MATERIALS AND METHODS:

Plant samples

In this present study, fifty-one individuals from twelve *Phyllanthus* species, *Phyllanthus amarus*, *P. urinaria*, *P. emblica*, *P. taxodiifolius*, *P. collinsiae*, *P. acidus*, *P. pulcher*, *P. debilis*, *P. virgatus*, *P. acutissimus*, *P. ankorensis*, and *P. reticulatus*, were collected from various locations in Thailand. Plant specimens were identified by Assoc. Prof. Dr. Nijsiri Ruangrungsi and Assoc. Prof. Thatree Phadungcharoen. Voucher specimens are deposited at the Museum of Natural Medicines, Faculty of Pharmaceutical Sciences, Chulalongkorn University, Thailand. Plant materials are presented in Table 1.

Table 1 List of *Phyllanthus* species used for RAPD in this study

No.	Species	Provinces	Date of Collection	Voucher ID
1-1	*P. amarus* Schum. & Thonn.	Bangkok	10.2005	MJT-4810101
1-2	*P. amarus* Schum. & Thonn.	Kanchanaburi	10.2005	MJT-4810102
1-3	*P. amarus* Schum. & Thonn.	Sakon Nakhon	10.2006	MJT-4910103
1-4	*P. amarus* Schum. & Thonn.	Nakhon Phanom	10.2006	MJT-4910106
1-5	*P. amarus* Schum. & Thonn.	Nong Khai	10.2006	MJT-4910107
1-6	*P. amarus* Schum. & Thonn.	Udon Thani	10.2006	MJT-4910108
1-7	*P. amarus* Schum. & Thonn.	Khon Kaen	10.2006	MJT-4910109
1-8	*P. amarus* Schum. & Thonn.	Chiang Mai	11.2006	MJT-4911110
2-1	*P. urinaria* L.	Bangkok	10.2005	MJT-4810201
2-2	*P. urinaria* L.	Nong Khai	10.2006	MJT-4910204
2-3	*P. urinaria* L.	Nakhon Pathom	03.2007	MJT-5003205
2-4	*P. urinaria* L.	Ratchaburi	09.2007	NSR-5009206
3-1	*P. emblica* L.	Bangkok	10.2005	MJT-4810301
3-2	*P. emblica* L.	Sakon Nakhon	06.2006	MJT-4906302
3-3	*P. emblica* L.	Nakhon Phanom	10.2006	MJT-4910303
3-4	*P. emblica* L.	Nong Khai	10.2006	MJT-4910304
3-5	*P. emblica* L.	Udon Thani	10.2006	MJT-4910305
3-6	*P. emblica* L.	Kalasin	10.2006	MJT-4910306
3-7	*P. emblica* L.	Khon Kaen	10.2006	MJT-4910307
3-8	*P. emblica* L.	Chiang Mai	11.2006	MJT-4911309
4-1	*P. taxodiifolius* Beille	Bangkok	10.2005	MJT-4810401
4-2	*P. taxodiifolius* Beille	Nakhon Pathom	01.2006	MJT-4901402
4-3	*P. taxodiifolius* Beille	Prachinburi	06.2007	TRP-5006403
5-1	*P. collinsiae* Craib	Bangkok	10.2005	MJT-4810501
5-2	*P. collinsiae* Craib	Bangkok	10.2005	MJT-4810502
6-1	*P. acidus* L.	Bangkok	10.2005	MJT-4810601
6-2	*P. acidus* L.	Sakon Nakhon	06.2006	MJT-4906602
6-3	*P. acidus* L.	Nakhon Nayok	10.2006	TT-4910603
6-4	*P. acidus* L.	Chiang Rai	10.2006	NR-4910604
6-5	*P. acidus* L.	Khon Kaen	10.2006	MJT-4910611
6-6	*P. acidus* L.	Nakhon Si Thammarat	10.2006	TT-4910612
6-7	*P. acidus* L.	Satun	10.2006	TT-4910613
6-8	*P. acidus* L.	Chiang Mai	11.2006	MJT-4911615
7-1	*P. pulcher* Wall. ex Müll.Arg.	Bangkok	10.2005	MJT-4810701
7-2	*P. pulcher* Wall. ex Müll.Arg.	Kanchanaburi	10.2005	MJT-4810702
7-3	*P. pulcher* Wall. ex Müll.Arg.	Chiang Mai	11.2006	MJT-4911703
7-4	*P. pulcher* Wall. ex Müll.Arg.	Nakhon Pathom	03.2007	MJT-5003704
7-5	*P. pulcher* Wall. ex Müll.Arg.	Rayong	08.2007	NSR-5008705
8-1	*P. debilis* Klein ex Willd.	Bangkok	10.2005	NSR-4810801
8-2	*P. debilis* Klein ex Willd.	Nakhon Pathom	01.2006	MJT-4901802
9-1	*P. virgatus* G.Forst.	Bangkok	10.2005	TRP-4810901
9-2	*P. virgatus* G.Forst.	Prachinburi	01.2006	TRP-4901902
9-3	*P. virgatus* G.Forst.	Pattalung	04.2007	TRP-5004903
9-4	*P. virgatus* G.Forst.	Chachoengsao	08.2007	TRP-5008904
9-5	*P. virgatus* G.Forst.	Ratchaburi	09.2007	NSR-5009905
10-1	*P. acutissimus* Miq.	Chachoengsao	01.2006	TRP-4901101
10-2	*P. acutissimus* Miq.	Ubon Ratchathani	04.2007	TRP-5004102
11-1	*P. ankorensis* Beille in Lecomte	Nakhon Pathom	01.2006	MJT-4901111
11-2	*P. ankorensis* Beille in Lecomte	Nakhon Pathom	03.2007	NSR-5008112
12-1	*P. reticulatus* Poir.	Bangkok	02.2006	MJT-4902121
12-2	*P. reticulatus* Poir.	Nakhon Pathom	03.2007	MJT-5003124

DNA extraction and PCR amplification

Sixty milligrams of fresh weight and twenty milligrams of silica gel-dried leaves were ground in liquid nitrogen for DNA extraction. Total genomic DNA was extracted by DNeasy™ Plant Mini Kit (QIAGEN, Germany) and purified using GENECLEAN® II Kit (Qbiogene Inc., Germany). The amount of DNA that was extracted from fresh leaves of *Phyllanthus* was approximately 5-40 ng/µl, which is adequate for RAPD analysis. In addition, we employed a DNA purification step to remove a large amount of polysaccharides and alkaloids, which from some plant species, insures a high DNA quality that is considered the major factor affecting the reproducibility of RAPD. However, one obvious disadvantage of RAPD is its sensitivity to slight changes in reaction conditions. Therefore, the reaction conditions and PCR cycling conditions should be stringently controlled to obtain high reproducibility[16]. For this reason, the PCR reaction step of RAPD was first investigated and optimized since it could affect the pattern of PCR products. The concentration of magnesium chloride varied from 2 to 5 mM. The optimum magnesium concentration was 3.5 mM since it could produce clear and reproducible RAPD bands. Gradient PCR was used to investigate the suitable annealing temperature that was set up at 36°C in this study.

PCR amplification were carried out using eighty decamer oligonucleotide primers including kit D, N, O, and S were obtained from Operon DNA Technologies (Alameda, USA). The PCR reaction mixture in 25 µl contained 10 mM Tris-HCl (pH 9.0), 50 mM KCl, 0.1% Triton® X-100, 3.5 mM $MgCl_2$, 0.2 mM each dNTP, 0.4 µM random primers, 0.6 U of *Taq* Polymerase (Promega, USA) and 5-40 ng of template DNA. RAPD amplification was carried out with the Eppendorf Mastercycler® (Perkin-Elmer, USA). Initial denaturation was at 95°C for 3 min, followed by 45 cycles of 95°C for 1 min, 36°C for 1 min, 72°C for 2 min and a final extension for 4 min. PCR products were separated on a 1.5% agarose gel and stained with ethidium bromide. RAPD fragments were analyzed using Gel Doc™ XR System PC/Mac (Bio-Rad Laboratories Inc., USA).

RESULTS AND DISCUSSION:

RAPD analysis

In a preliminary screening of eighty random deca-nucleotide primers, eighteen primers could be used to amplify DNA from *Phyllanthus* species (Table 2). Only nine, OPS-01, OPS-03, OPS-07, OPS-08, OPS-12, OPS-19, OPD-02, OPD-04 and OPD-07, out of eighteen primers produced clear and reproducible polymorphic RAPD band across all twelve *Phyllanthus* species (Figure 1). From the RAPD data obtained by using different primers, the bands ranged from 0.3 to 2.5 kb in size. The largest number of RAPD bands was detected for primer OPD-07 (64 bands, Figure 1C), while the lowest was scored for OPS-08 (29 bands, Figure 1D). In addition, there is a risk of misinterpretation in a genetic analysis from different RAPD patterns with similar size. This can be minimized by the use of several RAPD primers[24]. For example, PCR amplification using primer OPS-19 (Figure 1F) showed a unique fragment approximately 590 bp in both *P. amarus* (Lane 1) and *P. debilis* (Lane 8). It was difficult to identify those two species by OPS-19. Meanwhile, amplification using OPS-03 (Figure 1B) produced four major fragments, 300, 580, 700 and 1000 bp, in *P. amarus* and three major fragments, 390, 420, and 1000 bp, in *P. debilis*, so it could be identified using this primer. One individual representative of each species was selected for construction of a phylogenetic tree since it showed the same pattern of the major RAPD band in RAPD analysis.

Table 2 List of deca-oligonucleotide primers used in this experiment

Primer code	Sequence (5′-3′)	Primer code	Sequence (5′-3′)
OPD-02	GGACCCAACC	OPN-16	AAGCGACCTG
OPD-04	TCTGGTGAGG	OPN-18	GGTGAGGTCA
OPD-07	TTGGCACGGG	OPO-18	CTCGCTATCC
OPD-11	AGCGCCATTG	OPS-01	CTACTGCGCT
OPN-04	GACCGACCCA	OPS-03	CAGAGGTCCC
OPN-06	GAGACGCACA	OPS-07	TCCGATGCTG
OPN-09	TGCCGGCTTG	OPS-08	TTCAGGGTGG
OPN-10	ACAACTGGGG	OPS-12	CTGGGTGAGT
OPN-15	CAGCGACTGT	OPS-19	GAGTCAGCAG

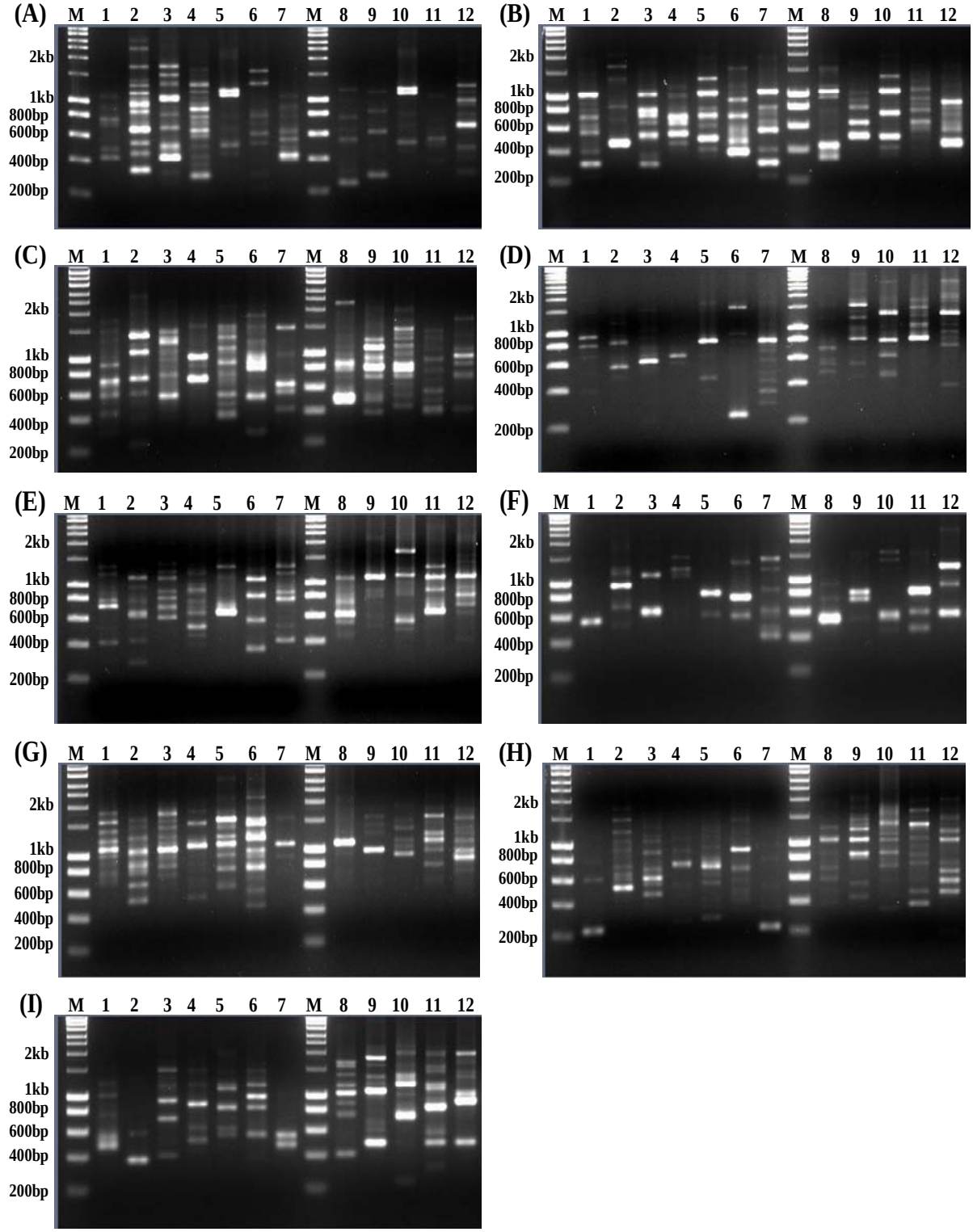


Figure 1 RAPD fingerprints of twelve *Phyllanthus* species generated with **(A)** OPS-01, **(B)** OPS-03, **(C)** OPS-07, **(D)** OPS-08, **(E)** OPS-12, **(F)** OPS-19, **(G)** OPD-02 (H) OPD-04, and **(I)** OPD-07, **M**: DNA marker. Lane 1: *P. amarus*, Lane 2: *P. urinaria*, Lane 3: *P. emblica*, Lane 4: *P. taxodiifolius*, Lane 5: *P. collinsiae*, Lane 6: *P. acidus*, Lane 7: *P. pulcher*, Lane 8: *P. debilis*, Lane 9: *P. virgatus*, Lane 10: *P. acutissimus*, Lane 11: *P. ankorensis*, Lane 12: *P. reticulatus*.

Phylogenetic analysis

To evaluate the genetic relationship between *Phyllanthus* species, RAPD bands of twelve *Phyllanthus* species produced by nine primers were scored and constructed as a phylogenetic tree. Standard DNA marker (Hyper Ladder I, Bioline USA Inc.) was used to assign the size of each RAPD fragment. Only fragments that could be accurately scored (0.3–2.5 kb) were chosen. RAPD fragments were assigned a DNA length and recorded in a binary matrix for each individual as presence (1) or absence (0) of a given band. Phylogenetic relationship of *Phyllanthus* species was constructed using Dice[25] clustering of NTSYS-pc version 2.11T (Exeter Software, Setauket, N.Y.) based on a UPGMA method (Figure 2). Among the twelve species of *Phyllanthus*, Dice similarity index (S.I.) ranged from 0.125 to 0.500 (Table 3) and could be divided into four clusters. Cluster I includes four species showing 0.375-0.500 similarity index (*P. amarus*, *P. pulcher*, *P. taxodiifolius*, and *P. reticulatus*). Cluster II includes two species showing similarity index 0.462 (*P. virgatus* and *P. ankorensis*) and cluster III includes five species with 0.258-0.500 similarity index (*P. urinaria*, *P. emblica*, *P. collinsiae*, *P. acidus*, and *P. acutissimus*. *P. debilis* is the distinct species in cluster IV. It was only 12% genetically similar to the rest of the 11 species. In addition, the four most closely related species were *P. pulcher* and *P. amarus* (cluster I) and *P. collinsiae* and *P. emblica* (cluster III) with the highest similarity index (0.500). *P. urinaria* and *P. emblica* fell into the same clade which

Table 3 Nei and Li's genetic similarity index of twelve species based on RAPD markers

Species		1	2	3	4	5	6	7	8	9	10	11	12
P. amarus	1	1.000											
P. urinaria	2	0.237	1.000										
P. emblica	3	0.419	0.479	1.000									
P. taxodiifolius	4	0.298	0.321	0.373	1.000								
P. collinsiae	5	0.393	0.400	0.500	0.302	1.000							
P. acidus	6	0.305	0.412	0.451	0.321	0.462	1.000						
P. pulcher	7	0.500	0.230	0.344	0.449	0.345	0.230	1.000					
P. debilis	8	0.227	0.151	0.286	0.146	0.240	0.340	0.174	1.000				
P. virgatus	9	0.296	0.286	0.212	0.196	0.333	0.381	0.286	0.125	1.000			
P. acutissimus	10	0.189	0.387	0.338	0.240	0.407	0.258	0.145	0.298	0.351	1.000		
P. ankorensis	11	0.426	0.171	0.329	0.207	0.358	0.400	0.381	0.255	0.462	0.344	1.000	
P. reticulatus	12	0.375	0.356	0.447	0.426	0.314	0.411	0.333	0.276	0.353	0.418	0.400	1.000

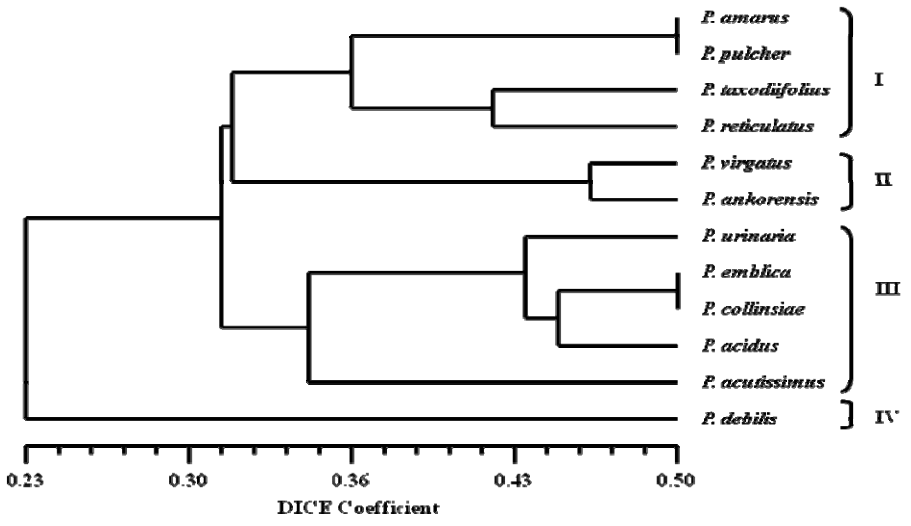


Figure 2 Unweighted pair group method with arithmetic average (UPGMA) tree showing the genetic relationships between twelve *Phyllanthus* species as determined by RAPD markers

correlated a previous report of *atp*B and *rbc*L partial gene sequences[3]. The four frequently misidentify herbaceous *Phyllanthus* species, *P. amarus*, *P. virgatus*, *P. urinaria*, and *P. debilis* were separated into different clusters (I, II, III, and IV, respectively) although their morphologies were similar. This analysis indicated that RAPD markers are a good choice for discrimination between them.

In summary, we discriminated twelve species in the genus *Phyllanthus* by RAPD with nine primers since they generated the unique DNA profiles. Sequencing characterized amplified region (SCAR) markers could be further developed as a tool to prevent misuse among *Phyllanthus* species.

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