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PHYLOGENETIC ANALYSIS AMONG FIVE MEDICINALLY SIGNIFICANT *PHYLLANTHUS* L. SPECIES IN BANGLADESH BASED ON TAXONOMIC AND MOLECULAR APPROACH

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Key words: Taxonomy, RAPD, Phylogenetic tree, Genetic polymorphism, *Phyllanthus*

Abstract

A combination of taxonomical and molecular technique had done for authentic characterization of medicinally important five *Phyllanthus* species *viz*. *P. acidus*, *P. emblica* (small and large fruit form), *P. niruri*, *P. reticulatus* and *P. urinaria*. They were analyzed with several taxonomical parameters such as branching pattern, morphology of bark, leaves, flowers, fruits and seeds. A dichotomous bracketed key is created for easy identification of the species. RAPD analysis of five *Phyllanthus* species displayed that *P. emblica* and *P. reticulatus* were closely related whereas *P. acidus* and *P. emblica* were genetically distantly related. The genus *Phyllanthus* are diverse, as seen by the 71.68% polymorphism among the five studied species determined by RAPD analysis. The two forms (small and large fruit forms) of *P. emblica* showed similarity as well as dissimilarity in taxonomic and molecular features. Thus, a subtle revision is necessary in the taxonomical point of view to update their taxa.

Introduction

The family Euphorbiaceae is recognized as one of the largest family among the dicot plants with about 8,000 species under 300 genera(1). This family has such a wide range of vegetative and floral diversity that morphometric analysis of this group has always been contentious since taxonomical data for these plants has yet to be collected systematically(2,3). *Phyllanthus* is one of the largest genera in the family Euphorbiaceae, which represents a group of medicinal plants used for various purposes. There are approximately 1200 species in the *Phyllanthus* genus, of which eleven have been reported from Bangladesh, namely *P. emblica* L., *P. acidus* (L.) Skeels, *P. maderaspatensis* L., *P. urinaria* L., *P. niruri* L., *P. reticulatus* Poir., *P. sikkimensis* Muell. -Arg., *P. boeobatryoides* Wall., *P. roxburghii* Muell. -Arg., *P. pendulus* Roxb. and *P. virgatus* Forst. f.(4).

Taxonomy is concerned with the identification, description, categorization, and naming of organisms at the species or taxonomic level(5). Morphological characteristics are components of external forms or appearance that are often utilized to hypothesize evolutionary connections and employed in plant systematics for far longer than cytogenetic

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and molecular evidence. The majority of plants, however, are classified primarily based on visible morphological characteristics such as size, shape, and color of different parts, which are employed in constructing taxonomic key. Classical taxonomy often failed to identify some species because of high morphological similarity and phenotypic plasticity for adaptation in different environmental conditions. These species must be identified using alternate approaches, such as cytogenetic data and molecular analysis. Most of the studies regarding this genus have been focused in taxonomical, pharmacological and chromosomal counts only[3]. In this context, taxo-molecular analysis could be considered as a powerful and informative tool to identify morphologically similar populations on the basis of DNA based marker and taxonomical status[6,7]. On the other hand, DNA fingerprinting by RAPD is one of the widely used molecular methods for characterizing species. Information on genetic diversity would provide advanced information for understanding the genetic diversity of different *Phyllanthus* species for appropriate management and conservation strategy. Extent of polymorphism among *Phyllanthus* species can be determined using the banding pattern obtained through PCR with RAPD markers. Bandyopadhyay and Raychaudhuri[8] employed Random amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP) and sequence characterized amplified region (SCAR) markers of five medicinally important species, namely *Phyllanthus emblica*, *P. reticulatus*, *P. amarus*, *P. fraternus* and *P. urinaria* for proper identification. Rout *et al.*[9] investigated twelve *Phyllanthus* species through PCR-based DNA (RAPD and ISSR) markers for identification and phylogenetic study. Manissorn *et al.*[10] used RAPD markers for constructing the genetic relationship between twelve *Phyllanthus* species from Thailand. Any attempt has neither been taken so far nor being initiated to combine taxonomical and molecular data for characterizing *Phyllanthus* species in Bangladesh. Therefore, the objective of the current research was to characterize and evaluate the evolutionary relationship among medicinally important five *Phyllanthus* species namely, *P. acidus*, *P. emblica*, *P. niruri*, *P. reticulatus* and *P. urinaria* of Bangladesh with taxo-molecular procedure and expand the database of DNA based marker of medicinal plants of Bangladesh.

Materials and Methods

Taxonomic analysis: Five *Phyllanthus* species of Bangladesh were used as plant materials in the present investigation. These were *P. acidus*, *P. emblica* (included two distinct forms, one bearing smaller fruits and the other bearing larger fruits), *P. niruri*, *P. reticulatus* and *P. urinaria*. Plant specimens were dried, pressed, and mounted on mounting sheets for Herbarium sheet production. Well–pressed and well dried specimens were mounted on 11.5 inch × 16.5 inch herbarium sheets with the help of gum. After mounting, the labeling was completed. Herbarium sheets that had been properly mounted and labeled were saved for future reference in Dhaka University Salar Khan Herbarium (DUSH). Specimens were organized using a recognized categorization system[11]. Morphological analysis of habits, barks, branches, leaves, flowers, fruits and seeds were also performed (Fig. 1, Table 1). A

taxonomic key to species was made for quick and authentic identification. A taxonomic key to species is consisting of a series of contrasting statements required for the identification and to make comparisons.

Fig. 1. External morphology of five *Phyllanthus* species. (a) Leaves, (b) flowers, (c) fruits and (d) seeds of *P. acidus*; (e) Leaves, (f) flowers, (g) fruits and (h) seeds of *P. emblica* (small fruit form); (i) Leaves, (j) flowers, (k) fruits and (l) seeds of *P. emblica* (large fruit form); (m) Leaves, (n) flowers, (o) fruits and (p) seeds of *P. niruri*; (q) Leaves, (r) flowers, (s) fruits and (t) seeds of *P. Reticulatus*; (u) Leaves, (v) flowers, (w) fruits and (x) seeds of *P. urinaria*.

Table 1. Comparative morphological analysis of five *Phyllanthus* species.

Morphology	*P. acidus*	*P. emblica* (small fruit form)	*P. emblica* (large fruit form)	*P. niruri*	*P. reticulatus*	*P. urinaria*
Habit	Tree	Tree	Tree	Herbs	Shrub	Herb
Branching Pattern	Robust, stout, leafless	Tawny pubescent	Tawny pubescent	Angular, less branched	Stout, much branched	Stem much branched
Morphology of bark	Very rough, hard, whitish gray	Smooth, thick, hard, blackish	Smooth, thin, hard, grayish	Smooth, thin, soft, light green	Rough, hard, thick, peeling or flaking, grayish-brown	Smooth, thin, soft, deep green
Phyllotaxy, Leaf shape and venation	Alternate, Simple, Pinnate	Alternate, Simple, Pinnate	Alternate, Simple, Pinnate	Alternate, Simple, Pinnate	Alternate, Simple, Pinnate	Alternate, Simple, Pinnate
Size of leaf blade	3-8×1-4 cm	8-20×2-6 mm	8-20×2-6 mm	5-12×2-5 mm	15-30×6-12 mm	5-20×2-9 mm
Inflorescence	Axillary	Axillary	Axillary	Axillary	Axillary	Axillary
Calyx aestivation	Imbricate	Valvate	Valvate	Imbricate	Valvate	Imbricate
Stamen number	4	3	3	3	5	3
Filaments	Free	Connate	Connate	Connate	Connate	Connate
Pistillode	Present	Present	Present	Present	Present	Present
Style branches	Bifid	Bifid	Bifid	Bifid	Bifid	Bifid
Style union	Connate	Connate	Connate	Connate	Connate	Connate
Ovule configuration	Hemitropous	Hemitropous	Hemitropous	Hemitropous	Hemitropous	Hemitropous
Embryo sac	Tetrasporic	Tetrasporic	Tetrasporic	Tetrasporic	Tetrasporic	Tetrasporic
Fruit type	Drupe	Drupe	Drupe	Capsule	Berry	Capsule
Seed type and size	Ecaranculate, Convex, 2-4×1-2 mm	Ecaranculate, planoconvex, 3-6×2-3 mm	Ecaranculate, planoconvex, 3-6×2-3 mm	Ecaranculate, trigonous, 1.0×0.6 mm	Ecaranculate, trigonous, 1.5-2.0 mm long	Ecaranculate, triangular, 1.0-1.2×0.8-1.0 mm

RAPD analysis: Total genomic DNA was isolated from the leaves using a DNA isolation Kit (Roti®-Prep Genomic DNA MINI, Carl Roth, Germany; Art.-Nr. 8472.2). A spectrophotometer was used to measure the concentration of DNA (Gel Documentation System (BioSciTec, Gelscan 6.0 Professional, German). The A 260/280 values for DNA samples ranged from 1.6 to 1.8. For subsequent usage, high-quality DNA was diluted to a concentration of 25 ng/1l. The 20 µl PCR reaction mixture containing template DNA (25 ng)

1 µl, master mixture (Pro-mega) 10 µl, PCR water 8.5 µl, primer (10 µM) 1.0 µl for RAPD analysis. PCR amplification was done in a 2720 thermal cycler (Applied Biosystems by Life Technologies). A total 12 primers were used for DNA fingerprinting and the details of the used primers and thermo-cycling conditions are presented in Table 2.

Table 2. Details of primers and thermo-cycling conditions for RAPD analysis.

Primer code	Sequence (5′—3′)	Annealing temperature (℃)	G+C content (%)
OPA-18	AGG TGA CCG T	32	60.00
OPB-19	ACC CCC GAA G	34	70.00
OPAB-5	CCC GAA GCG A	34	70.00
OPAB-6	GTG GCT TGG A	32	60.00
OPC-13	AAG CCT CGT C	32	60.00
OPC-15	GAC GGA TCA G	32	60.00
OPC-96	ACC AAG AAA GGG	36	50.00
OPD-69	CGC TCC AAA TCA	36	50.00
OPF-22	AAG ATC AAA GAC	32	33.33
OPH-12	ACG CGC ATG T	32	60.00
OPG-5	AGT CGT CCC C	34	70.00
OPG-7	GAA CCT GCG G	34	70.00

For RAPD analysis, the amplified products were separated by electrophoresis on 1.5% agarose gel. The gel was produced at 50 volts and 100 mA for 1.0 hour using 1.5 g agarose for RAPD with 8 µl (8 mg/ml) ethidium bromide and 100 ml 1 × TAE buffer. As a marker, a 1 kb DNA ladder was electrophoresed alongside the RAPD. DNA bands were photographed using a gel documentation system after being seen on a UV trans-illuminator. The size of bands and polymorphism were analyzed from the gel electrophoresis photographs of PCR products. In this case, 1 considered the presence of bands and 0 indicates bands absence. Single data matrixes in each case were prepared from all primers of RAPD and SSR markers. Useful and reliable software 'POPGENE 32' (Version 1.32) were used for conducting Nei's[12] genetic distance (D) and constructing UPGMA dendrogram among six *Phyllanthus* species.

Results and Discussion

Taxonomical diversification in five species of Phyllanthus : In the present investigation, five species of *Phyllanthus* differed in several taxonomical parameters such as branching pattern, morphology of bark, leaves, flower, fruits, seeds, and flowering and fruiting time (Fig. 1, Table 1). *Phyllanthus acidus* and *P. emblica* are tree, these two species differed in respect of some other characteristic features such as lamina ovate to ovate-lanceolate, flowers red, stamens 4, free, fruits drupe, depressed-globose in *P. acidus* and lamina oblong or linear-oblong, flowers greenish white or greenish red, stamens 3, filaments connate in a column in *P. emblica.* These two species also differed in cytological point of view *i.e. P. acidus* diploid

(2n = 2x = 26) and *P. emblica* polyploid (2n = 10x = 100)[3]. *Phyllanthus reticulatus* showed several distinct characteristics such as habit shrubs, stamens 5 and black coloured ripe fruit. *Phyllanthus niruri* and *P. urinaria* both is herb but differed regarding some features such as lamina shape, fruit shape. They also differed in somatic chromosome number and ploidy status (2n = 2x = 26 in *P. niruri* and 2n = 6x = 48 in *P. urinaria*)[3]. Plants belonging to *P. emblica* may be divided into two groups. Group-I containing plants yielding smaller fruits (size 2.0-2.2 cm and average weight 5.30 gm), may be regarded as small fruit form. Group-II containing plants yielding larger fruits (size 3.0-3.3 cm and average weight 25.18 gm), may be regarded as large fruit form. However, these two forms showed some dissimilarity (Table 1). The prominent external morphological differences are i) bark colour was blackish in small fruit form and grayish in large fruit form, ii) young leaves colour was green in small fruit form and purplish green in large fruit form, iii) flower colour was greenish white in small fruit form and greenish red in large fruit form, iv) flowering time was March to September for small fruit form and January to March for large fruit form.

Key to species of five Phyllanthus species: A key to species from five *Phyllanthus* species was prepared based on the external morphology and ploidy level which are presented below-

1. Plants tree- 2
- Plants herbs or shrubs- 3
2. Lamina ovate to ovate-lanceolate; flowers red, stamens 4, free; fruits drupe, depressed-globose. Plant diploid- *P. acidus*
- Lamina oblong or linear-oblong; greenish white or greenish red; stamens 3, filaments coherent into a central column. Plant polyploid- *P. emblica*

3. Plants shrubs; stamens 5; ripe fruit black. Plant diploid- *P. reticulatus*
- Plant herbs; stamens 3; fruit greenish or yellowish- 4
4. Lamina elliptic-oblong to elliptic-lanceolate; fruit trilobate-subglobose. Plant diploid- *P. niruri*
- Lamina linear or oblong to oblong-obovate; fruit globose. Plant polyploidy- *P. urinaria*

DNA fingerprinting by Random Amplified of Polymorphic DNA (RAPD): RAPD is a PCR based marker technique that has been used for estimation of genetic diversity of populations and for studying the genetic relationships among different genotypes[13]. In this investigation, 12 oligonucleotide primers for RAPD were utilized to study the genetic relationship among the five *Phyllanthus* species. The primer sequence, band size and banding pattern of five *Phyllanthus* species were shown in Tables 2 and 3. The 12 primers generated 141 distinct bands of which 97 were considered as polymorphic. Band size ranging from 150–4000 bp of PCR amplification products scored for all primers. The literature has several molecular marker-based researches on diverse *Phyllanthus* species.

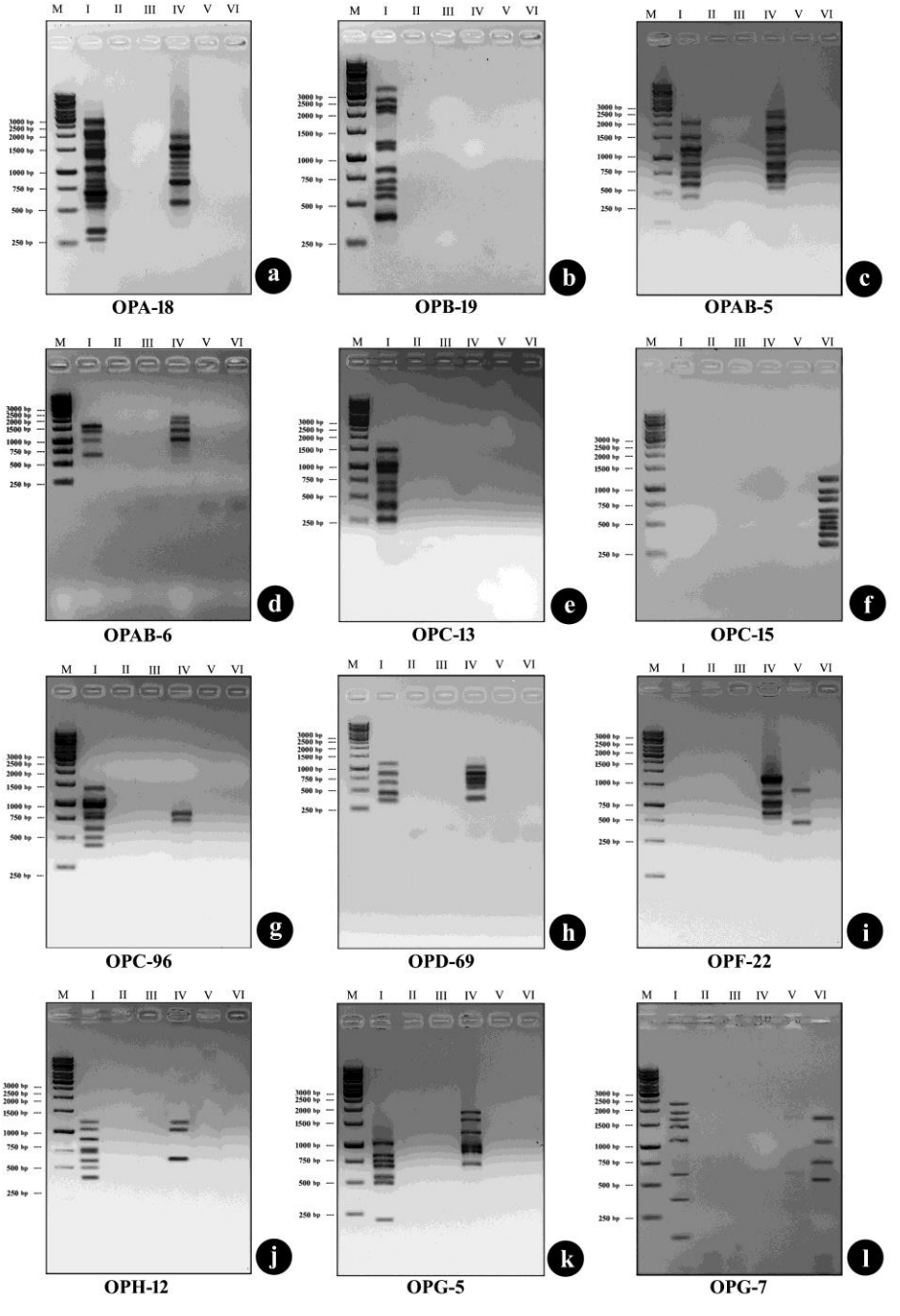

Fig. 2. RAPD profile of five *Phyllanthus* species showing amplification of PCR bands with 12 different random primers. M = ladder, I = *P. acidus*, II = *P. emblica* (small fruit form), III = *P. emblica* (large fruit form), IV = *P. niruri*, V = *P. Reticulatus* VI = *P. urinaria*.

Jain *et al.*[14] used RAPD markers to study 33 *P. amarus* accessions and discovered 65% variation between them. Chaurasia *et al.*[15] distinguished various types of *P. emblica* in another investigation utilizing RAPD markers. Rout *et al.*[9] used RAPD and ISSR markers to investigate the relationships between 12 *Phyllanthus* species. The results of the present investigation showed about 71.68% polymorphism among five species of *Phyllanthus*. The broad range of polymorphism revealed wide diversity among studied *Phyllanthus* species (Fig. 2, Table 3).

Table 3. Compilation of RAPD analysis in five *Phyllanthus* species.

Primer codes	Total bands	Size ranges (bp)	Number of polymorphic bands	Number of species specific unique bands	Polymorphisms (%)
OPA-18	22	300-3000	14	10 in *P. acidus* and 4 in *P. niruri*	63.64
OPB-19	11	400-4000	11	11 in *P. acidus*	100.00
OPAB-5	21	400-2800	13	5 in *P. acidus* and 8 in *P. niruri*	61.90
OPAB-6	8	700-2500	4	2 in *P. acidus* and 2 in *P. niruri*	50.00
OPC-13	7	250-1500	7	7 in *P. acidus*	100.00
OPC-15	9	300-1200	9	9 in *P. urinaria*	100.00
OPC-96	9	450-1400	5	5 in *P. acidus*	55.56
OPD-69	11	400-1100	5	2 in *P. acidus* and 3 in *P. niruri*	45.45
OPF-22	8	450-1100	8	6 in *P. acidus* and 2 in *P. reticulatus*	100.00
OPH-12	10	400-1300	4	4 in *P. acidus*	40.00
OPG-5	13	200-2000	9	5 in *P. acidus* and 4 in *P. niruri*	76.92
OPG-7	12	150-2400	8	6 in *P. acidus* and 2 in *P. urinaria*	66.67
Grand Total	141	150-4000	97	97	71.68

Unique RAPD markers: A number of species specific unique bands were observed in *Phyllanthus* species (Fig. 2, Table 3) such as 10 in *P. acidus* and 4 in *P. niruri* (OPA-18), 11 in *P. acidus* (OPB-19), 5 in *P. acidus* and 8 in *P. niruri* (OPAB-5), 2 in *P. acidus* and 2 in *P. niruri* (OPAB-6), 7 in *P. acidus* (OPC-13), 9 in *P. urinaria* (OPC-15), 5 in *P. acidus* (OPC-96), 2 in *P. acidus* and 3 in *P. niruri* (OPD-69), 6 in *P. acidus* and 2 in *P. reticulatus* (OPF-22), 4 in *P. acidus* (OPH-12), 5 in *P. acidus* and 4 in *P. niruri* (OPG-5), 6 in *P. acidus* and 2 in *P. urinaria* (OPG-7). A sequence that is found in one species using a certain primer but not in another is referred to as unique sequence. It was possible to employ the distinct bands as a characterization tool because they were stable and specific to that particular species.

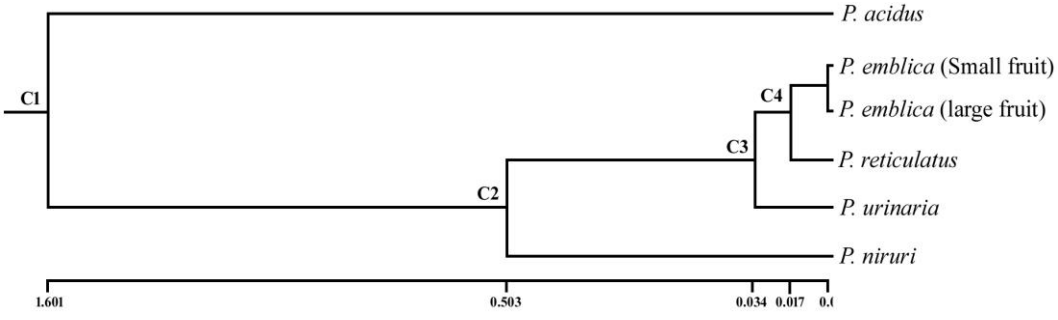

Fig. 3. UPGMA dendrogram based on Nei's (1972) genetic distance summarizing the data on differentiation among five *Phyllanthus* species according to RAPD analysis.

Table 4. Summary of Nei's (1972) genetic distances of five *Phyllanthus* species according to RAPD analysis.

pop ID	*P. acidus*	*P. emblica* (small fruit form)	*P. emblica* (large fruit form)	*P. niruri*	*P. reticulatus*	*P. urinaria*
P. acidus	****					
P. emblica (small fruit form)	1.3451	****				
P. emblica (large fruit form)	1.3451	0.0000	****			
P. niruri	1.6011	0.5025	0.5025	****		
P. reticulatus	1.4118	0.0169	0.0169	0.5306	****	
P. urinaria	1.3451	0.0342	0.0342	0.5596	0.0517	****

Genetic relationships and Cluster analysis of studied Phyllanthus species: A cluster analysis on the basis of DNA fingerprinting by RAPD was carried out. Dendrogram based on Nei's[12] genetic distance using UPGMA (Unweighted Pair Group Method of Arithmetic Means) segregated five *Phyllanthus* species into two major clusters C_1 and C_2 (Fig. 3).

According to dendrogram, *P. acidus* is separated from other four species with high genetic distance 1.6011 and placed in a separate cluster C_1 (Fig. 3, Table 4). On the other hand, other four species were placed in cluster C_2. The lowest genetic distance 0.0169 was found between *P. reticulatus* and *P. emblica* whereas the highest genetic distance 0.5025 was found between *P. niruri* and *P. emblica* within cluster C_2. So among five *Phyllanthus*, *P. emblica* and *P. reticulatus* were closely related whereas *P. acidus* and *P. emblica* were genetically distantly related.

The two dimensional aspects of the genus Phyllanthus assembled with taxonomic and molecular point: A taxonomic study with RAPD analysis in the genus *Phyllanthus* has been done in present investigation. Based on dendrogram prepared from RAPD analysis, *P. emblica* (both forms), *P. reticulatus* and *P. urinaria* were closely associated. Since *P. emblica* and *P. urinaria* were polyploid and *P. reticulatus* was the only diploid species among these three, it could be suggested that they might have shared some common genome that passing through the polyploid complex of *P. emblica* and *P. urinaria*[3]. The two forms (small and large fruit forms) of *P. emblica* displayed similarity in some taxonomic parameters and somatic chromosome numbers[3]. These two forms also stayed closely in the dendrogram of RAPD. The size of fruits is a constant and good taxonomic character. In addition, they were different in terms of important external features such as bark, leaves, and flower colour as well as flowering and fruiting times. These are also heritable characters, and the distinctions are discontinuous. Therefore, these two forms may be given distinct varietal ranks. In order to update their taxa, a strategic revision is therefore required from a taxonomic perspective.

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