Interrelationships Among Species of *Dioscorea* Revealed by Morphological Traits and RAPD Markers

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This present study assessed the interrelationships among the 12 species of *Dioscorea* by morphological traits and RAPD analysis using 14 decamer primers. In the molecular profiling of the 12 species of *Dioscorea* a total of 133 amplified products were obtained. Of the 133 amplified products all were found to be polymorphic showing high degree of genetic variability between species of *Dioscorea*. Morphologically related species such as *D. oppositifolia, D. spicata* and *D. wightii* got separated in RAPD analysis. Moreover, both RAPD and morphology separated *D. hispida* of the Section Lasiophyton from the other two species such as *D. pentaphylla* and *D. tomentosa*. Out of the 12 species studied, 9 species were grouped into 2 clusters and *D. bulbifera* remained as a separate entity. The results were in agreement with the earlier classification except *D. hispida* and *D. pubera*, which remained as distinct entities from the rest. But on the basis of RAPD data, nine species were grouped into two major clusters with each species showing distinct genetic distance with others, while the rest of the three *D. hispida, D. oppositifolia* and *D. wightii* remained as separate entities.

Key words: *Dioscorea* species, Interrelationship, Morphological characterization, RAPD

Yams belonging to the genus *Dioscorea* of the family Dioscoreaceae, are the major staple food in many tropical and subtropical countries and also a major source of pharmaceutical products such as diosgenin, botogenin, Kryptogenin, etc. Out of the 600 species reported, 23 species occur in the Indian subcontinent and 44% of these are endemic (Prain and Burkill, 1938). Based on the morphological characterization, the genus *Dioscorea* has been divided into different Sections *viz.*, Borderea, Stenophora, Stenocorea, Combilium, Illigerastrum, Paramecocarpa, Shannicorea, Opsophyton, Lasiophyton and Enantiophyllum (Prain and Burkill, 1936). The present study focused on the 12 selected species of *Dioscorea* collected from Southern Western Ghats, belonging to 3 different sections. The species *D. bulbifera* which belongs to the section Opsophyton is characterized by the stem twining to the left, other three species *viz.*, *D. hispida, D. pentaphylla, D. tomentosa* belonging to the section Lasiophyton are also characterized by left twining stem but with compound leaves and eight species belonging to the section Enantiophyllum are right twining.

Morphological variations, cytogenetic relationships, breeding systems and biochemical markers used in elucidating the relationships among species suffer some of the inherent limitations as these marker types depends on gene expression, which may be sensitive to environmental influence, genetic background, developmental stage and tissue type. Hence more reliable protein or DNA profiling is now gradually replacing the traditional method of identifying cultivars by morphological characters. RAPD markers are suitable for determining similarities among inbreds, genotype identification, species classification, establishing phylogenies, fingerprinting varieties, tagging desirable genes and mapping plant genomes.

Materials and Methods

Materials analysed

Twenty-four accessions, two each from the twelve species, procured from National Bureau of Plant Genetic Resources (NBPGR) Regional Station, Thrissur, Kerala were grown in the green house of the Department of Botany during 2001-04 (Table 1). For RAPD analysis, one accession each from the 12 species having the species specific morphological traits was selected (Table 1). Young leaf samples were used for DNA extraction and analysis.

Methodology

Morphological Characterization

Morphological characterization was done using the standard descriptors published by the International Plant

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Table 1. Materials characterized morphologically and by RAPD

Species	Accession number	Locality of collection
***Dioscorea pentaphylla* L.**	IC202352	Nilambur, Malappuram, Kerala
	IC202367*	Peechi, Thrissur, Kerala
Dioscorea oppositifolia L.	IC202386*	Valpara, Palghat, Kerala
	IC210336	Coimbatore, Tamil Nadu
Dioscorea hispida Dennst	IC202369	Nilambur, Malappuram, Kerala
	IC202370*	Nilambur, Malappuram, Kerala
Dioscorea tomentosa Heyne	IC202376	Parambikulam, Palghat, Kerala
	IC202379*	South Canara, Karnataka
Dioscorea belophylla Voight	IC248181*	Devakolly, Coorg, Karnataka
	IC248181A	Thirunelli, Wynad, Kerala
Dioscorea wightii Hk. f.	IC210447A	Papanasam, Mundanthurai, Tamil Nadu
	IC214855*	Mundanthurai, Tamil Nadu
Dioscorea intermedia Thw.	IC202384*	Santhanpara, Idukki, Kerala
	IC210409	Kattappana, Idukki, Kerala,
Dioscorea wallichii Hk. f.	IC202312*	Courtallum, Tirunelveli, Tamil Nadu
	IC202320	Vellani Hills, Thrissur, Kerala
Dioscorea hamiltonii Hk.	IC202326	Courtallum, Tirunelveli, Tamil Nadu
	IC202328*	Vadakkancherry, Thrissur, Kerala
Dioscorea spicata Roth.	IC202383*	Thirthahally, Coorg, Karnataka
	IC248245	Uttara Kannada, Karnataka
Dioscorea bulbifera L.	IC202349*	Peechi, Thrissur, Kerala
	IC298648	Madathara, Trivandrum, Kerala
Dioscorea pubera Bl.	IC202382*	Begur, Wynad, Kerala
	IC298641	Elangium, Trivandrum, Kerala

* Accessions characterized by RAPD

Genetic Resources Institute on *Dioscorea* spp. (IPGRI, 1997). Due to the absence of flowering or erratic nature of flowering in the species under *ex-situ* conditions, only vegetative traits were taken into consideration. In order to find out the interrelationships among the 12 species, tuber raised plants at the vegetative growth stage of 120 to 180 days were scored for morphological traits. Data of above ground vegetative traits such as stem, leaves and bulbils were recorded at the maximum growth period during September-October and that of below ground tubers at the time of harvest during January.

The qualitative character states were scored as present (1) or absent (0) across all the accessions. The two-way data matrix of accessions x traits was used to calculate pair-wise similarity coefficients following Jaccard (1908). This matrix of similarity coefficients was subjected to unweighted pair-group method analysis (UPGMA) to generate a dendrogram using average linkage procedure. The numerical taxonomic analyses were carried out using the software, NTSYS-pc, Version 1.80 (Exeter Software, New York).

Molecular Profiling using RAPD

Genomic DNA Extraction

Total genomic DNA was extracted from fresh young leaves of the *Dioscorea* species using CTAB with appropriate modifications (Murray and Thompson, 1990). 1.2% PVP was added to the extraction buffer to remove phenolic contaminants followed by double chloroform extraction at 10000 rpm. DNA after ethanol precipitation was resuspended in 100 µl of 10:1 TE buffer and quantified using spectrophotometer at 260 nm.

PCR Analysis

Fourteen random 10-base primers were used in this study (Table 2). PCR was carried out in a 25 µl reaction mixture containing 2.5 µl 10x amplification buffer, 200 µM each of dATP, dTTP, dGTP, dCTP, 1.0 U Taq DNA polymerase (10 mM Tris HCl, pH 9.0, 1.5 mM $MgCl_2$, 50 mM KCl and 0.01 % gelatine) from Finnzymes, 15 pmoles of 10mer primer (Operon Technologies Inc, USA & Biogene, USA) and 50 ng of genomic DNA. Amplification was performed in a Perkin-Elmer DNA Thermal Cycler (DTC 480). The sequential steps involved

were: 2 minutes at 94°C, 2 minutes at 36°C and 2 minutes at 72°C. This cycle was followed by 38 cycles of 1 minute at 94°C, 1 minute at 36°C and 2 minutes at 72°C. The last cycle was followed by 7 minutes extension at 72°C.

After completion of the PCR, the amplified products were resolved in 1.5% agarose gel (1xTBE) followed by ethidium bromide staining. 3 µl of the amplified sample was loaded into the wells and electrophoresis started with 94 V from a power pac (Pharmacia Biotech, USA). After electrophoresis, the bands were detected and documented using Alpha Chemi Imager Gel Documentation System. Amplified products that were reproducible and consistent in performance were chosen for data analysis. The two-way data matrix of species x traits was used to calculate pair-wise similarity coefficients following Jaccard (1908). This matrix of similarity coefficients was subjected to unweighted pair-group method analysis (UPGMA) to generate a dendrogram using average linkage procedure. The data was analyzed using NTSYS-pc, Version 1.80 (Exeter Software, New York).

Results

Simple Leaf Bearing Species

The nine different species of *Dioscorea*, which bear simple leaves, showed variations in qualitative juvenile traits. Waxy stem in *D. wallichii, D. oppositifolia, D. intermedia* and *D. bulbifera*, angular stem in *D. bulbifera* and *D. belophylla* and dense hairy stem in *D. pubera* are distinct variations in *Dioscorea* species which enable the taxonomic identity for each taxa. Stem spines were recorded only in *D. wallichii* at early stages and at the same time stem was waxy in *D. wallichii, D. oppositifolia, D. belophylla* and *D. intermedia.* The anti clock-wise stem twining is a unique feature in *D. bulbifera.* Juvenile leaf pubescence was recorded in *D. spicata, D. bulbifera* and *D. pubera.*

Table 2. Primers used for RAPD analysis and their results

#	Primers	Primer sequence 5' → 3'	No. of bands	No. of polymorphic bands
1	OPP 02	TCGGCACGCA	10	10
2	OPP 09	GTGGTCCGCA	6	6
3	C63	GGGGGTCTTT	5	5
4	C64	CCGCATCTAC	8	8
5	C66	GAACGGACTC	14	14
6	C67	GTCCCGACGA	11	11
7	C68	TGGACCGGTG	12	12
8	C69	CTCACCGTCC	9	9
9	C70	TGTCTGGGTG	10	10
10	C71	AAAGCTGCGG	8	8
11	C74	TGCGTGCTTG	13	13
12	C75	GACGGATCAG	12	12
13	C78	TGAGTGGGTG	6	6
14	C79	GTTGCCAGCC	9	9
	Total number of bands		133	133
	Mean per primer		9.5	9.5

The leaf traits of the species form important criteria in identification of the species in natural conditions. Arrangement of leaves is alternate in all except *D. oppositifolia* and *D. spicata* where it is alternate at base and opposite above. Leaf shape ranges from ovate in *D. oppositifolia* and *D. spicata*, followed by cordate long in *D. intermedia* and *D. pubera*, cordate broad in *D. bulbifera* and *D. wightii*, sagittate long in *D. hamiltonii* and *D. belophylla* and deltoid in *D. wallichii.*

The vegetative propagating unit, aerial bulbils recorded in different species also showed variation in bulbil traits. Bulbil production *in situ* was noticed only in four species studied in this group namely *D. bulbifera, D. hamiltonii, D. belophylla* and *D. pubera.* The shape ranges from round in *D. bulbifera,* callus like in *D. belophylla* and *D. hamiltonii* to ovate in *D. pubera.* The outer skin colour is grey in *D. pubera* and brown in others. Surface is rough with presence of roots in all except *D. pubera.* Bulbil flesh colour varied between orange-yellow in *D. pubera,* light green in *D. bulbifera* and *D. hamiltonii* and white in *D. belophylla.* Bulbil number ranges between an average of nine in *D. pubera,* 30 in *D. bulbifera* to small and many in *D. hamiltonii* and *D. belophylla.*

Tuber traits are the most variable among the different species of *Dioscorea.* Highly branched, elongated thin tubers, fused at knot and having prickly appearance, is a characteristic feature of *D. wallichii.* Tuber flesh colour is white in all except for orange in *D. pubera,* yellow in *D. bulbifera*, cream in *D. oppositifolia* and *D. wallichii.* Inner skin colour is white in all except for purple in *D. oppositifolia*, light yellow in *D. bulbifera,* cream in *D. wallichii* and *D. intermedia* and light orange in *D. pubera.*

Compound Leaf Bearing Species

The three different compound leaf bearing *Dioscorea* species *viz., D. tomentosa, D. hispida* and *D. pentaphylla* also showed distinct variations in morphological traits at various growth stages. Stem is pubescent in *D. tomentosa* but glabrous in others. Spine on the stem varied between short and curved downwards in

D. pentaphylla, long and curved upwards in *D. hispida* to short and straight in *D. tomentosa*. The identifying unique feature of *D. pentaphylla* and *D. hispida* is the presence of coalescent spines.

The number of leaflets showed wide range of variation in this group. The leaf is trifoliate in *D. hispida*, tri- to penta-foliate in *D. pentaphylla*, while, variable with simple, bilobed and trilobed in *D. tomentosa*. The lamina and petiole are highly tomentose in *D. hispida* and *D. tomentosa*. The leaf margin is serrate in *D. tomentosa*. The shape and size of leaflets in compound leaf bearing forms is of considerable importance. In *D. pentaphylla*, the terminal leaflet is elliptic and laterals inequilateral, whereas, in *D. tomentosa* the leaflets are ovate-broadly elliptic with laterals oblique at base and in *D. hispida*, ovate to abruptly acuminate. Among these 3 species, *D. pentaphylla* alone produces aerial bulbils, is elongated in shape with dark brown rough skin having roots on the surface. The tubers were elongated and cylindrical in *D. pentaphylla* and *D. tomentosa* while spherical and amorphous with numerous hairs in *D. hispida*. In *D. hispida*, the inner and outer skin colour is cream with light yellow tuber flesh. The tuber flesh colour and inner skin colour was white to cream in *D. pentaphylla* and *D. tomentosa* and the outer surface looks prickly in appearance by the presence of stiff hairs.

Interrelationship Based on Morphology

The sections of the genus *Dioscorea* comprising of different species based on the classification by Prain & Burkill (1936) is taken as the basis for numeric taxonomic studies. The resulting dendrogram obtained by morphologic characterization is summarized in Fig. 1, which depicts the detailed clustering pattern of all the 12 species. A comparative account of the earlier classification by Prain & Burkill (1936) along with the present clustering of individual species is presented in Table 3a.

Cluster Analysis

The numerical taxonomic studies of the 12 species selected for study resulted in the identification of 2 major clusters having a single sub-cluster. The dissimilarity coefficients ranged between 7.391 and 19.12%. Based on the dendrogram formed from the similarity matrix of 143 characters, the cluster I included *D. pentaphylla* and *D. tomentosa* with a similarity coefficient of 84.22%. The II cluster included most of the species coming under section Enantiophyllum, except *D. pubera* and the similarity level ranged between 83.8 to 92.6%. The exceptions, *D. hispida* of cluster I (Lasiophyton group) and *D. pubera* of cluster II (Enantiophyllum group), was similar to the I as well as the II cluster at a similarity level of 82.7% and 82.5%, respectively.

Interrelationships among Dioscorea species Based on RAPD

RAPD profiles of the representative samples of 12 species using 14 decamer primers generated 133 amplified products all of which were found to be polymorphic. Of the 14 primers, the number of scored markers produced by each primer ranged from a maximum of 14 in case of C66 (Plate) to a minimum of 5 as in the case of C63, with an average of about 9.5 markers per primers (Plate). The data showed a mean value of 9.5 products per primer (Table 2). Further the total number of products detected in individual species ranged from 13 to 51 with an average of 35.3 per primer. The maximum number of bands were produced by *D. hispida* (51) followed by *D. oppositifolia* (50), *D. intermedia D. belophylla* (49), *D. tomentosa* (47), *D. hamiltonii* (44), *D. pentaphylla* (35), *D. wightii* (33), *D. spicata* (29) and *D. bulbifera* (13).

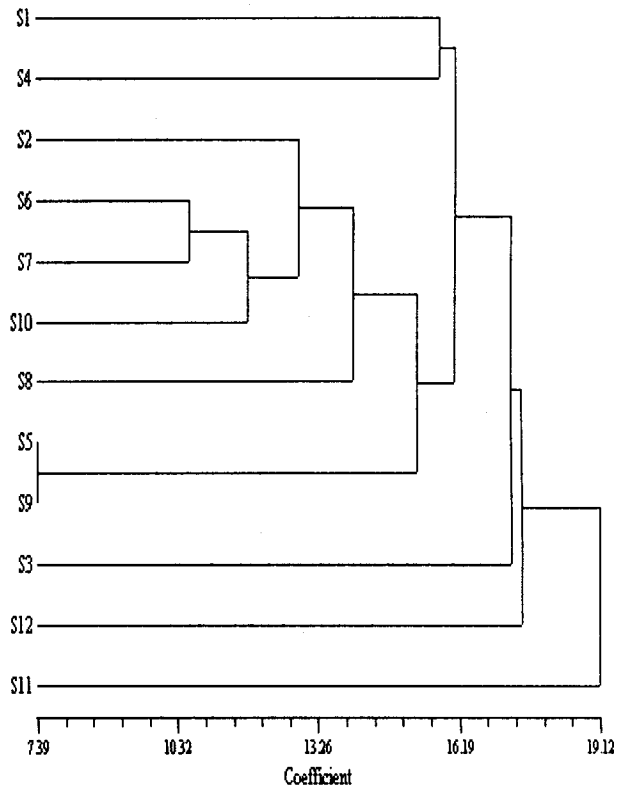
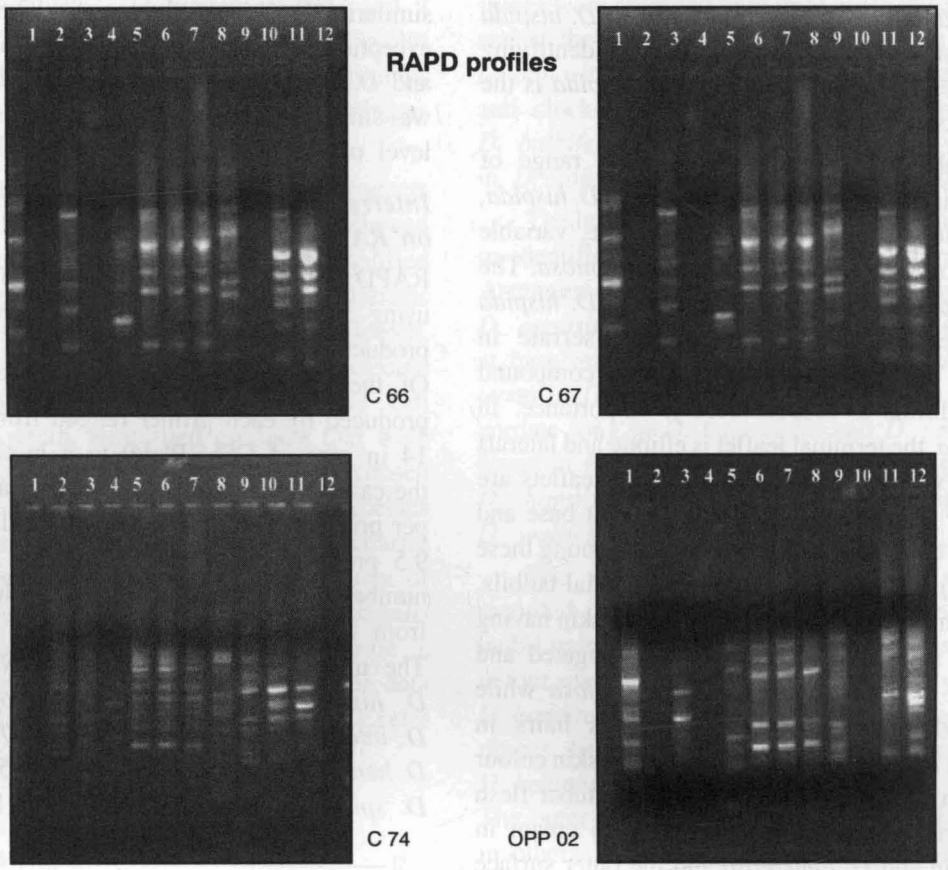


Fig. 1: *Dendrogram generated by morphological characterization*



Plate

Cluster Analysis

The dendrogram generated based on the genetic distance from RAPD data is shown in Fig. 2. Cluster analysis of the data shows grouping of 9 out of the 12 species into 2 major clusters. A comparative account of the earlier classification by Prain & Burkill along with the present clustering of individual species based on RAPD data is presented in Table 3b.

Cluster I

Cluster I have only 2 species *D. tomentosa* and *D. pentaphylla* of the section Lasiophyton with a similarity coefficient of 86.13%. Here *D. hispida* of the same section gets separated.

Cluster II

Cluster II comprised of 7 species *viz.*, *D. pubera, D. bulbifera, D. wallichii, D. spicata, D. intermedia, D. belophylla* and *D. hamiltonii.* All the species in this cluster coming under the section Enantiophylum and *D. bulbifera* of the section Opsophyton were grouped together within a range of 85.17 to 94.08 similarity having a ball cluster consisting of *D. intermedia* and

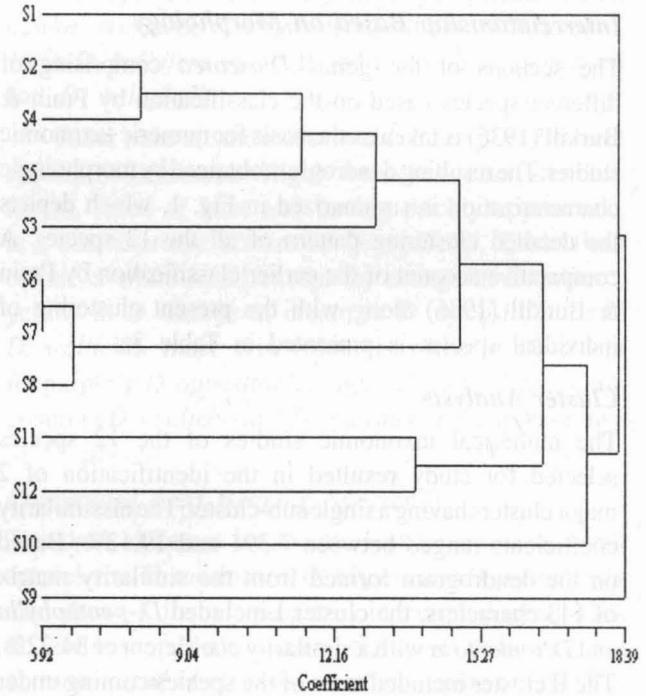


Fig. 2: *Dendrogram generated by RAPD characterization*

Table 3a. Comparison of Sections of *Dioscorea* based on morphology

Sections	Species	Cluster	Species
Lasiophyton	*D. tomentosa* *D. pentaphylla* *D. hispida*	I	*D. pentaphylla* *D. tomentosa*
	—	—	*D. hispida*
Enantiophyllum	*D. wightii* *D. intermedia* *D. spicata* *D. hamiltonii* *D. wallichii* *D. belophylla* *D. oppositifolia* *D. pubera*	II	*D. oppositifolia* *D. wightii* *D. intermedia* *D. spicata* *D. wallichii* *D. belophylla* *D. hamiltonii*
	—	—	*D. pubera*
Opsophyton	*D. bulbifera*	—	*D. bulbifera*

Table 3b. Comparison of Sections of *Dioscorea* with RAPD cluster analysis

Sections	Species	RAPD cluster	Species
Lasiophyton	*D. pentaphylla* *D. tomentosa* ***D. hispida***	II	*D. pentaphylla* *D. tomentosa*
	—	—	*D. hispida*
Enantiophyllum	*D. wightii* *D. intermedia* *D. spicata* *D. hamiltonii* *D. wallichii* *D. belophylla* *D. oppositifolia* ***D. pubera***	I	*D. pubera* *D. bulbifera* *D. wallichii* *D. spicata* *D. intermedia* *D. belophylla* *D. hamiltonii*
		—	*D. wightii*
		—	*D. oppositifolia*
Opsophyton	*D. bulbifera*	—	—

D. belophylla with a similarity % of 94.084, determined to be the closest among the species studied.

The analysis of the morphovariability and interrelationships among species of *Dioscorea* at the molecular level using RAPD revealed that among the 12 species studied, 9 of them were distinctly grouped in 2 clusters and the rest 3 species *viz. D. oppositifolia, D. hispida* and D. *wightii* stood apart. Of the 3 divergent species, *D. hispida* shows closeness to *D. pentaphylla* and *D. tomentosa. D. oppositifolia* seems to be the most divergent member from all the others with a mean similarity value of 81.61%. The mean value of the coefficients of similarity for cluster II is 90.41% and for cluster I is 84.28%. Analysis of RAPD data indicated that the extend of inter specific variation in the genus is relatively less as evidenced by the coefficient of similarity values that ranges from 81.61 to 94.08, with a mean value of 87.84%.

Discussion and Conclusion

A critical observation of the present clustering showed that out of the 12 species selected for the present study, 10 of them retained their integrity as in the grouping by Prain and Burkill, except for *D. hispida* in the group Lasiophyton and *D. pubera* in Enantiophyllum. *D. bulbifera* was retained as a separate entity and is in conformity with the earlier classification. As far as the species diversity and geographical distribution are concerned, the present clustering pattern did not show any strict relation as the species from different geographical region are grouped in one cluster.

The grouping of the two varied compound leaf bearing species *D. tomentosa* and *D. pentaphylla* with similar leaf venation pattern into a single cluster depicts the importance of leaflet number in the classification of species. Wilkin (1999) on the basis of leaflet number and vein characters grouped the six African yams into two sections.

The divergence of *D. hispida* from Cluster I and *D. pubera* from cluster II needs further analysis on molecular basis to confirm its position in Prain & Burkill's Sections Lasiophyton and Enantiophyllum, respectively. In effect it can be concluded that the species holds distinct variability with respect to vegetative and tuber characteristics and there exist 2 major clusters and 3 distinct species from the rest among the 12 species available in Southern Western Ghats.

In molecular profiling using RAPD, of the 133 amplified products obtained from 14 decamer primers showing 100% polymorphism suggests a high degree of genetic variability between species of *Dioscorea*. Polymorphisms arise because sequence variations in the genome alter the primer binding sites. A similar result of high polymorphism suggesting intraspecies variability was suggested among 18 cultivars/species of *Dioscorea* (Lay *et al.*, 2001). Polymorphisms resulted from mutations or rearrangements either at or between the primer binding sites are detected as presence or absence of a particular RAPD band. Intervarietal and interspecific polymorphisms of RAPD data enabling reliable discrimination of Jamaican cultivars of *Dioscorea* were reported by Asemota *et al.* (1996). Out of the 12 species studied, 9 species were grouped into 2 major clusters, while the rest of the three remained as separate entities on the basis of RAPD data. Moreover the closely related species based

on morphological grouping such as *D. oppositifolia, D. spicata* and *D. wightii* gets separated in RAPD analysis.

In morphological clustering, *D. hispida*, the trifoliate species remained distinct which according to the classification by Prain & Burkill (1938) comes along with the other compound leaf bearing species such as *D. pentaphylla* and *D. tomentosa.* The separation of *D. hispida* in morphological grouping may be due to its trifoliate leaflet condition than the varied leaf nature of *D. pentaphylla* and *D. tomentosa.* The RAPD result also supports the separation of *D. hispida* from cluster I.

The grouping of the 2 compound leaf species *D. pentaphylla* and *D. tomentosa* in the same cluster shows that morphology and RAPD are equally effective in the elucidation of interrelationship of *Dioscorea* species.

In morphological grouping *D. pubera, D. hispida* and *D. bulbifera* are the most divergent species whereas in RAPD *D. oppositifolia, D. hispida* and *D. wightii* shows distinctiveness. In this *D. pubera* and *D. bulbifera*, which gets separated in morphological classification, comes along with the simple leaf forms in RAPD clustering. Moreover, *D. wightii* and *D. oppositifolia*, which were grouped together into a single group along with 5 species by morphological clustering, were found to be divergent in the RAPD analysis. Even though *D. intermedia* did not form a close cluster with *D. belophylla* and *D. hamiltonii* in morphology, they form a single group in RAPD. The results of the present study showed that RAPD analysis is a rapid and useful approach for distinguishing closely related species of *Dioscorea* as well as for estimating genetic distance among the species.

As the study reveals varied nature of interrelationships and divergences among the species, there is a need for in-depth molecular studies using more primers and also using other molecular markers such as RFLPs, ISSRs and AFLP etc. for conclusively elucidating the genetic diversity and assessing the phylogenetic relationships among *Dioscorea* species.

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