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Biosystematics of the *Dioscorea* species of western ghats using chemical and molecular tools

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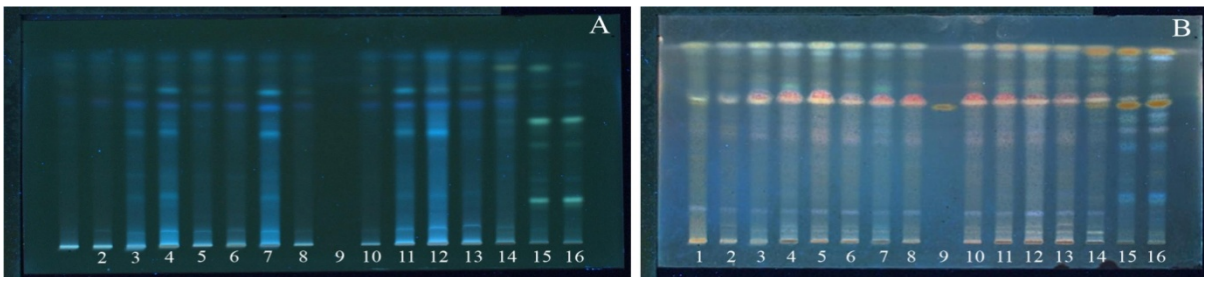
ABSTRACT



The genus *Dioscorea,* a vital group in the evolution of monocotyledons, has a significant place in the systematic studies. The present study reports the biosystematics and phylogenetic relation among 15 *Dioscorea* species collected from south India; *D. alata*, *D. belophylla*, *D. bulbifera*, *D. composita*, *D. esculenta*, *D. floribunda*, *D. hispida, D. kalkapershadii*, *D. oppositifolia*, *D. pentaphylla*, *D. pubera*, *D. rotundata*, *D. tomentosa, D. wallichii*, *D. wightii* based on the morphological, chemical and molecular studies. HPTLC chemical profile using standard marker compound diosgenin, groups the two exotic species *D. composita* and *D. floribunda* into one clad with *D. rotundata* and *D. esculenta* as allied species, while the true native species forms the second clad. The genetic diversity analysis of *Dioscorea* species were carried out using the universal DNA markers, *mat*K and chloroplast spacer region *rpl36-infA-rps8*. The dendrograms obtained using 'neighbour joining' methods of the two sets of markers distinctly delimit the 15 species and placed them into 2 main clusters; the native species and the exotic species. The study revealed that the two exotic species, *D. floribunda* and *D. composita* were distinct in their chemical and molecular constitution compared to the Indian *Dioscorea* species, suggesting the correlation of phytogeography as well as evolutionary phenology to the secondary metabolite distribution and genetic constitution.

Keywords: Dioscorea species, Western Ghats, diosgenin, HPTLC, matK, rpl36-infA-rps8

INTRODUCTION

The genus *Dioscorea* L. (family *Dioscoreacea*) is an important tuber crop, represented by 850 species in tropical or sub tropical regions, with Central America, South America, Africa and Southeast Asia as the centres of diversity. A few species are also distributed in the temperate areas of Europe and North America. Around 50 *Dioscorea* species are reported from India, including the greater yam or the Asiatic yam (*D. alata* L.) and potato yam (*D. bulbifera* var. *sativa*), along with the introduced white yam (*D. rotundata* Poir.) and lesser yam (*D. esculenta* Lour. Burk.). The Western Ghats, an important biodiversity hot spot in India, is rich in wild relatives of tuber crops and hosts more than 20 *Dioscorea* species.[1] The *Dioscorea* species are important source of secondary staple food as its tubers, commonly known as 'yam' have high nutritional value and were probably the main source of sustenance for the tribal people world over**.** Yams have a reputed place in traditional herbal medicinal practices, especially due to their potency in enhancing fertility in males. *Dioscorea* species are

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rich source of diosgenin, a steroidal sapogenin used as the precursors for corticosteroids and anticonceptional hormones.[2] Several wild and cultivated species of *Dioscorea* are used for treatment of leprosy, cancer, dyspepsia, diarrhoea, dysentery, psoriasis etc. Many species are used in ayurvedic, unani and other systems of medicine. It is used as purgatives, laxatives, expectorants, and for the treatment of poison bites and skin diseases.[3-6] The tribals of Assam use toxic tubers of *Dioscorea* to poison the arrows while hunting animals and fighting with enemies.

Being one among the most primitive of the angiosperms and a vital group in the evolution of monocotyledons, *Dioscorea* is by far the most critical taxa in monocot systematics. *Dioscorea* has presented a challenge to systematists for many years due to its great morphological diversity, dioecy and small flowers,[7] and several attempts have been made by classical taxonomists to divide *Dioscorea* species based on the morphology, ecology and biogeography.[8]

Along with morphological characters, several tools such as anatomy, palynology and cytology have also been explored for the systematics of the *Dioscorea* species and recently, higher level classifications of the group have been successfully demonstrated based on DNA sequence data.[9] This molecular level approach has been effective in addressing many phylogenetic questions that had not been solved only by using phenotypic characters. A variety of loci have been suggested to solve complicated taxonomic problems and to infer phylogenetic relationships of plants, including coding genes and non-coding spacers in the nuclear and plastid genomes. Among the different loci, the non-coding plastid *rpl36-infA-rps8* intergenic spacer region and the coding *matK* gene are two successfully demonstrated loci for *Dioscorea* species.[10,11] Partial dataset for plastid *rbcL* has already been demonstrated by L.R. Caddick et al,[9] and *matK* was selected to provide further resolution following its successful use in other studies of monocot taxa.[12]

The steroidal sapogenins particulary diosgenin is valuable as the precursors for corticosteroids and anticonceptional hormones. Sapogenins, the 27-carbon steroids, are widely distributed in plants, especially in a number of monocotyledonous families such as *Liliaceae*, *Amaryllidaceae* and *Dioscoreaceae*. *Dioscorea* species are rich source of sapogenins, especially diosgenin and *D. composita* and *D. floribunda* are widely exploited as source of diosgenin. It is interesting to note that no appreciable amount of diosgenin has been found in Old World or temperate region *Dioscorea* species.[13]. Further, steroidal saponins in *Dioscorea* species have already been used as standard marker compounds in botanical products due to their chemotaxonomical significance and their important biological activities.[14]

Though more than 50 *Dioscorea* species are reported from India, the taxonomic identity of some of the taxa are still in ambiguity, mainly due to the reliance on morphological characters that are highly polymorphic. The present work exploits chemical and molecular tools for the biosystematics and phylogenetic relations of *Dioscorea* species of Western Ghats for the first time. The approach could also be helpful in searching for wild relatives of the important indigenous yams, and will be useful for plant breeders, pathologists, phytochemists and other applied biologists.

MATERIALS AND METHODS

1.1. Plant materials: The tubers of 11 native *Dioscorea* species were collected from various regions of the south Western Ghats, while the four cultivated species *D. composita*, *D. floribunda*, *D. rotundata* and *D. esculenta* (table 2) were collected from JNTBGRI campus. Voucher herbarium specimens were deposited in the JNTBGRI herbarium (TBGT).

1.2. Morphological studies: Different species of *Dioscorea* from both sexes were collected in flowering and fruiting conditions preferably in. Special attention was paid to gather data pertaining to habit, habitat, altitude and other features like colour of the flowers and indumentums which cannot be deduced from the examinations of the herbarium specimens. The taxonomic identities of the collected materials were confirmed with the help of various floras and also by consulting with authentic specimens depositories in various national herbaria like MH, TBGT and MSSH.

1.3. HPTLC profiling: Diosgenin extraction was carried out according to reported procedure.[15] Dried and powdered tubers (2g each) were refluxed with 10 ml 2N HCl for 2h to hydrolyse the saponins to sapogenins and sugars. The residue separated by filtration was washed with 50 ml of water twice, dried at 65^{o}C for 2h and extracted over night with chloroform in a Soxhlet apparatus. The extracts were filtered through 0.45µm nylon membrane filters (PALL Gelman Laboratory, India) and made up to 10 ml. HPTLC analysis was done using Camag (Switzerland) HPTLC system. The extracts (5µL each) were applied to HPTLC plate (Silica gel, Merck 60, F_{254}) of 20 cm x 20 cm dimension and 0.2 mm layer thickness along with 1 µl standard diosgenin (0.2 mg /ml in chloroform). The spots were applied with automatic Linomat V sample applicator, fitted with a Camag micro syringe in N_2 flow. The plates were developed in Camag twin trough glass chamber using the mobile phase, hexane: ethyl acetate (8:2 v/v), after saturating with the same solvent system for 30 min. After derivatisation with anisaldehyde-H_2SO_4 spray reagent, the peak areas of the bands were measured at 254 and 366nm densitometrically using TLC scanner 3 and the data were analyzed with WinCATS Software version 4.03. For estimation purpose, the standard stock solution of diosgenin (Sigma-Aldrich, St. Louis, USA) was prepared by dissolving 10 mg diosgenin in 10 ml methanol, and diluting with methanol to get the final concentration of 100 ng/l. Standard diosgeninin the range 0.1–1.0 µg/band gave linear response. The experiments were done in triplicate and the average value was taken. The calibration curves were prepared by plotting the amount of diosgenin spotted (ng/band) as independent variable (X) and the respective peak area as dependent variable (Y). The diosgenin contents of the extracts were calculated from the calibration graph.

1.4. Molecular studies: DNA was extracted from young fresh or silica gel-dried leaves using a modified CTAB procedure.[16]

The primers for *matK* region (MF and MR) were designed based on the *matK* sequence of *D. alata* L. (AB040208),[11] and one intergenic spacer *rpl36-infA-rps8* (rpl36-f and rps8-r) based on reported method,[10] (table 1) with the aid of the software Oligo (Molecular Biology Insights, Inc., Cascade, Colorado, USA). Detailed sequences of all the primers are listed in Table 1. The polymerase chain reaction (PCR) was conducted with the following programs: a premelt of 5 min at 95°C, followed by 40 cycles of 30 s denaturation at 95°C, 40 s annealing at 58.7°C, 1 min extension at 72°C, plus a final extension of 5 min at 72°C for *mat*K, and a premelt of 5 min at 94°C , followed by 40 cycles of 30 s denaturation at 94°C, 30 s annealing at 55°C, 30 s extension at 72°C, plus a final extension of 5 min at 72°C for *rpl36-infA-rps8*. Each 20 µL reaction contained 1X PCR buffer (150mM TrisHCl, pH-8; 500mM KCl), 0.2mM each dNTPs (dATP, dGTP, dCTP and dTTP), 2.5mM $MgCl_2$, 20ng DNA, 1 unit of AmpliTaq Gold DNA polymerase enzyme (hot start DNA polymerase from applied biosystems, USA), 0.1 mg/ml BSA to avoid PCR inhibiting substances and 4% DMSO for removing secondary structure formation, 5pM of forward and reverse primers. The PCR products were checked in 1.2% agarose gels prepared in 0.5X TBE buffer containing 0.5 µg/ml ethidiumbromide. 1 µl of 6X loading dye was mixed with 5 µl of PCR products and was loaded and electrophoresis was performed at 75V power supply with 0.5X TBE as electrophoresis buffer for about 1-2 hours, until the bromophenol blue front had migrated to almost the bottom of the gel. The gels were visualized in a UV transilluminator (Genei) and the image was captured under UV light using Gel documentation system (Bio-Rad) (Figure 1). The PCR products were purified by ExoSAP-IT (GE Healthcare). Five micro litres of PCR product is mixed with 2 µl of ExoSAP-IT and incubated at 37°C for 15 minutes followed by enzyme inactivation at 80°C for 15 minutes Sequencing reaction was done in a PCR thermal cycler (GeneAmp PCR System 9700, Applied Biosystems) using the BigDye Terminator v3.1 Cycle sequencing Kit (Applied Biosystems, USA) following manufactures protocol. The sequencing PCR temperature profile consisted of a 1st cycle at 96°C for 2 minutes followed by 30 cycles of 30 sec at 96°C, 40 sec of 50°C and 4 minutes of 60°C for all the primers.

Table 1. Primers used to amplify and sequence DNA

Primer	Direction	Primer sequence (5' to 3')
matK(MF)	Forward	ATT TGC GAT CTA TTC ATT CAA T
matK(MR)	Reverse	TGA GAT TCC GCA GGT CAT T
rpl36-infA-rps8	Forward	CACAAATTTTACGAACGAAG
rpl36-infA-rps8	Reverse	TAATGACAGAYCGAGARGCTC GC

1.5. Phylogenetic analyses: The sequence quality was checked using Sequence Scanner Software v1 (Applied Biosystems). Sequence alignment and required editing of the obtained sequences were carried out using Geneious Pro v5.6.Preliminary phylogenetic analyses were done using the parsimony criterion for both the markers, and found no incongruent grouping among any of the comparisons. The data were combined to form a neighbor joining (NJ) analyses on this 3-gene matrix (*matK*, *rpl36-infA-rps8* and their concatenated sequence).

RESULTS

The *Dioscorea* species studied inhabit in the forest margins and open areas up to 2000m above sea level. The ariel stems twines on the neighboring plants and the distinctly petiolate leaves are with variable phyllotaxy. The plants are usually dioecious and the inflorescences are spicate with many or few actinomorphic flowers. Among the 15 *Dioscorea* species studied, the ariel parts of *D. esculenta* and *D. bulbifera* twines left with simple leaves, while all other species twines right with simple or compound leaves. Among the 15 species studied, 11 species were with simple leaves while *D. Pentaphylla*, *D. Kalkapershadii*, *D. hispida* and *D. tomentosa* were with compound leaves. The tubers were deeply buried underground except for *D. hispida*, where the tubers are near the soil surface. The tuber skin is comparatively thin in all species except the exotic species *D. composita* and *D. floribunda*. The colour of tuber flesh varies from white to lemon yellow or purple, except for *D. floribunda*, where the tuber flesh is deep yellow in colour. Bulbils, a specially adapted characteristic for vegetative propagation in the ariel portion, are present among 6 *Dioscorea* species studied.

Of the 15 *Dioscorea* species studied, *D. floribunda* and *D. composita* are truly exotic species, while *D. esculenta* and *D. rotundata* are exotic, but now widely distributed and naturalized

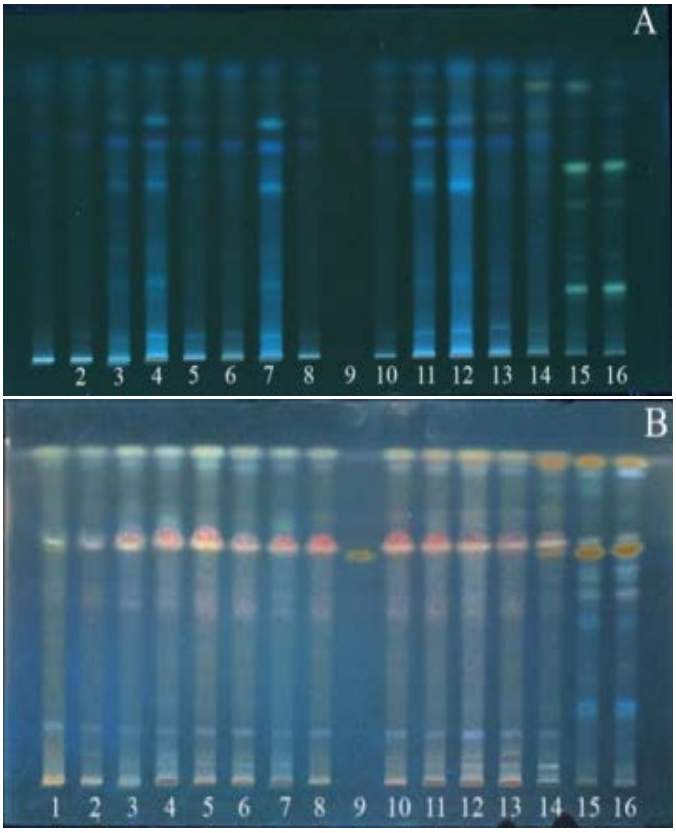

Figure 1. HPTLC profile

Table 2. *Dioscorea* species studied

Sl. No.	*Dioscorea* species	Distribution	Collection locality	Voucher specimen No. (TBGT)
1	*D. alata* L.	Cultivated, common	Palode, Thiruvananthapuram	68205
2	*D. belophylla* Voigt.	Wild, rare, endemic to Western Ghats	Kattunaika settlement, Wayanad	68254
3	*D. bulbifera* L.	Wild, common	Thirunelli, Wayanad	68267
4	*D. composita* Hemsl.	Cultivated, exotic	JNTBGRI campus, Thiruvananthapuram	68281
5	*D. esculenta* (Lour.) Burkill	Cultivated, exotic but naturalized in India	JNTBGRI campus, Thiruvananthapuram	71319
6	*D. floribunda* Mart. & Gal.	Cultivated, exotic	JNTBGRI campus, Thiruvananthapuram	68282
7	*D. hispida* Dennst., Schlussel	Wild, rare	MSSRF campus, Wayand	68288
8	*D. kalakapershadii* Prain & Burkill	Wild, endemic to Western Ghats	Silent valley National park	71311
9	*D. oppositifolia* L.	Wild, common	Mankulam, Idukki	68289
10	*D. pentaphylla* L.	Wild, common	Munnar, Idukki	68258
11	*D. pubera* Blume	Wild, rare	MSSRF campus, Wayanadu	60816
12	*D. rotundata* Poir.	Cultivated, exotic but naturalized in India	JNTBGRI campus, Thiruvananthapuram	71335
13	*D. tomentosa* Koenig ex Spreng.	Wild, common	Tholpetty, Wayanad	68243
14	*D. wallichii* Hook. f.	Wild, common	Agastyamala, Thiruvananthapuram	68225
15	*D. wightii* Hook. f.	Wild, endemic to Western Ghats	Agastyamala, Thiruvananthapuram	68252

in India. HPTLC estimation showed high diosgenin content in the two exotic species *D. floribunda* (15.42mg/g) and *D. composita* (8.64mg/g), followed by *D. rotundata* (0.12mg/g) and *D. esculenta* (0.07mg/g). The diosgenin content was not in detectable quantity in the native species (Figure 1). In addition to the high content of doisgenin, the HPTLC profile at UV366 nm revealed characteristic similarity between *D. floribunda* and *D. composita* in the distribution of other secondary metabolites. The distribution of secondary metabolites also revealed *D. rotundata* as more allied to the exotic species than *D. esculenta*.

All the 15 species from both the sexes were successfully amplified with the two sets of primers for *matK* and *rpl36-infA-rps8*, exception where only in *D. Kalkapershadii* with only one accession. Most *matK* sequences recovered the full length of the barcode region which was trimmed to a final aligned length of 1033bp (Figure 2). There were no insertions or deletions. Based on the sequence analysis of A-T rich *matk* gene we could find 14 specific sites for two exotic *dioscorea* species, *D. composita* and *D. floribunda*. But these two species were distinguished based on the single nucleotide at 128th position (T for *D. composita* and C for *D. floribunda*). The *rpl36-infA-rps8* sequences recovered length ranging from 519bp to 524bp, which were aligned to a final length of 529bp (Figure 2). There were 16 indels in the final alignment. Like that *matK* in A-T rich *rpl36-infA-rps8* gene we could find 6 specific sites for two *Dioscorea* species, *D. composita* and *D. floribunda*. But these two species where distinguished based on the single nucleotide at 101st position (C for *D. composita* and T for *D. floribunda*).

Three different types of dendrograms were obtained from the cluster analysis using neighbor joining methods for two sets of markers. The dendrograms distinctly delimits the 15 *Dioscorea* species studied (Figure 3).

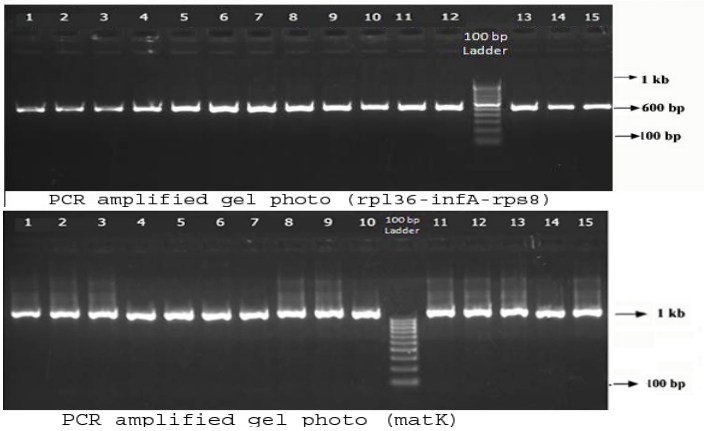

Figure 2. PCR amplified products alog with 1 Kb. Ladder

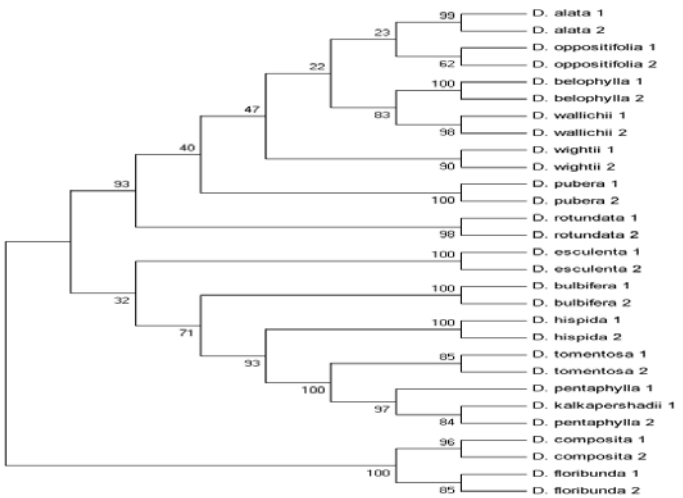

Figure 3. Combined neighbour joining (NJ) tree based on the *matK* and *rpl36-infA-rps8* sequence data.

All the *Dioscorea* species selected were clustered into two major groups; cluster 1 with 13 peninsular Indian species and cluster 2 with the two exotic species, *D. Composita* and *D. floribunda* (Figure 3). In combined pair-wise analysis, genetic divergence of *D. composita* was minimum with *D. floribunda* (0.26%), while the maximum genetic divergence was observed for *D. composita* and *D. floribunda* with *D. kalkapershadii* (3.45% and 3.32% respectively).

DISCUSSION

3.1. Morphological features: The genus *Dioscorea* is considered to be among the most primitive of the angiosperms and a vital group in the evolution of monocotyledons. They exhibit a number of morphological, anatomical and embryological characters reminiscent of dicotyledons such as petiolate leaves with reticulate venation and non-sheathing base.[17] Tuber morphology, stem twining direction, dioecy, fruit shape and seed wing shape are among the most important characters in the systematics of *Dioscorea* species. In the present study, evaluation of the morphological features revealed that the twining character, and number and nature of leaflet are strong characters that differentiate *Dioscorea* species. However, among the *Dioscorea* species with compound leaves, *D. pentaphylla* showed high degree of variation in number of leaves by displaying 3 to 5 leaves in accordance with the varieties. *D. tomentosa* is with compound leaves but at the younger stage simple leaves are displayed at the basal part of the stem. *D. hispida* is also with compound leaves but at the apical part it bears simple leaves when it attains maturity. *D. oppositifolia* is not always with opposite leaves as its name indicates.

Though the tubers of *Dioscorea* are considered as modified from rhizomes,[18] observed that the old world tuber bearing species are quite distinct from the rhizomatous forms. In the case of the old world *Dioscorea* species the multiple noded rhizomes appears to be suppressed, or it may be represented by the perennial bud. The organization of the tuber in the new world *Dioscorea* species into ventral and dorsal sides, however suggests the resemblance of the tuber to the ancestral rhizome,[19] described two types of tubers, one with continuous secondary growth, covered by a thick coat of cork,[20] found in species from the New World and another type that develops a new tuber after sprouting, usually found in plants from the Old World.[21] Among the 15 *Dioscorea* species studied, the perennial and exotic species *D. composita* and *D. floribunda* possess thick corky tuber tuber compared to the native *Dioscorea* species.

3.2. Biogeographic history: The genus *Dioscorea* is by far the most geographically widespread group in the family *Dioscoreaceae*. It is almost ubiquitous in tropical and sub tropical regions, and a few species extends to the temperate areas. Among the 15 *Dioscorea* species, *D. composita* and *D. floribunda* are newly introduced species in India from Mexico and Central America respectively,[22] while the other introduced species *D. esculenta* and *D. rotundata* are now widely distributed and naturalized in India. Comparison of *D. composita* and *D. floribunda* suggests that these two species are well separated morphologically.[23] The high diosgenin content and particularly its adaptability to the agro-climatic conditions of the vast Indian plains attracted attention for its wide propagation in India.[6]

3.3. Distribution of diosgenin: Among the *Dioscorea* species, sapogenin bearing species are separated morphologically, geographically and by season of bloom. A systematic effort for the study of diosgenin as a marker compound among *Dioscorea* species will supplement the hypothesis of biogeographical demarcation among the group as proposed by Knuth and Burkill.[8] There exists a clear demarcation between Old and New World *Dioscorea* species in the diosgenin content and the crossing data also suggest that the reproductive barriers between old and new world *Dioscorea* species are strong and difficult to circumvent.[23] The reason for successful crossing among sapogenin-bearing species, in contrast to less success in other crosses, may suggest a closer relationship of these species than has been previously recognized, or than would be suggested on the basis of morphological data alone.

The present study showed that in addition to tuber morphology, the Old World and New World *Dioscorea* species were well delineated in their diosgenin content also. The HPTLC profile using diosgenin as marker compound groups the two exotic species *D. composita* and *D. floribunda* into one clad with *D. composita* and *D. esculenta* as allied species to the group, while the true native species forms the other clad. Further, the HPTLC profiles of the exotic species *D. rotundata* and *D. composita* showed close similarity in the distribution of other secondary metabolites (Figure 1). The steroidal sapogenin, diosgenin can be considered as a biogeographical marker compound, demarkating the exotic and native *Dioscorea* species. The chemical profile thus suggests the correlation of phytogeography as well as evolutionary phylogeny to the secondary metabolite distribution.

3.4. Molecular phylogeny

Though morphology, cytology, palynology, and other traditional means of identification have been explored for the systematics of *Dioscorea* species, classification at the higher level remains obscure due to the paucity of methods adopted. With the development of molecular biology, however, some DNA sequences, such as *rbc*L, *mat*K and *trn*L-F, have been used to solve complicated taxonomic problems and to infer phylogenetic relationships among organisms, including members of the genus *Dioscorea*. A variety of loci have been suggested for phylogenetic analysis of plants, including coding genes and non-coding spacers in the nuclear and plastid genomes.

The efficiency of DNA isolation, PCR amplification and sequencing are the first and most important indicators for evaluating the applicability of DNA barcodes. The result agreed that the isolation is somewhat difficult in *Dioscorea* species due to the presence of phenolics and mucilage. For flowering plants the non-coding plastid *rpl36-infA-rps8* intergenic spacer region and the coding *matK* gene are two of the leading candidates.[10, 11] These two suggested locus were demonstrated to be successful in *Dioscorea*. Insertions or deletions appear to be a common

characteristic of this genetic region, even in closely related species.[24] The variable lengths of this region make sequence alignment difficult. Large insertion or deletion was also found in different populations of *Dioscorea*. For example, *Dioscorea zingiberensis* C.H. was collected from China by Wight had a 234 bp insertion segment at 183 bp when compared to other populations. In contrast with the problems of indels for sequence alignment, indels will ultimately enrich the information needed for species discrimination.[10] In our study the indels were only detected in the *rpl36-infA-rps8*

Even though, *matK* is a recommended single DNA barcode candidate gene because of its high rate of evolution. In our study showed that the *matK* is a strong, although not perfect, candidate for identification of *Dioscorea* species. But compared to *rpl36-infA-rps8* and their combined sequence analysis suggested *matK* is the best in species identification of this genus. Using NJ tree and their unique conserved site or sites for these species reveals, this is the best barcoding marker to identify the native and exotic species. In NJ tree the position of the two species, *D. floribunda D. composita* and single nucleotide polymorphism in these two species that delimits the plants native to Mexico, Central America, from peninsular Indian species.[22] It strongly delimit the monophyletic diosgenin rich group *D. floribunda*, and *D. Composita* from other native species

CONCLUSION

The study revealed characteristic differences in the chemical and molecular profile of native *Dioscorea* species compared to the exotic species suggesting the correlation of phytogeography as well as evolutionary phylogeny to the secondary metabolite distribution and genetic constitution. The exotic species were rich in diosgenin while in the Indian species diosgenin content was negligible or absent. This is the first report on the biosystematics of the *Dioscorea* species of Western Ghats, including 3 Western Ghats endemic species, incorporating morphological, chemical and molecular tools.

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REFERENCES AND NOTES

1. V. Balakrishnan, M.K. Ratheesh Narayanan, N. Anilkumar. Ethnotaxonomy of *Dioscorea* among Kattunaikka people of Wyanadu district, Kerala, India. *IPGRI Newsletter*, **2003,** 135, 24-32.
2. D.G. Coursey. Yams.An account of the Nature, Origins, Cultivation and Utilisation of the Useful Members of the *Dioscoreaceae*. *Tropical Agricultural Series. Longmans, Green and Co. Ltd. Londres, UK.* **1967,** 230.
3. K.R. Kirthikar, B.D. Basu. *Indian Medicinal Plants.* Bishen Singh Mahendrapal Singh. Dehra Dun. **1918,** 2479-2490.
4. R.N. Chopra, S.L. Nayar, I.C. Chopra. *Glossary of Indian Medicinal plants*. Council of Scientific & Industrial Research, New Delhi **1956,** 97-98.
5. H. Santapau, A.N. Henry. *A Dictionary of the flowering plants in India*. Council of Scientific and Industrial Research. New Delhi. **1968,** 55.
6. L.V. Asolkar, Y.R. Chadha. *Diosgenin and other steroid drug precursors.* Publication and Information Directorate, CSIR New Delhi. **1979,** 2-17.
7. P. Wilkin, P. Schols, M.W. Chase, K. Chayamarit, C.A. Furness *et al.* A plastid gene phylogeny of the yam genus, *Dioscorea*: roots, fruits and Madagascar. *Systematic Botany*. **2005,** 30, 736-749.
8. R. Knuth. *Dioscoreaceae*. In: Das Pflanzenr **1924**. 43. 4 (Heft 87), 1-318.
9. L.R. Caddick, C.P. Rudall, J.P. Wilkin, A.J. Hedderson, M.W. Chase. Phylogenetics of Dioscoreales Based on Combined Analyses of Morphological and Molecular data. *Botanical Journal of the Linnean Society* **2002**, 138, 123-144.
10. W.J. Kress, K.J. Wurdack, E.A. Zimmer, L.A. Weigt, D.H. Janzen Use of DNA barcodes to identify flowering plants. *Proceedings of the National Academy of Sciences*, USA, **2005**, 02, 8369-8374.
11. Xiao-Qin Sun, Ying-Jie Zhu, Jian-Lin Guo, Bin Peng, Ming-Ming Bai, Yue-Yu Hang DNA Barcoding of the *Dioscorea* in China, a Vital Group in the Evolution of Monocotyledon: Use of *mat*KGene for Species Discrimination. *PLoS One*, **2012**, 7(2), e32057.
12. S. Fuse, M.N. Tamura A phylogenetic analysis of the plastid *mat*K gene with emphasis on Melanthiaceae *sensu lato.Pl. Biol*. **2000,** 2, 415-427.
13. J.J. Willaman, C.S. Fenske, D.S. Correll Occurrence of alkaloids in Dioscorea. *Science* **1953**, 118(3064), 329-330.
14. K.D. Yoon, Y.W. Chin, M.H. Yang, J. Choi, J. Kim Application of High-speed Countercurrent Chromatography–Evaporative Light Scattering Detection for the Separation of Seven Steroidal Saponins from *Dioscorea villosa. Phytochemical Analysis*, **2012**, 23 (5) 462-468.
15. D. Drapeau, Y. Sauvaire, H.W. Blanch, C.R. Wilke. Improvement of diosgenin yield form *Dioscorea deltoidea* plant cell cultures by use of a nontraditional hydrolysis method. *Planta Med*., **1986**, 52, 474-478.
16. J.J. Doyle, J.L. Doyle. A rapid DNA isolation procedure for small quantities of fresh leaf tissue. *Phytochem. Bull.* **1987**, 19, 11-15.
17. Okagami. Dormancy in *Dioscorea*: Different temperature adaptation of seeds, bulbils and subterranean organs in relation to north-south distribution. *J. Plant Res*., **1986**, 99, 15-27.
18. I.H. Burkill. The organography and evolution of Dioscoreaceae, the family of the yam. *Jour. Linn. Soc.* **1960,** 56, 319-412.
19. F.W. Martin, S. Ortiz, New chromosome numbers in some *Dioscorea* species. *Cytologia* **1966**, 31, 105-107.
20. R. Dahlgren, H.T. Clifford, P.F. Yeo. The families of the monocotyledons. Structure, evolution, and taxonomy. Springer-Verlag, Berlin, **1985**.
21. I.C. Onwueme The tropical tuber crops.Yams, cassava, sweet potato and cocoyams. John Wiley & Sons, Chichester *John Wiley, New York*, **1978,** 234.
22. D.G. Correll, H.S. Schuber *et al.*, The search for plant precursors of cortisone. *Econo. Bot*. **1955**, 9, 305-375.
23. W. Franklin, Martin, Sonia Ortiz. New Chromosome Numbers in Some *Dioscorea* Species *Cytologia* **1966,** 31 no. 1, 1-107.
24. J. Aldrich, B.W. Cherney, E. Merlin, L. Christopherson. The role of insertions/deletions in the evolution of the intergenic region between *psbA* and *trnH* in the chloroplast genome. *Current Genetics* **1988,** 14, 137-146.