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Assessment of genetic relationships among *Dioscorea* spp. of Melghat Tiger Reserve Maharashtra of Central India, by using RAPD and ITS sequences

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ABSTRACT

Order Dioscoreales belongs to Monocotyledons, but with many more characters such as reticulate venation pattern, nervation between primaries, twinning habit, ring vascular bundles, compounding of leaf, trimerous flowers, rhuminate endosperm, and lateral cotyledon, which shows affinities of Dioscoreales to Dicotyledons. The genus *Dioscorea*, which belongs to the family Dioscoreaceae is widely distributed in MTR, Maharashtra, India and represents five species namely *D. bulbifera*, *D. oppositifolia*, *D. hispida*, *D. pentaphylla*, and *D. belophylla*. A total of 122 decamer random primers were scrolled for RAPD profile and 61 out of them gave amplification in all five *Dioscorea* species. The UPGMA created dendrogram on the basis of RAPD showed two clusters. The cluster I included two subclusters - subcluster I (*D. bulbifera* and *D. oppositifolia*) and subcluster II (*D. hispida* and *D. pentaphylla*). *D. belophylla* was completely outgrouped from rest of the species and was grouped under cluster II. However, ITS sequence data reveal that five species of *Dioscorea* were placed in two major clusters.

Key words: *Dioscorea*, Dioscoreaceae, RAPD, ITS, Genetic analysis

Introduction

Genus *Dioscorea* belongs to the family Dioscoreaceae. It comprises 10 genera and 650 species, distributed mainly in the tropical, subtropical, and semi-temperate regions of the world. The species are pantropical of both humid and arid regions, with a few temperate outliers in Europe, Asia and North America (Raz, 2003).The family Dioscoreaceae is a natural group of tuber forming, tropical vines. The family is usually allied with the Liliales and placed near Amaryllidaceae. The family is divided into two tribes: the Dioscoreae, including six genera, all of which have unisexual flowers, and the Stenomeridae with three genera, which produce hermaphroditic flowers (Smith, 1937). *Dioscorea* occurs in the humid tropics and extended beyond countries with hot summers. It reaches for instance the Amur river in Asia and the Southern border of Canada in North America, and South-eastern Australia, the 34th degree in South Africa and the Argentine in South America. (Burkill, 1939; 1960). Species of *Dioscorea* are widespread in the tropics and subtropics and are most frequently encountered as climbers which perennate by rhizomes or tubers in the forest margins and more open habitats (Wilkin, 2001).

The molecular methods are used as important tools for the investigation of population structure and phylogenetic relationships (Schwarzbach & Ricklefs, 2001). The technique of random amplified polymorphic DNA (RAPD) to study the variation at DNA level was first introduced by Williams et al., (1990). According to them RAPD markers hold promise for the automation of genome mapping, extending the power of genetic analysis to organisms, which lack an ample number of phenotypic markers to completely describe their genome. Genetic mapping using RAPD markers have several advantages over other methods because an universal set of primers can be used for genomic analysis. Simple and reproducible fingerprints of complex genomes can be generated by using single arbitrarily chosen primers by polymerase chain reaction (Welsh & McClelland, 1990).

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RAPD technology has quickly gained widespread acceptance and application because it has provided a tool for genetic analysis in biological systems that have not previously benefited from the use of molecular markers (Tingey & Tufo, 1993, Williams et al., 1993). RAPD can be used for classification at taxonomic levels ranging from individuals, to cultivars and species (Demeke et al., 1992). RAPD technique can be used in molecular ecology to determine taxonomic identity, to assess kinship relationships, to analyze mixed genome samples, and to create specific probes (Hadrys et al., 1992). The initial effort in yam genomics was devoted to the development of polymorphic DNA markers and assessment of their potential application in yams (Mignouna et al., 2003). In yam, cultivar identification via molecular marker-based analyses would be very useful because genetic variation is fixed within a line (Dansi et al., 2000). The RAPD analysis is a rapid and useful approach for distinguishing closely related species in the *Dioscorea* as well as for estimating genetic distance among the species (Asha et al., 2006; Lay et al., 2001).

Phylogenetic relationships in monocotyledons have been evaluated with DNA data (Chase et al., 1995a, Duvall et al., 1993) and have been resolve more or less systematic crunch in monocotyledon, a major clade in the angiosperms. The ribosomal internal transcribed spacer (ITS) sequences have been popularly utilized to study phylogenetic and genomic relationships of plants under lower taxonomic groups (Sang et al., 1995; Volkov et al., 1999; Feliner et al,. 2004; Liu et al., 2006). In the genome of all land plants there are multiple copies of ribosomal gene sequences and have been absorb rapid evolutionary pressure, but they appear to undergo rapid concerted evolution and all copies appear to be virtually identical (Baldwin et al.,1995; Álvarez & Wendel, 2003). The ITS sequences have exhibited evolutionary conservation, therefore, this region utilized for phylogenetic inference in plants (Baldwin, 1992). The ITS region of the 18S–5.8S–26S nuclear ribosomal cistron is widely utilized for construction of phylogenetic tree (Baldwin, 1993). ITS2 sequence data and RAPD analysis have been performed to determine the phylogenetic status of the genus *Dioscorea*. Although Dioscoreales belongs to Monocotyledons, there are many more characters such as reticulate venation pattern, nervation between primaries, twinning habit, ring vascular bundles, compounding of leaf, trimerous flowers, rhuminate endosperm, and lateral cotyledon, which shows affinities of Dioscoreales to Dicotyledons. The genus *Dioscorea* has net-veined rather than parallel-veined leaves and climbing habit like other monocots, which have presumably evolved as convergent adaptations to forest understory conditions (Chase et al., 1995b). The present investigation was undertaken in order to resolve this taxonomic ambiguous situation. The genus *Dioscorea* in the forest of MTR is represented by five species viz. *D. bulbifera* under section Opsophyton, however, *D. hispida* and *D. pentaphylla* under Lasiophyton and *D. oppositifolia* and *D. belophylla* under section Enantiophyllum.

Materials and Methods

Plant material

The genus *Dioscorea* is widely distributed in MTR, Maharashtra, India (Latitudes 21°15'N and 21°45'N, Longitudes 76°57'E and 77°33'E and altitude 312 m to 1178 m above mean sea level) and represents five species namely *D. bulbifera*, *D. oppositifolia*, *D. hispida*, *D. pentaphylla* and *D. belophylla*. A total 5 specimens of each species were randomly collected from different geographical locations. At least 10 locations were selected on the basis of morphological diversity and geographical distribution. During survey, wherever morphological variation occurred within a species the specimens were collected for study. During full flourish season i.e. from June to October plant saplings were collected along with its underground part, which was carried to Botanical Garden of the University.

Isolation of DNA

Isolation of DNA for RAPD analysis was performed by two methods. The species such as *D. bulbifera, D. hispida*, and *D. pentaphylla* contained low polysaccharides, polyphenols and mucus. The DNA from these species was isolated by adopting the CTAB method proposed by Doyle & Doyle (1990). *D. oppositifolia* and *D. belopylla* had high gummy polysaccharides, polyphenols and mucus that were difficult to remove by the protocol proposed by Doyle & Doyle. Hence, for DNA isolation pertaining to these species was performed according to the method proposed by Deshmukh et al. (2007), which is a modified SDS method in order to remove these gummy contents. From each location 50 mg young leaf plucked from each plant, from five plants 250 mg of leaves were randomly collected. The DNA was isolated separately from each location and latter DNA from all 10 locations was pooled and treated as one species sample. Likewise, DNA samples of five *Dioscorea* species were prepared. However, the total genomic DNA was isolated

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from fresh leaf tissue using GenElute™ Plant Genomic DNA Miniprep Kit (Sigma-Aldrich).

PCR Amplification

The RAPD deccamer primer amplification was performed according to the protocol outlined by Williams et al., (1990). Amplification was carried out in 25 µl volume reaction mixture. The reaction mixture contained 25-50 ng template DNA, 2.5 U *Taq* DNA polymerase enzyme (Qiagen, USA), 0.4 µM each dNTP (Qiagen, USA), 2.5 mM $MgCl_2$ (Qiagen, USA), 1X Taq DNA polymerase buffer, which contains 15 mM $MgCl_2$ (Qiagen, USA), 1X Q solution (Qiagen, USA), and 0.4 µm decamer primer (Operon, Technologies, Alameda, USA). The DNA of all the species of *Dioscorea* was amplified in a programmable thermocycler (T personal Biometra, GMBh, Gottingen, Germany) by applying following conditions with initial denaturation at 94°C for 5 min (Step-I), followed by denaturation at 94°C for 1 min (Step-II), annealing at 38°C for 1 min (Step-III), extension at 72°C for 3 min (Step-IV), program switch to step II followed by 49 cycles and final extension at 72°C for 7 min (Step-V). This PCR program requires 5 hrs 16 min for its completion. PCR amplification for ITS2 was performed using five pooled DNA samples representing five species of *Dioscorea* .The polymerase chain reaction (PCR) was conducted in eppendorf mastercycler gradient (Munchen, Germany) with the following program: a premelt of 5 min at 94°C, followed by 35 cycles of 30 s denaturation at 94°C , 30 s annealing at 56°C, 45 s extension at 72°C, plus a final extension of 10 min at 72°C. Each 50 µL reaction contained 1× Mg-free Dream Taq DNA polymerase buffer, 2.5 mmol/L $MgCl_2$, 1 U dream *Taq* DNA polymerase, 40 ng template DNA, 0.2 µmol/L each primer (ITS2-F – GCTGCGTTCATCGATGC and ITS2-R – TCCTCCGCTTATTGTATGC) and 0.12 mmol/L dNTPs. Amplified products were checked on 1.2% agarose gel. Purification and bidirectional sequencing were completed by Chromus Biotech pvt. Ltd. Banglore India**.** using the amplification primers. The sequences of ITS2 were deposited on NCBI's GenBank database (Table.1).

Phylogenetic analysis:

Genetic similarity (GS) for RAPD primers between individuals was estimated according to the formula given by Nei & Li (1979). The phylogenetic analysis was carried out by constructing unitary and binary data matrix as per the method of Nei & Li (1979). The binary data was analyzed for each marker by using NTSYS-pc version 2.0 (Rohlf 1987, 1998) to generate similarity coefficient. The matrix was subjected to Unweighted Pair Group Method for Arithmetic average analysis (UPGMA) (Sokal & Micluner, 1958; Sneath & Sokal, 1973) to generate a dendrogram using average linkage procedure. For ITS2 sequences the phylogenetic analysis was performed by using Mega 6.06 Software by applying Maximum Parsimony method (MP). Sequences were carefully checked on chromatograms with the MEGA 6 software program (Kumar et. al., 2008).

Table 1. *List of Dioscorea spp. with GPS location and Gene Bank accession numbers.*

No.	Species	GPS location	Gene Bank accession number
1	*Dioscorea pentaphylla*	21^{O} 23' 45.83" N / 77^{O} 12' 06.09" E	KR920023
2	*Dioscorea belophylla*	21^{O} 25' 20.55" N / 77^{O} 18' 06.27" E	KR920022
3	*Dioscorea hispida*	21^{O} 23' 39.74" N / 77^{O} 07' 40.39" E	KR920021
4	*Dioscorea bulbifera*	21^{O} 22' 07.58" N / 77^{O} 06' 21.77" E	KR869112
5	*Dioscorea oppositifolia*	21^{O} 23' 29.09" N / 77^{O} 19' 23.91" E	KR869111

Results

RAPD analysis

A total of 122 decamer random primers were scrolled for RAPD profile. Only 61 out of them gave amplification in all the five species of *Dioscorea* (Table 2). The 34 primers produced reproducible banding pattern, and unitary data was taken for phylogenetic analysis (Table 3). The data was analyzed by using NTSYS-pc version 2.0 to generate Ni and Li similarity coefficient (Table 4). The matrix was subjected to UPGMA to generate a dendrogram using average linkage procedure. 429 bands were generated totally by the 34 primers. Out of them, 394 were polymorphic and 35 were monomorphic; average bands per primer were calculated to be 27.88. The Figure 1 shows RAPD pattern of *Dioscorea* spp. with primer OPA12, OPA11, OPB 15, OPP 10, OPW 15 and OPW 16.The mean value of percent polymorphism was 91.55. OPP 4, OPP 10, OPA 3, OPA 9, OPA 12, OPA 13, and OPB 11 showed 100 % polymorphism. The lowest (80%) was in OPB 5 (Table 3).

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Table 2. *List of primer sequences amplified across five Dioscorea spp with amplified bands.*

Sr. No.	Primer	Sequence	No. of bands	Sr. No.	Primer	Sequence	No. of bands
1	OPAP 1	5'AACTGGCCCC 3'	07	**32**	OPW 8	5'GACTGCCTCT 3'	20
2	OPAP 4	5'CTCTTGGGCT 3'	10	**33**	OPW 12	5'TGGGCAGAAG 3'	18
3	OPAP 5	5'GACTTCAGGG 3'	11	**34**	OPW 14	5'CTGCTGAGCA 3'	15
4	OPP 1	5'GTAGCACTCC 3'	13	**35**	OPW 15	5'TTCCGAACCC 3'	18
5	OPP 2	5'TCGGCACGAC 3'	20	**36**	OPW 16	5'CAGCCTACCA 3'	14
6	OPP 3	5'GTGATACGCC3'	12	**37**	OPW 17	5'GTCCTGGGTT 3'	14
7	OPP 4	5'GTGTCTCAGG 3'	12	**38**	OPW 18	5'TTCAGGGCAC 3'	12
8	OPP 5	5'CCCCGGTAAG 3'	11	**39**	OPQ 4	5'AGTGCGCTGA 3'	14
9	OPP 8	5'ACATCGCCTA 3'	11	**40**	OPB 1	5'GTTTCGCTCC 3'	11
10	OPP 10	5'TCCCGCCTAC 3'	12	**41**	OPB 2	5'TGATCCCTGG 3'	07
11	OPP 12	5'AAGGGCGAGT 3'	12	**42**	OPB 3	5'CATCCCCCTG 3'	07
12	OPP 15	5'GGAAGCCAAC 3'	11	**43**	OPB 4	5'GGACTGGAGT 3'	06
13	OPP 17	5'TGACCCGCCT 3'	09	**44**	OPB 5	5'TGCGCCCTTC 3'	05
14	OPP 18	5'GGCTTGGCCT 3'	12	**45**	OPB 6	5'TGCTCTGCCC 3'	07
15	OPP 19	5'GGGAAGGACA 3'	07	**46**	OPB 7	5'GGTGACGCAG 3'	08
16	OPA 1	5'CAGGCCCTTC 3'	14	**47**	OPB 8	5'GTCCACACGG 3'	11
17	OPA 2	5'TGCCGAGCTG 3'	11	**48**	OPB 9	5'TGGGGGACTC 3'	11
18	OPA 3	5'AGTCAGCCAC 3'	11	**49**	OPB 10	5'CTGCTGGGAC 3'	12
19	OPA 4	5'AATCGGGCTG 3'	13	**50**	OPB 11	5'GTAGACCCGT3'	12
20	OPA 5	5'AGGGGTCTTG 3'	07	**51**	OPB12	5'CCTTGACGCA 3'	17
21	OPA 6	5'GGTCCCTGAC 3'	12	**52**	OPB 13	5'TTCCCCCGCT 3'	10
22	OPA 8	5'GTGACGTAGG 3'	07	**53**	OPB 14	5'TCCGCTCTGG 3'	18
23	OPA 9	5'GGGTAACGCC 3'	14	**54**	OPB 15	5'GGAGGGTGTT 3'	16
24	OPA 10	5'GTGATCGCAG 3'	14	**55**	OPB 16	5'TTTGCCCGGA 3'	13
25	OPA 11	5'CAATCGCCGT 3'	08	**56**	OPB 17	5'AGGGAACGAG 3'	14
26	OPA 12	5'TCGGCGATAG 3'	11	**57**	OPB 18	5'CCACAGCAGT 3'	14
27	OPA 13	5'CAGCACCCAC 3'	10	**58**	OPB 19	5'ACCCCCGAAG 3'	12
28	OPA 15	5'ACACCGGAAC 3'	09	**59**	OPB 20	5'GAACCCTTAC 3'	12
29	OPA 16	5'AGCCAGCGAA 3'	11	**60**	Primer 1	5'CCCTTGGGGG 3'	11
30	OPW 5	5'GGCGGATAAG 3'	15	**61**	Primer 2	5'ACAGGGGTGA 3'	16
31	OPW 6	5'AGGCCCGATG 3'	11				
Total no. of bands							**733**

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Table 3. *List of primers produced reproducible banding patterns and polymorphism generated by the primers.*

Sr. No.	Primers	Total bands	Polymorphic bands	Monomorphic bands	% Polymorphism
1	OPAP 1	7	06	1	85.71
2	OPP 1	13	12	1	92.30
3	OPP 2	20	18	2	90.00
4	OPP 3	12	11	1	91.66
5	OPP 4	12	12	0	100
6	OPP 8	11	10	1	90.90
7	OPP 10	12	11	1	91.64
8	OPP 12	12	12	0	100
9	OPP 15	11	09	2	81.81
10	OPP 17	09	07	2	77.77
11	OPP 18	12	11	1	91.66
12	OPA 1	14	13	1	92.85
13	OPA 3	11	11	0	100
14	OPA 9	14	14	0	100
15	OPA 12	11	11	0	100
16	OPA 13	10	10	0	100
17	OPA 15	09	08	1	88.88
18	OPA 16	11	10	1	90.90
19	OPW 5	15	14	1	93.33
20	OPW 6	11	10	1	90.90
21	OPW 8	20	19	1	95.00
22	OPW14	15	14	1	93.33
23	OPW15	18	16	2	88.88
24	OPB 5	05	04	1	80.00
25	OPB 8	11	10	1	90.90
26	OPB 9	11	10	1	90.90
27	OPB 11	12	12	0	100
28	OPB 13	10	09	1	90.00
29	OPB 14	18	15	3	83.33
30	OPB 15	16	14	2	87.50
31	OPB 17	14	13	1	92.85
32	OPB 18	14	13	1	92.85
33	OPB 19	12	10	2	83.33
34	Primer-1	16	15	1	93.75
Total		**429**	**394**	**35**	**91.55**

Table 4. *Similarity matrix of Dioscorea spp. based on RAPD data*

	D. bulbifera	***D. oppositifolia***	***D. hispida***	***D. pentaphylla***	***D. belophylla***
D. bulbifera	1				
D. oppositifolia	0.55	1			
D. hispida	0.48	0.48	1		
D. pentaphylla	0.45	0.48	0.45	1	
D. belophylla	0.47	0.52	0.51	0.52	1

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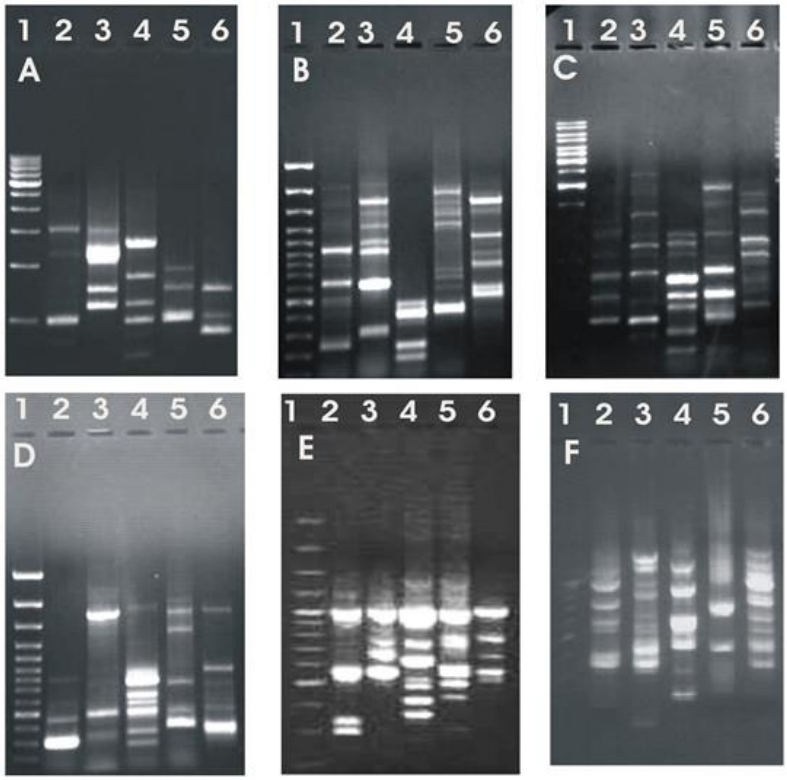

Figure 1. *RAPD genetated DNA fingerprinting patterns of five Dioscorea species. (A) OPA 12 Seq. 5'TCGGCGATAG3' (B) OPA 11 Seq.5'CAATCGCCGT3' (C) OPB 15 Seq. 5'GGAGGGTGTT3' (D) OPP10 Seq. 5'TCCCGCCTAC3' (E) OPW 15 Seq. 5'CAGCCTACCA3' (F) OPW 16 Seq. 5'CAGCCTACCA3' Lane-1- 10000 bp Ladder, 2 - D. bulbifera, 3 - D. oppositifolia, 4 - D. hispida, 5 - D. pentaphylla, 6 - D. belophylla.*

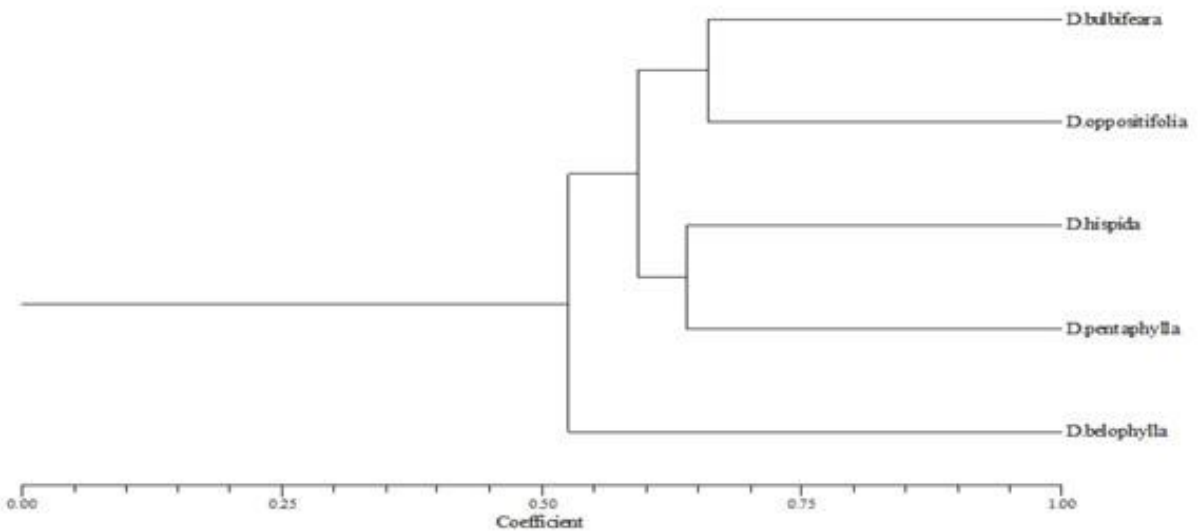

Figure 2. A *Dendogram developed from UPGMA analysis showing genetic relationships between Dioscorea spp. by RAPD*.

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From the dendrogram can be seen that the five species were placed in two major clusters (Figure. 2). The species *D. bulbifera* and *D. oppositifolia* were grouped in subcluster I, while the *D. hispida* and *D. pentaphylla* were grouped in subcluster II. *D. belophylla* was completely outgrouped from rest of the species and was separated as cluster II (Figure. 2). The highest dissimilarity was observed between *D. bulbifera*-*D. pentaphylla*, and *D. hispida*-*D. pentaphylla* (0.55). The lowest dissimilarity was observed between *D. bulbifera* and *D. oppositifolia* (0.45).

ITS Sequences

The dendrogram was obtained from ITS sequence data comparisons. It reveals that five species of *Dioscorea* were placed in two major clusters (Figure. 3). The species *D. oppositifolia* and *D. belophylla* were grouped in subcluster I of cluster-I, while the *D. hispida* was outgroupend in the same subcluster. However, *D. bulbifera* was maintaining its separate entity under this cluster. Moreover, *D. pentaphylla* was found to be outgrouped from rest of the species and placed in cluster II (Figure. 3).

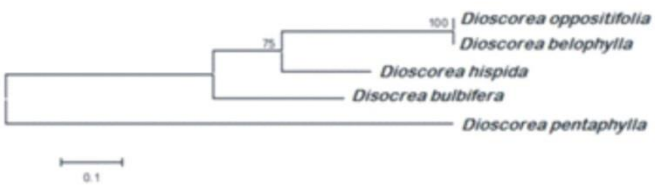

Figure 3. *ITS sequence generated dendrogram showing genetic relationship among Dioscorea spp.*

Discussion

There are at least three main contaminants associated with plant DNA that can cause considerable difficulties while conducting restriction enzyme analysis and PCR amplification: polyphenolic compounds, polysaccharides, and RNA (Jobes et al., 1995). It seemed that the different types of chemical constituents in more or less proportion would be present in species of *Dioscorea* of MTR. These chemical constituents interfere with the PCR process as they act as PCR inhibitors. To resolve this problem we performed isolation of DNA from *Dioscorea* species of MTR by two methods. The species such as *D. bulbifera, D. hispida,* and *D. pentaphylla* had low polysaccharides, polyphenols and mucus contents. Hence, the DNA of these species was isolated by CTAB method. Unlikely, *D. belophylla* and *D. oppositifolia* had high gummy polysaccharides, polyphenols and mucus, and for those species we have applied a modified SDS method.

For any particular genome, a certain percentage of RAPD primers do not produce satisfactory amplification products. Therefore, the best strategy to be used with RAPD technology is to screen many primers and select only those that give only highly reproducible bands for scoring rather than trying to optimize every primer/ template combination (Bowditch et al., 1993). In present study we screened 122 decamer primers for amplification of genomic DNA from five species of *Dioscorea*. Out of 122 primers only 61 produced good amplification in all five species of *Dioscorea*; the 34 primers produced reproducible results. Total 429 bands were generated by 34 primers. Out of them 394 were polymorphic and 35 were monomorphic. OPP 4, OPP 10, OPA 3, OPA 9, OPA 12, OPA 13, and OPB 11 exhibited 100% polymorphism. Most of the primers showed polymorphism (80-100%) in their banding patterns, resembled high degree of genetic diversity among five species of the genus *Dioscorea* in the forest of MTR. Highly reproducible banding patterns were obtained for nine of the fifteen primers, while the others produced either a few bands superimposed on a smear, or no bands at all in Jamaican yam (Asemota et al., 1996). Twelve primers generated a total of 63 bands of which 47 were polymorphic in *D. cyenensis* / *D. rotundata* complex (Dansi et al., 2000). This is an indicator to use non-ambiguous primers for RAPD analysis. RAPD profiles of the representative samples of 12 *Dioscorea* species using 14 decamer primers generated 133 amplified products, all of which were found to be polymorphic (Asha et al., 2006). In the present study, 50% of the primers failed to amplify the genomic DNA of divergent species of *Dioscorea* from MTR.

The dendrogram was prepared by applying UPGMA method for the establishment of genetic relationships within five species of *Dioscorea* of MTR using average linkage procedure. The two species, *D. rotundata* and *D. cyenensis* cultivars were not grouped according to their species designation on the basis of RAPD patterns (Asemota et al., 1996). But later on the yellow (*D. cyenensis*) and white (*D. rotundata*) form of Guinea yam were divided into two clearly defined groups according to their species designation (Ramser et al., 1997). The simple leaf viz. species *D. bulbifera* of section Opsophyton and *D. belophylla*, *D. pubera*, *D. wallichii*, *D. spicata*, *D. intermedia* and *D.*

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hamiltonii of section Enantiophyllum were grouped in same cluster on the basis of RAPD pattern (Asha et al., 2006). Likewise, in present investigation *D. bulbifera* of section Opsophyton and *D. oppositifolia* of Enantiophyllum were grouped in subcluster I of cluster I, indicating greater similarity between them and supported by their genetic similarity index. However, ITS and ITS2 have been also proposed to be the most promising universal DNA barcode in plants (Chen et al., 2010; China Plant BOL, 2011), but little research was available for establishment of phylogenetic relationships among *Dioscorea* species according to the internal transcribed spacer (ITS) regions, as well as to identify the efficiency of the generated ITS as the DNA barcode in *Dioscorea* spp. In this context, some species of genus *Dioscorea* were analyzed by using ITS sequences – such as *Dioscorea japonica*, *Dioscorea alata*, *Dioscorea elephantipes*, *Dioscorea communis*, *Dioscorea caucasia*, *Dioscorea sylvatica*, *Dioscorea polystachya* and *Dioscorea hispida* (Merckx et al., 2008; Munirah et. al., 2012; 2014). Moreover, the present investigation aims for establishment of phylogenetic relationship on the basis of ITS2 sequences. Analyses on the basis of ITS2 sequence data placed *D. oppositifolia* and *D. belophylla* of section Enantiophyllum under the Cluster-I and exhibited great morphodiversity. The placing of these two species under section Enantiophyllum by Prain & Burkill (1936) was strongly supported by the ITS2 sequence data. But some species like *D. hispida* (Lasiophyton) and *D. bulbifera* (Opsophyton) were placed closer to each other under Cluster-I. Moreover, *D. pentaphylla* of section Lasiophyton was outgrouped and these three species overruled the sections proposed by Prain & Burkill (1936).

Although, *D. belophylla* and *D. oppositifolia* belonged to the same section as under Enantiophyllum, but because of their genetic similarity index, in the dendrogram they are segregated into two different clusters as on the basis of RAPD, such as cluster II and subcluster-I of cluster-I respectively, showing genetic dissimilarity. When compound leaf species, *D. tomentosa* and *D. pentaphylla* of the section Lasiophyton are clustered together, *D. hispida* in spite of belonging to the same section was placed in another cluster, showing its genetic dissimilarity with above two species (Asha et al., 2006). But in the present study the grouping of two varied compound leaf species *D. hispida* and *D. pentaphylla* of section Lasiophyton in subcluster II of cluster I, thereby maintaining the integrity with classification proposed by Prain & Burkill (1936) and Duthie (1960) and also supported by dendrogram generated from SDS-PAGE profile (Gawande et. al., 2014). It appeared that *D. belophylla* of section Enantiophyllum is completely outgrouped in cluster II from rest of the species. The supporting of outgrouping of *D. bellophylla* was also as on the basis of protein profile analysis (Gawande *et. al*., 2014). The grouping of species under different sections and placement of the same section species as outgrouped species have been experienced by the present study: *D. oppositifolia* of Enantiophyllum exhibited close genetic relationship with *D. pentaphylla* of Lasiophyton. Moreover, *D. hispida* of section Lasiophyton out grouped from rest of the species under this investigation. According to Li et al., (2011) the ITS region is not suitable for the discrimination of the same species, but it is very efficient in the discrimination of different species because of low intraspecific variation and high interspecific variation. The present study has exhibited great interspecific variation in relation to ITS2 region and the ITS2 region found to be more significant to resolve taxonomic hierarchy of the *Dioscorea* species from MTR. When the genus subjected molecular phylogeny study has been the incongruence between the phylogeny of genes and the phylogeny of taxa faced difficulties for resolving taxonomic status, because of specific sequence variation in the genes and non-specific sequence variation in the genome resulted in to different phylogenetic status (Wendel & Doyle, 1998; Sang, 2002). Similarly, present investigation has also exhibited different taxonomic status of the genus *Dioscorea* for RAPD and ITS sequences.

In conclusion, the comparative study of RAPD and ITS2 sequences of *Dioscorea* species has opened new avenues as on the basis of sequence data generated by RAPD and ITS primers and will be taken for future reference to establish genetic relationship in the genus *Dioscorea*, but unfortunately these two data exhibited different results and needs to resolve this crunch by applying structural gene sequences like *Mat*K, *trn* L-F, *rbc*L etc. for generation of additional barcodes.

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References

Álvarez I, Wendel JF. 2003. Ribosomal ITS sequences and plant phylogenetic inference. Mol. Phylogenet. Evol., 29: 417–434.

DOI: 10.69085/jbb20153303

RESEARCH ARTICLE

Asemota HN, Ramser J, Lopez-Peralta C, Weising K, Kahl G. 1996. Genetic variation and cultivar identification of Jamaican yam germplasm by random amplified polymorphic DNA analysis. Euphytica, 92: 341-351.

Asha KI, Nair GM, Padmesh M, Nair MC, Reji JV. 2006. Interrelationships among species of *Dioscorea* revealed by morphological traits and RAPD markers. Indian J. Plant Genet. Resour., 19(1): 40-46.

Baldwin BG. 1992. Phylogenetic utility of internal transcribed spacers of nuclear ribosomal DNA in plants: An example from the Compositae. Molecular Phylogentics and Evolution., 1(1): 3-16.

Baldwin BG. 1993. Molecular phylogenetics of *Calycadenia* (Compomtae) based on ITS sequences of nuclear ribosomal DNA: choromosomal and morphological evolution reexmained. Am. J. Bot., 80: 220–238.

Baldwin BG, Sanderson MJ, Porter JM. 1995. The ITS region of nuclear ribosomal DNA: a valuable source of evidence on angiosperm phylogeny. Ann. Mol. Bot. Gard., 82: 247–277.

Bowditch BA, Albright DG, Williams GK, Braun M. 1993. Use of randomly amplified polymorphic DNA markers in comparative genome studies. Method. Enzymol., 224: 295-309

Burkill IH. 1960. The organography and the evolution of Dioscoreaceae, the family of the yams. J. Linn. Soc. (Bot.)., 56(367): 319-412.

Burkill IH. 1939. Notes on the genus *Dioscorea* in the Belgian Congo. Bull. Jard Bot. Etat. Brux., 15(4): 345-392.

Chase MW, Duvall MR, Hills HG, Conran JG, Cox AV, Egularte E, Hartwell J, Fay MF, Caddik LR, Cameron KM, Hoot S. 1995a. Molecular phylogenetics of Lilianae. In P.J. Rudall, P. J. Cribb, D. F. Cutler, and C. J. Humphries [eds.], Royal Botanic Gardens, Kew, London, UK. Monocotyledons: Systematics and evolution., 109–137.

Chase MW, Stevenson DW, Wilkin P, Rudall PJ. 1995b. Monocot systematics: a combined analysis. In P. J. Rudall, P. J. Cribb, D. F. Cutler, and C. J. Humphries [eds.], Royal Botanic Gardens, Kew, London, UK. Moncotyledons: Systematics and evolution., 685–730.

Chen SL, Yao H, Han JP, Liu C, Song JY. 2010. Validation of the ITS2 region as a novel DNA barcode for identifying medicinal plant species. PLoS ONE, 5: 8613.

China Plant BOL Group, Li DZ, Gao LM, Li HT, Wang H. 2011. Comparative analysis of a large dataset indicates that internal transcribed spacer (ITS) should be incorporated into the core barcode for seed plants. Proc. Natl. Acad. Sci. U S A., 108 (49): 19641–19646.

Dansi A, Mignouna HD, Zoundjihekpon J, Sangare A, Ahoussou N, Asiedu R. 2000. Identification of some Benin Republic's Guinea yam (*Dioscorea cayenensis / D. rotundata* complex) cultivars using random amplified polymorphic DNA. Genet. Resour. Crop Evol., 47: 619-625.

Demeke T, Adams RP, Chibbar R. 1992. Potential taxonomic use of random amplified polymorphic DNA (RAPD): a case study in Brassica. Theor. Appl. Genet., 84: 991-994.

Deshmukh VP, Thakare PV, Chaudhari US, Gawande PA. 2007. A simple method for isolation of genomic DNA from fresh and dry leaves of *Terminalia arjuna* (Roxb.) Wight and Arnot. Electronic J. Biotechnol., 3: 468-472.

Doyle JJ, Doyle JL. 1990. Isolation of plant DNA from fresh tissue. Focus, 12: 13-15.

Duthie JF. 1960. Flora of the Upper Gangentic plain Vol III. Botanical Survey of India, p. 321- 326.

Duvall MR, Clegg MT, Chase MW, Lark WD, Kress WJ, Hills HG, Egularte LE, Smith JF, Gaut BS, Zimmer EA, Laren GH. 1993. Phylogenetic hypotheses for the monocotyledons constructed form rbcL sequences. Annals of Missouri Botanical Garden., 80: 607–619.

Feliner GN, Larena BG, Aguilar JF. 2004. Fine-scale geographical structure, intra-individual polymorphism and recombination in nuclear ribosomal internal transcribed spacers in *Armefia* (Plumbaginaceae). Ann. Bot., 93: 189–200.

Gawande PA, Deshmukh VP, Choudhary US, Thakare PV. 2014. Establishment of phylogenetic relationship on the basis of SDS-PAGE among the *Dioscorea* species under section Opsophyton, Enantiophyllum and Lasiophyton from Melghat Tiger Reserve Maharashtra, India. Int. J. of Life Sciences, Special Issue, A2 : 77-80.

Hadrys H, Balick M, Schierwater B. 1992. Applications of random amplified polymorphic DNA (RAPD) in molecular ecology. Mol. Ecol., 1: 55-63.

Jobes DV, Hurley DL, Thien LB. 1995. Plant DNA isolation: a method to efficiently remove polyphenolics, polysaccharides, and RNA. Taxon, 44: 379-386.

Kumar S, Stecher G, Peterson D, Tamura K. 2008. MEGA: a biologist-centric software for evolutionary analysis of DNA and protein sequences. Brief Bioinform., 9: 299–306.

Lay HL, Liu HJ, Liao MH, Chen CC, Liu SY, Sheu BW. 2001. Genetic identification of Chinese drug material in yams (*Dioscorea spp.*) by RAPD analysis. J. Food Drug Anal., 9(3): 132-138.

Li M, Cao H, But PPH, Shaw PC. 2011. Identification of herbal medicinal materials using DNA barcodes. Journal of Systematics and Evolution., 49: 271-283.

Liu Q, Ge S, Tang H. 2006. Phylogenetic relationships in *Elymus* (Poaceae: Triticeae) based on the nuclear ribosomal internal transcribed spacer and chloroplast trnL-F sequences. New Phytol., 170: 411–420.

Merckx V, Chatrou LW, Lemaire B, Sainge MN, Huysmans S., Smets EF. 2008. Diversification of myco-heterotrophic angiosperms: evidence from Burmanniaceae. BMC Evolutionary Biology, 8: 178.

Mignouna HD, Abang MM, Asiedu R. 2003. Harnessing modern biotechnology for tropical tuber crop improvement: yam (*Dioscorea spp*.) molecular breeding. Afr. J. Biotechnol., 2(12): 478-485.

Munirah S, Nashriyah M, Nor-Zuhailah M, Siti NIO, Salmah T, Norhayati N, Ali AM. 2012. Internal Transcribed Spacer (ITS) as a molecular marker for identification of *Dioscorea hispida* Dennst. (Dioscoreaceae). Journal of Agribiotech., 3: 47-55.

Munirah S, Nashriyah M, Nur FHN, Nor-Zuhailah M, Arumugam N, Bakar MFA, Wahid MAA, Ali AM. 2014. The distribution of *Dioscorea hispida* Dennst. Germplasm in Terengganu and phylogenetic relationships of *Dioscorea spp*. using Internal Transcribed Spacer (ITS). Journal of Agribiotech., 5: 31-47.

Nei M, Li WH. 1979. Mathematical model for studying genetic variation in terms of restriction endonucleases. Proc. Natl. Acad. Sci. USA., 76(10): 5269-5273.

Prain D, Burkill IH. 1936. An account of the genus *Dioscorea* in the East, Part 1: The species which twine to the left. Ann. Royal Bot. Gard., Calcutta, 14: 1-210.

DOI: 10.69085/jbb20153303

RESEARCH ARTICLE

Ramser J, Weising K, Lopez-Peralta C, Terhalle W, Terauchi R, Kahl G. 1997. Molecular marker based taxonomy and phylogeny of Guinea yam (*Dioscorea rotundata – D. cayenensis*). Genome, 40: 903-915.

Raz L. 2003. Dioscoreaceae. Flora of North America, 26: 11-12, 479-484. Available via www.eFloras.org

Rohlf FJ. 1987. NTSYS-pc Micro-computer programme for numerical taxonomy and multivariate analysis. Amer. Statistician, 41: 330.

Rohlf FJ. 1998. NTSYS-pc: Numerical taxonomy and multivariate analysis system version 2.0. Department of Ecology and Evolution. State University of New York.

Sang T, Crawford DJ, Stuessy TF. 1995. Documentation of reticulate evolution in peonies (Paeonia) using internal transcribed spacer sequences of nuclear ribosomal DNA: implications for biogeography and concerted evolution. Proceedings of the National Academy of Science USA., 92: 6813-6820.

Sang T. 2002. Utility of low-copy nuclear gene sequences in plant phylogenetics. Critical Reviews in Biochemistry and Molecular Biology, 37: 121–147.

Schwarzbach AE, Ricklefs RE. 2001. The use of molecular data in mangrove plant research. Wetland Ecology and Management, 9: 195-201.

Smith BW. 1937. Notes on the cytology and distribution of the Dioscoreaceae. Bull. Torrey Bot. Club, 64(4): 189-197.

Sneath PHA, Sokal RR. 1973. Numerical Taxonomy. W. H. Freeman and company, San Francisco.

Sokal RR, Micluner CB. 1958. A statistical method for evaluating systematic relationships. Univ Kans Sci Bull., 38: 1409-1438.

Tingey SV, Tufo JP. 1993. Genetic analysis with random amplified polymorphic DNA markers. Plant Physiol., 101: 349-352.

Volkov RA, Borisjuk NV,Panchuk II. 1999. Elimination and rearrangement of parental rDNA in the alloteraploid nicotiana tabacum. Mol. Biol. Evol., 16: 311–320.

Welsh J, McClelland M. 1990. Fingerprinting genomes using PCR with arbitrary primers. Nucl. Acids Res., 18(24): 7213-7218.

Wendel JF, Doyle JJ. 1998. Phylogenetic incongruence: window into genomes history and molecular evolution. In: Soltis DE, Soltis PS, Doyle JJ eds. Molecular systematic of plants.II. DNA sequencing. Boston: Kluwer Academic Publications.

Wilkin P. 2001. Dioscoreaceae of South-Central Asia. Kew bulletin., 56: 361-404.

Williams JGK, Kubelik AR, Livak KJ, Rafalski JA, Tingey SV. 1990. DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. Nucl. Acids Res., 18(22): 6531-6535.

Williams JGK, Hanafey MK, Rafalski JA. Tingey SV. 1993. Genetic analysis using random amplified polymorphic DNA markers. Methods Enzymol., 218:704-740.

DOI: 10.69085/jbb20153303