Identification of Different Yam (*Dioscorea*) Species by Random Amplified Polymorphic DNA

D.J.M.N. J.JAYAMAHA[1], P.K.SAMARAJEEWA [2] and D.P.S.T.G. ATTANAYAKA[1]

[1]*Department of Biotechnology, Faculty of Agriculture and Plantation Management, Wayamba University of Sri Lanka, Makandura, Gonawila (NWP), Sri Lanka.*
[2]*Plant Genetic Resources Centre, Gannoruwa, Peradeniya*

ABSTRACT

Yam (*Dioscorea*) is a monocotyledonous tuber crop, which belongs to the family Dioscoreaceae. Commercially important *Dioscorea* species are *Dioscorea esculenta, D. opposita, D. alata, D. cayenensis* and *D.rotundata.* Knowledge about the diversity of the crop is essential for conservation of germplasms. Duplication of *Dioscorea* varieties and lack of knowledge in genetic diversity are major problems in establishing those in collection and conservation. DNA marker techniques can be successfully used in the identification of probable duplication among accessions and analyze germplasm. The DNA samples from seven *Dioscorea* varieties were subjected to RAPD analysis using five primers to differentiate the varieties. *D.bulbifera* species known as 'Udala' (U) characterized with two specific bands of 500bp and 2000bp in size produced by OPC 09 primer. Close genetic relationship between *D.esculenta* and *D.alata* species was observed. The best primer to detect gene diversity was OPK16 primer. The highest number of effective alleles was also produced by this primer. OPK16 primer differentiated *D.esculenta* species. The *Dioscorea* accessions used for this study were identified as genetically different varieties. One accession, which was believed to be of *D.esculenta*, was identified, as *D.alata*.

KEY WORDS: Accession, *Dioscorea*, Genetic Diversity, PopGene 32, RAPD, Yams

INTRODUCTION

Yam (*Dioscorea*) is a tuberous, monocotyledonous plant, which belongs to the family Dioscoreaceae. Cultivations in Sri Lanka are still a major source of food in Jaffna specially *Dioscorea cayenensis*, *D. alata, D.rotundata, D. esculenta* and *Dioscorea hispida*. Basically yam has 70% moisture, 24.4% starch, 2.5% proteins, 0.9% minerals and 0.3% lipids. Taxonomic distinctions in this genus are often difficult to make because of their high morphological variation. Major differences among *Dioscorea* species are method of tuber production and the color of tubers. Classification of some species specially *D. alata* in this genus is considered to be problematic, which is attributed to its high continuous variability of morphological characters, specially of aerial parts, such as leaves and bulbils. Commercially important *Dioscorea* species are *D. esculenta, D. opposita, D. alata, D. cayenensis, D. trifida, D.japonica* and *D. rotundata*. A genetic resource survey was conducted (Lynch and Milligan, 1994) to asses the extent of genetic diversity of yam (*Dioscorea*). The survey showed that yams have the 36 numbers of varieties in Sri Lanka. Three selected *Dioscorea* species has different morphological characteristics (Table 2). Duplication of *Dioscorea* varieties and lack of knowledge in genetic diversity are major problems in establishing those in collection and conservation. Application of DNA finger printing techniques like Random Amplified Polymorphic DNA (RAPD), Random Fragment Length Polymorphism (RFLP), Simple Sequence Repeats (SSR) are useful to separate different germplasms than morphological markers. DNA finger printing can be successfully used in the identification of probable duplication among accession, identification of species and cultivars and determination of phylogenetic relationship among cultivars and wild species. These markers, specially the DNA markers have many advantages, such as, they aren't influenced by the environment, development stage or by gene interaction. Among those DNA marker techniques RAPD technique is relatively inexpensive, fast, and reliable and no need of sequence information. It can be detected by using small quantity of DNA with low development cost and start up cost. In the present study seven *Dioscorea* accessions previously classified by morphological characters were analyzed by RAPD method.

MATERIALS AND METHODS

The experiment was carried out at the molecular biology laboratory of the Plant Genetic Resources Centre (PGRC), Gannoruwa, Peradeniya during the 6 months period commencing from 4th January 2006.

Plant Materials

Leaves of the three selected *Dioscorea* species were collected from the field at Horticultural Crop Research and Development Institute (HORDI) including seven *Dioscorea* accessions (Table 1).

Table 1 - The *Dioscorea* accessions used in the study:

D. alata	*D. esculenta*	*D. bulbifera*
Ledantha (H)	Heen Hingurala (X)	Udala (U)
Ini Ala (I)	Suta Hingurala (Y)	
Raja Ala (J)	Heen Angiliala (Z)	

Table 2 - Characteristic features of three selected *Dioscorea* species:

Character	*D. alata*	*D. esculenta*	*D. bulbifera*
Leaf	Ovate, opposite, larger, lighter green in color	Broadly cordate, alternative, few, simple, light green in color	Simple, large
Pigment	Present	Absent	Absent
Twining	Anticlockwise (Right)	Anticlockwise	Anticlockwise
Cross section of stem	Stellate, winged stem	Circular, cylindrical stem	Circular, herbaceous stem
Spines	Absent	Present	Absent
Bulbils	Occasionally	Absent	Present
Number of tubers	Large 1-2	10-15	Large 1-2
Yield	30-35 t/ha	28-30 t/ha	
Age	8 months	8 months	
Examples	Ledantha, Ini Ala, Raja Ala	Heen Hingurala,Suta Hingurala,Heen Angiliala	Udala

DNA Extraction

DNA was extracted from the leaves according to the modified CTAB (4%) procedure described by Rogers & Bendich (1985) and Doyle & Doyle (1987). Leaf sample either fresh or at -20°C were used for the extraction. 1.5g of leaves without veins were selected for DNA extraction with 1000μl of DNA extraction buffer (1M Tris-HCl, 0.5M EDTA, 250 mM NaCl, P^H 8). Samples were mixed with 2μl of 0.1% beta-mercaptoethanol and tubes were sealed well with piece of parafilm. Samples were kept in 67°C water bath for 45 minutes. Tubes were then centrifuged at 13000 rpm for 10 minutes to remove cell debris and other unwanted compounds. Supernatants were separated to new eppendoff tubes. 2.5μl of 10 mg/ml RNAase was added and mixed thoroughly on shaker about 15-20 minutes. Samples were centrifuged at 13000rpm for 10 minutes and supernatants were separated again. 600 μl of chilled iso-propanol was added and slightly inverted until DNA cluster was formed. If not, DNA samples were centrifuged again at 13000 rpm for 10 minutes. The supernatants were removed and the pellets were washed with 500μl of 70% ethanol to remove CTAB residuals. (Tubes were kept with ethanol for 30 - 60 minutes). DNA samples were centrifuged again at 13000 rpm for 10 minutes. Ethanol was discarded and samples were air dried until ethanol is completely removed without DNA cluster was over dried. The pellets were dissolved in 100μl of TE (p^H 8) buffer. (1M Tris-HCl, 0.5 EDTA, pH 8) and stored at 4°C until used.

Purity Analysis of Extracted DNA Samples

A DNA sample of 8μl was mixed with 2μl of gel loading buffer. The mixture was loaded on 150 volumes 0.8% agarose gel. Gel was run at 135v and 55mA using 0.5% X TBE buffer until the dark blue bromophenol blue dye has migrated to two-thirds from length of the gel. The DNA was stained with ethidium bromide solution for 15 minutes an de- stained in distilled water for 5 minutes. Then gel was visualized under UV light through BIORAD gel documentation system with quantity one software package.

Polymerace Chain Reaction (PCR)

PCR was performed using 4.2μl of autoclaved distilled water, 0.8μl of 2.5mM dNTP, 1μl of 10Xbuffer, 0.25μl of 5μ/μl Taq polymerace, 1μl of 20ng/μl primer and 3μl of DNA template. Amplification was done at 94°C for 5 minutes as denaturation step. Annealing step was carried out at 93°C for 1 minute, 35°C for 3 minutes and 72°C for 2 minutes repeating 40 cycles. Finally it was kept at 72°C for 10 minutes to facilitate hybridization.

Primers

Amplification was carried out using different primers. The most suitable primers were selected for the detailed studies based on polymorphic and well-resolved amplification bands. Repeating the experiment twice tested the reproducibility of these results.

Amplified Products Resolving and Visualization

PCR products were loaded on 150ml volumes 1.4% agarose gel and stained with ethidium bromide solution for 15 minutes. After de-stained with distilled water, amplified products were visualized under UV light through BIORAD gel documentation system, using quantity one software package.

Data Analysis

The data were analyzed using PopGene 32 computer software package.

RESULTS AND DISCUSSION

Selection of Samples

Initially attempts were made to extract DNA from twenty samples of *Dioscorea* for the RAPD analysis. But due to the unavailability of good quality leaf materials most of them yielded unclear bands upon the conducting gel electrophoresis. Therefore only seven samples were selected for further studies,

three from *Dioscorea alata*, another three from *Dioscorea esculenta* and one from *Dioscorea bulbifera*.

Selection of Primers

Primers were selected by studying the preliminary work done at PGRC and by studying the various research articles related to *Dioscorea* (Williams *et al*., 1990). Initially six primers were selected for PCR. Among those primers five primers were selected to continue the RAPD analysis because they produced well-resolved amplification profiles and polymorphic banding patterns (Table 3).

Screening of Genotypes

In this study, the *Dioscorea bulbifera* species that collected by common name 'Udala' (U) was characterized 500bp and 2000bp two fragment with OPC 09 primer. No other samples had those two bands. Therefore other accessions were considered as separate species. H and J samples from *Dioscorea alata* species resulted most probably same banding patterns with this OPC 09 primer. Different banding patterns were detected by different primers in this study (Figure 1). The most of bands obtained with OPK16 primer for *Dioscorea esculenta* were identical. The specific bands, which resulted between 1500bp - 2000bp fragments, were the key to differentiate *Dioscorea esculenta* from other species. No amplified bands were resulted in *Dioscorea alata* (I) with this primer. Genetic differentiation with a fewer number of RAPD primers was possible because of high heterogeneity of the yam accessions. Yam is highly heterozygous plant species. Their chromosome numbers vary from 2n = 40, 60, 80,100,140 specially in *Dioscorea bulbifera* (Westman and Kresovich, 1997). The number of polymorphic loci obtained from this analysis is 198, the percentage being 96.59. *Dioscorea alata* and *D.esculenta* (Y and J) species were found to be genetically related (Figure 2). But *Dioscorea bulbifera* species that is used sample name as "Udala" has highest genetic distance between other two *Dioscorea* species (Table 4). One *Dioscorea esculenta* (Z) was more genetically different from the other two accessions. From 205 total alleles produced, 79 numbers of alleles were present only in 14.3% of the population indicating its usefulness in genetic differentiation. In that analyzing *D.alata* accessions H, J and I formed a separate cluster along with one of the *D.esculenta* accession (Y). This implies that the sample Y of *D.esculenta* to be a better candidate to include as a member of *D.alata* species though previously they have been categorized by morphological characters as two separate species. Analyzing those 79 numbers of alleles it could able to differentiated *Dioscorea esculenta* species (Z) from others (Figure 3). In that analysis, *Dioscorea alata* (H and J) species also can be differentiated. But it related with population 3(Y). This genetic relatedness suggests the possible use of cross breeding programme involving these two accessions for the future crop improvement. Highest gene diversity can be obtained from OPK16 primer, which resulted 0.5000 for OPK16-3, OPK16-7, OPK16-8 and OPK16-22 alleles. So the highest number of effective alleles also present for these four alleles in this population.

Table 1 - Detail of the RAPD profiles obtained by five selected primers:

Primer	Sequence 5' to 3'	Number of amplified bands
OPK 16	GAGCGTCGAA	34
OPC 19	GTTGCCAGCC	52
OPC 20	ACTTCGCCAC	26
OPC 09	CTCACCGTCC	46
OPA 09	GGGTAACGCC	47

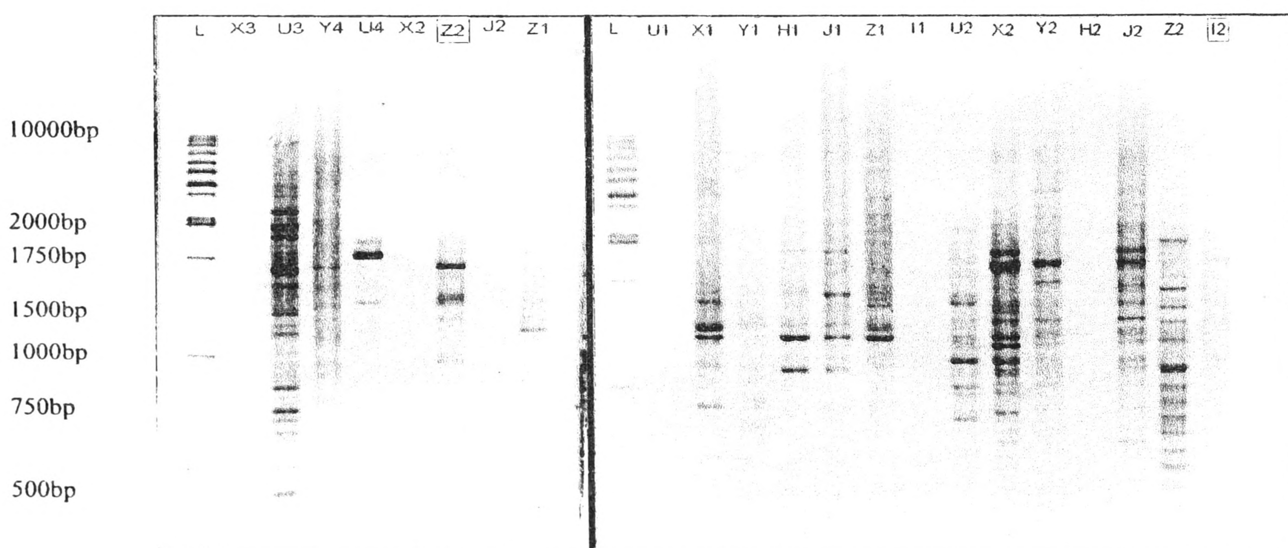

Figure 1 - Banding patterns obtained with different primers in this study:
L-10kb ladder, U-D.bulbifera , X,Y,Z- D.esculenta , H,I,J- D.alata X3,U3,Y4,U4,X2-I,Z2-I,J2-I,Z1-I for OPC09 Primer , U1,X1,Y1,H1,J1,Z1,I1 for OPA09 Primer, U2,X2-II,Y2,H2,J2-II,Z2-II,I2 for OPC19 Primer.

Table 2 -Genetic Distance of seven *Dioscorea* accessions:

Between	And	Length
6	Pop1	32.65940
6	5	4.17509
5	4	4.54043
4	3	1.90955
3	Pop2	22.03433
3	2	5.22083
2	1	3.15580
1	Pop3	13.65770
1	Pop7	13.65770
2	Pop5	16.81350
4	Pop6	23.94388
5	Pop4	28.48431

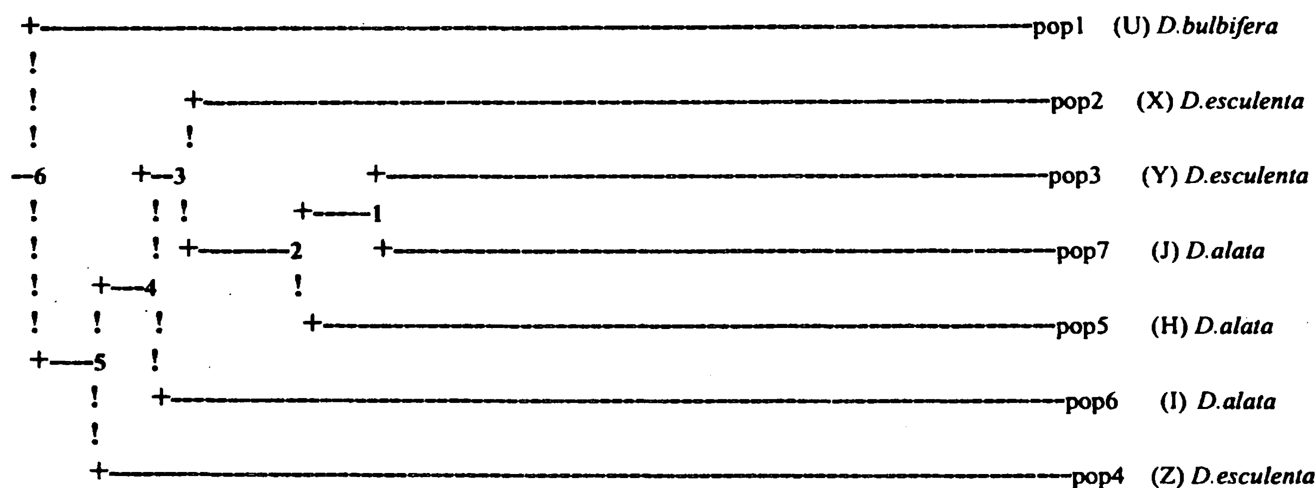

Figure 2 - The cluster diagram showing the genetic relatedness of the seven *Dioscorea* accessions:

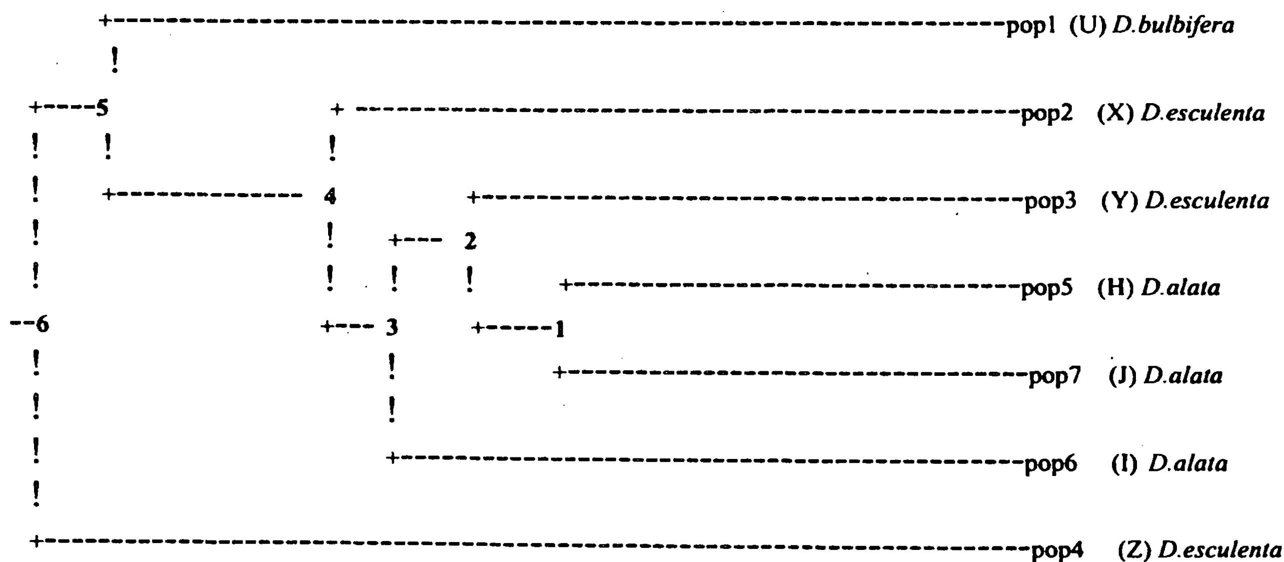

Figure 3- The cluster diagram by analyzing selected 79 alleles that showing genetic relatedness:

CONCLUSIONS

The *Dioscorea bulbifera* species 'Udala' (U) can be differentiate by using OPC 09 primer. Close genetic relationship between *Dioscorea esculenta* and *Dioscorea alata* species was observed. OPK16 primer differentiated *Dioscorea esculenta* species. The sample name Y that was morphologically categorized as one of accession in *Dioscorea esculenta* was identified as *D.alata* species. One *D.esculenta* (Z) was more genetically different from the other two accessions in the same species. The RAPD technique can be efficiently used to differentiate closely related accessions and analyze genetic diversity with the same species. The OPK16 primer was identified to be useful in detecting genetic diversity in the *Dioscorea* species. The accessions use in this study showed that there is no genetic duplication in the collection.

ACKNOWLEDGEMENTS

The authors wish to express their gratitude to Dr W.L.G.Samarasinghe and Ms.D.M.K.K.Dissanayake, Biotechnology and Tissue Culture division, Plant Genetic Resources Centre, Gannoruwa, Peradeniya and Dr K.P.U.De Silva, Head, Root and Tuber Crop division, Horticultural Crop Research and Development Institute, Gannoruwa, Peradeniya for there excellent guidance and encouragement throughout the study. Deep appreciationis extended to Dr.W.J.S.K.Weerakkody, Director and Mr.K.H.M.I.Karunarathne, Computer Instructor, Computer Service Unit, Faculty of Agriculture and Plantation Management, Wayamba University of Sri Lanka.

REFERENCES

Doyle, J.J. and J.J. Doyle. (1987). "A rapid DNA isolation procedure for small quantities of fresh leaf tissues." (pp 11-19).

Lynch, M. and B.G Milligan (1994)."Analysis of population genetic structure with RAPD markers." *Molecular Ecology*(pp.250-263).

Perera, A.L.T. and H.Jayasinghe, (1998). "PCR based DNA fingerprinting." A laboratory manual, University of Peradeniya,Sri Lanka.

Rogers, S.O. and A.J. Bendich.(1985). "Extraction of DNA from milligram of fresh, herbarium and mummified plant tissue." *Plant Molecular Biology* (pp 69-76).

Samarasinghe, W.L.G., J.P.D. Rukshanthi, A.M Nahfees, R. Muhunthan and A.L.T. Perera. (2001)." A laboratory manual on DNA typing using RAPD and SSR technologies with silver stained PAGE." PGIA, University of Peradeniya, Sri Lanka.

Westman, A.L. and S.Kresovich. (1997). "Use of molecular marker techniques for description of plant genetic variations." In J.A. Callow, B.V. Ford-Lloyd and H.J.Newury (eds.), *Biotechnology and plant genetic resource conservation and use*(pp 658-667).

Williams, J.G., A.R. Kubelic, K.J. Livack, J.A. Rafalski and S.V. Tingey .(1990) ."DNA polymorphism amplified by arbitory primers are useful as geneticmarkers." (pp 6531-6535)

Williams, J.G., R.S. Raiter, R.M. Young and P.A. Scolnic.(1993). "Genetic mapping of mutation using phenotypic pools and mapped RAPD markers." (pp 2697-2702).