Genetic Diversity in Pointed Gourd (*Trichosanthes dioica* Roxb) Revealed by Random Amplified Polymorphic DNA (RAPD) Markers

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Abstract

Genetic variation in 64 pointed gourd accessions was investigated using the Randomly Amplified Polymorphic DNA (RAPD). Out of 45 random primers screened five were selected, which gave 38 clear and bright fragments, out of which 30 (79.5%) fragments were considered polymorphic. The proportion of polymorphic loci across all loci was 96%. The number of bands per primer was five to eleven. The highest genetic distance 0.6419 was observed between the accession PG035 and PG051, PG035 and PG056 and PG035 and PG021. While the lowest genetic distance 0.00 was observed between the accessions PG042 and P043 and between PG042 and PG044. The UPGMA dendogram constructed based on RAPD analysis in 64 pointed gourd accessions were found to be grouped in twelve major clusters. Cluster VIII is a broad one which includes 21 accessions and only a single accession formed in cluster VII (PG021). RAPD analysis showed promise as an effective tool in estimating genetic polymorphism in different accessions of pointed gourd.

Introduction

Pointed gourd (*Trichosanthes dioica* Roxb) is an economically important cucurbit and is extensively propagated through vegetative means, viz vine and root cuttings. Because of judicious selection, considerable variability is available in pointed gourd. As the accessions are poorly characterized it is important at the beginning of a breeding program to discriminate among available genotypes to establish the level of genetic diversity and there by, identify the most suitable materials for crossing. Genetic diversity among individuals or populations can be determined using morphological and molecular markers. In contrast, molecular markers based on DNA sequence polymorphism, are independent of environmental conditions. Identification of genotypes based on morphological markers implies culture inspection at different stages and is not very reliable because many traits of interest have low heritability and are genetically very complex. Molecular markers provide a quick and reliable method for estimating genetic relationships among genotypes of any organism (Thormann et al., 1994).

Among the different types of molecular markers available, random amplified polymorphic DNA (RAPD) are useful for the assessment of genetic diversity because of their simplicity, fast and easy to perform and comparatively cheaper and requires no prior knowledge of DNA sequences (Williams et al., 1990 and Welsh et al., 1991). RAPD markers have been used to classify accessions (Horejsl and Staub, 1999), identify cultivar and hybrids (Sharma et al., 1995, in lentil Meng et al., 1996 and Bustos et al., 1998 in hordeum) and genetic diversity (Sureja et al., 2006). Additionally, RAPDs are also advantageous over isozyme analysis since they detect more polymorphism at about the same cost of analysis (McDonald et al., 1994). Hence the present investigation was carried out for analyzing the amount of genetic variation in pointed gourd accessions and classifying them to assist in selection of parent genotypes in a breeding

program. They can facilitate rapid screening of large numbers of genotypes for polymorphic loci.

Materials and Methods

Sixty four pointed gourd accessions were used in this study (Table 1). All the accessions were obtained from Regional Agricultural Research station, BARI, Ishurdi, Pabna during the month of November 2005. The accessions were different in their origin and their phenotypic character. In order to carryout RAPD analysis DNA was extracted from young growing leaves from each accession using following phenol: chloroform: isoamyl alcohol purification and ethanol precipitation method. Finally, the DNA samples were stored at -20^{o}C. DNA concentrations were determined at 260 nm spectrophotometrically and the quality verified by electrophoresis on a 1.4% garose gel.

Initially, 45 decamer primers from three kits (20 from kit A, 20 from kit B and 5 from kit C) of random sequence (Operon Technologies, Inc., Alameda, California, USA) were screened on a sub sample of three randomly chosen pointed gourd accession to test their suitability for amplifying pointed gourd. Primers were evaluated on the basis of intensity or resolution of RAPD bands, repeatability of markers consistency within individuals and potential to differentiate accessions (polymorphism).

A final subset of five primers (Table 2) exhibiting better quality banding patterns were tested three times on sub samples to be certain that the bands obtained were not mere artifacts of the RAPD method but rather true amplified products; they were then selected for analyses of the entire sample set of 64 accessions. The amplification condition was based on Williams et al. (1990) with some modification. PCR reactions were performed on each DNA sample in a 10 μL reaction mix containing 1 μL of 10X ampli Taq polymerase buffer, 2 μL of 10μM primer, 1 μL of 250 μM dNTPs (Gene, Banglar, India), I unit of Ampli Taq polymerase (Gene, Banglar, India), 1.5 mM $MgCl_2$ and 50 ng genomic DNA made up to 10 μL with sterile deionized water.

DNA amplification was performed in an oil-free thermal cycler (Master Cycler Gradient, Eppendorf). The reaction mix was preheated at 94^{o}C for 3 min followed by 45 cycles of one min denaturation at 94^{o}C, one min annealing at 34^{o}C and elongation or extension at 72^{o}C for two minutes. After the last cycle, a final cycle of seven minutes at 72^{o}C was added to allow complete amplified fragments. The amplified product from each sample was separated electrophoretically on 1.4% agarose gel (Fisher Biotech, USA) containing ethidium bromide in TBE buffer at 120 V for 1½ hrs. Molecular weight DNA markers (pUC18/ Sau 3A I-pUC18/Taq I Digest and 100 bp DNA Ladder) were electrophoresed alongside the RAPD reactions. DNA bands were observed on UV-transilluminator and photographed by a Gel Cam Polaroid camera (Type 667).

The RAPD markers were scored visually on the basis of their presence (1) or absence (0), separately for each accession and each primer. For more accuracy, band scoring was performed by two independent persons. Bands not identified by the two readers were considered as non scorable. The scores obtained using all primers in the RAPD analysis were then pooled for constructing a single data matrix. This was used for estimating polymorphic loci, (Nei's, 1973) and gene diversity (Nei's, (1972) genetic distance (D) and constructing an unweighted Pair group Method of arithmetic mean (UPGMA) dendogram among accessions using POPGENE (version 1.31) (Yeh et al., 1999) computer program.

Estimation of gene frequencies of RAPD loci was based on the assumption of a two allele system. Of the two alleles, only one is capable of amplification of a RAPD band by primer annealing at an unknown genomic position (locus). The other is the 'null' allele incapable of amplification, mainly because of loss of the primer annealing site by mutation. The two allele assumption was in most cases acceptable, because co dominant loci showing band shifts are few (Elo et al., 1997; Welsh and McClelland 1990). In this system only a null homozygote was detectable as negative for the RAPD band of interest. Under the assumption of Hardy-Weinberg equilibrium the null allele frequency (q) may be (N/n) ½, where N and n are the number of band negative individuals observed and the sample size, respectively. The frequency of the other allele (P) is 1-q. The assumption of the two allele system enables us to calculate the Nei's genetic distance (Nei's, 1972) from the RAPD pattern.

Table 1 Different character of 64 accessions of pointed gourd and their sources of collection.

Accession	Leaf color	Leaf type	Leaf margin	Fruit color at marketable stage	Fruit stripe	Fruit shape	Fruit curvature	Source
PG001	Green	Pointed	Entire	Green	GWS	Spindle	Curved	Rangpur
PG002	Green	Pointed	Serrated	Green	GWS	Oval	Curved	Rangpur
PG003	LG	Pointed	Entire	White	NS	Spindle	Straight	Natore
PG004	Green	Pointed	Serrated	Green	GWS	Cylindrical	Curved	Rangpur
PG005	LG	Pointed	Entire	Light green	WS	Oval	Straight	Kushtia
PG006	DG	Blunt	Entire	Dark green	GWS	Cylindrical	Straight	Pabna
PG007	LG	Pointed	Serrated	Light green	WS	Cylindrical	Straight	Kushtia
PG008	LG	Pointed	Entire	Light green	WS	Cylindrical	Straight	Kushtia
PG009	LG	Pointed	Entire	Light green	WS	Oval	Straight	Kushtia
PG010	DG	Blunt	Entire	Dark green	GWS	Oval	Straight	Pabna
PG011	DG	Blunt	Entire	Dark green	GWS	Cylindrical	Straight	Pabna
PG012	LG	Pointed	Serrated	Light green	WS	Cylindrical	Straight	Kuishtia
PG013	LG	Pointed	Serrated	Light green	WS	Cylindrical	Straight	Kushtia
PG014	LG	Pointed	Entire	Light green	WS	Cylindrical	Straight	Kushtia
PG015	LG	Pointed	Entire	Light green	WS	Spindle	Straight	Kushtia
PG016	Green	Pointed	Entire	Green	GWS	Cylindrical	Straight	Bogra
PG017	Green	Pointed	Entire	Green	GWS	Spindle	Straight	Rangpur
PG018	Green	Pointed	Entire	Green	GWS	Spindle	Straight	Rangpur
PG019	Green	Pointed	Entire	Green	GWS	Oval	Straight	Rangpur
PG020	Green	Pointed	Entire	Green	GWS	Cylindrical	Straight	Natore
PG021	Green	Pointed	Serrated	Green	GWS	Cylindrical	Straight	Rangpur
PG022	Green	Pointed	Entire	Green	GWS	Cylindrical	Straight	Rangpur
PG023	Green	Pointed	Undulated	Green	GWS	Oval	Straight	Pabna
PG024	Green	Pointed	Serrated	Green	GWS	Cylindrical	Straight	Pabna
PG025	Green	Pointed	Entire	Green	GWS	Cylindrical	Straight	Jessore
PG026	Green	Pointed	Undulated	Green	GWS	Cylindrical	Straight	Jessore
PG027	Green	Pointed	Undulated	Green	GWS	Cylindrical	Straight	Jessore
PG028	Green	Pointed	Entire	Green	GWS	Cylindrical	Straight	Jessore
PG029	Green	Pointed	Entire	Green	GWS	Cylindrical	Straight	Meherpur
PG030	Green	Pointed	Entire	Green	GWS	Cylindrical	Straight	Jessore
PG031	Green	Pointed	Entire	Green	GWS	Cylindrical	Straight	Jessore
PG032	Green	Pointed	Entire	Green	WS	Cylindrical	Straight	Bogra
PG033	Green	Pointed	Entire	Green	GWS	Cylindrical	Straight	Jessore
PG034	LG	Pointed	Entire	Green	GWS	Spindle	Straight	Pabna
PG035	Green	Pointed	Entire	Green	GWS	Cylindrical	Straight	Jessore
PG036	Green	Pointed	Entire	Green	GWS	Cylindrical	Straight	Pabna
PG037	Green	Pointed	Entire	Green	GWS	Cylindrical	Straight	Jessore
PG038	Green	Pointed	Entire	Dark green	GWS	Oval	Straight	Bogra
PG039	Green	Pointed	Entire	Dark green	GWS	Cylindrical	Straight	Jessore
PG040	Green	Pointed	Entire	Dark green	GWS	Cylindrical	Straight	Jessore

Table 1 Cont.

Accession	Leaf color	Leaf type	Leaf margin	Fruit color at marketable stage	Fruit stripe	Fruit shape	Fruit curvature	Source
PG041	Green	Pointed	Entire	Dark green	GWS	Cylindrical	Straight	Jessore
PG042	Green	Pointed	Entire	Dark green	GWS	Cylindrical	Straight	Jessore
PG043	Green	Pointed	Entire	Dark green	GWS	Cylindrical	Straight	Jessore
PG044	Green	Pointed	Entire	Dark green	GWS	Cylindrical	Straight	Jessore
PG045	Green	Pointed	Entire	Dark green	GWS	Cylindrical	Straight	Jessore
PG046	Green	Pointed	Entire	Dark green	WS	Cylindrical	Straight	Jessore
PG047	Green	Pointed	Entire	Light green	WS	Cylindrical	Straight	Gaibandha
PG048	Green	Pointed	Entire	Light green	WS	Cylindrical	Straight	Gaibandha
PG049	Green	Pointed	Entire	Light green	WS	Cylindrical	Straight	Gaibandha
PG050	Green	Pointed	Entire	Light green	WS	Cylindrical	Straight	Gaibandha
PG051	Green	Pointed	Entire	Light green	WS	Cylindrical	Straight	Gaibandha
PG052	Green	Pointed	Entire	Light green	GWS	Cylindrical	Straight	Gaibandha
PG053	Green	Pointed	Entire	Green	GWS	Spindle	Curved	Bogra
PG054	Green	Pointed	Entire	Green	GWS	Cylindrical	Straight	Gaibandha
PG055	Green	Pointed	Entire	Green	GWS	Spindle	Curved	Rangpur
PG056	Green	Pointed	Entire	Green	GWS	Spindle	Curved	Chuadanga
PG057	Green	Pointed	Entire	Green	GWS	Spindle	Curved	Chuadanga
PG058	Green	Pointed	Entire	Green	GWS	Spindle	Curved	Natore
PG059	Green	Pointed	Entire	Green	GWS	Spindle	Curved	Rajshahi
PG060	Green	Pointed	Entire	Green	GWS	Spindle	Curved	Rajshahi
PG061	Green	Pointed	Entire	Green	GWS	Spindle	Curved	Rajshahi
PG062	Green	Pointed	Entire	Green	GWS	Spindle	Curved	Rajshahi
PG063	Green	Pointed	Serrated	Green	GWS	Cylindrical	Straight	Rajshahi
PG064	Green	Pointed	Entire	Green	GWS	Spindle	Curved	Rajshahi

GWS = Green white stripe, WS = White stripe, PG= Pointed Gourd (*Tricosanthes dioica).*

Table 2 Parameters of the Operon random primers used for pointed gourd accessions.

Primer codes	Sequence (5' to 3')	(G+C) %
OPA16	AGCCAGCGAA	60
OPA20	GTTGCGATCC	60
OPB01	GTTTCGCTCC	60
OPB05	TGCGCCCTTC	70
OPB08	GTCCACACGG	70

Results and Discussion

Five primers used in RAPD analysis of 64 pointed gourd accessions amplified 38 different reproducible bands (Table 3). The five different primers generated various banding patterns ranging from 5 (OPA-16) to 11 (OPB-08) and the average bands per primer was 7.6. The size of the amplified products varied from 202 to 2320 bp. and of the 38 bands scored, 30 (79 %) were found to be polymorphic (either occurring in or absent in less than 95% of all accessions) and eight bands (1.6%) were found to be monomorphic in nature.

Table 3 RAPD primers with corresponding bands scored and their size range together with polymorphic bands observed in 64 pointed gourd accessions.

Sl. No.	Primer cod	No. of total band	Size range (bp)	Number of monomorphic band	Number of polymorphic band	Proportion of polymorphic loci (%)
1	OPA-16	5	202-1441	1	4	80
2	OPA-20	7	227-1443	1	6	86
3	OPB-01	7	505-2320	3	4	57
4	OPB-05	8	225-1510	1	7	88
5	OPB-08	11	220-1605	2	9	82
	Total	38		8	30	393
Average		7.6		1.6	6	79

The frequencies of polymorphic bands obtained varied from primer to primer. Frequencies of maximum number of polymorphic loci were found to be high with the exception of OPA16-4 (0.078), OPA20-2 (0.078), OPB05-5 (0.047) and OPB08-1 (0.047) (Table 4). Though no accession-specific marker was identified, the high level of polymorphism revealed by the proportion of polymorphic loci (79%) indicated that RAPD markers could be considered as effective tools for estimating genetic diversity in different accessions of pointed gourd. Accessions could be distinguished by a combination of fragments and differences between clusters reflected differences in frequencies rather than presence or absence of accession specific fragments.

Estimates of Nei's (1973) gene diversity (0.220 ± 0.177) and Shannon information index (0.342 ± 0.247) across all loci (Table 5) also supported the existence of high level of genetic variation in 64 accessions of pointed gourd.

There was a high level of genetic variation among the studied accessions of pointed gourd as indicated by the proportion of polymorphic loci. In a previous survey, Gwanama et al. (2000) analyzed genetic variation in sweet gourd (*Cucurbita moschata*) by RAPD markers and they found only 23.2% polymorphism in 31 accessions of the species. Estimation of higher level of genetic variation in the pointed gourd might be consistent with the fact that it is a highly cross pollinated dioecious plant. Beside this, vegetative means of propagation of pointed gourd is a common practice

Table 4 Frequency of polymorphic RAPD markers in pointed gourd accession.

No.	Loci	Frequency
1	OPA16-1	0.484
2	OPA16-2	0.688
3	OPA16-4	0.078
4	OPA16-5	0.781
5	OPA20-1	0.813
6	OPA20-2	0.078
7	OPA20-3	0.531
8	OPA20-4	0.891
9	OPA20-5	0.703
10	OPA20-7	0.266
11	OPB01-1	0.172
12	OPB01-2	0.828
13	OPB01-6	0.484
14	OPB01-7	0.266
15	OPB05-1	0.109
16	OPB05-3	0.172
17	OPB05-4	0.922
18	OPB05-5	0.047
19	OPB05-6	0.516
20	OPB05-7	0.750
21	OPB05-8	0.969
22	OPB08-1	0.047
23	OPB08-2	0.938
24	OPB08-4	0.984
25	OPB08-5	0.203
26	OPB08-6	0.156
27	OPB08-7	0.094
28	OPB08-8	0.750
29	OPB08-10	0.984
30	OPB08-11	0.359

among the farmer by which the genetic variability among the different accessions remains same.

The degree of RAPD polymorphism detected was relatively higher than that reported in melon (18%) (Garcia-mas et al., 2000), watermelon 21% (Lee et al., 1998), pumkin 23% (Gwanama et al., 2000) and ash gourd 28% (Sureje et al., 2006). Among the five primers OPB-08 resulted in the maximum number of polymorphisim bands (9) thus it showed a higher level of polymorphism. This was higher than the number of polymorphic bands reported in diversity analysis in melon (Mo et al., 1998) and in ash gourd (Sureje et al., 2006). On the other hand, the primer OPA-20 and OPB-05 generated 6 and 7 polymorphic bands respectively. These results gave an average of 6 polymorphic bands per primer. The lowest number polymorphic band (4) was produced by the primer OPA-16 and OPB-01 (Table 4). In contrast, while analyzing the genetic diversity among some accession of the *Cucurbita maxima*, *Cucurbita pepo*, *Cucurbita ficifolia* and *Lagenaria siceraria* using 26 primers, Ferriol et al. (2003) observed 57% polymorphic bands from a total of 92 consistent bands. Such a high level of polymorphism may be attributed to the use of different species and genus rather than use of cultivars or inbred lines within a species. A typical example with primer OPB-08 is shown in Figure 1.

Table 5 Estimation of genetic variability.

Number of polymorphic loci	Proportion of polymorphic loci (%)	Nei's (1973) gene diversity	Shannon's Information index
30	79	0.220 ± 0.177	0.342 ± 0.247

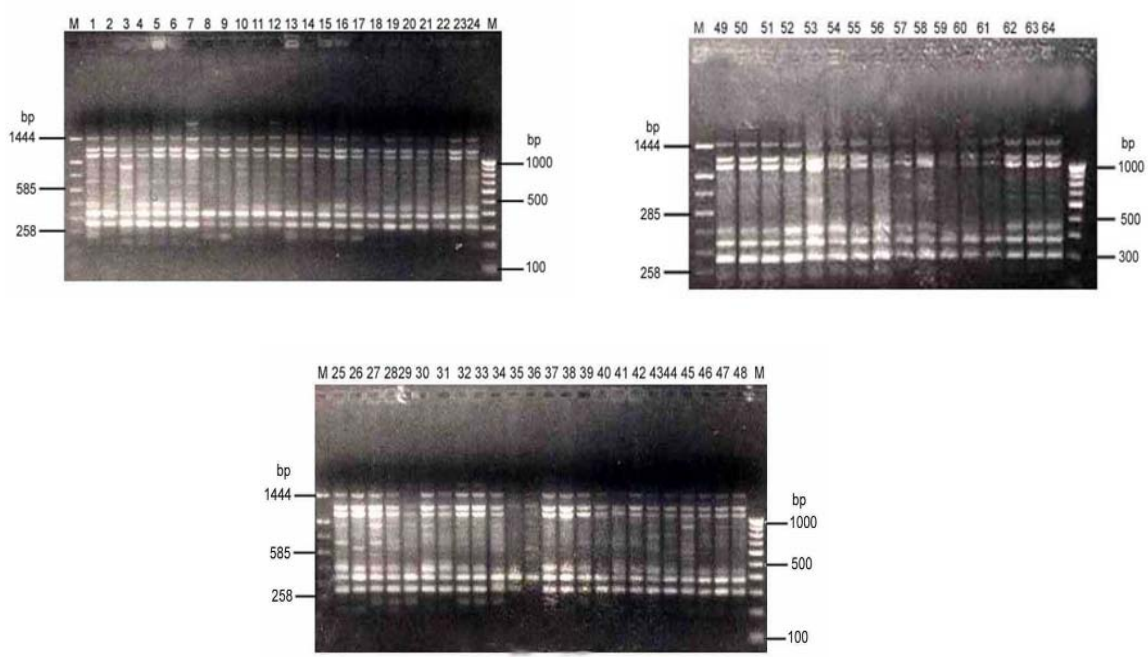

Figure 1 RAPD application patterns of 64 pointed gourd accessions with primer OPB-08. The lane numbers writwen on the top correspond to accessions. The lane labeled M is the moleculer weight mrker (100 bp DNA ladder and pUC 18/ Sau 3A1-pUC 18/Taq 1 Digest.

The highest genetic distance 0.6419 was observed between the accession PG035 and PG051, PG035 and PG056 and PG035 and PG021. While the lowest genetic distance 0.00 was observed between the accessions PG042 and P043 and between PG042 and PG044 (Figure 2). The difference between the highest and lowest genetic distance indicated the presence of variability among the 64 accessions of pointed gourd. Gwanama et al. (2000) reported a range of 0.13 to 0.41 genetic distances of 31 accessions of sweet gourd (Cucurbita moschata). Another reason for greater diversity of pointed gourd accessions might be that the accessions were collected from distant geographical regions (Table 1).

All the accessions were found to be grouped in twelve major groups designated as I, II, III, IV, V, VI, VII, VIII, IX, X, XI and XII (Figure 1). The distribution of the cluster members is shown in Table 6. Cluster VIII is broad which includes 21 accessions. Cluster IV, V and IX contained six accessions each. Five accessions constituted cluster VI.

Four accessions were grouped in cluster III, X and XI followed by 3 accessions in cluster I and II. Only a single accession formed in cluster VII (PG021) and XII (PG036). The accessions collected from the same location were grouped into different clusters.

Divergent accessions may have good breeding value. In spite of representing heterogenetic accessions among different clusters, accessions in the same cluster may also represent members of one heterotic group. Maximum variability for selection in segregating populations may be achieved by utilizing accessions from different clusters as parents of crosses.

Conclusions

The RAPD analysis discovered sufficient genetic variations among the pointed gourd accessions. No information on genetic structure of the pointed gourd was available in Bangladesh. This is the first attempt to study genetic structure of pointed gourd. Only 30 polymorphic markers were generated which was possibly not sufficient to cover pointed gourd genome particularly the regions influencing the expression of quantitative traits. Hence, sufficient and more efficient RAPD

Table 6 Distribution of 64 pointed gourd accessions under different cluster.

Cluster no.	Total no. of accession in cluster	Accessions included in different clusters
1	3	PG001, PG034 and PG064
II	3	PG002, PG012 and PG063
III	4	PG004, PG007, PG030 and PG031
IV	6	PG006, PG015, PG016, PG018, PG023 and PG026
V	6	PG017, PG028, PG029 PG032, PG033 and PG040
VI	5	PG022, PG024, PG049, PG051 and PG052
VII	1	PG036
VIII	21	PG038, PG039, PG041, PG042, PG043, PG044, PG045, PG046, PG047, PG048, PG050, PG053 PG054, PG055, PG056 PG057, PG058, PG059, PG060, PG061 and PG062
IX	6	PG003, PG010, PG013, PG014, PG019 and PG020
X	4	PG005, PG025, PG027 and PG037
XI	4	PG008 PG009 PG011and PG035
XII	1	PG021

Figure 2 Unweighted Pair Group Method of Arithmetic Mean (UPGMA) dendrogram based on Nei's (1972) genetic distance, summarizing data on differentiation in 64 accessions of pointed gourd according to RAPD analysis.

primers showing maximum number of polymorphic bands or other available markers systems could be utilized for analysis of pointed gourd. The result of the present study indicated that RAPD can be, used for identification of the available pointed gourd gremplasm. It can also be used to study relationship of pointed gourd germplasm among each other for further improvement through cross breeding.

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