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Fingerprinting of Sugarcane Using RAPD Markers

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ABSTRACT

The investigation was undertaken at Biotechnology Laboratory, Bangladesh Sugarcane Research Institute (BSRI), during cropping season 2005-2006. Two BSRI released sugarcane varieties such as Isd 16, Isd 29 and two accessions such as CP 70-1133 and Poj 2878 were used as plant materials. An efficient and easy method was adopted for isolation of quality DNA from meristem cylinder without liquid nitrogen. The amount of recovered DNA was enough for Polymerase Chain Reaction (PCR) amplification and marker studies such as Randomly Amplified Polymorphic DNA (RAPD). DNA fingerprinting was performed using ten RAPD markers such as OPA-01, OPA-03, OPA-08, OPA-12, OPB-05, OPB-09, OPB-18, OPB-20, OPC-02 and OPC-04 primers. Amplified DNA fragments by primers showed 58.72% polymorphism. Dendrogram based on linkage distance using Unweighted Pair Group Method of Arithmetic Means (UPGMA) indicated segregation of the four verities of sugarcane into two main clusters. Varieties Isd 29, CP70-1133, and Poj2878 grouped in cluster 1 followed by sub-clusters again however, variety Isd 16 alone was in cluster 2. It is revealed that the sugarcane variety Isd 16 a sustainable cultivated variety under field is genetically different from other varieties and accessions used in this investigation.

Key words: Sugarcane, DNA Fingerprinting, RAPD marker.

INTRODUCTION

In Bangladesh selection characters of sugarcane varieties are based on agronomic and morphological traits. Breeders are required relevant data based on morphological characters for release of variety and identification. Such traits are highly affected by the environment. As such no break-through could be expected in sustainable varietal improvement in future. With the introduction of Plant Variety and Farmers Right Protection Act (PVFRPA) of the country, there is a need for adequate and well characterization and documented information of the varieties already approved and new varieties for the production in the country. Therefore, it is important to start Genetic Fingerprinting of germplasms as well as of the varieties developed using molecular markers so that it can indicate the varietal position in addition to its morpho-physiological traits that are now being used as criteria for variety release.

Molecular markers form the basis of current strategies for genome analysis, gene mapping and germplasm identification. It is also argued that molecular markers with significant linkage to the phenotypic characters could be useful in the breeding programme since they would facilitate accurate, rapid and early screening of progeny independent of environmental or ontogenic factors. A number of markers linked to dominant genes in important crops have been characterized (Martin *et al.*,1991; Haley et al.,1993; Adam-Blondin et al.,1994). Little is known about the structure of the sugarcane genome (Roach and Danniels, 1987; Harvey et al., 1994) and as a result genetic distance between varieties cannot be calculated by comparing the nucleotide sequences of conserved gene regions. However, the techniques like RAPD may be carried out with no prior knowledge of the genome.

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Genetic Fingerprinting increasingly are being based upon information at the DNA level by various molecular markers such as, Randomly Amplified Polymorphic DNA (RAPD), Amplified Fragment Length Polymorphism (AFLP), Restriction Fragment Length Polymorphism (RFLP), Simple Sequence Repeat (SSR) or Micro satellite etc. Among them, a RAPD marker, generated by the polymerase chain reaction (PCR) is widely used for various purpose which include identification and classification of accession (Fukuoka *et al.*, 1992; Virk et al., 1995) as well as varieties. Therefore, the investigation was undertaken for fingerprinting sugarcane accession and varieties using RAPD markers.

MATERIALS AND METHODS

The experiment was carried out at the Biotechnology Laboratory, Bangladesh Sugarcane Research Institute (BSRI), Ishurdi, Pabna, Bangladesh in the cropping season 2005-2006.

Plant materials and sample collection: Two BSRI released sugarcane varieties (Isd 16, Isd 29) and two accessions (Poj 2878, CP 70-1133) from germplasm collection were used for DNA isolation. Top of the field grown 8 month old sugarcane was cut and placed vertically in a bucked with water for remain alive and fresh. Then the top in the bucket was taken in the laboratory. The outer leaf sheaths were removed leaving inner spindle to get meristem cylinder. Then the meristem cylinder (spindle base) was cut down into small pieces (about 5 mm long with 3 mm dia) with sterile scissors and weight required amount (0.2 g) using a fine balance. Care was taken during sampling from injury of meristem cylinder after removing of outer leaf sheaths.

DNA isolation: A modified method of Aljanbi *et al.* (1999) was used to isolate total genomic DNA from sugarcane varieties Isd 16, Isd 29 and accessions CP70-1133, Poj 2838. Cut pieces of meristem cylinder (about 5 mm long with 3 mm dia) 0.2 g was taken in small mortar (dia 6.5 cm and 3.5 cm depth) homogenized with pestle in 800 μl of extraction buffer (200mM Tris HCl, pH 8.0; 50 mM EDTA.H_2O, pH 8.0; 2.2 M NaCl; 2% CTAB; 0.06% Sodium Sulfite) until finely shredded within 40-50 second. Total grinded sample was taken into 2 ml eppendorf tube then added 150 μl of each 5% SDS, 10% PVP, 20% CTAB mixed well quickly and incubated at 65 °C at water bath for 40 min during incubation 3-4 times inversion were done. After incubation the samples were cooled to room tempetrature and equal volume of Phenol : Chloroform : Isoamyl Alcohol (25 : 24 : 1) was added mixed well by inversion and centrifuzed at 10,000 rpm for 30 min at room temperature. Then aqueous phase (about 800 μl) was recovered and transferred to a fresh ice-cold 2 ml eppendorf tube and an equal volume of ice-cold isopropanol was added followed by 120 μl of 6M NaCl. The samples were incubated at -20°C for at least 1hr and centrifuged at 10,000 rpm for 20 min at room temperature. The upper layer of solution was discarded carefully by using adjustable micropipette and 70% ice-cold ethanol about 2.5 times of the solution was added. Again centrifuged at 10,000 rpm for 10 min at room temperature. After pellet formation the solution was discarded from the tube carefully so that the DNA pellet remains constant and undisturbed. Then 70% ethanol was added up to the slant of the tube. The ethanol was discarded from the tube carefully so that the DNA pellet remains constant and undisturbed. DNA pellet was dried for at least 30 min by putting the tubes upside down on a filter paper. Then the DNA pellet was re-suspended in 50 μl of TE buffer (10 mM Tris HCl, pH 8.0; 1mM EDTA.H_2O pH 8.0) and stored at -20°C for lateral use.

Primers used: Selected ten decamer primers (Operon Technologies, Inc., USA) of random sequence (Table 1) were used for the whole sample set of four varieties/accessions of sugarcane. Primers were evaluated on the basis of intensity or resolution of bands, repeatability of markers and consistency within individual and potential to differentiate varieties/accessions (polymorohism).

PCR amplification and electrophoresis: PCR amplification was done in an oil-free thermal cycler (Master Cycler Gradient, Eppendorf) following the PCR profile of 94ºC for 4 min followed by 35 cycles of 1 min denaturation at 94ºC, 1 min annealing at 36ºC and elongation or extension at 72ºC for 2 min. After the last cycle, a final step of 7 minutes at 72ºC was added to allow complete extension of all amplified fragments. After completion of cycling programme, reactions were held at 4ºC. PCR reactions were performed on each DNA sample in a 10 μl reaction mixture containing 1μl of 10X Ampli Taq polymerase buffer, 1 μl of 10μM Primer, 1 μl of 2.5 mM DNTPs, 0.2 μl of 3 U/μl Ampli Taq DNA polymerase (Bangalore Genei Pvt. Ltd., India), 4 μl of 25 ng/μl genomic DNA and a suitable amount (2.8μl) of sterile deionized water. After amplification 2 μl loading dye was added to the amplification product and separated on 1.4% agarose gel (SRL, India) containing ethidium bromide (0.8 μg/ml from 10 mg/ml in 1xTBE buffer). Electrophoresis was performed at 100V for 2 hrs. DNA ladder 100 bp (Bangalore Genei Pvt. Ltd., India) was run alongside the RAPD reactions. DNA bands were observed on UV-transilluminator in Gel Documentation System (uvitec, DBT-2000LS), printed and saved on floopy disket for lateral use.

Data analysis: All distinct bands or fragments (RAPD markers) were thereby given identification numbers according to their position on the gel and scored visually on the basis of their presence (1) or absence (0), separately for each individual and each primer. The scores obtained using all primers in the RAPD analysis were then combined to create a single data matrix. This was used for estimating linkage distance (D) and constructing a UPGMA (Unweighted Pair Group Method of Arithmetic Means) Dendogram among the populations using computer programme Statistica.

RESULTS AND DISCUSSION

The RAPD technology has provided a quick and efficient for DNA sequence-based polymorphisms at a very large number of loci. The major advantage is that no prior DNA sequence information is required. The vast range of potential primers that can be used gives the technique great diagnostic power. Reproducible RAPD bands can be found by a careful selection of primers, optimization of PCR conditions for the target species, and replication to ensure that only the reproducible bands are scored.

The size of amplification products was estimated by comparing the migration of each amplified fragment with that of a known size fragments of molecular weight marker (100bp DNA ladder). All distinct bands or fragments (RAPD marker) were thereby given identification numbers according to their position on the gel and scored visually on the basis of their presence (1) or absence (0), separately for each individual and each primer.

Among the ten primers, six primers, OPA-01, OPA-12, OPB-05 OPB-18, OPC- 02, and OPC-04 produced comparatively maximum number of high intensity bands with minimal smearing. Ten primers generated 109 bands with size ranging from 400-1400bp. Out of the 109 bands, 64 bands were polymorphic bands. The ten different primers generated various banding patterns, ranging from 5(OPA-09) to 16(OPA-01). The primer OPA-12 and OPB-18 produced the 12 polymorphic bands. Thus it showed higher level of polymorphism. On the other hand, the primer OPC-02 did not generate any polymorphic bands (Table 2). The banding patterns of four sugarcane varieties/accessions using primers OPA-01, OPA-03, OPA-08, OPA-12, OPB-05, OPB-09, OPB-18, OPC-02 and OPC-04) are shown in Figures 1 and 2.

The 10 arbitrary primers generated 109 RAPD bands across four sugarcane varieties of which, 58.52% were found to be polymorphic and 6.4 polymorphic bands were produced per primer. Similar proportion of polymorphic RAPD bands per primer, was reported by Ko *et al.*, (1994) in Australian rice varieties. This is a relatively high level of polymorphism expressed by

arbitrary primers compared to reports of other RAPD studies, e.g. in *Brassica* spp.(Demeke *et al.*, 1992) *Allium* spp. (Wilkie *et al.*,1993), Sweet potato (Connolly *et al.*, 1994), sorghum (Tao *et al.*, 1993) alfalfa (Yu and Pauls, 1993) and celery (Yang and Quros, 1993).

The values of pair-wise comparisons of linkage distances analyzed by using computer software "Statistica" between varieties were computed from combined data for the ten primers, ranged from 26.0 to 37.0 (Table 3). Comparatively higher distance was observed between lsd-16 vs. Poj 2878; lsd16 vs. lsd 29; lsd 29 vs. Poj 2878 variety-pairs than the other variety combination. The lowest linkage distance (26.0) was found in CP 70-1133 vs. Poj 2878 variety pair.

Dendrogram (Fig 3) based on linkage distance using Unweighted Pair Group Method of Arithmetic Means (UPGMA) indicated segregation of the four verities of sugarcane into two main clusters: lsd 29, CP 70-1133, and Poj 2878 grouped in cluster 1 and lsd 16 alone is laid in a separate cluster 2. In cluster 1, CP 70-1133 and Poj 2878 formed subcluster 1 and lsd 29 alone formed subcluster 2. Again, among the verities of subcluster 1, CP 70-1133 and Poj 2878 grouped together with lowest linkage distance of (26). Results indicate that lsd 16 a sustainable high yielding variety of sugarcane is different from other varieties used in this investigation. It is well experienced that field performances of variety lsd 16 is superiorly different from other cultivated varieties. This is perhaps due to genetically different identity of lsd 16 from other varieties.

Results revealed that the sugarcane variety lsd 16 a sustainable cultivated variety under field is genetically different from other varieties and accessions used in this investigation.

Table 1. Parameters of the Operon random primers used in the present study.

Primer Code	Sequence (5′-3′)	G+C Content (%)
OPA-01	CAGGCCCTTC	70%
OPA-03	AGTCAGCCAC	60%
OPA-08	GTGACGTAGG	60%
OPA-12	TCGGCGATAG	60%
OPB-05	TGCGCCCTTC	70%
OPB-09	TGGGGGACTC	70%
OPB-18	CCACAGCAGT	60%
OPB-20	GGACCCTTAC	70%
OPC-02	GTGAGGCGTC	70%
OPC-04	CCGCATCTAC	60%

Table 2. RAPD primers with corresponding bands scored and their size range together with polymorphic bands in four sugarcane varieties.

Primer codes	Sequences (5′-3′)	Total number of bands scored	Size ranges (bp)	Number of polymorphic bands
OPA-01	CAGGCCCTTC	16	500-1300	3
OPA-03	AGTCAGCCAC	5	500-1300	4
OPA-08	GTGACGTAGG	12	400-1400	9
OPA-12	TCGGCGATAG	14	400-1400	12
OPB-05	TGCGCCCTTC	12	700-1300	9
OPB-09	TGGGGGACTC	5	700-900	2
OPB-18	CCACAGCAGT	13	500-1000	12
OPB-20	GGACCCTTAC	12	500-1100	10
OPC-02	GTGAGGCGTC	12	500-900	0
OPC-04	CCGCATCTAC	8	700-1400	3
Total		109		64

Table 3. Summary of linkage distance values for different cultivar pairs of sugarcane.

Variety	Isd 29	Isd 16	CP 70-1133	Poj-2878
Isd 29	0.00	36.0	27.0	35.0
Isd 16	36.0	0.0	31.0	37.0
CP 70-1133	27.0	31.0	0.0	26.0
Poj 2878	35.0	37.0	26.0	0.0

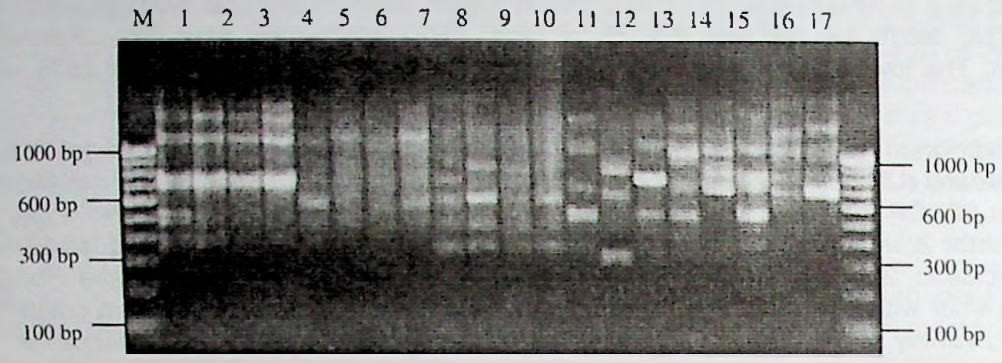

Figure 1. RAPD fingerprints of four sugarcane varieties Isd 29, Isd 16, CP 70-1133 and Poj 2878 against each of primer OPA-01 (lane 1-4), OPA-03 (lane 5-8), OPA-08 (lane 9-12), OPA-12 (lane 13-16) and OPB-05 (lane 17-20). M: Molecular weight marker (100 bp DNA ladder).

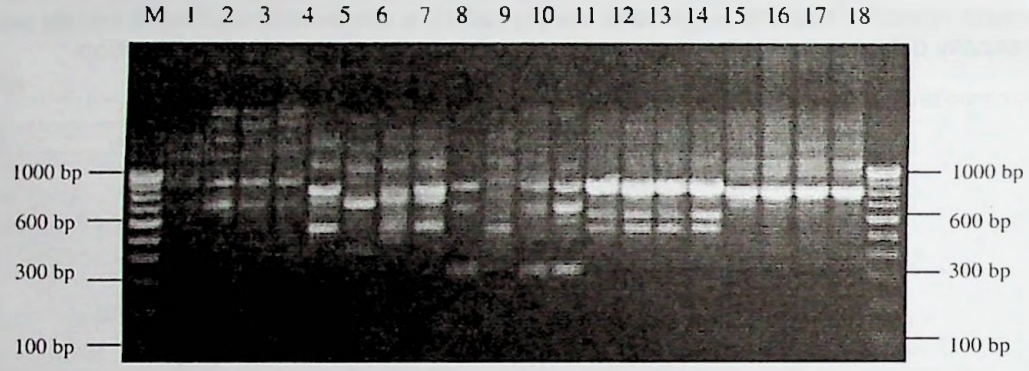

Figure 2. RAPD fingerprints of four sugarcane varieties Isd 29, Isd 16, CP 70-1133 and Poj 2878 against each of primer OPB-09 (lane 1-4), OPB-18 (lane 5-8), OPB-20 (lane 9-12), OPC-02 (lane 13-16) and OPC-04 (lane 17-20). M: Molecular weight marker (100 bp DNA ladder).

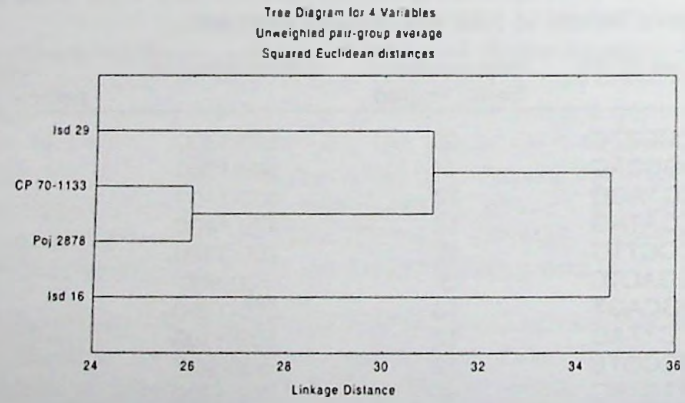

Figure 3. Cluster analysis by UPGMA of four sugarcane varieties based on 10 RAPD markers.

REFERENCES

Adam-Blondin, A.F.; Sevignac, M.; Bannerot, H.; and Dron, M.1994. SCAR, RAPD, and RFLP markers linked to a dominant gene (Are) conferring resistance to a dominant gene (Are) conferring resistance to anthracnose in common bean. *Theor. Appl. Genet.* **88**:865-870.

Aljanabi, S.M.; Forget, L. and Dookun, A. 1999. An improved rapid protocol for the isolation of polysaccharide and polyphenol-free sugarcane DNA. *Plant Mol. Biol.* Rep. **17**:1-8.

Connolly, A.G.; Godwin, I.d.; Cooper, M. and DeLacy, I. H. 1994. Interpretation of randomly amplified polymorphic DNA marker data for fingerprinting sweet potato (*Ipomoea batatas* L.) genotypes. *Theoritical and Applied Genetics*, **88**: 332-336.

Demeke, T.; Adams, R.P. and Chibbar, R. 1992. potential taxonomic use of randomly amplified polymorphic DNA(RAPD); a case study in *Brassica*. *Theoritical and Applied Genetics*, **84**: 990-994.

Fukuoka, S.; Hosaka, K. and Kamijima, O. 1992. Use of random amplified polymorphic DNAs (RAPDs) for identification of rice accessions. *Japanese Journal of genetics*, **67**:243-252.

Haley, S.D.; Miklas, J.R.; Stavely, J.R.; Byrum, J. and Kelly, J.D.1993. Identification of RAPD markers linked to a major rust resistance gene block in common bean. *Theor. Appl. Genet.*, **86**: 505-512.

Harvey, M.B.; Huckett, B.J. and Botha, F.C. 1994. Use of the polymerase Chain Reaction (PCR) and random amplification of polymorphic DNAs (RAPDs) for the determination of genetic distances between 21 sugarcane varieties. *Proc. S.A. Sugar Technol assn.*, **68**:36-40.

Ko,H.L.; Cown, D.C.; Henry, R.J.; Graham, G.C.; Blakeney, A. B. and Lewin, L.G. 1994. Random amplified polymorphic DNA analysis of Australian rice (*Oryza sativa* L.) varieties. *Euphytica*, **80:** 179-189.

Martin, G.B.; Williams, J.G.K.and Tanksley, S.D. 1991. Rapid identification of markers linked to a Pseudomonas resistance gene in tomato by using random primers and near isogenic lines. *Proc. natl. Acad . Sci.*, USA, **88**: 2336-2340.

Roach, B.T.; and Daniels, J. 1987. A review of the origin and improvement of sugarcane. In: *Cpersucar International Breeding Workshop.* PP. 1-32, S.P Copersucar (ed), Copersucar, Brazil.

Tao, Y.; Manners, J.M.; Ludlow,M.M. and Henzell, R.G. 1993. DNA polymorphism in grain shorghum (*Sorghum bicolor*). *Theoretical and Applied Genetics*, **86**: 679-688.

Virk, P. S.; Newbury, M.; Jackson, T. and Ford, B.V.L. 1995. The identification of duplicate accessions within a rice germplasm collection using RAPD analysis. *Theoretical and Applied Genetics*, **90**: 1049-1055.

Wilkie, S.E.; Issac, P.G. and Slater, R.J. 1993. Random amplified polymorphic DNA (RAPD) markers for genetic analysis in Allium. *Theor. Appl. Genet.* **86**:497-504.

Yang and Quiros C.1993. Identification and classification of celery cultivars with RAPD markers. *Theoretical and Applied Genetics*, **86**: 205-212.

Yu, K. and Pauls, K.P. 1993. Rapid estimation of genetic relatedness among heterogenous populations of alfalfa by random amplification of bulked genomic DNA samples. *Theoretical and Applied Genetics*, **87(6)**: 788-794.