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Assessment of genetic diversity analysis in contrasting sugarcane varieties using random amplified polymorphic DNA (RAPD) markers

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Sugarcane is an important crop in the country economically, politically and sociologically. It is the second largest agro-industry next to textiles. The selection and combination of parents for crossing rely on an understanding of their genetic structures and molecular diversity. In the present study, 28 sugarcane genotypes were used for genomic diversity analyses based on 30 randomly amplified polymorphic DNA markers (RAPD). These 30 sets of RAPD marker generated a total of 277 discernible and reproducible bands which included 179 polymorphic and 98 monomorphic bands. The unweighted pair group method with arithmetic average (UPGMA) analysis revealed six distinct clusters: I, II, III, IV, V and VI within the 28 genotypes. The polymorphic information content value per locus ranged from 0.21 for the OPA12 locus to 0.53 for OPH05, with an average of 0.40 for all loci. The range of genetic distance or coefficient of similarity among sugarcane genotypes were 0.08-1.00. The analysis of these similarities matrix revealed that greater similarity between Co 05011 and Co 0237, Co 05011 and Co 0241 (1.00), and lowest similarity between CoSe 03234 and CoS8432 (0.08). The knowledge obtained in this study will be useful to future breeding programs for increasing genetic diversity of sugarcane varieties and cultivars to meet the demand of sugarcane cultivation for sugar and bioenergy use.

Key words: Sugarcane, random amplified polymorphic DNA (RAPD) marker, genetic diversity, unweighted pair group method with arithmetic average (UPGMA).

INTRODUCTION

Sugarcane is a commercial crop that belongs to genus *Saccharum officinarum* L. (Poaceae), and contributes nearly 70% sugar production worldwide. The crop is cultivated in more than 90 countries all over the world. Sugarcane is mainly used for white sugar production but recent emphasis is also being given on production of biofuel like ethanol. It is an alternate source of energy and its production from sugar and or by products like molasses also makes sugarcane a future biofuel plant (Singh et al., 2010). Sugarcane is among the major

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Table 1. Characteristics of selected twenty eight sugarcane genotypes.

Variety	Parent	Type
CoS 08272	CoSe 92423 GC cross	Early
CoSe 98239	CoS 7927 x Co 775	Early
CoS 8436	MS 6847 x Co 1148	Early
CoSe 03234	Bo 91 x PCGC cross	Early
Co 98014	Co 8316 x Co 8213	Early
Co 0118	CoS 8347 x Co 86011	Early
CoSe 01424	Bo 91 x Co 453	Early
Co 0238	CoLk 8102 x Co 775	Early
CoC 671	Q 63 x Co	Early
CoJ 64	Co 976 x Co 617	Early
Co 1148	P4383 x Co 312	Unknown
CoS 95255	Co 1158 x Co 62198	Early
UP 9530	CoSe 1084/86 x CoSe 22/85	Midlate
CoSe 1434	Co 880239 x Co 775	Midlate
CoS 08279	CoLk 8102 x Co89803	Midlate
CoS 7250	CoS 8436 x Co 775	Midlate
Co 05011	CoS 8436 x Co 89003	Midlate
CoSe 92423	Bo 91 x Co 435	Midlate
CoS 96275	CoS 8119 x Co 62198	Midlate
CoS 96463	Unknown	Early
UP 49	CoSe 92423 x UP 9742	Midlate
CoS 8432	MS6847xCo1148	Unknown
CoSe 98231	CoS7927xCo775	Unknown
Co 0237	Co 93016 GC	Unknown
Co 0240	Unknown	Midlate
Co 0241	Unknown	Midlate
CoS 6287	Unknown	Midlate
CoS 8276	Unknown	Midlate

economic crops in the world and is the raw material for sugar and ethanol production as also as other by-products having the most efficient energetic balance (input over output) when compared with other energetic crops such as maize, sorghum and wheat (Lam et al., 2009). Brazil is the world leader in sugarcane production next to India with approximately 570 million tonnes of sugarcane, 31 millions of tonnes of sugar and 26 billions of litters of ethanol per year (UNICA, 2011).

Genetic diversity can be estimated based on different methods, morphological traits, pedigree record and molecular markers. The use of molecular markers for evaluation of genetic diversity is receiving much more attention. Molecular marker is potentially a valuable tool for crop improvement. Molecular markers play a role to portray genetic variability in several crops. Nowadays, fingerprinting system based on random amplified polymorphic DNA (RAPD) analysis have been increasingly utilized for detecting polymorphism in those genera which has no prior sequence information. Due to technical simplicity and speed, RAPD methodology has been used for diversity analysis in many plant species (Tonk et al., 2011). RAPD markers have several advantages over other polymorphism detection techniques including RFLP and other markers. These include quickness, relatively easy assay, and requirement for small amount of template DNA, no requirement of DNA sequence information and use of fluorescence. Because of these advantages, RAPD are commonly used to characterize variability (Vijay et al., 2009). There have been substantial reports of genetic diversity analysis of different crops including sugarcane plants using RAPD molecular markers (Singh et al., 2010a, b; Zhang et al., 2004, 2008; Pandey et al., 2012; Tabasum et al., 2010).

MATERIALS AND METHODS

Plant material and DNA isolation

Pot experiment was laid out by planting 28 sugarcane genotypes (Table 1), based upon highly contrasting morphological feature obtained from gene pool of Sugarcane Breeding Institute, Regional Station (SBI-RS) Karnal, Haryana and Sugarcane Research Station, Muzaffarnagar, U.P. India. Fresh leaves collected, were dipped in liquid nitrogen and used to isolate DNA applying CTAB method (Hoisington et al., 1994) with some modifications. 500 mg leaf tissues were ground in liquid N_2 and mixed in 8 ml of

Table 2. RAPD primer pairs used in this investigation.

Primer code	Primer sequence	Amplicon size (Kb)	Total bands	Monomorphic bands	Polymorphic bands	% Polymorphism	PIC value
OPH-03	5'-AGACGTCCAC-3'	0.3-2.5	8.0	3.0	5.0	62.5	0.28
OPH-04	5'-GGAAGTCGCC-3'	0.2-1.5	10.0	3.0	7.0	70.0	0.51
OPH-05	5'-AGTCGTCCCC-3'	0.3-2.5	7.0	2.0	5.0	71.4	0.53
OPH-07	5'-CTGCATCGTG-3'	0.2-3.0	11.0	2.0	9.0	81.8	0.44
OPH-09	5'-TGTAGCTGGG-3'	0.4-1.5	8.0	3.0	5.0	62.5	0.33
OPH-12	5'-ACGCGCATGT-3'	0.3-2.0	10.0	5.0	5.0	50.0	0.27
OPH-19	5'-CAAACGTCGG-3'	0.3-1.7	9.0	3.0	6.0	66.7	0.53
OPA-03	5'-AGTCAGCCAG-3'	0.4-1.8	12.0	4.0	8.0	66.7	0.27
OPA-02	5'-GAGGATCCCT-3'	0.2-2.5	8.0	3.0	5.0	62.5	0.33
OPC-19	5'-GTTGCCAGCC-3'	0.2-1.5	11.0	4.0	7.0	63.6	0.29
OPE-02	5'-GGTGCGGGAA-3'	0.3-2.8	9.0	3.0	6.0	66.7	0.39
OPA-01	5'-CAGGCCCTTC-3'	0.3-2.5	10.0	4.0	6.0	60.0	0.37
OPA-04	5'-AATCGGGCTG -3'	0.4-1.5	12.0	4.0	8.0	66.7	0.35
OPA-05	5'-AGGGGTCTTG-3'	0.2-2.0	10.0	3.0	7.0	70.0	0.37
OPA-07	5'-GAAACGGGTG-3'	0.2-1.5	8.0	4.0	4.0	50.0	0.45
OPA-09	5'-GGGTAACGCC-3'	0.2-2.5	5.0	1.0	4.0	80.0	0.43
OPA-10	5'-GTGATTCGCAG-3'	0.3-2.5	9.0	4.0	5.0	55.6	0.51
OPA-11	5'-CAATCGCCGT-3'	0.3-3.0	11.0	4.0	7.0	63.6	0.35
OPA-12	5'-TCGGCGATAG-3'	0.2-2.5	8.0	4.0	4.0	50.0	0.21
OPA-13	5'-CAGCACCCAC-3'	0.4-3.0	12.0	5.0	7.0	58.3	0.29
OPA-15	5'-TTCCGAACCC-3'	0.2-2.5	7.0	2.0	5.0	71.4	0.37
OPA-16	5'-AGCCAGCGAA-3'	0.3-2.8	8.0	3.0	5.0	62.5	0.51
OPA-17	5'-GACCGCTTGT-3'	0.2-2.4	10.0	3.0	7.0	70.0	0.55
OPA-18	5'-AGGTGACCGT-3'	0.2-1.8	10.0	4.0	6.0	60.0	0.43
OPA-19	5'-CAAACGTCGG-3'	0.2-2.0	8.0	2.0	6.0	75.0	0.51
OPE-05	5'-TCAGGGAGGT-3'	0.3-2.4	8.0	3.0	5.0	62.5	0.39
OPA-20	5'-GTTGCGATCC-3'	0.3-2.5	10.0	2.0	7.0	70.0	0.51
OPA-20	5'-GTTGCGATCC-3'	0.2-3.0	7.0	3.0	4.0	57.1	0.44
OPAB-8	5'-GTCCACACGG-3'	0.3-3.2	11.0	4.0	8.0	72.7	0.37
OPAB-11	5'-GTAGACCCGT-3'	0.2-2.7	10.0	4.0	6.0	60.0	0.52
	Average		9.2	3.3	6.0	64.7	0.40

prewarmed CTAB extraction buffer (2% (w/v) CTAB, 20 mM EDTA, 1.4 M NaCl, 100 mM Tris-HCl (pH 8.0), and 0.2% (v/v) β-mercaptoethanol with 2% PVP (w/v) added immediately prior to use and incubated at 65°C for 1 h. The content was mixed with equal volume of chloroform : isoamyl alcohol (24:1) and subjected to centrifugation for 10 min at 12,000 rpm at 4°C. Sample was taken out from ultracentrifuge and aqueous phage was pipetted out in a 25 ml autoclaved tube. Equal volume of chilled iso-propanol was added in aqueous phage to precipitate the DNA. Precipitated threads of DNA were pipetted out in a 2 ml appendoff using wide bore tip followed by centrifugation at 12,000 rpm for 10 min. Milky white pellet was washed with 70% alcohol; air dried, and resuspended in 10 mM Tris, at pH 8.0. To degrade RNA content, RNAse treatment was given for 1 h at 30°C. The quality and quantity of the genomic DNA was checked on 0.8% agarose gel and diluted appropriately for working concentration of 25 ng/µl.

RAPD marker genotyping

The polymerase chain reaction was performed in a thermal cycler (MyGene, MG96G") using 30 RAPD primers synthesised by Banglore Genei, India. The PCR was carried out in 25 µl reaction volume containing genomic DNA (50 ng), 1.5 mM $MgCl_2$, 1.2 mM dNTPs, 0.7 µl (1U/ µl) *Taq* DNA polymerase and 30 ng primer (Table 2). The cycling conditions included initial cycle of denaturation at 94°C for 4 min followed by repeated 35 cycles of denaturation at 94°C for 45 s, annealing differ for each primers for 45 s and extension at 72°C for 60 min. After completion of 35 cycles, a final extension at 72°C for 5 min was carried out and finally held at 4°C. The amplified products resulting from different primers were resolved on 1.4% agarose gel and analyzed by standard ethidium bromide staining and finally photographed using gel documentation system (Alfa Innotech, USA). The PCR amplification was performed twice with each primers and band scoring was done accordingly.

Amplicon scoring

The bands resulting from different sets of RAPD primers for different populations of *S. officinarum* were scored on agarose gel photograph in gel documentation system for its presence and absence across the populations collected (Figures 1 and 2). The

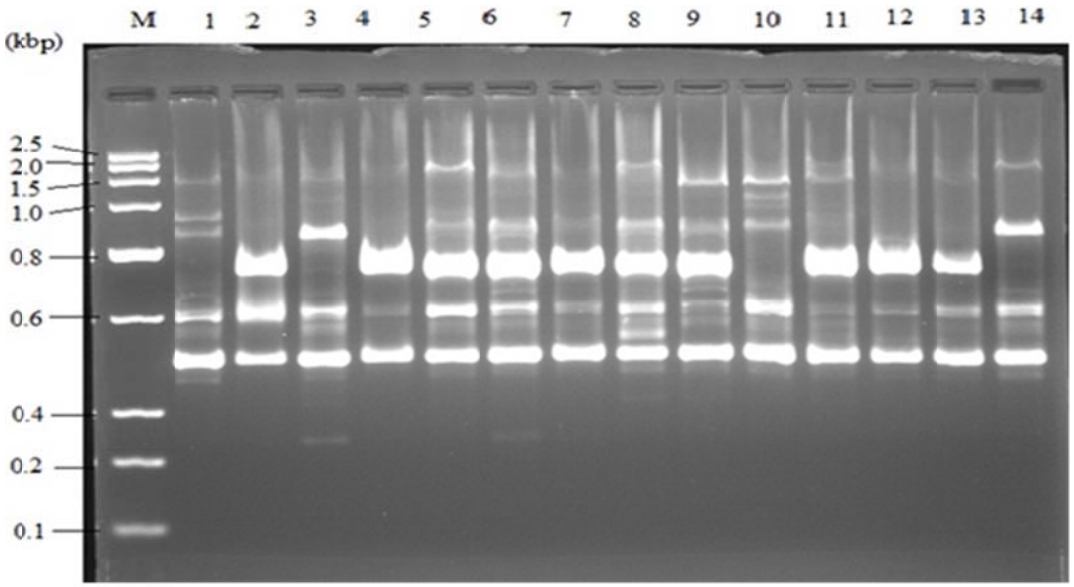

Figure 1. RAPD amplification of genomic DNAs of *Saccharum* and related genera used in this study using RAPD primer OPA-19. M, gene ruler 100 bp DNA ladder; Lane 1, CoS 08272; 2, CoSe 98239, 3, CoS 8436; 4, CoSe 03234; 5, Co 98014; 6, Co 0118; 7, CoSe 01424; 8, Co 0238; 9, CoC 671; 10, CoJ 64; 11, Co 1148; 12, CoS 95255; 13, UP 9530; 14, CoSe 1434.

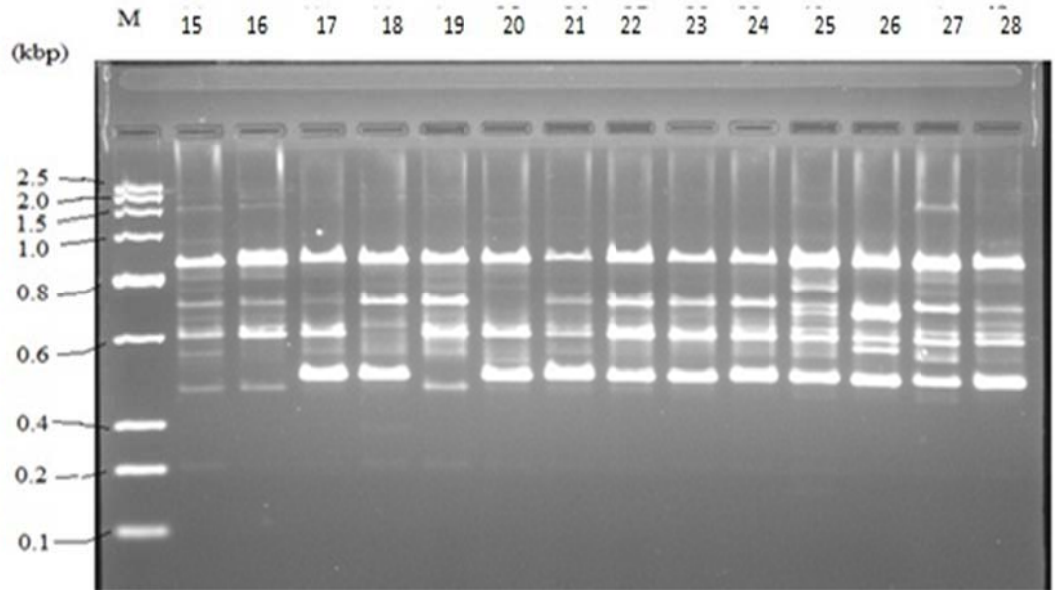

Figure 2. RAPD amplification of genomic DNAs of *Saccharum* and related genera used in this study using RAPD primer OPA-19. M, gene ruler 100 bp DNA ladder; Lane 15, CoS 08279; 16, CoS 7250; 17, Co 05011; 18, CoSe 92423; 19, CoS 96275; 20, CoS 96463; 21, UP 49; 22, CoS 8432; 23, CoSe 98231; 24, Co 0237; 25, Co 0240; 26, Co 0241, 27; CoS 6287; 28, CoS 8276.

image profiles of banding patterns were recorded and molecular weight of each bands were determined by DNA ladder. The results were analysed based on the principle that a band is considered to be polymorphic if it is absent in at least one individuals or accessions. Similarity index of bands which were common between two accessions was estimated by Nei and Li (1979). The final RAPD data generated with 30 RAPD primers were used to calculate pair wise similarity coefficients using Jaccard's coefficient of similarity (Jaccard, 1908). The cluster analysis and dendrogram construction were performed with NTSYS-PC (version 2.02e)

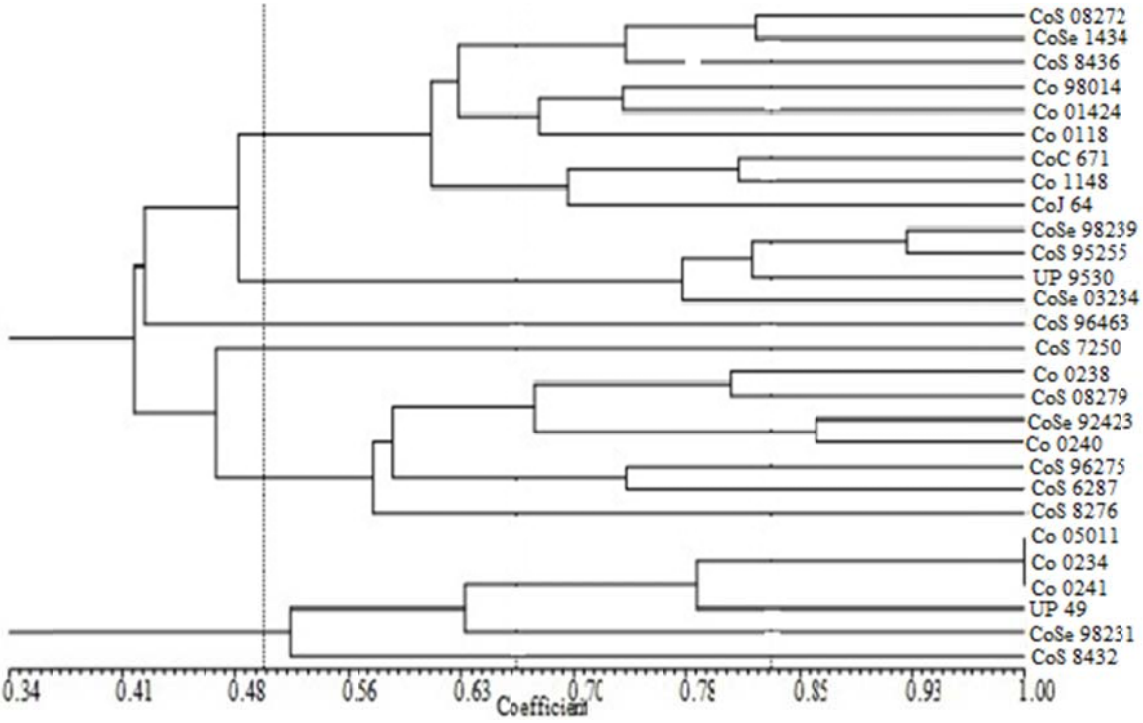

Figure 3. Dendrogram for 10 sugarcane genotypes showing the genetic similarities derived from UPGMA cluster analysis using the jaccard similarity coefficient.

software for generating phylogenetic tree using the unweighted pair group method with arithmetic average (UPGMA) method (Nie and Li, 1979).

RESULTS AND DISCUSSION

For RAPD analysis, a total of 30 primer pairs were screened, and used for polymerase chain reaction (PCR) amplification (Table 2). The PCR product was electrophoresed on 1.4% agarose gels (Figures 1 and 2). Bands were counted and the presence and absence of bands were scored as 1 and 0, respectively. The PCR amplicons sizes ranged from 0.26 to 3.2 Kb. These sets of primers revealed intra-specific variations. A total of 277 bands were scored with 179 polymorphic and 98 monomorphic bands (Table 2). The 28 sugarcane genotypes were clustered based on the matrix of genetic similarities using UPGMA. The cluster analysis and dendrogram construction were performed with NTSYS-PC (version 2.02e). Using 30 RAPD markers, a total of 277 alleles were detected among 28 sugarcane genotypes studied. The average number of allele per locus was 9.2. Dendrogram was constructed based on genetic distance calculated from 277 alleles generated from 28 sugarcane genotypes. The UPGMA cluster tree analysis led to the grouping of the 28 sugarcane genotypes into six major clusters (Figure 3). Cluster I, the largest cluster comprised nine sugarcane genotypes among selected 28 sugarcane genotypes. Cluster II comprised four sugarcane genotypes. Clusters III and IV were the smallest cluster comprised only one sugarcane genotype, CoS96463 and CoS7250 respectively. Cluster V, the second largest cluster comprised seven sugarcane genotypes among selected 28 sugarcane genotypes while cluster VI the third largest cluster comprised six sugarcane genotypes among selected 28 sugarcane genotypes. The PIC value per locus ranged from 0.21 for the OPA12 locus to 0.53 for OPH05, with an average of 0.40 for all loci (Table 2). The range of genetic distance or coefficient of similarity among sugarcane genotypes were 0.08-1.00. The analysis of these similarities matrix revealed that greater similarity between Co 05011 and 24 Co 0237, Co 05011 and 26 Co 0241 (1.00), and lowest similarity between CoSe 03234 and CoS8432 (0.08) (Figure 3). Those cultivars that display similar coefficient of matrix are genetically close to one another and vice-versa. Similar studies or observation was reported by many researchers worldwide previously (Pandey et al., 2012; Tabasum et al., 2010).

Therefore, the present investigation reported the results of a study on the genetic diversity among 28 accessions of sugarcane revealed by RAPD. Using RAPD as genetic markers, as high as 81.8% polymorphic bands were detected in 28 accessions of sugarcane; similar studies were conducted previously by Burner et al. (1997) and Nair et al. (2002). This study will facilitate in marker-assisted applications in sugarcane breeding. The present investigation is an initial step to evaluate the molecular

diversity of this critically complex genome and polyploidy nature of different sugarcane accessions. Therefore, further work is required to promote molecular markers like genomic simple sequence repeats (gSSRs) and expressed sequence tagged based simple sequence repeats (EST-SSRs) can be utilized. This study will be a boon to conserve sugarcane *in vitro* and *ex-situ* as well identification of parents for breeding improvement programme and also to fulfil the growing demand at national and inter-national market.

Conflict of Interests

The author(s) have not declared any conflict of interests.

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