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Genetic variability in sugarcane (*Saccharum* spp. hybrid) genotypes through inter simple sequence repeats (ISSR) markers

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Abstract: In the present study, 14 sugarcane (*Saccharum* spp. hybrid) genotypes were used for genomic diversity analysis based on nineteen inter simple sequence repeat (ISSR). These nineteen sets of ISSR markers generated a total of 164 discernible and reproducible bands including 109 polymorphic and 55 monomorphic bands. The unweighted pair group method with arithmetic average (UPGMA) analysis revealed three distinct clusters: I, II and III within the 14 genotypes. The polymorphic information content (PIC) value per locus ranged from 0.14 (UBC811) to 0.53 (ISSR1) locus with an average of 0.42 for all loci**.** The range of genetic distance or coefficient of similarity among sugarcane genotypes varied 0.14 - 0.78. The analysis of these similarities matrix revealed that greater similarity between CoS03234 and CoSe1424 (0.78), and lowest similarity between CoS03234 and Co0118 (0.14). The knowledge gained in this study would be useful to future breeding programs for increasing genetic diversity of sugarcane varieties and cultivars to meet the increasing demand of sugarcane cultivation for sugar and bio energy uses.

Keywords: Genetic diversity, Inter simple sequence repeat (ISSR) marker, Unweighted pair group method with arithmetic average (UPGMA)

INTRODUCTION

Sugarcane (*Saccharum* spp. hybrid) is an important industrial crop for the tropical and subtropical region of the world. It is produced in more than 100 countries, with global production of 175.1 million tonnes of sugar. It accounts for about 80 percent and sugarbeet for about 20 percent of total sugar produced (FAOSTAT, 2015). Economically sugarcane is an important industrial raw material for sugar and allied industries producing alcohol, acetic acid, butanol, paper, plywood, industrial enzymes, animal feed and bioethanol as a biofuel alternative to petrol. Brazil is the largest sugarcane producer country, contributing with 40.4% of the world production (768 MT) followed by India 17.9% (341.43 MT), (FAOSTAT, 2015).

The complexity and size of the sugarcane genome is a major limitation in genetic improvement. 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 a potentially valuable tool for crop improvement. Molecular markers play a role to portray genetic variability in several crops. Now a day, fingerprinting system based on Inter simple sequence repeat (ISSR) analysis have been increasingly utilized for detecting polymorphism in those genera which has no prior sequence information. ISSR markers have several advantages over other polymorphism detection techniques including RFLP, AFLP and other markers. ISSR marker system is another newly developed method, which relies on one primer for PCR that anneals to an SSR region and amplifies region between inversely oriented adjacent SSRs (Pandey *et al.,* 2012). ISSR assay can be undertaken for any species that contains a sufficient number and distribution of SSR motifs and has the advantage that genomic sequence data is not required. This technique amplifies large numbers of DNA fragments per reaction, representing multiple loci from across the genome; it is an ideal method for fingerprinting varieties. There have been substantial reports of genetic diversity analysis of different crops including sugarcane plants using molecular markers (Singh *et al*., 2010; Zhang *et al.,* 2008; Tabasum *et al.,* 2010; He *et al.,* 2011; Rao *et al.,* 2014) included ISSR in sugarcane (Costa *et al.,* 2011; Kalwade *et al.,* 2012; Devarumath *et al.,* 2012). The present investigation was conducted to study the genetic variability in sugar-

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cane (*Saccharum* spp. hybrid) genotypes through inter simple sequence repeats (ISSR) markers.

MATERIALS AND METHODS

Pot experiment was laid out by planting 14 sugarcane genotypes (Table 1), based upon highly contrasting morphological feature obtained from gene pool of Sugarcane Breeding Institute, Regional Station, Karnal, Haryana, India. Fresh leaves were collected and dipped in chilled liquid nitrogen and used to isolate DNA applying CTAB method (Hoisington *et al.*, 1994) with own modifications. The 500 mg leaf tissues were ground in liquid N_2 and mixed in 5 ml of 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 pipette 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 pipette out in a 2ml appendroff tubes 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 re-suspended in 100 µl TE buffer (10 mM Tris, 1mM EDTA, 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% (w/v) agarose gel and diluted appropriately for working concentration of 25 ng/µl.

ISSR marker genotyping: The Polymerase chain reaction was performed in a thermal cycler (MyGene, 96G) using 19 ISSR primers synthesised by Bangalore Genei, India. The PCR was carried out in 25 µl reaction volume containing genomic DNA (50 ng), 1.5 mM MgCl2, 1.2 mM dNTPs, 0.7 µl (1U/ µl) *Taq* DNA polymerase and 19 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 6 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 2.4% (w/v) 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.

Statistical analysis: The bands resulting from different sets of ISSR primers for different populations of *Saccharum* spp. hybrid were scored from agarose gel photograph (Fig. 1). The 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 (Nei and Li, 1979). The ISSR data generated with 19 ISSR 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) software for generating phylogenetic tree using the unweighted pair group method with arithmetic average (UPGMA) method (Nie and Li, 1979).

RESULTS AND DISCUSSION

For ISSR analysis, a total of nineteen primers were used on 14 genotypes of sugarcane for polymerase chain reaction (PCR) amplification (Table 2). The PCR product was electrophoresed on 2.4% agarose gels (Fig. 1). 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 164 bands were scored with 109 polymorphic and 55 monomorphic bands (Table 2). The 14 sugarcane genotypes were clustered based on the matrix of genetic similarities using the Unweighted Pair Group Method with Arithmatic Mean (UPGMA). The cluster analysis and dendrogram construction were performed with NTSYS-PC (version 2.02e). Using 19 ISSR markers, a total of 164 alleles were detected among 14 genotypes studied. The average number of allele per locus was 8.83. Dendrogram was constructed based on genetic distance calculated from 164 alleles generated from 14 genotypes. The UPGMA cluster tree analysis led to the grouping of genotypes into three major clusters (Fig. 2). Cluster I was the largest one and comprised eight genotypes, cluster II was the smallest and comprised only one genotype while cluster III comprised of five genotypes among selected 14 sugarcane genotypes (Table 4). The PIC value per locus ranged from 0.14 (UBC811) to 0.53 (ISSR1) locus, with an average of 0.42 for all loci (Table 2).

The two-dimensional plot generated from PCoA also supported the clustering pattern of the UPGMA dendrogram (Fig. 3). This reflected a higher genetic diversity in the studied collection, which was confirmed by a principle component analysis of the genotype data. Results of this analysis showed a wider genetic distribution of genotypes in the studied collection. In the three-dimensional PCoA plot, generally, similar groupings with the UPGMA dendrogram and additional information were also revealed. The first three principal axis accounted for the total variation indicating the complex multidimensional nature of ISSR variation (Fig. 4). The range of genetic distance or coefficient of

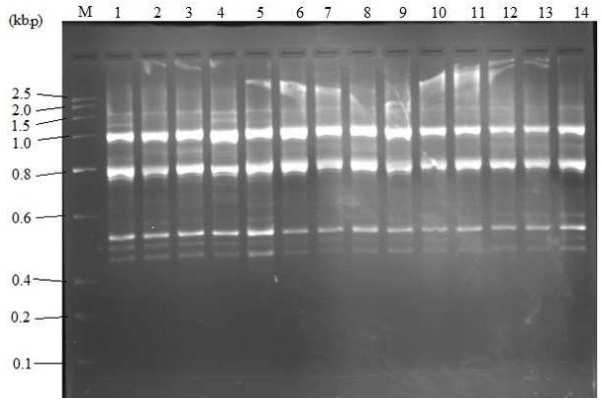

Fig. 1. *ISSR amplification of genomic DNAs of Saccharum spp. hybrid used in this study through ISSR primer UBC814 and depicting species specific amplicons (accession numbers 1, 4 and 5). M gene ruler 100 bp DNA ladder, Lane 1. CoS03234, CoSe 01424, Co0118, Co0238, CoC671, CoJ64, Co1148, CoS08272, CoS 95255, BO91, UP9530, CoSe1434, UP49 and CoS7250*

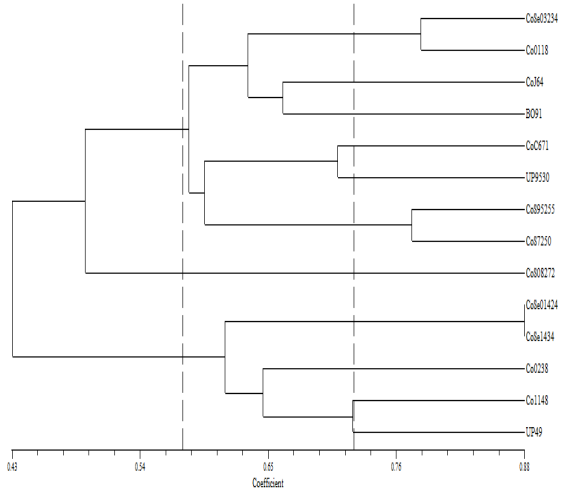

Fig. 2. *Dendrogram for 14 sugarcane genotypes showing the genetic similarities derived from UPGMA cluster analysis using the Jaccard similarity coefficient.*

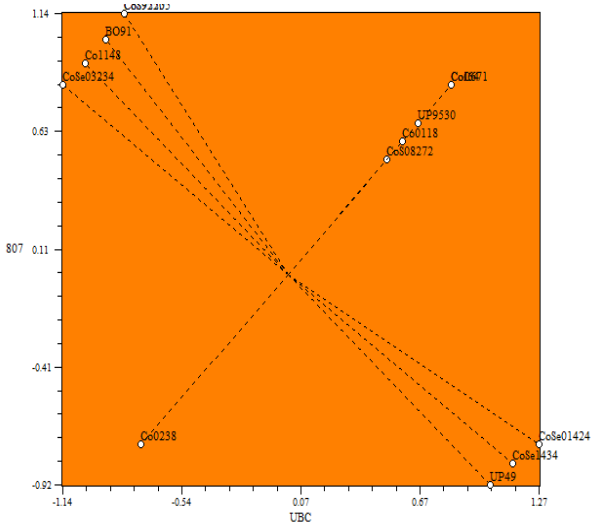

Fig. 3. *Two dimensional plot showing relationships among 14 sugarcane accessions using 164 ISSR bands and extracting the first three PCA components.*

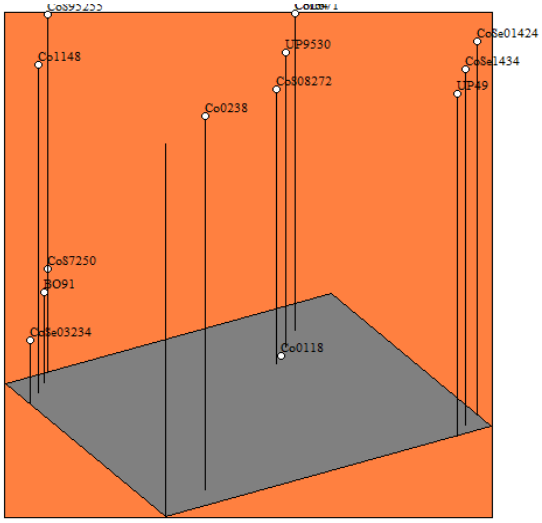

Fig. 4. *Three dimensional plot showing relationships among 14 sugarcane accessions using 164 ISSR bands and extracting the first three PCA components.*

Table 1. Characteristics of selected fourteen sugarcane genotypes.

S.N.	Varieties	Parents	Maturation type
1	CoS 03234	Bo 91 X PCGC cross	Early
2	CoSe 01424	Bo 91 X Co 453	Early
3	Co 0118	CoS 8347 X Co 86011	Early
4	Co 0238	CoLK 8102 X Co 775	Early
5	CoC 671	Q 63 × Co 775	Early
6	CoJ 64	Co 976 X Co 617	Early
7	Co 1148	P 4383 X Co 191	Early
8	CoS 08272	CoSe 92423 GC cross	Early
9	CoS 95255	Co 1158 X Co 62198	Early
10	BO 91	Bo 55 × Bo 43	Midlate
11	UP 9530	CoSe 1084/86 x CoSe 22/85	Midlate
12	CoS 01434	Co 880239 X Co 775	Midlate
13	UP 49	CoSe 92423 X UP 9742	Midlate
14	CoS 7250	CoS 8436 X Co 775	Midlate

Table 2. ISSR primer pairs used in this investigation.

Primer name	Sequence (5'-3')	Amplicon Size (Kb)	Total bands	Monomorphic bands	Polymorphic bands	% Polymor-phism	PIC value
UBC 807	5'AGAGAGAGAGAGAGAGT3'	0.3-1.7	7	2	5	71.4	0.27
UBC 810	5'GAGAGAGAGAGAGAGAT3'	0.2-3.0	10	3	7	70.0	0.31
UBC855	5'ACACACACACACACACYT3'	0.3-2.5	9	4	5	55.6	0.51
UBC 811	5'GAGAGAGAGAGAGAGAC3'	0.3-2.5	7	2	5	71.4	0.14
UBC 814	5'TGTGTGTGTGTGTGTGA3'	0.4-1.5	10	2	8	80.0	0.39
UBC823	5'TCTCTCTCTCTCTCTCC3'	0.2-1.5	9	5	4	44.4	0.45
UBC 812	5'GAGAGAGAGAGAGAGAA3'	0.2-2.5	9	3	6	66.7	0.53
UBC 815	5'CTCTTCTCTCTCTCTCTG3'	0.2-1.5	10	3	7	70.0	0.51
UBC 848	5'CACACACACACACACARG3'	0.3-3.2	7	2	5	71.4	0.37
UBC 834	5'AGAGAGAGAGAGAGAGYT3'	0.3-2.8	9	3	6	66.7	0.37
UBC 857	5'ACACACACACACACACYG3'	0.2-3.3	8	2	6	75.0	0.52
ISSR1	5'AGAGAGAGAGAGAGAGT3'	0.2-2.4	10	3	7	70.0	0.53
ISSR2	5'AGAGAGAGAGAGAGAG3'	0.2-2.7	9	4	5	55.6	0.37
ISSR3	5'GAGAGAGAGAGAGAGAT3'	0.2-1.8	10	4	6	60.0	0.43
ISSR4	5'AGTGTGTGTGTGTGTGT3'	0.2-2.5	7	3	4	57.1	0.44
ISSR5	5'ACTCTCTCTCTCTCTCT3'	0.2-2.0	8	2	6	75.0	0.52
ISSR6	5'ATCTCTCTCTCTCTCTC3'	0.3-2.5	12	4	8	66.7	0.51
ISSR7	5'AGAGAGAGAGAGAGAGGC3'	0.3-2.4	8	3	5	62.5	0.39
ISSR8	5'AGAGAGAGAGAGAGAGCC3'	0.2-1.5	5	1	4	80.0	0.44
		Sum	164	55	109	1269.5	8.00
		Average	8.83	3.00	5.83	66.08	0.42
		Minimum	5	1	4	44.4	0.14
		Maximum	12	5	8	80.0	0.53

Table 3. Average similarity coefficient among different accessions of sugarcane studied.

	CoS 03234	CoSe 01424	Co 0118	Co 0238	CoC 671	CoJ 64	Co 1148	CoS 08272	CoS 95255	BO 91	UP 9530	UP 49	CoS 7250	CoS 1434
CoS 03234	1.00													
CoSe 01424	0.14	1.00												
Co 0118	0.78	0.31	1.00											
Co 0238	0.38	0.55	0.31	1.00										
CoC 671	0.57	0.50	0.56	0.38	1.00									
CoJ 64	0.57	0.50	0.66	0.38	0.57	1.00								
Co 1148	0.61	0.54	0.50	0.70	0.61	0.61	1.00							
CoS 08272	0.52	0.37	0.61	0.37	0.62	0.44	0.47	1.00						
CoS 95255	0.58	0.50	0.46	0.36	0.58	0.58	0.50	0.43	1.00					
BO 91	0.72	0.25	0.57	0.25	0.58	0.58	0.50	0.35	0.60	1.00				
UP 9530	0.57	0.63	0.56	0.38	0.83	0.69	0.61	0.62	0.72	0.58	1.00			
UP 49	0.35	0.87	0.37	0.66	0.58	0.46	0.63	0.43	0.45	0.23	0.58	1.00		
CoS 7250	0.53	0.60	0.53	0.60	0.53	0.66	0.72	0.41	0.41	0.41	0.53	0.70	1.00	
CoS 1434	0.72	0.36	0.57	0.25	0.56	0.58	0.50	0.43	0.77	0.77	0.77	0.72	0.33	0.41	1.00

Table 4. Cluster of dendrogram based on ISSR markers in fourteen accession of sugarcane.

Clusters	Fre-quency	Genotypes
Cluster-1	8	CoS 03234, Co 0118, CoC 671, CoJ 64, CoS 95255, BO 91, UP 9530, CoS 7250
Cluster-2	1	CoS 08272
Cluster-3	5	CoSe 01424, CoS 01434, Co 0238, Co 1148, UP 49

similarity among sugarcane genotypes were 0.14-0.78. The analysis of these similarities matrix revealed that greater similarity between CoSe03234 and CoSe1424 (0.78), and lowest similarity between CoSe03234 and Co0118 (0.14) (Table 3). Those cultivars that display similar coefficient of matrix are genetically close to one another and vice-versa. Using ISSR as genetic markers, as high as 66.08% polymorphic bands were detected in 14 accessions of sugarcane; similar studies were conducted previously using 37 sugarcane varieties with 8 ISSR primers. A total of 33 bands were amplified, of which 39.4 % were polymorphic. The genetic similarity coefficients amongst 37 sugarcane varieties ranged from 0.67 to 0.97, with the average of 0.873, by He *et al.* (2011). Seventeen sugarcane genotypes were carried out by using 27 ISSR markers. Out of the 252 amplicons amplified by 27 ISSR primers, 212 were polymorphic (84.13%) with an average of 9.3 alleles per locus by Kalwade *et al.* (2012). Eighty one sugarcane genotypes were used to characterize by ISSR primers. A total of 13 ISSR primers used and produced 65 amplified fragments, of which 63 (96.5 %) were polymorphic. The Polymorphic Information Content (PIC) value ranged from 0.11 (UBC824) to 0.45 (UBC825) primers with an average value of 0.28 by Devarumath *et al.* (2012) and Costa *et al.* (2011). High level of polymorphism has been reported in wheat through ISSR markers by Sadigova *et al.* (2014). The average polymorphism produced by 11 selected ISSR primers performed to estimate genetic diversity among 27 rice varieties was more than 75% which may be due to wide range of pedigrees of varieties (Al-Turki, 2015). Therefore, the present investigation reported the results of a study on the genetic diversity among 14 accessions of sugarcane revealed by ISSR.

Conclusion

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. It would be helpful to conserve sugarcane ex-situ as well identification of parents for breeding improvement programme and also to fulfil the growing demand at national and international market.

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