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DIVERSITY AND DNA FINGERPRINTING OF SUGARCANE (*SACCHARUM OFFICINARUM* L.) GENOTYPES USING MICROSATELLITE MARKERS

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Abstract

Large genome size and deficiency of adequate informative molecular markers bottlenecked genetic improvement in sugarcane. DNA fingerprinting and diversity analysis of sugarcane genotypes provide essential genetic evidences that breeders could utilize in crop improvement program. To investigate the genetic diversity based on 46 microsatellite markers, 16 promising exotic sugarcane genotypes were utilized. Twenty (20) out of 46 microsatellite markers were examined at Germplasm Evaluation Lab of BCI, National Agricultural Research Centre, Islamabad, whereas the remaining 26 were tested at the genomics lab of SIU-Carbondale, USA. The genotypes portrayed substantial level of genetic polymorphism. Ratio of monomorphic loci was 28.66% out of 164, whereas polymorphic loci were 71.34% with an average 3.57 alleles/locus. Out of 46 microsatellite markers, 10 (21.74%) produced monomorphic, 13 (28.26%) produced polymorphic bands and 23 (50%) produced both monomorphic and polymorphic bands. SSR markers SCM16 and UGSM574 produced maximum number of bands (10), whereas markers SMC7CUQ, SMC1604SA, MCSA053C10, SOMS118, UGSM154, UGSM312, mSSCIR3, SMC851MS, SOMS156, SMC336BS and SMC1751CLproduced the least number of band i.e., 1. In all 16 sugarcane genotypes, the PIC value of the polymorphic loci ranged between 0.009 and 0.947 with the mean value of 0.490/locus. Mean number of alleles/polymorphic locus was 3.30, whereas mean number of alleles/locus was calculated as 3.57. Through similarity matrix extent of genetic relatedness among the sugarcane genotypes was determined. Genetic similarity as pair-wise ranged between 71 to 93%. Minimum genetic similarity was noted 71% between genotypes CP89831 and MS94CP15, while the maximum between genotypes S97CP288 and MS99HO391. The phenogram categorized the 16 cultivars into main four (4) clusters/groups. Cluster-1/group-1 consisted two (2) genotypes only, 2nd cluster consisted of five (5) genotypes, whereas 3rd cluster consisted only one genotype (MS92CP979) which was branched solitary. The 4th cluster was comparatively a large one and consisted of eight genotypes. This was suggested that the genotypes showed maximum level of genetic polymorphism might be further utilized in sugarcane varietal development and breeding plans.

Key words: Cluster analysis, DNA fingerprinting, Genetic diversity, PIC, SSR markers, Sugarcane genotypes.

Introduction

The prime objective of any sugarcane breeding program is to enhance the crop yield. As compared to other crops, genetic improvement in sugarcane crop is very slow process due to complexity and large genome size besides other limiting factors. In crop improvement programs, sugarcane germplasm characterization provides genetic diversity information that breeders might easily exploit for their specific objectives. Variations in morphological attributes among genotypes of sugarcane are highly minor and significantly vary with changes in environmental conditions. Therefore, morphological attributes can't be applied to reliably distinguish amongst commercially released varieties. In contrast, molecular markers have the distinctive characteristic to differentiate among the cane germplasm and guide the researchers to investigate even minor genomic differences. To find out the genetic variations in sugarcane varieties belongs to diverse origins of the world; numerous molecular markers are being employed. Molecular markers such as RFLPs, AFLPs, RAPDs, SSRs, TRAP and SNP have been extensively utilized for the taxonomic and genetic classification of sugarcane genotypes and many other crops (Powell *et al*., 1996b; Masood *et al*., 2005; Rabbani *et al*., 2010; Akbar *et al*., 2011).

In the genome of eukaryotic organisms, microsatellites markers exist which repeats tandemly from one to six nucleotides base pairs (1-6 bp) showing variations in the repeats of these units (Shah *et al*., 2015). Among the various molecular markers, SSRs became a suitable choice for the investigation of agricultural crops genetic diversity (Ullah *et al*., 2017). In plant genomes, as compared to other molecular marker systems, microsatellites are rich and comparatively easy to be handled during analysis of DNA fingerprinting and diversity in agronomic crops. SSRs became more popular and obtained significant position in genomic research studies for their vast genomic distribution, reproducibility, hyper-variability, co-dominant inheritance, multi-allelic nature and chromosome specific location (Glynn *et al*., 2009).

Due to this variability SSRs utilized in sugarcane more specifically for the studies of genotyping and finger printing, genetic diversity, useful genes mapping, varietal identification, marker assisted selection (MAS), evolutionary or phylogenetic relationships among various species (Akbar *et al*., 2019). The advancement of SSRs DNA genotyping technology in sugarcane crop enabled breeders to accurately and efficiently detect phylogenetic relationship and distinction among various genotypes of sugarcane (Glynn *et al*., 2009; Singh *et al*., 2010).

The aims of current research studies were to assess the usefulness of sugarcane SSRs markers in sugarcane breeding program to characterize and detect variations in sugarcane germplasm/genotypes to provide necessary genetic information on DNA level and to investigate interrelationship/linkages among sugarcane genotypes based on genetic diversity for exploitation in future sugarcane breeding strategies.

Materials and Methods

Sugarcane plant samples: Sixteen (16) promising sugarcane genotypes in the final varietal development stage were selected for investigation of genetic diversity and DNA fingerprinting through SSR molecular markers. These sugarcane genotypes were introduced from different internationally advanced organizations of sugarcane research. Table 1 is representing genotypes list with their concerned origin. For the purpose of DNA extraction, the genotypes were sown during November, 2011. The trial was planned and laid out in the National Agricultural Research Center (NARC), Islamabad screen house. Forty-six (46) microsatellite markers in 16 sugarcane genotypes had been tested for investigation of diversity on genetic basis. Twenty (20) of forty-six (46) microsatellite markers were tested at Germplasm Evaluation Lab of PGRI, NARC, Islamabad, whereas the rest of twenty-six (26) had been tested at the genomics lab of SIU-Carbondale, USA. Table 2 is representing detail list of 46 microsatellite markers.

Table 1. List of 16 sugarcane genotypes used during present study.

Sr.No.	Genotypes	Source
1.	MS91CP272	USDA-ARS Station at Canal Point, USA
2.	MS94CP15	USDA-ARS Station at Canal Point, USA
3.	MS91CP238	USDA-ARS Station at Canal Point, USA
4.	MS92CP979	USDA-ARS Station at Canal Point, USA
5.	MS99HO391	USDA-ARS Station, Houma, Louisiana, USA
6.	S97CP288	USDA-ARS Station at Canal Point, USA
7.	MS99HO317	USDA-ARS Station, Houma, Louisiana, USA
8.	RS97N45	South African Research Institute, Natal, South Africa
9.	MS99HO388	USDA-ARS Station, Houma, Louisiana, USA
10.	MS99HO675	USDA-ARS Station, Houma, Louisiana, USA
11.	MS99HO93	USDA-ARS Station, Houma, Louisiana, USA
12.	S96SP1215	São Paulo, Brazil
13.	Hoth127	USDA-ARS, Houma, Louisiana, USA and Sugarcane Research Institute, Thatta
14.	CP89831	USDA-ARS Station at Canal Point, USA
15.	CP77400 (Check-I)	USDA-ARS Station at Canal Point, USA
16.	Mardan93 (Check-II)	USDA-ARS Station at Canal Point, USA

MS: Mardan Selection, Hoth: Houma-Thatta, SP: São Paulo, HO: Houma, N: Natal
USDA-ARS: United States Department of Agriculture-Agriculture Research Service

Genomic DNA isolation/separation from tissue samples of fresh sugarcane leaf: Total genomic DNA from fresh tissues of sugarcane plant was extracted through CTAB method of Doyle & Doyle (1990) with a little alteration for this study. UV spectrophotometer was used to quantify DNA concentrations by using 1.00% (w/v) electrophoresis of agarose gel (Sambrook *et al*., 1989). Disease free small samples of sugarcane leaf pieces (approximately 500 mg) had been cut and crashed in a pre-chilled mortar and pestle. Two to three (2-3 ml of 2.00 X CTAB buffer (2.00% (w/v) CTAB, 20mM EDTA, 1.4M NaCl, 100mM Tris-HCl (pH 8.0) has been mixed along with 1.00% mercaptoethanol. Leaf samples were vigorously crushed and ground to emulsify by mortar and pestle. The emulsion having volume of 750 µl was shifted into a new microcentrifuge tube and was incubated in a water bath at 65˚C for 30 minutes. 750µl of chloroform:isoamyl alcohol (24:1) was added to the tube and mixed slightly. This was followed by centrifugation of the samples for 10 minutes at 10,000 rpm. About 0.6ml of the upper aqueous portion was carefully pipetted out in a fresh microcentrifuge tube. For precipitation of DNA ice chilled iso-propanol (2-propanol) in equal volume was added to the tube. The samples were then incubated at 4˚C for 10 minutes. After this, the samples were once again centrifuged for 10 minutes at 4˚C at 12,000 rpm. The samples upper portion was removed and the DNA pellet was washed with 70% ethanol. Finally, the samples tubes were again centrifuged at 12,000 rpm, for 10 minutes and 4˚C. To get the solid and dried DNA pellet, the samples upper portion was removed and dried in air. TE buffer (50/100µl) containing one micro letter (1µl) of RNase-A (10mg/ml) was added to the dried DNA pellet to degrade the content of RNA. The quality and quantity of the genomic DNA was tested on 1.00% agarose gel and the concentration was diluted with ddH_2O appropriately (20ng μl^{-1}) prior using in PCR master mix reaction and stored the samples at -20°C.

Amplification of PCR and gel electrophoresis: The isolated DNA samples of sixteen (16) sugarcane genotypes were tested using 46 microsatellite markers for diversity and DNA fingerprinting (Table 2). The nucleotide microsatellite makers sequences had been selected from Pan (2010) and Singh *et al*., (2010). PCR reaction was carried out in a total volume of 20µl consisted of 1µl of 20ng template DNA, 0.2µl of 1unit of Taq DNA polymerase, 2µl of 10X PCR buffer, 1.6µl of 10mM dNTPs, 1.2µl of 1.5mM of $MgCl_2$,12µl ddH_2O and 1µl of 10 pecomole μl^{-1} of each reverse and forward primer. Thermal cycler was used for amplifications such that the program was set as initial denaturation at 94°C for 5 min. The total amplification cycles were 35 and each cycle of amplification was denatured initially at 94°C for 1 min. This was followed by annealing at 55°C for 1 min and then 72°C for 2 min. The final extension was at 72°C for 7 min. The amplified PCR products was stored at 4°C and separated the product on 3% agarose gel electrophoresis in 1.00X TBE buffer. Confirmation and visualization of the amplified DNA-SSR bands was carried out by 0.5µg/ml ethidium bromide staining. GelDoc System (Alpha Innotech) was used for gel photographs under UV light.

Allele scoring and data analysis for detection of genetic diversity: The stained Ethidium bromide agarose gels showed several bands. The size of most intensively amplified band of each SSR marker was find out based on its electrophoretic mobility relative to molecular weight marker (100bp ladder). Qualitatively SSR marker amplified products were scored for absence and presence of each marker allele-genotype combination. Using MS Excel sheet, data of each genotype SSR marker were entered into a binary matrix as discrete variables, 0 for absence of the attribute and 1 for presence. Based on the extent of polymorphism, the most informative primers were tagged. The Polymorphism Information Content (PIC) value of a marker was determined using below formula:

$$\text{PIC} = 1 - \sum_{j=1}^{n} P^2 ij$$

As P_{ij} is the *j*th allele frequency for the *i*th marker and aggregated over *n* alleles. To measure the genetic similarity, pair-wise genotypes comparisons based on the proportion of unique and shared alleles were used. Fraction of polymorphic loci (β) was computed by using formula: $\beta = (np/n_p+n_{np})$, where n_p represents number of alleles that showed polymorphism and n_{np} is the number of alleles that were non-polymorphic (Powell *et al*., 1996a). Number of loci per assay unit (n_u) was worked out by using formula: $n_u = L/U$, where L is representing number of loci and U represents number of assay units (Maras *et al*., 2008). Marker Index (MI) represents the product of Effective Multiplex Ratio (EMR) and Polymorphic Information Content (PIC) and computed using formula: MI = EMR x PIC. Effective Multiplex Ratio (EMR) is the number of polymorphic loci in the germplasm set of interest analyzed per experiment and computed from the formula: EMR = β × n (Powell *et al*., 1996b). Genetic similarity estimates were computed between all pairs of the genotypes according to Nei & Li (1979) based on the formula: Similarity (F) = 2nab /(na+nb), where nab is representing the number of fragments shared by individuals 'a' and 'b', na is the total number of fragments detected in individual 'a' and nb is the total number of fragments shown by individual 'b'. The final data of similarity matrix was employed to create a phenogram based on Un-weighted Pair-Group Method with an Arithmetic Mean (UPGMA) to exhibit genetic relationships among genotypes/germplasm. The data was analyzed using computer software NTSys-pc, Version2.2 package (Rohlf, 2005) and MS Excel, 2010.

Results

Genetic diversity analysis based on molecular marker (SSR) was obtained in sixteen (16) genotypes of S*accharum* cultivars introduced from research institutes of sugarcane cultivating countries. In presence of available resources, using agarose gel, results of the experiment were repeated twice. Forty six (46) cleared and well amplified unambiguous SSR markers bands were considered for scoring of data. Figures 1 and 2 are representing the gel photographs of SSR marker. Forty six (46) SSR primers produced a total of 164 loci in 16 sugarcane genotypes. Out of these loci, 28.66% were monomorphic, whereas71.34% were polymorphic with an average of 03.57 alleles per locus of SSR marker (Table 2). Out of forty-six (46) primers, only 10 (21.74%) generated monomorphic, 13 (28.26%) produced polymorphic bands and 23 (50%) generated both monomorphic and polymorphic. In all 16 sugarcane genotypes, the PIC value of the polymorphic loci of the each SSR marker ranged from 0.009 to 0.947 with an average value of 0.490/locus. Maximum PIC value of 0.947 has been recorded in SSR marker mSSCIR5, followed by 0.939 in UGSM667, 0.893 in UGSM565, 0.889 SMC486CG and 0.793 in SMC336BS, whereas the minimum 0.009 was recorded in UGSM542, followed by 0.031 in SCM27, 0.051 in SOMS118 and 0.047 in UGSM574 (Table 3). The maximum MI value of 0.893 was recorded in UGSM565 due to maximum EMR component value. Mean number of alleles/locus was calculated as 3.57. Similarly, mean number of alleles per polymorphic locus were 3.30. Maximum polymorphic alleles (9/10) were recorded for SSR marker SCM16, while the minimum for UGSM154, mSSCIR74, mSSCIR74, MCSA042E08, SOMS120, SMC334BS and mSSCIR5. Complicated allelic patterns were recorded in sugarcane microsatellite markers having number of amplified fragments ranging from 1 (MCSA053C10, SMC336BS, SMC1751CL, SOMS118, mSSCIR3, SMC1604SA, SMC7CUQ, SOMS156, SMC851MS, UGSM312 and UGSM154) to 10 (UGSM574 and SCM16) (Table 2). The highest numbers of alleles (10) were produced by microsatellite primer UGSM574 and SCM16, whereas the least one (1) by MCSA053C10, SMC336BS, SMC1751CL, SOMS118, SMC7CUQ, mSSCIR3, SMC851MS, SOMS156, UGSM312, SMC1604SA and UGSM154. Amplified fragments size ranged from 42bp (MCSA042E08) to 1237bp (SOMS58) in length.

Genetic similarity among the sixteen (16) sugarcane genotypes: Among 16 sugarcane genotypes the level of relatedness had been worked out using genetic similarity matrix formulated by Nei's (1972) which was ranged from 71 to 93% (Table 4). The maximum genetic similarity (93%) was determined between SSR markers S97CP288 and MS99HO391, similarly 92% between MS99HO317 and S97CP288, 91% between MS99HO317 and MS99HO391, 90% between Mardan93 and CP77400, 89% between CP89831 and Hoth127and MS99HO93 and MS99HO675. The minimum genetic similarity (71%) was shared between CP89831 and MS94CP15. The lower values being worth mentioning were recorded to be 73% between MS91CP272 & CP89831, MS91CP272 & Mardan93, and MS94CP15 & Mardan93, 74% between Mardan93 andMS92CP979 and 75% between MS94CP15 & MS99HO388.

Cluster analysis: Using Dice coefficient, the generated phenogram categorized the sixteen (16) genotypes of sugarcane into four major clusters based on 46 derived microsatellite primer pairs (Fig. 3). Cluster-1 composed of two genotypes of sugarcane i.e., MS94CP15 and MS91CP272, which might be assumed to have the same genetic background. Second cluster was a bit larger one and was further classified into two sub-clusters groups i.e., sub-cluster II-A and II-B. Sub-cluster II-A composed of four genotypes MS99HO391, MS91CP238, MS99HO317 and S97CP288 which exhibited uniform allelic pattern. Genotype RS97N45 was grouped alone into sub-cluster II-B which might be assumed to have diverse genetic background. The 3rd cluster composed of only on genotype i.e., MS92CP979 and did not showed similarity to rest of genotypes which further confirmed to have a very diverse origin and genetic background. The 4th cluster was the largest cluster as compared to the others which consisted of eight genotypes. Again this cluster was further categorized into two sub-clusters i.e., sub-cluster IV-A and IV-B. The genotypes MS99HO675, MS99HO93, MS99HO388, Mardan93 and CP77400 were present in sub-cluster IV-A as these genotypes are comparatively more genetically similar than the others. The possible reason might be sharing a common origin (Canal Point, USA). On other hand, predominantly sub-cluster IV-B composed of three genotypes i.e., CP89831, Hoth127and S96SP1215.

Table 2. Details of SSR markers with their annealing temperature (Tm), sequences (forward and reverse), range of product size, total number of alleles (n_a), number of polymorphic (n_p) and monomorphic alleles (n_{np}) given by the primer.

No.	Primers	Forward primer (5'-3')	Reverse primer (5'-3')	Tm	Allele size range (bp)	n_a	n_p	n_{np}
1.	SCM4	CATTGTTCTGTGCCTGCT	CCGTTTCCCTTCCTTCCC	64.5	105-134	8	5	3
2.	SCM15	GGAGATGTTTGAGAGGGAA	AGAGTAGCATAAAGGAGGCAG	65.4	145-624	5	4	1
3.	SCM16	GTGCGAGAGGAACTGTGT	AGCCCTGCCTAACAAGGA	66.8	123-792	10	9	1
4.	SCM18	CATCAGTATCATTTCATCTTGG	CAGTCACAGTCGGGTAGA	65.7	199-694	1	0	1
5.	SCM21	CCCTCCCATAACACACAC	TTGACAGCCCAAAGAGTT	66.8	514-774	2	2	0
6.	SCM27	TTCTCTGACTTCCAATCCAA	ATCAAGCACGCCCGCCTC	64.2	279-714	4	3	1
7.	SCM32	GATGAAGCCGACACCGAC	AGTTGCCTGTTCCCATTT	69.1	156-770	4	4	0
8.	SOMS58	CCGCTTTCAACCTCTACAC	GGCTTGGTGATTCTTCTCT	67.6	99-1237	6	5	1
9.	SOMS118	GAGGAAGCCAAGAAGGTG	TAGAGCGAGGAGCGAAGG	66.8	82-1018	8	7	1
10.	UGSM60	CGACTCCACACTCCACTC	CCGAACACCACCTTCTTG	69.1	92-759	6	5	1
11.	UGSM193	AGATATAACACACACACACAAA	GGCCATCGAGGAGGAGTTCAAG	67.0	53-787	8	3	5
12.	UGSM296	ATTATCTACATTCAGACACGTCAC	ATCTTTGTTAGCAATCCATTAAG	68.8	357-1054	3	3	0
13.	UGSM301	GAAGAAGAAGAAGAAGAAGAAGAA	ACTCGTCCTACAACCACGACTAC	67.0	79-725	3	3	0
14.	UGSM302	GAAGAAGAAGAAGAAGAAGAAGAA	ACTCGTCCTACAACCACGACTAC	67.0	82-738	2	2	0
15.	UGSM312	AACGTATCTTTATTTCCATTCTTC	CTTTCAGTTCAACTTTGGATAAAT	65.3	200-583	1	0	1
16.	UGSM504	TAGAGGAAATAGCAGAACAGG	AGACTGACACCTTTGAGATGA	66.9	168-224	4	4	0
17.	UGSM542	ACCTCCACCTCCACCTCAGTTC	CGTTCAGCTTCAGGGTGTCGAT	75.1	53-1123	6	6	0
18.	UGSM565	CATAGCAAGCACCACCTC	TCTTCTTCTCGTCCACCC	66.8	348-539	2	2	0
19.	UGSM574	GCTTCCTCGCTCCTCCTC	TACTTCTACCTCGTCTGCTTC	71.3	65-1026	10	6	4
20.	UGSM575	CTGTTTCCTTCCTTCTCGT	CAATCATAGCCCAGACACC	65.4	66-901	9	7	2
21.	UGSM671	TCCCTACTTCTATGAATATCCTTC	TTGACAAATTGCTTGATGTAGT	59.4	96-571	2	2	0
22.	UGSM681	ACACATCGCTTTCCCACA	GCATACCTGTCGTCGTCT	57.6	94-592	6	4	2
23.	UGSM667	CTATCCTCTTGTTGGGTCCT	TCCGCACCTCCGTTCACC	60.4	54-1063	4	3	1

Table 2. (Cont'd.).

No.	Primers	Forward primer (5'-3')	Reverse primer (5'-3')	Tm	Allele size range (bp)	n_a	n_p	n_{np}
24.	UGSM665	GTTACCATCCCATCCCAC	TGTCCCTCGTTCACAGAC	59.9	147-770	6	4	2
25.	UGSM585	GAAGAGGAGGAGAGGAGAAG	TGGGATGGTTGTTGACTG	62.4	62-648	6	6	0
26.	UGSM154	CTCGTTTCATAGCAGACCTT	GCAACTGGAGGAACTGATG	58.4	54-1101	1	1	0
27.	SOMS156	ATCGTCTCTGGTTGTTGGT	ATCCTCCATTTCCACCTC	58.0	62-593	1	0	1
28.	mSSCIR43	ATTCAACGATTTTCACGAG	AACCTAGCAATTTACAAGAG	53.7	169-252	5	3	2
29.	SMC486CG	GAAATTGCCTCCCAGGATTA	CCAACTTGAGAATTGAGATTCG	58.4	224-247	2	1	1
30.	SMC851MS	ACTAAAATGGCAAGGGTGGT	CGT GAG CCC ACA TAT CAT GC	58.4	128-157	1	0	1
31.	SMC119CG	TTCATCTCTAGCCTACCCCAA	AGCAGCCATTTACCCAGGA	60.6	95-210	3	2	1
32.	SMC1604SA	AGGGAAAAGGTAGCCTTGG	TTCCAACAGACTTGGGTGG	60.2	106-126	1	0	1
33.	mSSCIR74	GCGCAAGCCACACTGAGA	ACGCAACGCAAAACAACG	62.2	209-337	3	1	2
34.	SMC7CUQ	GCCAAAGCAAGGGTCACTAGA	AGCTCTATCAGTTGAAACCGA	62.6	158-171	1	0	1
35.	mSSCIR3	ATAGCTCCCACACCAAATGC	GGACTACTCCACAATGATGC	60.4	171-187	1	0	1
36.	SMC1751CL	GCCATGCCCATGCTAAAGAT	ACGTTGGTCCCGGAACCG	60.4	134-153	1	0	1
37.	SMC334BS	CAATTCTGACCGTGCAAAGAT	CGATGAGCTTGATTGCGAATG	58.7	144-165	2	1	1
38.	SOMS143	TGACTTGGAATAACACAAAGAA	ATGGGATGGATAATAAGCAGT	55.2	137-555	2	2	0
39.	SOMS135	TCTTCAACTTCCTCTGCCT	GTTCCTGACTGTTCCCTTG	58.0	210-929	3	2	1
40.	SOMS118	GAGGAAGCCAAGAAGGTG	TAGAGCGAGGAGCGAAGG	59.9	82-1018	1	0	1
41.	SOMS120	GCATCTATCGGTCTTCTGG	ATCCAATCCTTCATCTTCTTC	60.2	84-1155	2	1	1
42.	MCSA053C10	CGAGCATGGCGAGGAGTCCG	GCAGGGCGAGGCGAGATCAG	68.6	53-153	1	0	1
43.	MCSA068G08	CTAATGCCATGCCCCAGAGG	GCTGGTGATGTCGCCCATCT	64.5	68-200	2	1	1
44.	mSSCIR5	GCAGCCTTGGTTCGGTCTATG	GCATCCCTCGCCCTTCCTC	64.5	378	2	1	1
45.	MCSA042E08	CTTGAGGGTGAAGCGGATGG	AGCCTCTGCCACCACTCCTC	64.5	42-197	2	1	1
46.	SMC336BS	ATTCTAGTGCCAATCCATCTCA	CATGCCAACTTCCAAACAGAC	58.9	141-239	1	1	0

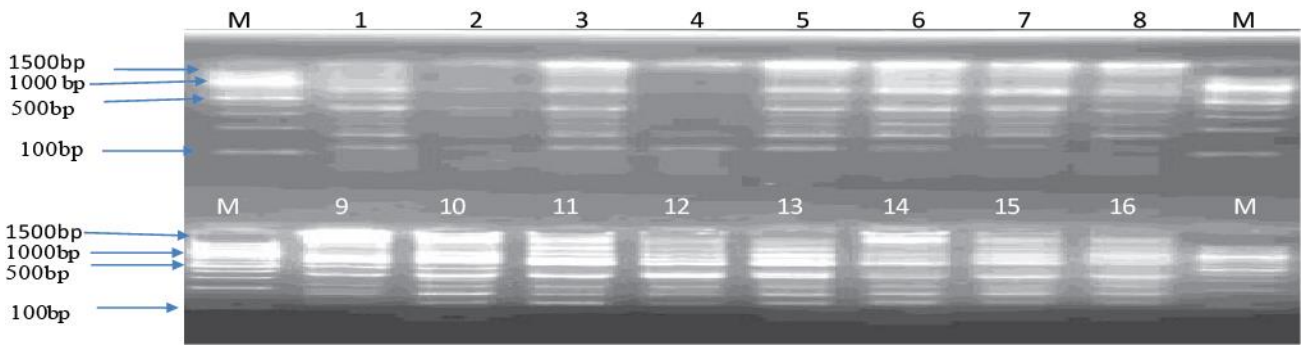

Fig. 1. Agarose gel banding pattern for microsatellite marker SCM16, M = 100bp DNA Ladder, 1= MS91CP272, 2= MS94CP15, 3= MS91CP238, 4= MS92CP979, 5= MS99HO391, 6= S97CP288, 7= MS99HO317, 8= RS97N45, 9= MS99HO388, 10= MS99HO675, 11= MS99HO93, 12= S96SP1215, 13= Hoth127, 14= CP89831, 15= CP77400, 16= Mardan93

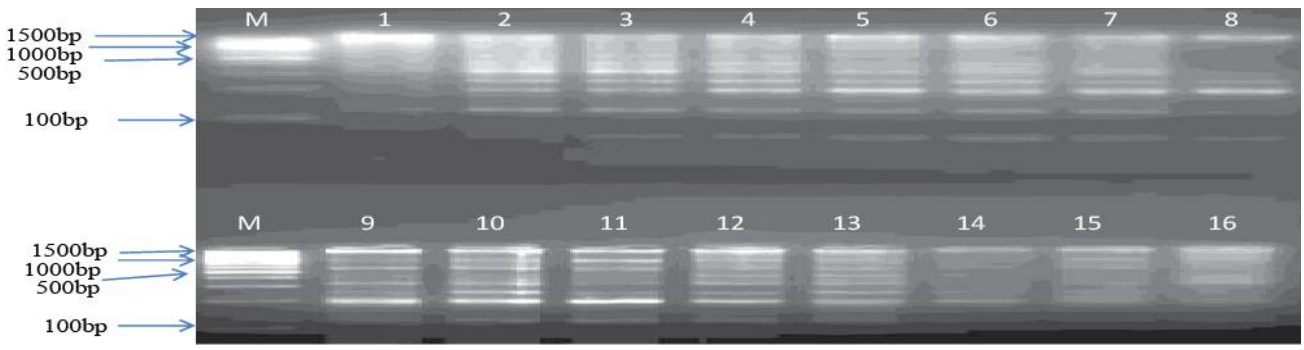

Fig. 2. Agarose gel banding pattern for microsatellite marker SOMS118, M = 100bp DNA Ladder, 1= MS91CP272, 2= MS94CP15, 3= MS91CP238, 4= MS92CP979, 5= MS99HO391, 6= S97CP288, 7= MS99HO317, 8= RS97N45, 9= MS99HO388, 10= MS99HO675, 11= MS99HO93, 12= S96SP1215, 13= Hoth127, 14= CP89831, 15= CP77400, 16= Mardan93

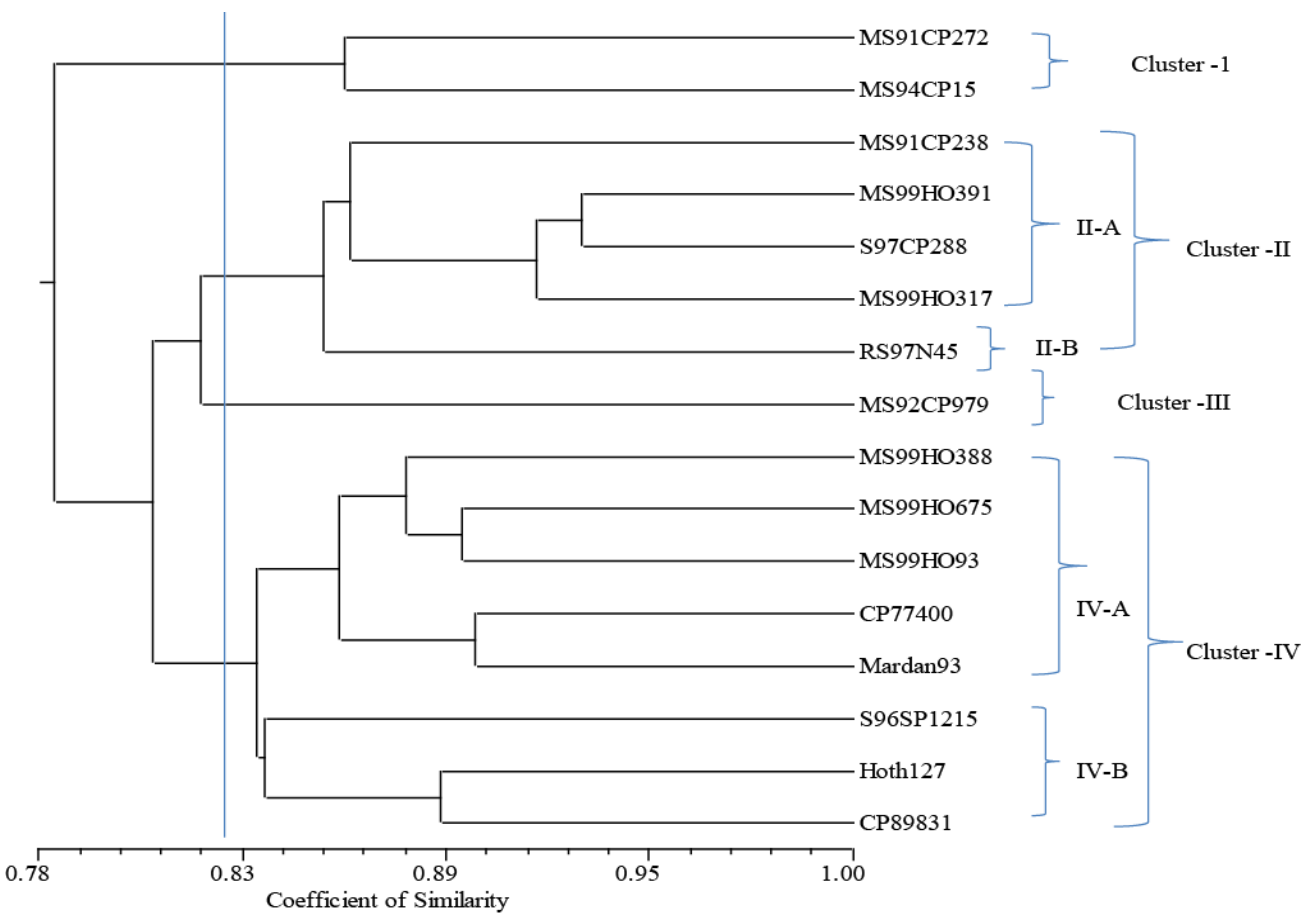

Fig. 3. Dendrogram of 16 sugarcane genotypes developed from 46 SSR markers based on Dice coefficient.

Table 3. Polymorphic alleles % (n_p%), polymorphic information content (PIC), fraction of polymorphic loci (β), number of loci per assay unit (n_u), Effective Multiplex Ratio (EMR), Marker Index (MI), calculated for each simple sequence repeat (SSR) locus count.

Sr. No.	Marker	n_p(%)	PIC	B	n_u	EMR	MI
1.	SCM4	63	0.100	0.625	1	0.625	0.063
2.	SCM 15	80	0.318	0.800	1	0.800	0.254
3.	SCM 16	90	0.174	0.900	1	0.900	0.156
4.	SCM 18	0	0.000	0.000	1	0.000	0.000
5.	SCM 21	100	0.391	1.000	1	1.000	0.391
6.	SCM 27	75	0.031	0.750	1	0.750	0.023
7.	SCM 32	100	0.613	1.000	1	1.000	0.613
8.	SOMS 58	83	0.313	0.833	1	0.833	0.261
9.	SOMS 118	88	0.051	0.875	1	0.875	0.045
10.	UGSM 60	83	0.249	0.833	1	0.833	0.207
11.	UGSM 193	38	0.476	0.375	1	0.375	0.178
12.	UGSM 296	100	0.571	1.000	1	1.000	0.571
13.	UGSM 301	100	0.476	1.000	1	1.000	0.476
14.	UGSM 302	100	0.433	1.000	1	1.000	0.433
15.	UGSM 312	0	0.000	0.000	1	0.000	0.000
16.	UGSM 504	100	0.222	1.000	1	1.000	0.222
17.	UGSM 542	100	0.009	1.000	1	1.000	0.009
18.	UGSM 565	100	0.893	1.000	1	1.000	0.893
19.	UGSM 574	60	0.047	0.600	1	0.600	0.028
20.	UGSM 575	78	0.382	0.778	1	0.778	0.297
21.	UGSM 671	100	0.529	1.000	1	1.000	0.529
22.	UGSM 681	67	0.413	0.667	1	0.667	0.276
23.	UGSM 667	75	0.939	0.750	1	0.750	0.704
24.	UGSM 665	67	0.318	0.667	1	0.667	0.212
25.	UGSM585	100	0.762	1.000	1	1.000	0.762
26.	UGSM154	100	0.793	1.000	1	1.000	0.793
27.	SOMS156	0	0.000	0.000	1	0.000	0.000
28.	mSSCIR43	60	0.736	0.600	1	0.600	0.441
29.	SMC486CG	50	0.889	0.500	1	0.500	0.444
30.	SMC851MS	0	0.000	0.000	1	0.000	0.000
31.	SMC119CG	67	0.682	0.667	1	0.667	0.455
32.	SMC1604SA	0	0.000	0.000	1	0.000	0.000
33.	mSSCIR74	33	0.395	0.333	1	0.333	0.132
34.	SMC7CUQ	0	0.000	0.000	1	0.000	0.000
35.	mSSCIR3	0	0.000	0.000	1	0.000	0.000
36.	SMC1751CL	0	0.000	0.000	1	0.000	0.000
37.	SMC334BS	50	0.889	0.500	1	0.500	0.444
38.	SOMS143	100	0.342	1.000	1	1.000	0.342
39.	SOMS135	67	0.789	0.667	1	0.667	0.526
40.	SOMS118	0	0.000	0.000	1	0.000	0.000
41.	SOMS120	50	0.395	0.500	1	0.500	0.198
42.	MCSA053C10	0	0.000	0.000	1	0.000	0.000
43.	MCSA068G08	50	0.640	0.500	1	0.500	0.320
44.	mSSCIR5	50	0.947	0.500	1	0.500	0.473
45.	MCSA042E08	50	0.640	0.500	1	0.500	0.320
46.	SMC336BS	100	0.793	1.000	1	1.000	0.793
			Hev = 0.490			**Mean alleles/locus = 3.57**	

Table 4. Summary of the Nei's (1972) genetic similarity for all loci among 16 sugarcane cultivars generated by SSR amplification.

Genotypes	MS91CP 272	MS94CP 15	MS91CP 238	MS92CP 979	MS99HO 391	S97CP 288	MS99HO 317	RS97N 45	MS99HO 388	MS99HO 675	MS99H O 93	S96SP 1215	Hoth 127	CP 89831	CP 77400	Mardan 93
MS91CP272	1.00															
MS94CP15	0.86	1.00														
MS91CP238	0.85	0.84	1.00													
MS92CP979	0.78	0.79	0.84	1.00												
MS99HO391	0.80	0.78	0.88	0.83	1.00											
S97CP288	0.82	0.80	0.84	0.82	**0.93**	1.00										
MS99HO317	0.82	0.82	0.87	0.81	0.91	0.92	1.00									
RS97N45	0.82	0.81	0.85	0.81	0.84	0.86	0.88	1.00								
MS99HO388	0.78	0.75	0.83	0.77	0.85	0.86	0.83	0.83	1.00							
MS99HO675	0.80	0.82	0.84	0.76	0.85	0.84	0.84	0.82	0.88	1.00						
MS99HO93	0.76	0.77	0.82	0.79	0.85	0.83	0.84	0.82	0.88	0.89	1.00					
S96SP1215	0.77	0.79	0.81	0.79	0.84	0.81	0.82	0.81	0.84	0.85	0.86	1.00				
Hoth127	0.78	0.75	0.82	0.75	0.82	0.80	0.78	0.80	0.86	0.84	0.83	0.86	1.00			
CP89831	0.73	**0.71**	0.79	0.77	0.80	0.81	0.78	0.80	0.85	0.84	0.83	0.82	0.89	1.00		
CP77400	0.79	0.78	0.85	0.79	0.85	0.84	0.83	0.83	0.89	0.89	0.88	0.83	0.87	0.85	1.00	
Mardan93	0.73	0.73	0.78	0.74	0.78	0.78	0.79	0.77	0.83	0.83	0.84	0.80	0.83	0.80	0.90	1.00

Discussion

Existing of insufficient genetic diversity availability in Pakistani cultivated sugarcane germplasm is primarily due to the deficit of solid true (hybridization) sugarcane breeding program which is related to the unavailability of favorable climatic conditions for sugarcane breeding. Although, sugarcane is a cross pollinating crop but this continued trend resulting in bottle nicking genetic diversity in sugarcane crop. The scarcity of sufficient informative molecular markers could be the second possible reason. Molecular markers are independent and are not influenced by growth practices and environmental factors like biochemical and morphological markers (Ovesna *et al*., 2002). For genome diversity analysis and loci mapping, microsatellite markers are the tremendous technique. The present research work was conducted with the objectives to search the genetic diversity using 46 microsatellite markers among 16 promising sugarcane genotypes. The results indicated an appropriate level of genetic variation among the all genotypes. Out of 164 loci, 28.66% were monomorphic, whereas 71.34% were polymorphic with an average of 3.57 alleles/locus of SSR. Singh *et al*., (2010) used 32 microsatellite markers and reported maximum average of 8.78 alleles/locus from a total of 281 loci in 84 sugarcane cultivars with 94% being polymorphic. The differences in the number of alleles observed/locus from our findings might be due to the use of more diverse and large number of germplasm. The complexity in the allelic patterns of sugarcane crop is due to its heterozygous nature and polyploidy level of the genome (Nawaz *et al*., 2010).

The PIC value varied among the loci and ranged from 0.009 and 0.947 with an average value of 0.490/locus. The maximum PIC value was observed in mSSCIR5 (0.947), whereas the minimum was recorded in UGSM542 (0.009). Hameed *et al*., (2012) used 21 SSR microsatellite markers and reported polymorphic information value ranging between 0.400 and 0.840 in a study diversity analysis and DNA fingerprinting of sugarcane (*Saccharum* sp) cultivars susceptible and resistant to red rot. Minimum PIC value might be the result of closely related genotypes and maximum PIC values were usually attributable to diverse nature of genotypes (Rahman *et al*., 2010). The number of alleles amplified by a primer and its PIC values depend upon the repeat number and repeat sequence of the microsatellite sequences (Ni *et al*., 2002; Rahman *et al*., 2010). Ni *et al*., (2002) reported that larger repeats and GA-repeats yield higher number of alleles and maximum values of PIC. The polymorphism level between any two genotypes showed that differences are possible with use of suitable SSR primer pair. Diversity in sugarcane germplasm was also examined by other researchers involving use of different molecular markers including Lima *et al*., (2002) who used 21 AFLP primer combinations and evaluated a total of 1121 polymorphic loci, however they couldn't detect maximum polymorphism among the analyzed germplasm. Similarly, Nair *et al*., (2002) who quoted diversity in 28 tropical and subtropical Indian sugarcane cultivars using microsatellite markers.

In this study, four (4) major clusters in phenogram were exhibited at the level of similarity 0.825. These findings are supported by the results of Singh *et al*., (2011) who used 30 sugarcane genotypes and got four main clusters. The genetic similarity in the recent study ranged between 0.71 and 0.93. Minimum level of genetic similarity (0.71) was detected between genotypes CP89831 and MS94CP15 exhibiting diversity among these two genotypes. Maximum level of similarity was obtained between genotypes S97CP288 and MS99HO391, which exhibited that these two genotypes could be having a very narrow genetic background. Harvey & Botha (1996) reported 77-95% similarities among 20 elite sugarcane genotypes, whereas Harvey *et al*., (1994) observed almost 80% genetic similarity among 21 South African sugarcane genotypes.

Conclusions

This is concluded from present research work that microsatellite markers showed highly polymorphism as they displayed high PIC values ranging from 0.009 to 0.947 with an average of 0.490 per locus. The genetic similarity ranged from 0.71 to 0.93. Minimum level of genetic similarity (0.71) was detected between genotypes CP89831 and MS94CP15, while maximum genetic similarity was observed between S97CP288 and MS99HO391 which suggested that these two genotypes could be having a very close genetic background. A genetic variation based on molecular markers provides more authentic information to breeders than the field observation alone which are vulnerable to environmental fluctuations. Findings of such type of studies could guide the breeders in planning breeding programs in a more reliable way. Based on results from this study, it is recommended that markers utilized in the present study could be utilized by breeders for genetic mapping and gene tagging of sugarcane. Genotypes that have maximum extent of polymorphism could be utilized in the future breeding programs for crop improvement and varietal development in sugarcane.

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