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MOLECULAR CHARACTERIZATION OF LOCALLY ADOPTED SUGARCANE (*Saccharum officinarum* L.) VARIETIES USING MICROSATELLITE MARKERS

M. S. Ahmed[1] and S. D. A. Gardezi[1]

[1]Department of Plant Breeding & Molecular Genetics, Faculty of Agriculture, University of Azad Jammu & Kashmir, Pakistan
*Correspondence author Email: ms.ahmedgenes@gmail.com

ABSTRACT

Sugarcane is an important crop that caters about 80 percent sugar requirement of the world. Modern day cultivated sugarcane is a complex hybrid between its few species like *Saccharum officinarum* L. and *S. spontaneum* L. having complex aneuploidy genomes with almost eight sets of basic chromosome number. Its flowering behaviours in the country and use of setts as seed narrow downs its genetic base. Molecular characterization provides the basis for determining its variability to start reasonable breeding program. Microsatellite markers have the unique ability to determine the extent of genetic divergence among sugarcane genotypes. In present study 49 SSR primers were utilized for selection of genetically diverse genotypes among 20 sugarcane cultivars adopted in Pakistan. A total of 420 bands were generated with a size range from 50 bp to 600 bp. The total number of bands generated by single primers pair ranged from 3 to 22. Polymorphic information contents (PIC) estimated ranged from 0.7 to 0.3. Cluster analysis grouped genotypes into four distinctive clusters at 70% genetic homology. Principal Coordinate analysis generated 50.04% variation from first 4 PCoA, of which PCoA-1 and PCoA-2 accounted 31% variability. PCoA divided genotypes into 4 groups in a similar pattern as generated with cluster analysis and authenticated the results of cluster analysis. Four genotypes *viz*; *S-03-US-694, S-05-FSD-307, S-08-FSD-19* and *HSF-240* were selected for future breeding endeavours involving crossing between genotypes that may provide valuable strategies for pyramiding beneficial gene(s) in novel sugarcane cultivars. It is concluded that use of SSR marker is very reliable approach for identification of diverse genotype(s) where phenotypic similarity of the cultivars leads to difficulty while selections of parents for hybridization.

Keywords: Genetic diversity, Sugarcane, SSR markers, PAGE, Cluster analysis, Principal Coordinate Analysis.

INTRODUCTION

Sugarcane (*Saccharum* spp.) is a tall, tropical, monocotyledonous, complex aneu-polyploid plant (2n = 8x or 10x = 100-130) that propagates asexually through planting of vegetative cuttings (setts) of mature stalks. It is one of the important commercial sugar producing crops and a major source of approximately 80% sugar and 50% ethanol in the world. It is the source of most of the sugar produced in the world, greatly exceeding sugar beet (Cordeiro *et al.,* 2001). Sugarcane is the second major cash crop of Pakistan and is used as a raw material for the production of sugar, paper, chip board and ethanol. Its share in agriculture and GDP is 3.7 and 0.8 percent, respectively. Sugarcane was cultivated on an area of 10.46 million hectares with cane production of 58.0 million tons, for the year 2011-12 (MNFS&R, 2012). Worldwide, Pakistan ranks 5th in cultivated area and 15th in cane yield (FAO, 2013). Thus, there is a big gap between ranking in cultivated area and cane yield. This is because some native sugarcane genotypes have been adopted over many decades for cultivation in the vast sugarcane growing areas in Punjab and Sind Province of Pakistan. However, repeated utilization of sugarcane clones as a seed for cultivation increase the narrowness of the genetic base of sugarcane cultivars, which leads to the loss of some important characteristics (Tew, 2003; Sreenivasan 2004 and Cheavegatti-Gianotto *et al*., 2011).

An essential first step in any varietal development program is to come up with germplasm that has sufficient genetic variability reflected on morphological bases. Accurate assessment of genetic diversity is very important in crop breeding as it helps in the selection of desirable genotypes, identifying diverse parental combinations for further improvement through selection in segregating populations, and introgression of desirable genes from diverse germplasm into the available genetic base (Mohammadi *et al.,* 2003). Therefore, genetically diverse germplasm is essential in breeding programs to enhance the productivity and stoutness of cultivars. In case of the non-availability of diverse germplasm, utilization of introduced germplasm with full knowledge of its genetic base also help in crop improvement programs (Malik *et al.,* 2010).

The efficiency of genetic introgression in Pakistani sugarcane cultivars has been low, due to the sporadic flowering behaviour, barriers in natural hybridization, self-incompatibility, technical difficulties in artificial crossing, prolonged selection and evaluation processes. Therefore, extensive breeding strategies are required to enhance the genetic base of cultivars and

close the gap between current cane yield and actual yield potential. For hybridization program the basic aim of plant breeder is to select genotypes with broad genetic base, for this purpose diversity assessment in available germplasm is prerequisite. To understand the extent of natural variation on a molecular basis it is important to set up new strategies for sugarcane improvement program. Molecular markers such as microsatellites have been used for this purpose. Morphological evaluation of sugarcane genotypes to know the extent of variability on the basis of some important matric traits under agro-climatic condition is essential to assess the genotype × environmental (G×E) interaction, biotic and abiotic stress response of genotypes. Unlike morpho-physiological characters that are affected by environmental fluctuations, molecular markers are considered stable and not influenced by geographical region or seasonal changes. Microsatellite markers, also known as simple sequence repeats (SSRs), are one of the most powerful genetic marker classes. SSRs are repeated DNA sequences of simple sequence motifs, each motif ranging from one to six nucleotides (Kalia *et al.,* 2011). Microsatellite markers are abundantly present in the genome of eukaryotic organisms, highly polymorphic, co-dominant (Xu and Crouch, 2008; Chen *et al.,* 2009), ubiquitous and owing to some of the spontaneous mutation affecting the number of repeat units. The hyper variability of SSRs among related organisms makes them an informative and excellent choice of markers for a wide range of applications in sugarcane, which include high-density genetic mapping (Chen *et al.,* 2007), molecular tagging of genes (Singh *et al.,* 2008), genotype identification, genetic analysis of diversity (Cordeiro *et al.,* 2003) and paternity determination (Tew 2003; Pan *et al.,* 2010). SSR markers are suitable for sugarcane molecular genotyping (Pan *et al.,* 2003) and genetic diversity estimation (Cordeiro *et al.,* 2001). Several studies have been conducted on sugarcane diversity analysis using SSR markers (Cordeiro *et al.,* 2001, 2002, 2003, 2007; Pan *et al.,* 2003; Chen *et al.,* 2007; Singh *et al.,* 2008; Chen *et al.,* 2009; Glynn *et al.,* 2009; Silva *et al.,* 2012; Hameed *et al.,* 2012; Devarumath *et al.,* 2012) reflecting the importance of SSR marker utility for assessment of its genetic diversity.

Utilization of SSR markers for molecular characterization, diversity assessment and molecular genotyping of sugarcane germplasm has been reported by many authors, Parida *et al*., (2008) revealed high degree of polymorphism in sugarcane species. Pan *et al.,* (2010) create database of molecular identities of sugarcane somaclones with SSR markers that provides molecular description for new cultivar registration. Shahid *et al.,* (2011) utilized SSR markers for the detection of polymorphism in red rot resistant somaclones and 67% variability was recorded among sugarcane somaclones. Perera *et al.,* (2012) characterized and identifies sugarcane genotypes by using SSR markers. Ramu *et al.,* (2013) assess high magnitude of variability in sugarcane by using SSR markers.

In the light of above mention studies an experiment was conducted with the objective to determine the molecular diversity of adopted local and exotic sugarcane genotypes in Pakistan using SSR markers and to select genotypes for future breeding programs.

MATERIALS AND METHODS

Plant Material: Twenty sugarcane cultivars used in this study were collected as setts from the germplasm collection at the Sugarcane Research Institute, AARI Faisalabad, Pakistan. The germplasm collection contained local and exotic (Canal Point, USA, Sao Paulo, Brazil and Barbados, West Indies) material evaluated for improved cultivars to be grown throughout sugarcane growing areas of Punjab, Pakistan. Vegetative sets of these cultivars were sown at Arja, Azad Kashmir, Pakistan under field conditions and leaf samples were collected for DNA isolation from one month old seedlings. Leaf samples were immediately put into isotherm bucket contained ice gel pads and brought to laboratory of Plant Breeding and Molecular Genetics, Faculty of Agriculture Rawalakot where samples were stored at -80°C freezer.

DNA extraction and quantification: DNA was extracted from 0.5 gm fresh young leaves according to the CTAB procedure of Doyle (1991) with modifications. One gram of leaf sample was macerated in a pre-autoclaved mortar and pestle containing liquid nitrogen. Fine powdered leaf tissue was transferred to 50 ml Falcon™ tubes and 5 ml 2X CTAB buffer (CTAB powder 20 gm, 100 mM Tris-HCl pH 8.0, 0.5 mM EDTA pH 8.0, 1.4 M NaCl, PVP 40 SIGMA-ALDRICH™. 10 gm, β-Mercaptoethanol 10 ml, d_3H_2O up to 1000 ml) was added to each tube. The contents were mixed and placed in a 65°C water bath for 30 min with the tubes shaken after 10 min. An equal volume of chloroform: isoamylalcohol (24:1, v/v) was added to each tube and the contents mixed gently by inversion and then incubated at room temperature for 5 min. Tubes were centrifuged at 6000 g (Ahmed *et al*., 2011) for 15 min and the aqueous upper layer supernatant was transferred into fresh tubes. An equal volume of ice chilled iso-propanol was added and the tubes were inverted 4-5 times to mix the contents. Tubes were centrifuged at 6000 g for 10 min to collect the precipitated nucleic acid at the bottom of the tube. The nucleic acid pellet was washed with 70% ethanol and air dried. The nucleic acid was resuspended in 200 µl Milli Q water and transferred to 1.5 ml microfuge tubes and incubated at 37°C for an hour after adding 5 µl RNAse (10mg/mL) Thermo Scientific™

to digest RNA. Quantification of DNA was carried out at Agricultural Biotechnology Research Institute, Faisalabad Pakistan using a Nano Drop® ND-1000 Spectrophotometer. From 200 µl DNA stock, 1 µl was used to measure the concentration at $A_{260\ nm}$ wavelength and 20 ng/µl final concentration of DNA for each sample was made for PCR amplification.

Primer selection: A total of 49 primer pairs were selected for this study. Among them 20 primer pairs were synthesised from already published sugarcane SSR primers (Chen *et al.,* 2009). The remaining SSR primers were designed and developed based on the microsatellite containing sequences of sugarcane by the International Consortium of Sugarcane Biotechnology (ICSB).

PCR amplification: Polymerase Chain Reactions were conducted following a procedure described by reagents manufacturer (Thermo Scientific™) with little modifications. Reaction volume was 20 µl containing reagents (Thermo Scientific™) 10X Taq Buffer 2.0 µl, 25 mM $MgCl_2$, dNTP's mix 2.5mM, 10 mM forward and reverse primers each, Taq DNA polymerase 1 U/µL, 20ng/µl DNA from each genotype and MilliQ H_2O 5.8 µl. The PCR amplification reactions were conducted in a Mini Opticon Real-Time PCR System BIO RAD™ under the programme of 105°C pre heating, 95°C for 5 min initial denaturation, 35 cycle of: 94°C denaturation for 30 secs, annealing ranging 48-68°C depending on primer length for 45 secs, and extension 72°C for 1 min. Final extension at 72°C for 10 min and hold at 4°C.

Electrophoreses and fragment analysis: The PCR products volume 10 µl mixed with 2 µl 6X loading dye (Thermo Scientific™) were analysed by electrophoresis on a 2% (w/v) agarose gel in TBE. Gel images were captured under gel documentation system (UV tech™). SSR fragments were normally in the range of 50 bp to 600 bp, so they did not resolve well on agarose gels. Polyacrylamide gels were used to clearly separate the SSR fragments. The procedure for PAGE was used as described by Anderson *et al.,* (2013) with modifications. Gel composition comprises of 0.5X TBE buffer, 10% APS (Ammonium persulphate), TEMED (Tetramethylethylenediamine Sigma Aldrich®) and PAGE gel solutions (Rotiphorese® Gel 30). The PCR products volume 1 µl diluted in 3 µl 6X loading dye was used to run on gel at 80 volts/cm for two hours. The banding pattern of amplified fragments was compared by running 1 µl of 50bp DNA ladder (Thermo Scientific™). Gel was dipped in the fixative solution (10% ethanol 80 ml, 10% acetic acid 40 ml, d_3H_2O 680 ml) for 15-20 min with gentle shaking then stained in silver nitrate solution ($AgNO_3$ 1.6 gm, d_3H_2O 800 ml) for 10-12 min with gentle shaking at electric shaker. The gel was washed twice with deionised double distilled water (d_3H_2O) and put in the developing solution (NaOH 12gm, formaldehyde 8ml and d_3H_2O 800 ml) till the appearance of bands. Gel images were taken under gel documentation system (UV Tech™) containing NTSYS SPc 2.2 software and save in JPEG mode. Totallab Quant ID gel image processing software (Totallab™) was used for band detection.

Statistical analysis: Polymorphic SSR marker's alleles were scored as a binary data: presence (1) and absence (0) in MS Office 2010® Excel Sheet. Only unambiguous and clearly resolved bands were used in the analysis. The genetic similarity coefficient was estimated by using NTSYS-pc v. 2.1 software (Rohlf *et al.,* 2004). A dendrogram for cluster analysis was constructed on NTSYS-pc v.2.1 software by using Unweighted Pair Group Method with Arithmetic Mean (UPGMA) as described by Sneath and Sokal (1973). To estimate the genetic association among genotypes Principal Coordinate analysis (PCoA) of the SSR data was performed by using the Simpson similarity index with PAST statistical software (Hammer *et al*., 2001). Polymorphic information content (PIC) was calculated by using following formula;

$$PIC=1-(p^2-q^2)$$

Where p is the presence of band frequency and q is the absence of band frequency as described by the Mondal *et al.,* (2009). The mean PIC value for a polymorphic primer was estimated by sum of PIC of all polymorphic bands divided by the number of polymorphic bands. PIC provides an account of the differentiation power of an allele and its value range from 0 to 1 "0" represents monomorphic allele and "1" represents very discriminative alleles (Smith *et al.*,1997). Diversity index (DI) values were calculated by the formula given by (Simpson, 1949);

$$D = \frac{\Sigma n\,(n-1)}{p\,N\,(N-1)} \text{ (Simpson's Index)}$$

1-D = Simpson's index of diversity

Where n represents the frequency of alleles present in genotypes tested and *N* represents the total number of alleles generated by each SSR primer pair. The value of Simpson's diversity index ranges between 0 and 1, the greater the value, the greater the sample diversity. The value of D is then subtracted from 1 to calculate diversity indices of each primer pair.

RESULTS

A total of 20 adopted sugarcane genotypes that are cultivated in most part of sugarcane growing areas of Pakistan were evaluated for selection of some diverse genotypes for future breeding programme using SSR markers. To test the general utility of 49 SSR primer pairs; genetic similarity coefficient, number of alleles, PIC value, polymorphism percentage and diversity index

(DI) were calculated. Cluster analysis following UPGMA was conducted to access the genetic similarity (GS) among genotypes while Principal Coordinate Analysis was conducted to estimate the genetic variation and confirmation of results generated with cluster analysis. Data obtained for each marker and genotype are presented in Table 2, that contained information about number of primer pairs used, their forward and reverse sequences, melting temperature optimized for each primer by doing gradient PCR, sizes of PCR amplified fragments, total number of loci generated by each primer, polymorphic loci, polymorphic information contents of markers and their diversity indices. PCR products generated from 49 SSR primer pairs ranged from 50 bp to 600 bp. In total, 420 SSR alleles were identified, of which 60 were monomorphic while remaining 380 were polymorphic. The total number of alleles generated by any single SSR primer pair ranged from 3 to 22. Among 49 SSR markers used in this study, three SSR markers namely; P-89, P-90 and P-100 showed higher polymorphism by generating more than 15 alleles (Fig. 2abc). Fourteen markers showed moderate polymorphism by producing 10 to 14 alleles. Eighteen SSR markers pairs produced polymorphic alleles between 7 and 9. The remaining 17 markers generated less than 7 alleles and showed lower level of polymorphism. Findings about these markers may be help while selection of SSR markers for future diversity analysis in sugarcane.

The mean diversity index (DI) value of 49 SSR markers ranged from 0.174 (P-114) to 0.728 (P-89). SSR markers with higher DI value lead to lower allelic frequency. Most likely, a marker is more useful to detect polymorphism if it generates large numbers of alleles with high value. Primer P-90 showed higher DI value and 20 polymorphic loci out of 21, followed by P-100 with 15 detectable alleles out of 15, P-137 with 10 polymorphic alleles out of 10 and mSSCIR58 with 11 polymorphic alleles out of 14. These SSR markers generated large numbers of polymorphic alleles.

Polymorphic information content (PIC) value of 49 SSR primer pairs tested ranged from 0.174 (P-114) to 0.728 (P-89) with a mean of 0.34. Only one marker, P-89, showed a greater PIC value (0.728). Nine markers showed PIC values of almost 0.4 and the other markers generated PIC values of 3 or less. The markers that revealed greater PIC values and generated large numbers of polymorphic alleles differentiated a large number of genotypes than SSR markers with lower PIC values but generates fewer polymorphic alleles. Out of 9 markers that showed PIC values of almost 0.4, only four markers viz; P-101, mSSCIR43, mSSCIR66 and SMs009 generated more than 90% polymorphic alleles.

Cluster analysis grouped twenty adopted sugarcane genotypes into four main clusters (I, II, III and IV) at 70% homology level (Fig. 1). Pairwise similarity coefficient values ranged from 64% (S-03-SP-93) to 88% (S-08-FSD-19 and HSF-240). Cluster-I contained nine genotypes. Cluster-I can be further partitioned into three sub-groups i.e., (Ia), (Ib) and (Ic). Sub-cluster (Ia) contained four genotypes viz; HSF-242, SPF-213, CPF-237 and BF-162 at 73% homology. Sub-group (Ib) included three genotypes viz; S-03-US-694 and S-05-FSD-307 at a level of 75% similarity. Sub-cluster (Ic) consisted of only two genotypes at a level of 74% similarity. Cluster-II included genotypes four genotypes namely; S-06-US-272, S-03-US-127, S-06-US-658 and S-03-US-778. Cluster-III contained six genotypes. This cluster can be further grouped into three sub-clusters i.e, (IIIa), (IIIb) and (IIIc). Sub-cluster (IIIa) consisted of only one genotype (S-05-FSD-317) at 70% similarity index. Sub-cluster (IIIb) had two genotypes (SPF-232 and LHO-83153) and they share 75% similarity. Sub-cluster (IIIc) contained three genotypes viz; S-08-FSD-23, S-08-FSD-19 and HSF-240 at almost 78% similarity, while within the same cluster two genotypes S-08-FSD-19 and HSF-240 shared 88% similarity. Cluster-IV contained only a single genotype, S-03-SP-93.

The data generated from 20 sugarcane genotypes on the bases of SSR polymorphic loci was also subjected to Principal Coordinate Analysis (PCoA) for conformation of results generated from cluster analysis (Fig.2). The first four PCoA showed Eigenvalues greater than 0.1 following the Simpson's similarity index. Eigenvalue (> 0.1) is considered as significant. Eigenvalue is a number that indicates how much variance there is in the data, a larger eigenvalue means PCoA explains large amount of variance in the data. First four coordinates; generated total 50.1% variability, to which PCoA-1 and PCoA-2 accented 31% variability while PCoA-3 and PCoA-4 generated 16% variability. Principal coordinate divided the 20 genotypes into 4 groups in a similar pattern as grouped in the cluster analysis diagram (Fig.1).

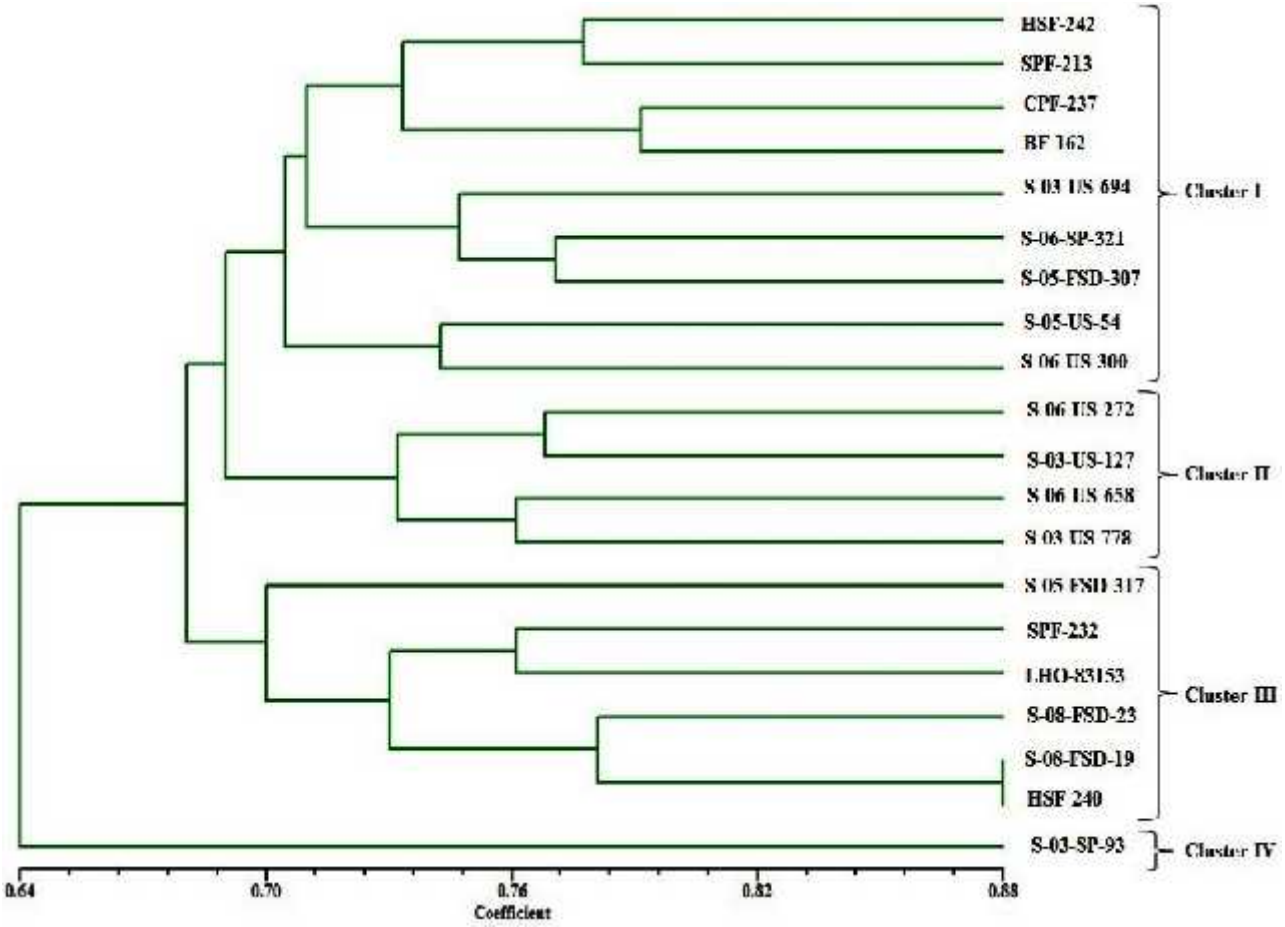

Fig. 1: A hierarchical homology tree constructed by the NTSYSpc (V2.0) software indicating the similarity coefficient (%age) among 20 sugarcane genotypes (*Saccharum officinarum* L.).

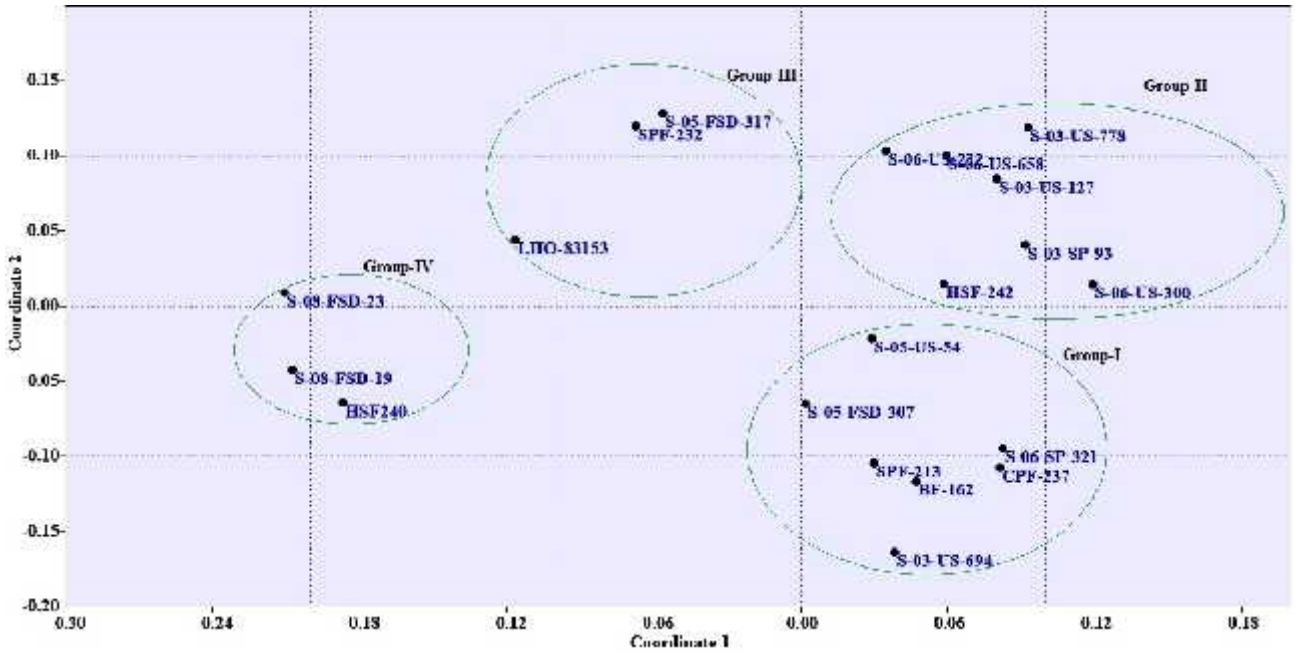

Fig.2: Principal Coordinate Analysis (PCoA) graph of the first two axes, that grouped 20 genotypes into 4 groups almost in a similar pattern as cluster analysis on the bases of linkage distances.

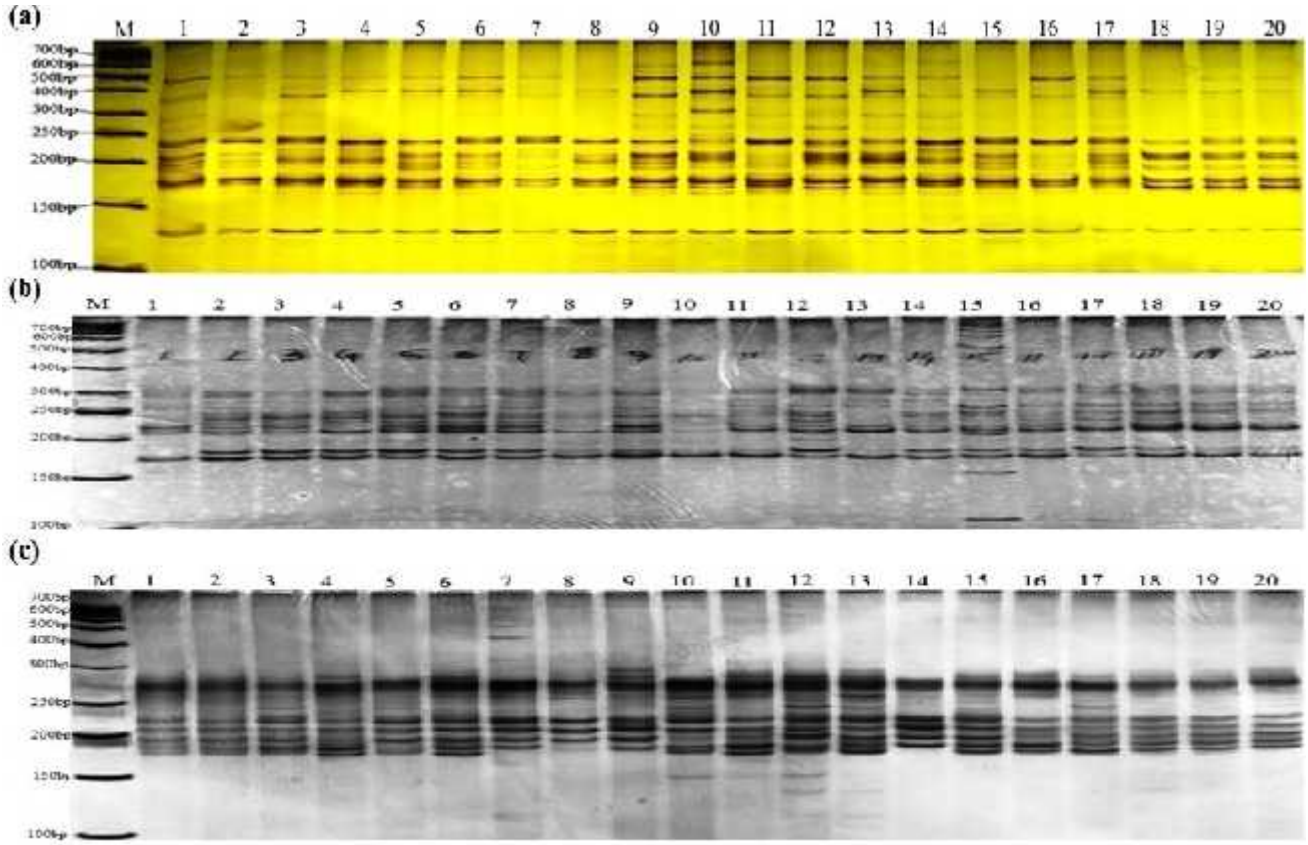

Fig. 2: Molecular profiling of 20 sugarcane genotypes generated with SSR primer P-90 (a), P-89 (b) and P-100 (c). Where 1-20 represents sugarcane genotypes/varieties and their names mentioned in Table 1 respectively.

Table. 1: Sugarcane genotypes used in the study, their names, nativity and parentage.

Sr. No	Genotypes/ varieties	Nativity	Female parent	Pollen parent
			SPHS- 89-2085	Poly cross
2	SPF-213	Sao Paulo, Brazil	SP -70-1006	Unknown
3	CPF-237	Canal Point, America	86P-19	CP 70-1133
4	BF-162	Barbados, West Indies	Co 1001	Unknown
5	S-03-US-694	Canal Point, America	CP87-1628	CP84-1198
6	S-06-SP-321	Sao Paulo, Brazil	Unknown	Unknown
7	S-05-FSD-307	Murree, Pakistan	Unknown	Unknown
8	S-05-US-54	Canal Point, America	CP92-1167	CP93-1634
9	S-06-US-300	Canal Point, America	Not known	Not known
10	S-03-SP-93	Sao Paulo, Brazil	Not known	Not known
11	S-06-US-272	Canal Point, America	Not known	Not known
12	S-03-US-127	Canal Point, America	CP89-879	CP90-956
13	S-06-US-658	Canal Point, America	Not known	Not known
14	S-03-US-778	Canal Point, America	CP -43-33	Unknown
15	S-05-FSD-317	Murree, Pakistan	Not known	Not known
16	SPF-232	Sao Paulo, Brazil	Not known	Not known
17	LHO-83153	Not known	Not known	Not known
18	S-08-FSD-23	Murree, Pakistan	Not known	Not known
19	S-08-FSD-19	Murree, Pakistan	Not known	Not known
20	HSF-240	Sindh, Pakistan	CP -43-33	open pollination

Table. 2: A description of 49 sugarcane microsatellite markers containing primer names, melting temperature, PCR Product range (bp), No. of loci, Polymorphic loci, % polymorphism, Polymorphic information contents (PIC), Diversity Index (DI).

S. No	Primer ID	Forward Primer (5'→3)	Reverse Primer (3'→5)	Tm (°C)	PCR Product range (bp)	No. of loci	Polymorphic loci	Polymorphism (%)	PIC	Diversity Index (DI)
1	**P-86**	CTGTCCATTCCCATCCTC	GCACCGATTCTCTTCTGG	57	200-300	6	5	83.30	0.29	0.79
2	**P-89**	AGAGAGAAAGAGAGGCGG	CTTCACGGAGCGAGAGAC	55	140-600	15	13	86.67	0.728	0.91
3	**P-90**	CTTCCACAACCAGAGCAG	GGAGACAGAGGCGAACAG	55	100-600	22	21	95.45	0.328	0.95
4	**P-92**	CTGGCTCTCCTGGTTTCC	CTGCTGTTGTTCCTGCTC	53	190-400	10	10	100.0	0.289	0.85
5	**P-94**	ATTCTTGTCTATGGCGGG	GCTATCCCTTCACTCTCCTC	53	100-400	5	4	80.00	0.265	0.69
6	**P-99**	GTCTGTCTCCTTCCTGCTC	TGTCTCCCTGCTGTTGTT	54	230-450	4	3	75.00	0.257	0.68
7	**P-100**	AACGCCTCCGACAGTGAG	CCGAGACCAACCAAGCAG	54	50-500	15	15	93.33	0.333	0.91
8	**P-101**	AGGAAATGGATTGCTCGG	CTTGTGGATTGGATTGGAT	53	130-400	11	11	100.0	0.405	0.90
9	**P-105**	TGATACACCATTGTTGATGC	ACACCACTCACATCCACTTG	59	220-500	6	6	100.0	0.201	0.71
10	**P-108**	TGCTTCTAAGTCAACCAAA	TGGTCTACTGAATTCGTG	51	110-250	8	8	100.0	0.241	0.77
11	**P-111**	GCCTTCTTTTGTTTTCCTC	CGTCTCTATGCACCCTATC	53	200-270	7	6	85.71	0.222	0.67
12	**P-114**	CAGGTTGCGTCTTCCACCT	AGCGATGGGTGCTGACAT	54	100-220	9	9	100.0	0.174	0.87
13	**P-126**	CCATAGCAACTACATACAGCATCT	TTACTAAAGGCACAACAAGAAC	57	150-210	7	7	100.0	0.331	0.84
14	**P-127**	CATGCCAACTTCCAATACAGACT	AGTGCCAATCCATCTCACAGA	57	150-300	12	12	100.0	0.31	0.88
15	**P-128**	GGATGAGCTTGATTGCGAATG	CAATTCTGACCGTGCAAAGAT	58	90-160	8	8	100.0	0.405	0.86
16	**P-129**	GCCAGAGAGAGAGAGAGTAGG	ATCGGCTTACATTCAGGTT	58	130-200	8	8	100.0	0.325	0.86
17	**P-132**	GAAATTCCTCCCAGGATTA	CCAACTTGAGAATTGAGATTCG	60	180-200	3	2	66.67	0.37	0.67
18	**P-133**	GTTGTTTATGGAATGGTGAGGA	GCCTTTCTCCAAACCAATTAGT	61	120-200	10	8	80.00	0.402	0.89
19	**P-137**	TGCCAGAAGTGGTTGTCCTCA	TTAAGAGACCCGCCTTTGGAA	61	130-300	14	10	71.43	0.343	0.91
20	**P-139**	CCAATCGTGCCACTGTAGTAAG	ACGCTTGCGTGCTCCATT	59	180-250	6	4	66.67	0.381	0.81
21	**P-141**	CTTCCCTCCCTCTCCTCT	AGCCTTCTAAACTATCTGCT	63	90-160	8	7	87.50	0.416	0.90
22	**P-142**	TAAGAATCGTTCGCTCCAGC	TTACTGGCTGGGTTTTGTTC	60	120-250	11	11	100.0	0.421	0.91
23	**P-143**	AGCTCTATCAGTTGAAACCGA	GCCAAAGCAAGGGTCACTAGA	62	160-210	5	4	80.00	0.364	0.77
24	**SMC851**	CGTGAGCCCACATATCATGC	ACTAAAATGGCAAGGGTGGT	58	80-150	11	10	90.91	0.397	0.88

25	**SMC 18SA**	ATTCGGCTCGACCTCGGGATAT	AGTCGAAAGGTAGCGTGGTGTTAC	62	140-200	6	4	66.67	0.404	0.82
26	**SMC1604SA**	AGGGAAAGGTAGCCTTGG	TTCCAACAGACTTGGGTGG	62	130-200	8	8	100.0	0.343	0.87
27	**SMC7CUQ**	GCCAAAGCAAGGGTCACTAGA	AGCTCTATCAGTTGAAACCGA	60	175-300	6	6	100.0	0.268	0.77
28	**SMC703BS**	GCCTTTCTCCAAACCAATTAGT	GTTGTTATGGAATGGTGAGGA	62	210-250	5	4	80.00	0.344	0.79
29	**SMC24DUQ**	CGCAACGACTTATACACTTCGG	CGACATCACGGAGCAATCAGT	64	120-200	9	8	88.89	0.272	0.87
30	**SMC36BUQ**	GGGTTT CATCTCTAGCCT ACC	TCAGTAGCAGAGTCAGACGCTT	64	110-250	7	6	85.71	0.359	0.80
31	**SMC119CG**	AGCAGCCATTTACCCAGGA	TTCTCTCTAGCCTACCCCAA	58	140-220	9	8	88.89	0.389	0.86
32	**SMC278CS**	TTCTAGTGCCAATCCATCTCAGA	CATGCCAACTTCCAAACAGACT	64	140-250	10	10	100.0	0.362	0.88
33	**SMC334BS**	CAATTCTGACCGTGCAAAGAT	CGATGAGCTTGATTGCGAATG	64	120-250	10	9	90.00	0.234	0.84
34	**SMC569CS**	GCGATGGTTCCTATGCAACTT	TTCGTGGCTGAGATTCACACTA	62	160-200	4	2	50.00	0.375	0.69
35	**SMC597**	GCACACCACTCGAATAACGGAT	AGCTGAATCGTGGTGAACAA	64	150-200	6	6	100.0	0.322	0.79
36	**SMC851MS**	ACTAAAATGGCAAGGGTGGT	CGTGAGCCCACTATCATGC	58	130-220	8	7	87.50	0.346	0.83
37	**SMC1751CL**	GCCATGCCCATGCTAAAGAT	ACGTTGGTCCCGGAACCG	60	130-180	6	6	100.0	0.320	0.84
38	**mSSCIR-3**	ATAGCTCCCACACCAAATGC	GGACTACTCCACAATGATGC	60	170-300	9	9	100.0	0.328	0.85
39	**mSSCIR17**	AGTTCTTTTCGTTCTCTGG	AGCATAGTTTTTGTGGAC	60	230-350	8	7	87.50	0.306	0.83
40	**mSSCIR24**	TTACTCCGCCTCTTTACT	AGATGAACCCAAAAACTTA	59	250-380	6	5	83.33	0.399	0.77
41	**mSSCIR-43**	AACCTAGCAATTTACAAGAG	ATTCAACGATTTTCACGAG	53	120-410	13	13	100.0	0.406	0.92
42	**mSSCIR58**	TGGTCTATCACTTAATCAGCAC	AGGCTACATGCTTACAGCCAT	61	110-250	14	11	78.57	0.343	0.91
43	**mSSCIR-66**	AGGTGATTTAGCAGCATA	CACAAATAAACCCAATGA	58	125-180	6	6	100.0	0.446	0.83
44	**mSSCIR78**	GCAACCGCGTCCTCATAC	CAGGTTCGTCTTCCAGCT	54	100-220	7	6	85.71	0.353	0.83
45	**SMs009**	TCATACAAGCAGCAAGGATAG	GAGCCGCAAGGAAGCGAC	51	100-300	12	9	75.00	0.435	0.91
46	**SMs012**	AAGGAGATGCTGATGGAGA	AAATGTCTTCGCACTAACC	58	150-800	7	6	85.70	0.200	0.60
47	**SMs016**	TCTGTCCTCTGGTAATCCTG	AGCACGGCACGCAATCAC	65	100-175	6	6	100.0	0.322	0.77
49	**SMs037**	AGTTGTAAGTCGTTCTGGTTT		64	200-400	6	6	100.0	0.000	0.90
					Average	8.6	7.8	89.12	0.34	0.83
					Min	3	2	50	0.174	0.60
					Max	22	21	100	0.728	0.95

DISCUSSION

Generally, the SSR markers are considered very useful in the determination of genetic diversity among the varieties and even genotypes because of their codominance. The SSR markers already proven their usefulness in sugarcane were used to evaluate the suitability of these markersto identify the genetically diverse genotypes form the material under investigation.

Polymorphism percentage and SSR loci: A total of 420 SSR alleles were identified with a mean polymorphism of 89.12% estimated for all markers. A range of loci from 50 to 280 were obtained by (Cardeiro *et al.,* 2003; Glynn *et al.,* 2009; Silva *et al.,* 2012; Devarumath *et al.,* 2012; Hameed *et al.,* 2012) by using SSR markers. Our results were in conformation with the overall findings of Cardeiro *et al.,* (2003); Glynn *et al.,* (2009); Silva *et al.,* (2012); Hameed *et al.,* 2012) with respect to average number of alleles generated by individual markers. SSR primer pairs viz; P-89, P-90 and P-100 generated more than 15 polymorphic alleles and found best for genotyping sugarcane germplasm.

Diversity index (DI): Diversity index (DI) values indicate allelic frequency of SSR microsatellite alleles. If the DI value is low, it means polymorphic alleles only exists in fewer genotypes and vice versa. Most probably, a marker is more feasible to identify in a genotype if it generates large numbers of alleles with high diversity index (Chen *et al.,* 2009). Number of alleles and DI value play an important role in the molecular distinctiveness of any genotype. DI values ranging from 0.60 to 0.95 were found in the germplasm of sugarcane under investigation. Eleven primers, namely P-89, P-90, P-100, P-101, P-137, P-141, P-142, mSSCIR43, mSSCIR58, SMc009 and SMs037 revealed DI values more than 0.9 (Table 2). This reflects the general usefulness of 11 SSR markers.

Polymorphic information content (PIC): Polymorphic information content (PIC) is a measure of the relative information content of a marker that indicates whether the marker is useful in determining polymorphism in germplasm (Cordeiro *et al.,* 2003). It generates information about the number of identifiable alleles and the distribution of their occurrence (Ni *et al.,* 2002). PIC measures the extent of a marker system to differentiate among genotypes (Weir 1990). PIC values of SSR markers used in our study ranged from 0.174 to as high as 0.728. The work of several scientists indicated that PIC values varied for SSR markers used in sugarcane (Cordeiro *et al*., 2000 and Singh *et al*., 2008). The differences might be due to the type of germplasm used, small set of genotypes as well as the method of detection. Irrespective of the coincidence, PIC values for any SSR marker may not be stable but give the differences for the capacity to detect variation. Finally, the outcome from this section (Table. 2) is concluded that 420 alleles generated 89.1% polymorphism with average diversity index 0.83 and average polymorphic information contents 0.72. The markers with higher PIC and DI values reflects their discriminatory ability among tested genotypes.

Cluster Analysis and Principal Coordinate Analysis: Cluster analysis grouped 20 genotypes into 4 clusters at 70% homology level (Fig.1). Cardeiro *et al.,* (2003) grouped sugarcane genotypes into two clusters at 37% genetic similarity index by using SSR markers. However, Cardeiro *et al.,* (2003) and Silva *et al.,* (2012) reported high genetic diversity in sugarcane species. Sugarcane share many of the same genomic regions (Cordeiro *et al.,* 2001; Pinto *et al.,* 2006; Glynn *et al.,* 2009; Duarte *et al.,* 2010), which may affect the efficiency of molecular markers to differentiate genotypes. The presence of genetic similarity (GS) or homology in the germplasm means less genetic diversity and vice versa. However, due to the occurrence of very unique patterns of sexual reproduction, sporadic flowering responses in different agro-climatic conditions, self-incompatibility mechanisms, less chance of transgressive segregation, clonal propagation and evolution of *Saccharum officinarum* L. from few common ancestors are the common factor that lead cultivated sugarcane to have less genetic divergence. Cluster-VI contains only single genotype S-03-SP-93 with 64% genetic similarity as compared to the other genotypes tested. This is an outlier with broad genetic background and can be used for hybridization programme while genotypes S-08-FSD-19 and HSF-240 showed 88% homology. Genotypes derived from their respective region grouped in the same clusters due to their common genetic base. In general, it can be suggested here that genotypes grouped in Cluster-I (CPF-237 and BF-162) and Cluster-III (S-05-FSD-317, S-08-FSD-19 and HSF-240) have higher genetic distance and relatively less homology and these genotypes can be used for future hybridization programmes but, this genotype did not flower at our experimental site (Arja, Azad Kashmir, East 73.97°-42 minutes, North 33.97°- 21 minutes, Altitude 797 m above sea level) while genotypes S-08-FSD-19 and HSF-240 showed 88% homology. Genotypes in Cluster-I (CPF-237 and BF-162) did not flower under local natural conditions according to author's personal assessment. Couple of genotypes from Cluster-I (S-05-FSD-307 and S-03-US-694) and Cluster-III (S-05-FSD-317, S-08-FSD-19 and HSF-240) respond flowering. From Cluster-I genotype S-03-US-694 has high genetic distance with S-08-FSD-19 and HSF-240 While S-05-FSD-307 has high genetic distance with S-08-FSD-19 and HSF-240. These genotypes can be successfully exploited for hybridization programme by adjustment of synchronization problems in some genotypes under natural/control conditions.

Principal coordinate analysis (PCoA) divided 20 genotypes into 4 groups almost in a similar pattern as grouped in cluster analysis. Group-I contained 7 genotypes viz; SPF-213, BF-162, S-03-US-694, S-06-SP-321, S-05-FSD-307 and S-05-US-54. Most of these genotypes were the introduction from Sao Paulo Brazil and Canal Point Florida USA, Group-II comprised of 7 genotypes; HSF-242, S-06-US-300, S-03-SP-93, S-06-US-272, S-03-US-127, S-06-US-658 and S-03-US-778. Group-III consist of three genotypes; S-05-FSD-317, SPF-232 and LHO-83153 while Group-IV have three genotypes; S-08-FSD-23, S-08-FSD-19 and HSF-240 and these genotypes were indigenous to Pakistan and made separate group having maximum genetic distance with genotypes like S-03-US-694 andS-05-FSD-307 from Group-I. Although, genotypes from Group-II and Group-I have high genetic distance however, some genotypes from Group-1 did not respond flowering under local conditions. Principal coordinate Analysis validated the results generated from cluster analysis.

It can be concluded that SSR markers are reliable tool for genotyping and diversity analysis in sugarcane. A considerable genetic diversity obtained from material resulted in the identification of our genotypes S-03-US-694, S-05-FSD-307, S-08-FSD-19, S-08-FSD-23 and HSF-240 that may provide valuable material for future hybridization programme and pyramiding beneficial genes in new sugarcane cultivars while retaining genetic diversity.

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