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Evaluation of microsatellite markers for genetic diversity analysis among sugarcane species and commercial hybrids

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Abstract

Currently, the main concern of sugarcane breeding program is to increase the sugar productivity by genetic modification but limited progress has been achieved due to size and complexity of sugarcane genome. Characterization of sugarcane germplasm provides essential information of genetic diversity for breeders to utilize it in crop improvement programs. The objective of this study was to evaluate the utility of 7 sugarcane cDNA derived microsatellite markers (SCM), 9 genomic microsatellites and 16 unigene sugarcane microsatellite markers (UGSM) to access the genetic diversity and inter-relationships among sugarcane germplasm collection. Genetic diversity among 83 genotypes of the *S. barberi*, *S. spontaneum*, *S. officinarum*, Indian and non-Indian commercial cultivars was estimated using SSR markers. The UGSM and SCM primers yielded a higher mean number of alleles per locus and superior polymorphism information content (PIC) values than the sugarcane genomic markers (SOMS). The number of amplified fragments ranged from 4 (UGSM312) to 14 (UGSM667) indicates high level of polyploidy and hetrozygosity in sugarcane genotypes used in the study. The *S. spontaneum* clones shown significantly higher number of amplified DNA fragments (20%) than S. barberi clones (14%). Based on cluster analysis, 83 *Saccharum* species clones and commercial cultivars were grouped into 10 distinct clusters. Each cluster is a groups of individuals having close genetic relationship among the sugarcane species clones, Indian and non-Indian sugarcane commercial cultivars. Genetic relationship has been explained by geographical origin, available pedigree information, adaptation zone and morphological characters. The cultivars B29-228 & *S. spontaneum* clones N-56 and N-75 & Inter-specific hybrids (ISH-112) were found as most diverse pairs but unfortunately these genotypes were not extensively utilize in past breeding programs for creating genetically modified sugarcane cultivars. These sources which have desirable agronomic characteristic and showing diversity should be used as progenitors for development of cultivars with a wider genetic base.

Keywords: SSR, RFLP, RAPD, TRAP, UPGMA, Jaccard's Similarity coefficient; Boot Strap Values

Abbreviations: UGSM- Sugarcane Unigene derived microsatellite markers; SCM- cDNA derived; microsatellite marker; SGM-sugarcane genomic microsatellite markers; ISH-interspecific Hybrids; NICH- non-Indian commercial hybrids.

Introduction

Sugarcane (*Saccharum* spp. hybrids) is a genetically complex crop of major economic importance in tropical and sub tropical countries. It is mainly used for sugar production but recently gained increased attention because of its employment generation potential and recent emphasis on bio-fuels production. Ethanol is an alternate source of energy and its production from sugary or starchy materials also makes sugarcane as a future bio-fuel plant. Considering the current needs of cane industry it is imperative to breed high sugar producing varieties with other desired agronomic traits. Modern sugarcane genome is a complex blend of aneuploidy and polyploidy. It is derived from the interspecific hybridization involving different *Saccharum* species particularly *S. officinarum* and *S. Spontaneum*. The importance of the wild species *S. spontaneum* L. (x=8, 2n=40-128) was realized after its successful hybridization with the domesticated sugar-producing species. It is characterized by low sugar content, thin stalks, high fiber,

high ratooning ability and high resistance to biotic and abiotic stresses. Among the five species of the genus *Saccharum, S. spontaneum* has the widest distribution, extending from Afghanistan in the west to the Malaya peninsula, Taiwan and the South pacific island in the east (Alexander, 1973). Whereas *S. officinarum* (x=10, 2n=8x=80) is best represented by two commercial hybrid varieties Co205 and Co285, which replaced the indigenous cultivated varieties from northern India (Sreenivasan et al., 1987). It represents cultivated sugarcane and is characterized by high sugar content, thick stalks, low fiber and low disease resistance.

Most of the sugarcane varieties in the word are breed of *S. spontaneum and S. officinarum*. To minimize the negative effects of *S. spontaneum* and to retain the high sucrose producing ability of *S. officinarum* during crosses, a series of backcrosses were made between the inter-specific hybrids and the *S. officinarum* parents. This led to the "nobilization" of *Saccharum spp*. hybrids (Sreenivasan et al., 1987). This was a major breakthrough in sugarcane varietal improvement programs in the terms of improved sugar productivity, high disease resistance and high ratooning ability. Although nobilization was highly successful but due to limits of the gene pool exploited during traditional breeding programs, very limited progress has been achieved in increasing sugar content (Sreenivasan et al., 1987; Lima et al*.,* 2002; Pan et al*.,* 2004).

The success of sugarcane breeding program lies in the proper choice of rich and genetically diverse parents. The search of genetically diverse parents can be based on geographical origin, agronomic traits, and pedigree data or molecular markers data (Melchinger, 1999). Evaluation of genetic diversity based on morphological characters is very limited and influenced by the environmental effects, therefore, techniques that measure the genetic relationship without any influence of environmental factors and phenotype properties are the need of breeding programs.

Molecular marker analysis offers an efficient measure of genetic relationships on the basis of genetic characteristics. Among these molecular marker techniques, DNA based markers which include restriction fragment length polymorphism (RFLP), random amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP), microsatellite or simple sequence repeat (SSR) and single nucleotide polymorphism (SNP) are of significance in crop improvement (Ali MA. et al., 2008, Akter J et al., 2008). These markers are being used to increase the understanding of genetic and taxonomic complexity of various agricultural crops. The desirable attributes of these markers encouraged its development (Cordeiro et al*.,* 2000*)* and utilization to achieve desirable agronomic traits in sugarcane (Rossi et al*.,* 2003*;* Aitken et al*.,* 2005). *Saccharum sp.*, which has a complex polyploid genome, requires more number of such informative markers for various applications in genetics and molecular breeding.

Microsatellites or Simple Sequence Repeats (SSRs) are tandem repeats of 1 to 6 nucleotides long DNA motifs, present in eukaryotic genome. Microsatellites has gained considerable importance in molecular breeding due to their desirable genetic attributes like hyper-variability, wide genomic distribution, co-dominant inheritance, reproducibility, multi-allelic nature and chromosome specific location. These markers are suitable for paternity determination, mapping of useful genes, marker-assisted selection and for establishing evolutionary relationship (Parker et al*.,* 2002*;* Aitken et al., 2005). SSR markers have been extensively used in genetic diversity studies in many plants, including sweet sorghum (*Sorghum bicolor L*; Ali ML. et al., 2008), triticale (*X-Triticosecale Wittmack*; Kuleung et al. 2004, wheat (*Triticum aestivum L.*; Fufa et al*.* 2005), and pearl millet (*Pennisetum glaucum L.*; Budak et al*.* 2003).

This work was focused on evaluation of SSR markers, their polymorphic potential and assessment of genetic diversity among 83 sugarcane genotypes. Two hundred and eighty three SSRs primer pairs were evaluated on a group of 14 randomly chosen genotypes. On the basis of their capacity to generate polymorphic bands only 32 pairs of highly polymorphic SSRs was used to estimate the diversity among all the 83 the accessions (Table 2). Out of these 32 microsatellite markers used, 7 were developed from sugarcane cDNA microsatellite markers (SCM), 9 sugarcane genomic microsatellite markers (SGM), and 16 unigene derived microsatellite markers (UGSM). Genetic relationships would be useful in utilization and management of the genotypes during breeding programs. The information obtained from diversity analysis will be utilized in making the crosses and selection of divergent parents to maximize heterosis in future breeding program.

Materials and method

Plant material

Genotypes used in the experiment were grown in the institute's farm. These 83 genotypes include 21 non-Indian commercial hybrid viz., B29-228, B34-104, B49-228, PoJ2818, PoJ2878, PoJ2883, CP33-130, CP33-320, CP349-377, CP36-105,CP44-120, CP5011, Q49, Q68, PR-1048, H6538, H5174, TuC521, HM-223, HP89 and H35-263, three *Saccharum sinense* clone viz., UbaWhite, Malani and Kheli, three *Saccharum barberi* clone, viz., Dhaulu, Khatuya and ManeriaIPM-1552, twenty one *S. spontenium* clone, viz., SES594, SES515/7, Calcutta, Ramsal, Gajraula, BG-10, BG-15, Pusa-2, Pusa-9, Lal-Kuan, Baheri-2, N91, N-129, N-56, N-144, N-290, N-75, N-58, N-87, N176 and WS-18 and five inter specific hybrids viz. ISH-112, ISH-135, ISH-143, ISH-163 and ISH-111 and rest 29 were the commercial cultivars of tropical and subtropical parts of the Indian subcontinents, viz., CoS510, CoS687, CoS767, CoS8432, CoS8436, CoS91269, CoSe92423, CoSe95422, CoS95255, CoS96269, CoS96275, CoSe96436, CoS97261, CoS97264, CoSe98231, CoSe01235, Co213, Co331, Co621, Co111, Co1148, Co1158, Co89003, CoH56, CoLk8102, UP39, BO52, BO128 and BO91 (table 1).

Table 1: The parentage, place of origin and the nature of genotypes used in the study.

Genotype(Origin)	Parent	Genotype (Origin)	Parent	Genotype (Origin)	Parent
***Saccharum Barberi* Jesw 2n= 81-124**		***Saccharum officinarum* L 2n= 80**		**National Commercial Hybrids**	
ManeriaIPM-1552 (India)	Wild	Dr Lal (India)	Wild	CoS97261 (UPCSR, India)	$70A_2GC$
Khatuya(India)	Wild	**Non-Indian Commercial Hybrids**		CoS8436 (UPCSR, India)	MS6847xCo1148
Dhaulu(India)	Wild	CP33-130 (USA)	NICH	CoS97264 (UPCSR, India)	$70A_2GC$
***Saccharum spontaneum L* 2n=42-128**		CP349-377(USA)	NICH	CoS510 (UPCSR, India)	Co453xCo557
Pusa-2 Bihar (India)	Wild	CP33-320(USA)	NICH	CoS95255 (UPCSR, India)	Co1158xCo62198
Pusa-9 Bihar (India)	Wild	CP36-105(USA)	NICH	CoS8432 (UPCSR, India)	MS6847xCo1148
Baheri-2 (UP, India)	Wild	CP44-120(USA)	NICH	CoS96269 (UPCSR, India)	BO108xCo1148
SES594 (UP, India)	Wild	CP5011(USA)	NICH	CoS96275 (UPCSR, India)	CoS8119xCo62198
SES515/7 (UP, India)	Wild	Q49 (Australia)	NICH	CoS91269 (UPCSR, India)	BO91xCo1158
BG-10 (UP, India)	Wild	Q68(Australia)	NICH	CoS767 (UPCSR, India)	Co419xCo313
BG-15 (UP, India)	Wild	H6538 (Hawai)	NICH	CoS687 (UPCSR, India)	Co976xCo312
N-91 (Bihar, India)	Wild	H5174 (Hawai)	NICH	CoSe95422 (UPCSR, India)	BO91xCo453
N-129 (Bihar, India)	Wild	PoJ2818 (Indonesia)	NICH	CoSe01235 (UPCSR, India)	CoS8119xCo62198
N-56 (Bihar, India)	Wild	PoJ2878 Indonesia	NICH	CoSe92423 (UPCSR, India)	BO91xCo453
N-75 (Bihar, India)	Wild	PoJ2883 Indonesia	NICH	CoSe96436 (UPCSR, India)	BO91xCo62198
N-58 (Bihar, India)	Wild	B49-228 (Barbados)	NICH	CoSe98231 (UPCSR, India)	CoS7927xCo775
N-87 (Bihar, India)	Wild	B29-228 (Barbados)	NICH	UP39 (UPCSR, India)	Wild
N-176 (Bihar, India)	Wild	B34-104 (Barbados)	NICH	Co89003(Coimbatore, India)	Co7314xCo775
N-290(Bihar, India)	Wild	TuC521 (Tucuman)	NICH	Co331 (Coimbatore, India)	Co213xCo214
N-144(Bihar, India)	Wild	PR10-48 (China)	NICH	Co1158 (Coimbatore, India)	Co421xCo419
WS-18 (WB, India)	Wild	HM-223 (Hawai)	NICH	Co621 (Coimbatore, India)	Unknown
Calcutta(WB, India)	Wild	HP89 (Hawai)	NICH	Co213 (Coimbatore, India)	PoJ213xCo291
Gajraula (India)	Wild	H35-263 (Hawai)	NICH	Co111 (Coimbatore, India)	Co421xCo312
Lal-kuan (India)	Wild	**Inter Specific Hybrids**		Co1148 (Coimbatore, India)	P4383xCo301
Ramsal (India)	Wild	ISH-112(Coimbatore, India)	ISH	CoH56 (Haryana, India)	Wild
***Saccharum sinense* Roxb; 2n=111-120**		ISH-135 (Coimbatore, India)	ISH	CoLk8102(Lucknow, India)	CO1158GC
Malani (P.R.China)	Wild	ISH-143 (Coimbatore, India)	ISH	BO52 (Bihar, India)	Unknown
Kheli (P.R.China)	Wild	ISH-163(Coimbatore, India)	ISH	BO128 (Bihar, India)	Unknown
Uba white (P.R.China)	Wild	ISH-111(Coimbatore, India)	ISH	BO91 (Bihar, India)	BO55xBO43

NICH; Non-Indian Commercial Hybrids
ISH; Inter Specific Hybrids

DNA extraction

For Genomic DNA extraction, disease free, whorl, young, immature leaves were collected from the institute farm. Sample was freeze-dried and then stored at -86^0 C. Genomic DNA was extracted from young leaf tissues of each genotype using modified CTAB method (Hoisington et al., 1994). 500 mg leaves from different sugarcane genotypes were separately grounded to fine powder in liquid nitrogen using pre-chilled mortar pestle and was transferred to 25ml sterilized tube containing 10ml pre-warmed CTAB buffer (Composition: 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). Sample was incubated in water bath up to one hour at 60^0 C followed by 15 minutes incubation at room temperature. Equal volume of choloroform : Isoamyl alcohol (24:1) was added and the mixture was set to centrifuge at 12,000 rpm for 10 minutes. After centrifugation aqueous phage was pipette out in a 25 ml autoclaved tube.

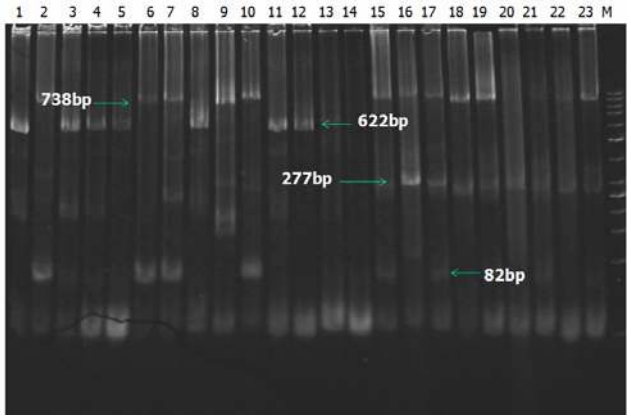

Fig 1. Molecular Diversity of *Saccharum* spp. clones along with non-Indian commercial hybrid showing polymorphism with UGSM-302, Lane 1-N-290, 2-Pusa 9, 3-Baheri, 4- Lalkuan, 5-N-144, 6-BG 15, 7-Pusa 2, 8-N 56, 9-N 129, 10-Gajraula, 11-N 91, 12-Ramsal, 13-BG 10, 14-Calcutta, 15-SES 515/7, 16-H-5174, 17-Q68, 18-SES 594, 19-Uba White, 20-H35-263, 21-CP44-120, 22-Khatuiya, 23- CP 36-105.

Table2. Details of SSR markers with their annealing temperature (Ta), sequences (forward and reverse), product size, range of product size and total number of amplified bands and total number of polymorphic bands given by the primer are shown in small bracket (*Parida et al., 2008*).

	Primer Name	Repeat Motif	Forward Primer (5' → 3') Reverse Primer (3' → 5')	Ta (^{0}C)	Range of Product Size(bp)	No of Bands
1	SCM4	(CGGAT)4	CATTGTTCTGTGCCTGCT CCGTTTCCCTTCCTTCCC	52	134-705	10(10)
2	SCM15	(CAG)5	GGAGATGTTTGAGAGGGAA AGAGTAGCATAAAGGAGGCAG	55	145-624	6(5)
3	SCM16	(TCCG)4	GTGCGAGAGGAACTGTGT AGCCCTGCCTAACAAGGA	50	123-792	11(11)
4	SCM18	(ATAC)3	CATCAGTATCATTTCATCTTGG CAGTCACAGTCGGGTAGA	55	199-694	10(10)
5	SCM21	(GGCC)3	CCCTCCCATAACACACAC TTGACAGCCCAAAGAGTT	50	514-774	8(7)
6	SCM27	(GCAA)4	TTCTCTGACTTCCAATCCAA ATCAAGCACGCCCGCCTC	55	279-714	6(6)
7	SCM32	(TCG)4	GATGAAGCCGACACCGAC AGTTGCCTGTTCCCATTT	50	156-770	11(11)
8	SOMS58	(ACC)7	CCGCTTTCAACCTCTACAC GGCTTGGTGATTCTTCTCT	52	99-1237	8(7)
9	SOMS118	(AT)9cacca...(A)12	GAGGAAGCCAAGAAGGTG TAGAGCGAGGAGCGAAGG	55	82-1018	11(10)
10	SOMS120	(GAG)6ctctga..(GTGA)6(GA)11	GCATCTATCGGTCTTCTGG ATCCAATCCTTCATCTTCTTC	52	84-1155	11(11)
11	SOMS124	(AT)6gatat...(A)87	TAGAGGAAATAGCAGAACAGG AGACTGACACCTTTGAGATGA	52	93-1057	8(8)
12	SOMS135	(TA)6aatat...(T)10	TCTTCAACTTCCTCTGCCT GTTCCTGACTGTTCCCTTG	52	210-929	6(6)
13	SOMS143	(AC)6cttc..(A)17	TGACTTGGAATAACACAAAGAA ATGGGATGGATAATAAGCAGT	55	137-555	8(7)
14	SOMS148	(GAA)5gggca...(GAG)5	GATGACTCCTTGTGGTGG CTTGACGACCCTGCTGCT	52	193-1008	8(7)
15	SOMS154	(AAG)5gaga..(CAG)5	CTCGTTTCATAGCAGACCTT GCAACTGGAGGAACTGATG	52	54-1101	5(5)
16	SOMS156	(CGG)5ctgg..(TTC)5	ATCGTCTCTGGTTGTTGGT ATCCTCCATTTCCACCTC	52	62-593	7(6)
17	UGSM60	(CGA)7	CGACTCCACACTCCACTC CCGAACACCACCTTCTTG	55	92-759	7(6)
18	UGSM193	(GA)8	AGATATAACACACACACACAAA GGCCATCGAGGAGGAGTTCAAG	55	53-787	13(13)
19	UGSM296	(TC)7	ATTATCTACATTCAGACACGTCAC ATCTTTGTTAGCAATCCATTAAG	55	357-1054	5(4)
20	UGSM301	(TC)7	GAAGAAGAAGAAGAAGAAGAAGAA ACTCGTCCTACAACCACGACTAC	55	79-725	8(7)
21	UGSM302	(AT)7	GAAGAAGAAGAAGAAGAAGAAGAA ACTCGTCCTACAACCACGACTAC	50	82-738	8(7)
22	UGSM312	(GA)7	AACGTATCTTTATTTCCATTCTTC CTTTCAGTTCAACTTTGGATAAAT	58	200-583	4(3)
23	UGSM504	(GCT)7	TAGAGGAAATAGCAGAACAGG AGACTGACACCTTTGAGATGA	56	168-224	5(5)
24	UGSM542	(GA)9	ACCTCCACCTCCACCTCAGTTC CGTTCAGCTTCAGGGTGTCGAT	55	53-1123	12(11)
25	UGSM565	(GCA)6	CATAGCAAGCACCACCTC TCTTCTTCTCGTCCACCC	53	348-539	8(8)
26	UGSM574	(ACG)6	GCTTCCTCGCTCCTCCTC TACTTCTACCTCGTCTGCTTC	53	65-1026	13(12)
27	UGSM575	(AGC)6	CTGTTTCCTTCCTTCTCGT CAATCATAGCCCAGACACC	53	66-901	12(11)
28	UGSM585	(CGT)6	GAAGAGGAGGAGAGGAGAAG TGGGATGGTTGTTGACTG	53	62-648	12(11)
29	UGSM665	(CAC)6	GTTACCATCCCATCCCAC TGTCCCTCGTTCACAGAC	53	147-770	10(10)
30	UGSM667	(CTC)6	CTATCCTCTTGTTGGGTCCT TCCGCACCTCCGTTCACC	56	54-1063	14(13)
31	UGSM671	(AGG)5	TCCCTACTTCTATGAATATCCTTC TTGACAAATTGCTTGATGTAGT	55	96-571	6(6)
32	UGSM681	(AC)8	ACACATCGCTTTCCCACA GCATACCTGTCGTCGTCT	55	94-592	10(9)

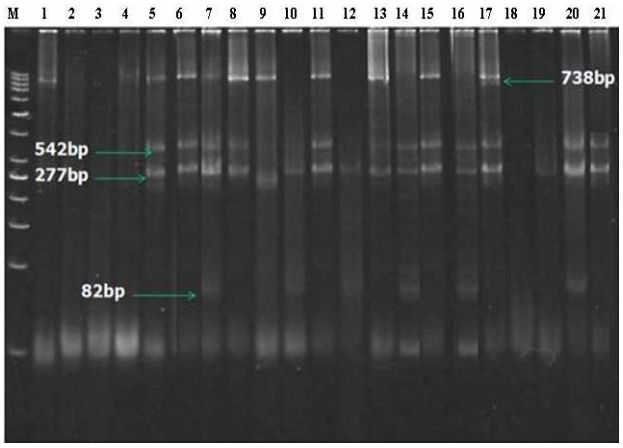

Fig 2. Molecular diversity of elite commercial clone of Indian and non-Indian commercial hybrid showing polymorphism with UGSM 302, Lane 1-CoS97261, 2-CoS8436, 3-CoS97264, 4-Co621, 5-Co89003, 6-CoS95255, 7-Co331, 8-CoS8432, 9-CoS96275, 10-CoS91269, 11-CoS767, 12-Co111, 13-Co1148, 14-CoS687, 15-CoH56, 16-B49-228, 17-CoSe01235, 18-CoSe92423,19- CoSe96436, 20-CoSe98231, 21-B34-104.

Equal volume of chilled iso-propanol was added to aqueous phage to precipitate the DNA. Precipitated threads of DNA were pipette out in a 2ml eppendorf 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 1hour at 30^0C. The quality and quantity of the genomic DNA was checked on 0.8% agarose gel and diluted appropriately for working concentration of 25ng/µl concentration.

PCR amplification and PAGE

A total of 32 SSR primer pairs were used to determine the diversity among sugarcane genotypes and cultivars. SSR primers were developed at National Research Centre on Plant Biotechnology (NRCPB), Indi- an Agricultural Research Institute (IARI), New Delhi, India under our collaborative 'National Network Project on Sugarcane Genome' funded by Department of Biotechnology, Govt. of India (Parida et al., 2008). Primers were synthesized by commercial services provider Bangalore GeNei™ on the demand of Sugarcane Research Institute, Shahjahanpur, India (Table 2). PCR reactions was carried out in a total of 10 µl volume containing 25 ng template DNA, 1.0 µl of each forward and reverse primer, 100 mM of dNTPs, 0.5 U of taq DNA polymerase, 1.0 µl of 10X PCR buffer and 2.5 mM of $MgCl_2$. Amplifications were performed in a Peltier thermal cycler (*MJ Research*) with fallowing parameters: initial denaturation at 94°C for 5 minute followed by 25 cycles amplifications. Each amplification cycle was initially at 94°C for 1 minute followed by annealing temperature (Ta) for 1 minute and then 72°C for 2 minute; final extension at 72°C for 7 minute was allowed. The amplified products were stored at 4°C. The amplified products were separated in 7.5% denaturing polyacrylamide gel electrophoresis in 0.5X TBE buffer. Visualization of bands was done by 0.5µg/ml ethidium bromide staining. Gel photographs were taken under UV light in GelDoc system (*Alpha Innotech*).

Genetic diversity estimation

Bands were scored for the presence (1) or absence (0) in all 83 genotype and this binary data was used to calculate the Jaccard's Similarity coefficient (JS) using implemented module in FreeTree (Hampl V et al., 2001). Genetic distances between each pair of lines were estimated as D = 1 - JS. Clustering is based on a similarity matrix using a heuristic and well resolved algorithm Unweighted Pair Group Method with Arithmetic average (UPGMA), of freeware program FreeTree (Hampl V. et al., 2001). Most universal re-sampling technique bootstrapping was used to estimate confidence levels of inferred relationships. TreeView, drawing software was used for interactive visualization of the dendrogram (Page RDM, 1996).

Results

Protocol for the use of sugarcane microsatellite markers as a potential cost effective method for molecular diversity analysis of sugarcane is primary outcome of the research where as diversity among 83 S*accharum spp.* genotypes and hybrids was estimated by established protocol. After repeating the result several times on PAGE (fig. 1 and fig. 2) only clear and unambiguous bands were considered in scoring. A total of 281 loci were generated, out of which 94.0% were polymorphic with a mean of 8.78 alleles per SSR locus (Table 2). The highest level of polymorphism was detected in UGSM followed by SCM and SGM. Complex banding patterns were encountered in sugarcane with the number of amplified fragments ranging from 4 (UGSM312) to 14 (UGSM667) (Table 2). Fragments size ranged from 62 (SGM156 and UGSM585) to 1237 bp (SGM58) in length.

Analysis of four *Saccharum* spp. and fifty five cultivars including inter-specific hybrids shown high level of genetic diversity in some cases. Estimated genetic similarities (Jaccard's similarity) of elite sugarcane germplasm were visualized in dendrogram (Fig. 3) and radial diagram (Fig. 4). Analysis of the SSR data using Jaccard's similarity coefficient showed that genetic similarity (GS) values ranged from 0.056 (B29-228 and N-75) to 0.778 (Co1158 and Co213) with a mean of 0.34 among sugarcane population, where as a low degree of similarity was also found between ISH-112 & N56 (0.059), ISH-112 & BG-15 (0.060), B29-228 &

Gajraola (0.063). In the dendrogram, B29-228 and N-75 are in different clusters, diverse from each other and easily distinguishable among the genotypes tested (Fig 3, 4)

The cluster analysis of 83 *Saccharum species* clones and commercial cultivars of sugarcane from tropical/subtropical part of India, PR China, Australia, Hawai, Indonesia, Barbados, Argentina and USA into 10 main groups. Different clusters of the tree indicated a clear pattern of division among the sugarcane genotypes. Internodes reveal a general complex structure between cultivars when examined comparatively in terms of pedigree information, geographical origin, adaptation zone and morphological characters.

Seven genotypes were clustered in group I, namely, Malani, CoH56, CoSe01235, CoSe92423, B49-228, Co1148 and CoS687, Most of them share a common genetic background while Malani (*Saccharum sinense*), is unrelated from rest of the entries with regard to the apparent pedigree relationship. Group II included accessions CoS767, CoS8432, CoSe96436, CoSe98231, CoS8436, CoS97264, CoS95255 and CoS96275. Out of these eight cultivars, CoS767, CoS8432, CoSe98231, CoS8436, CoS95255 and CoS96275 have common parent. All of them were high sugar, high yielding varieties which were very popular in subtropical part of India. In group III, out of nine, four genotypes, ISH-111, ISH-135, ISH-163 and ISH-143, developed at Sugarcane Breeding Institute, Coimbatore (TN), India. They clustered together as they shared the common parent in their pedigree. They are the product of second 'nobilization' because they also have genes for red rot resistance and high sugar content. Inter-specific crosses have been predominantly responsible for nobilization in sugarcane. Inclusion of Dr Lal (*S. officinarum*) in this group could not be explained by parentage information. Rest of the genotypes in this cluster has been developed from common *S. officinarum* ancestors.

In group IV out of four, three cultivars, BO128, BO52 and BO91, have common parent and origin. They were developed at Sugarcane Research Institute, Pusa, Bihar, India. In the group V, 4 genotypes were clustered together even they were from different geographical origin. But the reason behind the close similarity in clustering is the common parents. In group VI, seven genotypes were included; out of which Kheli and Khatauya are the basic clones of *S. sinense* and *S. barberi* respectively and closely related to CoLk8102; because Kheli is one of the parents of CoLk8102 (Table 1, Fig. 3, 4). Similarly grouping of two foreign commercial hybrids CP36-105 and CP44-120 together could be explained by their parental relationship.

The group VII clusters included twenty six genotypes of which twenty one were wild S. *spontaneum* clones collected from different states of the Indian subcontinent. Four non-Indian commercial cultivars viz; HP35-263, CCP50-11, Q-68 and H-5174 were from the different biographical origin but they have *S. spontaneum* dominated genetic characteristics. Presence of UbaWhite (*S. sinense*) could not be explained based on their genomic identity. In group VIII, national and foreign commercial hybrids were clustered together due to involvement of common parents during interrogation (Table1, Fig 3, 4). Although some of the commercial cultivars in this cluster are from different geographical origin but due to the use of same *S. spontaneum* clones in their development, this may be reason that they share a close similarity. In group IX only two foreign commercial hybrids HP89 and PR10-48 were clustered together which have common ancestor but different geographical origin. Last group X have only two wild clones, namely, Dhaulu and MineriaIMP-1552, which are showing similarity (0.32) in the dendrogram, because of their origin from same species (*S. barberi*).

These results indicate presence of large amount of genetic diversity among majority of genotypes analyzed. Closely related commercial cultivars from tropical and subtropical Indian subcontinent yield similarities among them and occupy their place close to each other in dendrogram. This behavior explains that recent cultivar has gained a saturation level in the characters and there is a need to identify desired traits for future breeding programs. The genotypes derived from the breeding program in Sugarcane Research Institute, Shahjahanpur (UP), India, exhibit a wide distribution, concentrated in the lower right portion of the tree in Fig. 4. Elite cultivars dispersed in the rest of the tree (Fig. 4), suggest the high ploidy level and the tradition of germplasm exchange among breeders in breeding programs.

Discussion

This is the first report on the use of genomic and cDNA derived microsatellite markers for fingerprinting and evaluation of genetic relationships in germplasm and commercial cultivars used at Sugarcane Research Institute, Shahjahanpur (UP), India. The present study determines the feasibility of the use of sugarcane derived genomic and cDNA microsatellite markers in establishing genetic relationship among the sugarcane species and commercial cultivars. This work supports use of SSR markers, as an excellent tool, for diversity analysis and loci mapping. This genetic relationship among individuals can be potentiality utilized in future sugarcane varietal improvement programs. Our study revealed a large degree of SSR polymorphisms within the genotypes under study; only 6% of the markers were found monomorphic. High levels of polymorphism and hetrozygocity was detected with an average number of 8.78 polymorphic fragments per primer pair. The level of polymorphism indicates that distinction between varieties is possible using appropriate SSR primer pair. Polymorphism had been also reported by Nair et al. (2002) using RAPD markers on twenty eight tropical and subtropical Indian sugarcane cultivars.

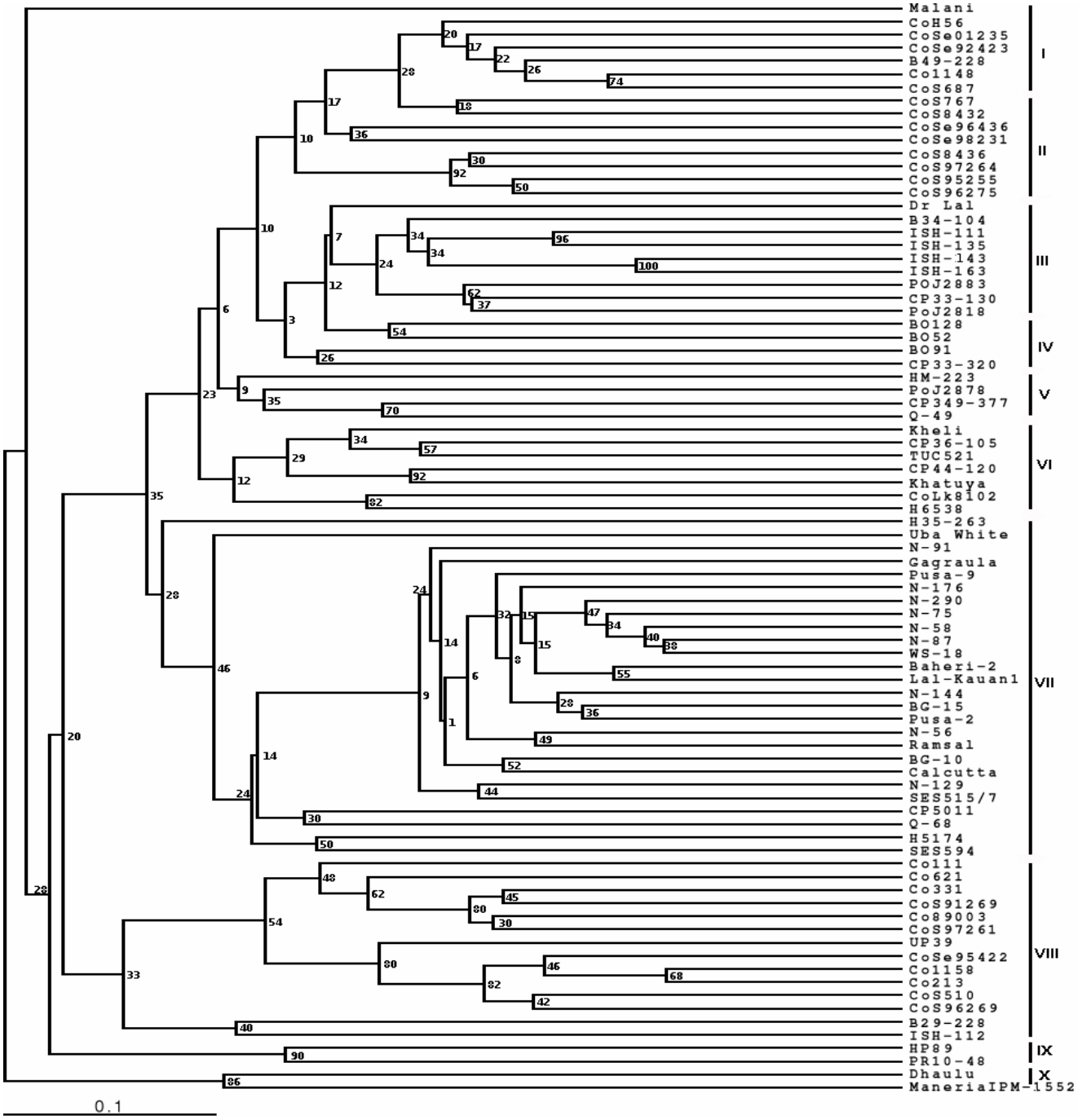

Fig 3. UPGMA tree showing genetic relationships among the sugarcane species clones, commercial cultivars of Indian and non-Indian commercial hybrids based on Jaccard's similarity coefficient. Numbers shown in the internodes of the tree represent percentage confidence level for particular branch, obtained in the bootstrap analysis.

With the objective of evaluating the genetic diversity among sugarcane cultivars, Lima et al. (2002) used 21 AFLP primers combination and evaluated a total 1121 polymorphic loci but they did not found such level of genetic diversity by AFLP. Afghan et al., (2005) used 38 RAPD primers, evaluating a total of 258 discrete markers in 10 sugarcane genotypes where as Ali et al.(2008) used forty one SSR markers that generated 132 alleles to estimate to estimate the genetic diversity among 72 sweet sorghum germplasm but reported SSR markers shown better polymorphism than previously reported. The necessity of a high number of the polymorphic marker for the complete mapping of sugarcane genome can be partially supported by these primers. This large amount of molecular variation obtained through SSR primers will allow a thorough analysis of the organization of quantitative traits in *Saccharum* species and the commercial cultivars studied.

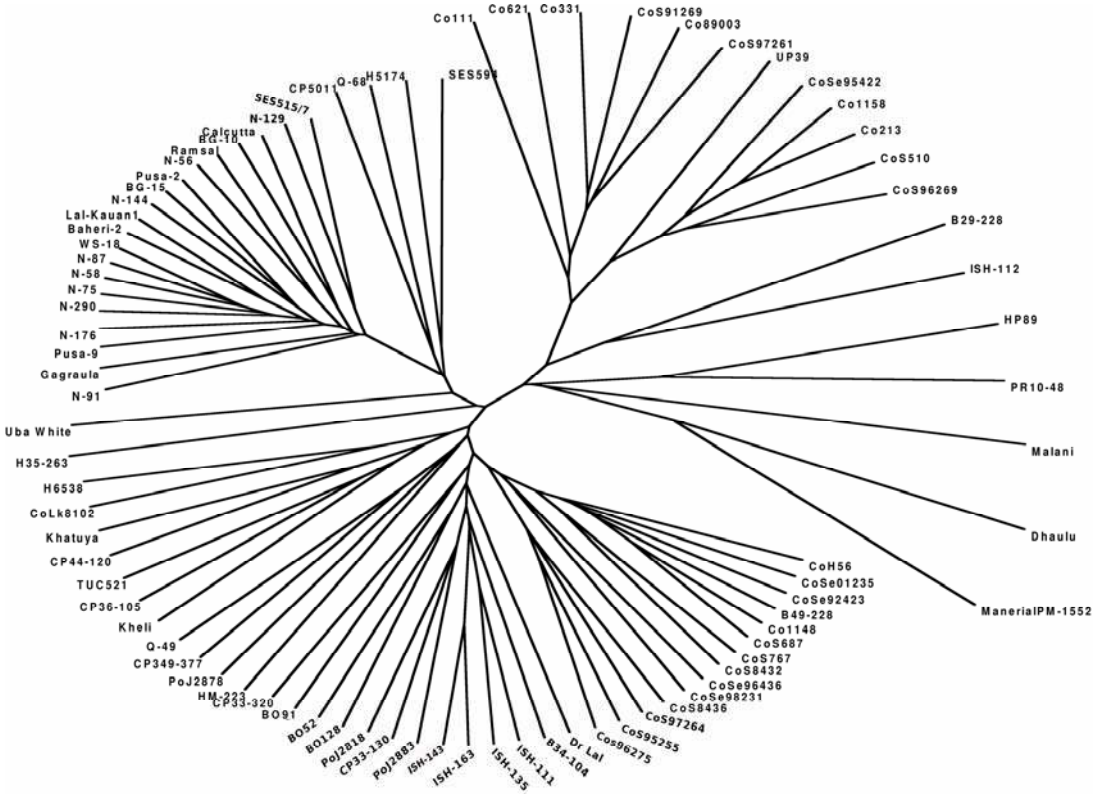

Fig 4. Unrooted UPGMA tree, calculated from SSR data (281 Loci), assembling 83 genotypes which were sugarcane species clones, commercial cultivars of Indian and non-Indian commercial hybrids.

The level of polymorphism detected by UGSM and SCM markers was comparatively higher than the microsatellite markers derived from the genomic sequences (SGM). It could be due the use of highly conserved nature of coding sequences as compared to genomic sequences. More Number of the polymorphic bands was observed in twenty one accessions of *S. spontaneum* which indicates that, it is the most variable and diverse species among the *Saccharum* species and the main structuring part of the variability among the cultivars is due to alleles inherited from *S. spontaneum*. This finding is in agreement with Mary *et al.,* (2006) where authors estimated genetic diversity among *S. spontaneum* taken from different geographical regions. Our results illustrate the large genetic contribution of *S. spontaneum* and indicate that it may be related to the specialization of the cultivars.

Many common bands obtained with inter-specific hybrids and commercial cultivars shows that they share a great fraction of their genome with *S. officinarum*. This was also explained earlier that why these cultivars shared more unique bands with *S. Officinarum.* (Sreenivasan *et al.,* 1987; d'Hont *et al.,* 1996; Suman *et al.,* 2008).The tropical and subtropical region's cultivar did not clustered together in dendrogram, individual clusters include cultivars from both of the places which supports that cultivar in both the region have genetic similarities and transfusion of germplasm has been made frequently. Among the cultivars analyzed, Co331, B29-228, PoJ2878 were found to be distinct and divergent from rest of the cultivars. Study point out few diverse species which can be used as new sources of germplasm in future breeding programs.

Commercial cultivars are showing more similarity with *S. officinarum* clones where as less similarity with *S. spontaneum* which shows that principal component of diversity between cultivars may be due to the genetic influence of *S. spontaneum*. A very weak global topology observed in the dendrogram may be due to profuse exchanges of parental materials between sugarcane breeding stations in the India. Traces of linkage disequilibrium can be attributed to the distribution of *S. spontaneum* chromosomes among sugarcane cultivars (Mary *et al.,*2006).

Genetic similarity coefficient ranging from 60.5 % to 88.5% has earlier been reported by Pan *et al.,* 2004 in a collection of *S. spontaneum* clones using RAPD markers. *S. spontaneum* clones proved their wide distribution because of the presence of morphological as well as cytological (2n = 40-128) variation in their genome. They are divergent among all the genotypes used in the study (Table 1, Fig 2, 3), supporting the earlier reports based on RFLP (Burnquist *et al.,* 1995).

Very low similarity observed between B29-228 and N-75 can be explained by the fact that farmer is an elite foreign commercial hybrid and later belongs to wild *Saccharum spontaneum* collection. This relationship

shows that these genotypes were geographically diverse and evolved independently either by interrogation (ISH-112 and BG29-228) or by their wild nature (wild collections of *S. spontaneum*). Both the genotypes had not been utilized in past breeding programs due to lack of the knowledge but have unique features for the improvement of high fiber, high ratooning ability and high resistance to biotic and abiotic stresses in sugarcane commercial cultivar.

Ten major clusters illustrated in the dendrogram were connected at a similarity level of 0.34 with GS values ranging from 0.056 to 0.778. Low degree of similarity between genotypes from different species and geographical origin like ISH-112 & N-56, ISH-112 & BG-15, B29-228 & Gajraola supports the use cultivars for future breeding programs. The *S. spontaneum* clones clustered in a clearly separate group, which is supported by previous findings of RAPD markers (Nair *et al.,* 1999 and 2002), TRAP markers (Alwala *et al.,* 2006) and SRAP markers (Suman *et al.,* 2008). Dr Lal (*S. officinarum* germplasm), shown great similarity with modern cultivated sugarcane cultivars and inter-specific hybrids. It supports the origin of modern sugarcane cultivars by crossing the *S. officinarum* clones with *S. spontaneum*, followed by a few backcrosses to *S. officinarum*.

Present clustering analysis based on the segregation at SSR loci can resolve the genetic background issues of the cultivars with unknown pedigree. These relationships between *Saccharum* spp. hybrids of unknown genetic origin and cultivars with known parentage will help sugarcane breeders to select appropriate parents in their breeding programs; to maximize yield as well as to maintain genetic diversity. Thus, it can be concluded that estimates of genetic diversity based on molecular markers may provide more accurate information to plant breeders than the pedigree method. It could help breeders in making reliable crossings on a short term basis or to strategically plan the breeding program on a long term basis.

Genetic analysis has been hindered in sugarcane due to lack of sufficiently informative markers. Less information is available about the genetic diversity within and between *Saccharum* cultivars which has been based mainly on morphological characteristic. Thus, it can be concluded that estimates of genetic similarity based on molecular markers may provide more accurate information to plant breeder. This data will support the breeders in exploitation of sugarcane germplasm on molecular basis. SSR markers used in the study may also be used by researcher for genetic mapping and gene tagging in sugarcane. Locus mapping ability of these SSR markers will provide more information and can be effectively utilize for construction of genetic map in sugarcane.

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