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Assessment of genetic diversity among sugarcane cultivars using novel microsatellite markers

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Genetic diversity based on the characterization of genetic makeup, using molecular markers is of utmost importance for breeders in crop improvement programme. A total of 26 microsatellite primers were used to determine the genetic diversity among 40 sugarcane genotypes including their parents. The polymerase chain reaction (PCR) products were examined for both size and polymorphism using these primers. Overall alleles are amplified with an average of 2.3 per locus in this study. Of the total 26 simple sequence repeat (SSR) markers, only 10 (38.4%) displayed polymorphism, with polymorphism index contents (PIC) values ranging from 0.15 to 0.67. The observed homozygosity (Ho) and gene diversity (Nei's) for individual loci varied from 0.0000 to 0.277 and 0.129 to 0.473, respectively. Shannon's informative index (I) was found to be highest (0.661) in SKM04 while the lowest was 0.252 in SKM01 SSR loci with an average of 0.524. Fixation index was also calculated which was in the range of -0.074 to 1.00. A genetic relationship among cultivars and parental genotype was also analyzed by cluster analysis using unweighted pair group method with arithmetic mean (UPGMA), the average-linkage method, with the similarity matrix as input data. The genetic relationship and genetic diversity among the cultivars depicted from this study can be used to select the parents in sugarcane breeding programme.

Key words: Sugarcane, microsatellite, genetic diversity, SSR.

INTRODUCTION

Sugarcane (*Saccharum* spp.) is an important cash crop in the tropical areas that is cultivated for its stalks, which accumulate sucrose. It contributes 60% of the raw sugar produced worldwide, the remaining 40% coming from sugar beet (Grivett and Arruda, 2002). Modern sugarcane varieties that are cultivated for sugar production are complex interspecific hybrids (*Saccharum* spec. hybrid), that have arisen through intensive selective breeding of species within the *Saccharum* complex, primarily involveing crosses between the species *Saccharum officinarum*

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L. and *Saccharum spontaneum* L. (Sajjad and Khan, 2009). Considering the current needs of cane industry, it is imperative to breed high sugar producing and disease resistant varieties with other desirable traits such as high tillering ability and high ratooning capacity. The success of sugarcane breeding program lies in the proper choice of rich and genetically diverse parents. On the flip side, though, genetic base of sugarcane varieties appears to be narrow and at present, this is reflected in slow progress in sugarcane improvement. This could possibly reflect the use of same and over exhausted genotypes as germplasm in the repeated sugarcane breeding programme. Regular selection of genetically diverse parents for crossing programme is therefore a crucial factor to enhance the efficiency of genetic improvement in sugarcane (Kanwar et al., 2009). The genetically diverse parents may be selected on the basis of diverse geographical distribution of the genotypes, information on agronomic characters or diversity analysis through molecular markers (Melchinger, 1998).

The study of genetic diversity is a key for successful breeding programme and it helps in inserting desirable characters into any genotype through crossing of diverse parents. Diversity naturally exists for different characters, to be used in breeding programme and the screening of genotype for these desirable characters is important to develop improved genotypes. Parameters which are considered to be useful for screening the genetic diversity are morphological, physiological and molecular. Although, morphological (agronomic) parameters are simple to use but they are time consuming and expensive and inaccurate, most of these parameters are being affected by the environment. Physiological characters are also influenced by external environment. However, molecular parameters are reliable, fast, and cost effective and are not under the influence of environment. Hence, techniques which measure the genetic diversity without the influence of environmental factors hold the key for successful breeding program. Thus, molecular marker offers an efficient measure of genetic diversity on the basis of genetic characteristics.

The use of molecular markers allows the assessment of genetic diversity at DNA level. Different molecular markers such as restriction fragment length polymorphism (RFLPs), random amplified polymorphic DNAs (RAPDs), sequence tagged sites (STS), amplified fragment length polymorphisms (AFLPs), simple sequence repeats (SSRs) or microsatellites, single nucleotide polymorphisms (SNPs) etc. have been developed and applied to wide range of crop species. These markers widely used for increasing the understanding of genetic and taxonomic complexity of various agricultural crops. The desirable attributes of these markers encouraged their development (Cordeiro et al., 2000) and utilization to achieve important agronomic traits in sugarcane (Rossi et al., 2003; Aitken et al., 2005). Among the PCR-based markers, microsatellites or SSR markers has proved to be the most powerful tool for diversity analysis in molecular breeding due to their abundant genetic distribution, high reproducibility, multi allelic nature co-dominant inheritance and cross transferability to closely related genera (Powell et al., 1996; Gupta and Varshney, 2000; Pan, 2006; Gupta and Prashad, 2009; Kalia et al., 2011; Yu et al., 2011). SSR markers can be developed either from the DNA sequence information available in the databases or by screening of genomic DNA libraries enriched for different repeat motifs including sequence based methods (Gupta and Varshney, 2000; Zane et al., 2002; Jones et al., 2002; Kalia et al., 2011).

In recent, SSR markers have been extensively used in genetic diversity study of many plants like maize (Yao et al., 2008; Selvi et al., 2003) and rice (Kibria et al., 2009), foxtail millet (Gupta et al., 2012) including sugarcane (Pandey et al., 2011). The present investigation aims for genetic diversity analysis of 40 important genotypes of sugarcane and polymorphic information content for 26 newly developed SSR markers for their subsequent use in molecular marker studies.

MATERIALS AND METHODS

Plant material

Sugarcane genotypes used in the present investigation were available in Norman E. Borloug Crop Research Centre, G. B. Pant University of Agriculture and Technology, Pantnagar, India. A total of 40 genotypes were used in the present investigation including 34 F_1 generation genotypes and 6 of their parents genotypes namely BO91, Co 0238, CoPant 99214, CoS 767, Co Pant 90223, CoS 8436.

Development and characterization of simple sequence repeat (SSR)

Genomic DNA of ISH 100 was extracted from fresh leaves, using CTAB method (Hoisington et al., 1994) and send to Genetic Identification Service Inc. (GIS, Chatsworth, CA) for the construction of microsatellite enriched library. Total genomic DNA was digested using *Eco*R1. The recombinant plasmids were produced by ligating restriction fragments from *Saccharum* DNA into the *Hin*d III site of pUC19 plasmid. The fragments were enriched for microsatellite motifs CA, GA, ATG and TAG prior to ligation. Ligated products were introduced into *E. coli* strain DH5α (ElectroMaxJ, Invitrogen) by electroporation. 2 μl of ligation mix was used for each of the libraries. After transformation and recovery incubation in SOC broth (Invitrogen), glycerol was added to a level of 20% of the final volume. Libraries were stored at approximately -70^{O}C. To isolate colonies for sequencing, cells from the glycerol stock were spread on X-gal/IPTG/ampicillin-LB agar plates. Sterilized toothpicks were used to transfer white colonies from the spread stock plates onto a X-gal/IPTG/ampicillin LB plate. The plate was incubated overnight, and colonies were selected from this plate. Plasmid DNA was isolated from the cultures using miniprep spin kit (Qiagen, Germany). Plasmids were sequenced by ABI 377 automated DNA sequencers (Applied Biosystems, Foster city, (CA)). Sequenced data from the clones containing SSRs were analyzed for primer selection and primers were designed from

Table 1. List of sugarcane genotypes were used for genetic diversity analysis.

Genotype code	Name of genotypes
C1,C2,C8-C12, C34	CoS 8436 X CoPant 97222
C3-C7	CoSe 92423 X CoS 767
C13-C16	Co 0238 X CoPant 97222
C17	CoPant 90223 X Bo 91
C18,C19,	CoS 8436 X CoPant 99214
C20-C28,C33	CoSe 92423 X CoS 8436
C29-C32	CoPant 99214 X CoS 8436
C35	Bo 91
C36	Co 0238
C37	CoPant 99214
C38	CoS 767
C39	CoPant 90223
C40	CoS 8436

flanking regions surrounding the SSR motif with Designer PCR ver. 1.03 (Table 2). All the primer pairs were first screened on DNA of sugarcane species and commercial varieties. The polymorphic primer pair with non-specific amplifications and too faint products was discarded using high throughput touchdown and gradient PCR.

DNA extraction and amplification

The genomic DNA was extracted from young leaves of each of the genotypes using modified CTAB method (Hoisington et al. 1994). 500 mg leaves from different sugarcane genotypes were separately grinded to fine powder in liquid nitrogen using pre-chilled mortar pestle and transferred to 25 ml sterilized tube containing 10 ml pre warmed CTAB buffer [2% (w/v) CTAB, 20 mM EDTA, 1.4 M NaCl, 0.5% sodium bisulfite, 100 mM Tris-HCl (pH 8.0) and 0.2% (v/v) β-mercaptoethanol] and purified by RNase treatment. The quantity of isolated DNA was determined spectrophotometrically; visualized on 0.8% agarose gel stained with ethidium bromide and a final concentration of 80 ng was used for PCR. For PCR amplification, 25 µl of reaction mixture containing 80 ng DNA, 1X Taq buffer, 1.5 mM $MgCl_2$, 10 pmoles each of forward and reverse primers, 0.2 mM dNTPs and 1 U of Taq DNA polymerase. A total of 26 SSR primer pairs were used to determine the diversity among sugarcane genotypes used in the present investigation. PCR amplification was carried out using the PCR profile; denaturation at 94°C for 40 s followed by annealing temperature (Ta) for 40 s at range of 54 to 58°C (according to the sequence of the specific primer) and extension at 72°C for 30 s and final extension at 72°C for 7 min in Thermal Cycler (Applied biosystem, USA). The amplified products were electrophoresized in 2.5% agarose gels in 1X TAE buffer and visualized by staining the gels in 0.5 µg/ml ethidium bromide and captured under UV light in gel documentation system (Avegene, Taiwan).

Data analysis and genetic diversity estimation

For determining the genetic relationships among genotype used in the present investigation, the profile of SSR markers were scored on the basis of their band size, either present (1) or absent (0) for each SSR loci. Polymorphic information content (PIC) was used to determine allele diversity at each locus and was calculated according to Roldan-Ruiz et al. (2001) as:

$$PIC_i = 2f_i(1 - f_i)$$

Where, f_i is the frequency of the amplified allele (Band present) and $(1 - f_i)$ is the frequency of null allele (band absent) of marker *i.*

The genetic similarities among the accession were calculated according to Jaccard's coefficient (Jaccard, 1908) using NTSYS-pc software package version 2.10d. A phylogenetic tree was constructed using UPGMA method. The observed heterozygosity (H_0), Nei's average gene diversity (Nei, 1973), fixation index (F_{IS}) and Shannon's informative index (*I*) were calculated using POPGENE 1.32 software (Yeh and Boyle, 1997).

RESULTS

In the present investigation, 26 SSR primers were designed and tested against 40 (6 parental and 34 of F_1 generation) genotypes of sugarcane to determine their genetic diversity (Table 1). PCR amplification with SSR primers revealed that 16 developed markers were monomorphic and 10 were polymorphic. The identified polymorphic SSR markers were further used to estimate the genetic diversity among the genotypes studied

Genetic diversity

The analysis of polymorphic markers (38.5%) in this study was carried out using tools described in material and methods section. A total of 22 alleles were found, averaging 2.3 alleles per locus varying from two (SKM01, SKM02, SKM05, SKM06, SKM07, SKM08, SKM09, SKM10), to three (SKM02, SKM03). Substantial variations in allelic polymorphism were also observed and the size range from 147 to 320 bp (Figure 1, Table 3). The PIC value extended from 0.15 (SKM03) to 0.67 (SKM07) with the mean of 0.34. Generally, PIC values increased proportionally with increasing heterozygosity at a locus.

The observed heterozygosity (Ho) for individual loci varied from 0.0000 to 0.277 with an average of 0.163 per locus. The expected heterozygosities or gene diversity (Nei's) ranged from 0.129 to 0.473 with an average of 0.336 per loci.

Fixation index (F_{IS}), a measure of genetic diversity was calculated, which is ranging from (-) 0.074 to 0.570 with an average of 0.515, positive value of fixation index represent excess of observed homozygotes where as negative value is demonstrating extra heterozygotes. The Shannon's informative index (I) of loci varied from 0.252 to 0.661 with the mean of 0.524 per locus. The data for PIC value, observed heterozygosity (Ho), expected heterozygosities or gene diversity (Nei's), Fixation index (F_{IS}), Shannon's informative index (I) of loci is shown in Table 2.

The genetic distance of genotypes under study on the basis of the current study was constructed according to Nei (1978), and relationships between genotypes were displayed graphically in the form of dendrogram (Figure 2).

Table 2. List of all 26 SSR primers with their Tm and PUID No.

Primer	Forward sequence	Reverse sequence	Tm (^{0}C)	PUID No
SKM01	TATGGAGAGAGCAACCTATCA	GACGGAAGATTGGGATTC	56.9	28374217
SKM02	GGCCTTCGATTAACCGAT	ACAGGACGCTGCTTCTTG	57.7	28374215
SKM03	CCTATCGAATTGTGCTACTC	GCATGTGTATTGTGTTAGAGAA	54.8	28374210
SKM04	TTATTTGTCCAACCTGCTTCTG	CATGGATGCTTTTGCGTTAG	56.9	28374225
SKM05	ACCACCACCACTTTGTCTT	GGATTGCTAAAGCATTGGT	57.3	28374233
SKM06	ACCACCACCACTTTGTCTT	CGTGAGAAGGTAGGGAAACA	56.7	28374240
SKM07	CCAAACCACATTGTAGCAG	CTTCTTGTCATCATCACTTGAG	56.6	28374252
SKM08	TTATCCCTTTCGTTCAGTAGAG	ATTTTGCGTAGGGTCTGAG	57.3	28374222
SKM09	GGTGGCTAACAGACAGGG	TTGCTGCCGAGAGTCATA	56.9	28374220
SKM10	GCGCCTATTTAATACCAGA	CTTTCCCTATACCCATGATAG	56.0	28374247
SKM11	TCAAAGTGGCTACAGAATAGGT	CAGCAAGGTTCCAAGTACC	56.7	28374214
SKM12	AGTTCCCTGTACTTGTCTACCA	TTGGTCGCTTAAAGTCAATC	56.4	28374204
SKM13	GGATACAAAGGAGAGCACAAC	CGAGGAATCAGTTCACCC	57.5	28374209
SKM14	CCCAGTAAGCTGTTGTTGC	TCTGCGATGTAACCCTATTTC	56.0	28374237
SKM15	CACCCAGCAGTTATTGGA	CAGCAATCAAGTGTTCACTG	56.6	28374228
SKM16	AATGGTTCACTGCATGATATG	GGACGACTTAAAGTTCTTGTGA	57.7	28374235
SKM17	GCTTTGAATGCCACACTC	CACCGTGCTAGTGAGGAC	56.1	28374249
SKM18	GAGGGTGTTGGAGACCAT	ATCCCAATTCAATCCGTC	56.2	28374250
SKM19	GGCTTCTTGTTGATAGCAATG	AGAGGGGCAAGTTTGAGAA	56.2	28374251
SKM20	GAGGTGATGAGTCCATACC	CCTTGAATACGGTGGTCT	56.4	28374244
SKM21	TGAGAACTTGATGGAGTATCTC	GAGCACTCACTTGATTAGTAGC	55.3	28374241
SKM22	CCCAAACTAACCCACATG	CACAACCTCTGCAAAGTGT	56.3	28374242
SKM23	ACCGTCATCGTCCACTAC	TGGAAGACCATGAGGATC	56.6	28374243
SKM24	GCTGAGGTGATGATGACA	GGAGAGCACAAAAGATAACTC	56.0	28374245
SKM25	CTTTGGTTTGTTGTAGCATATC	GAGGCCAAGATGACATTC	56.8	28374246
SKM26	AAGGGAAGAGCAGGAGAG	CGGGAGGTCAAAATGTTA	56.8	28374248

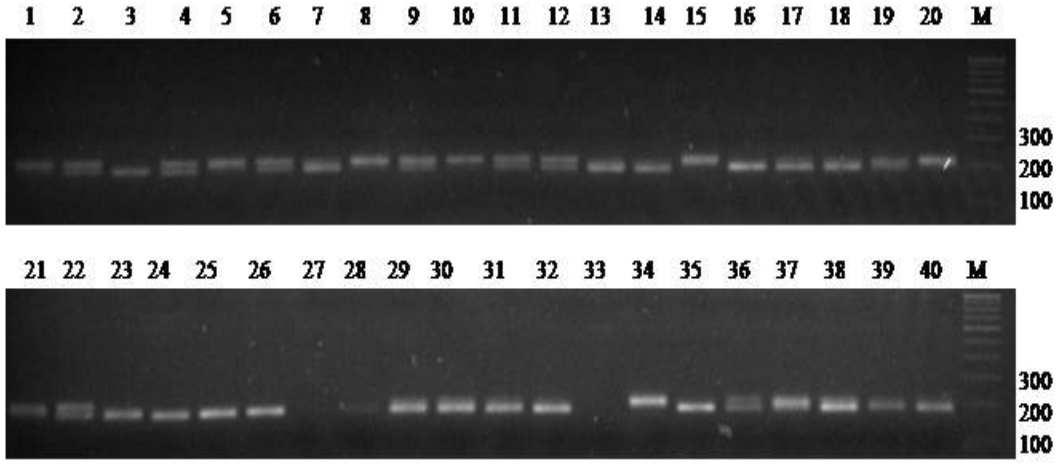

Figure 1. Molecular profiting of 40 genotypes of sugarcane with SSR primer SKM10 as given in table 1 and 3 with 100-bp DNA ladder.

Genetic relationship

Genetic similarity was minimum (0.13) between C1 and C40 genotypes and maximum (1.00) between C20 and C24/C22 and C27 genotypes calculated via genetic similarity matrix. The UPGMA clustering method based on Nei's (1978), unbiased genetic distance classified all the sugarcane accessions into two major groups (I and II)

Table 3. Summary of genetic diversity of 40 sugarcane genotypes using 10 microsatellite markers.

Marker	SSR motif	N_A	Allele size range (bp)	H_o	Nei	F_{Is}	I	PIC
SKM01	$(CA)_{25}$	2	210-243	0.138	0.129	-0.074	0.252	0.19
SKM02	$(GA)_{17}$	3	150-256	0.277	0.387	0.282	0.649	0.26
SKM03	$(CA)_{16}$	3	147-320	0.128	0.210	0.391	0.436	0.15
SKM04	$(CT)_{22}$	2	149-200	0.250	0.468	0.466	0.661	0.43
SKM05	$(CT)_{25}$	2	287-300	0.000	0.438	1.000	0.630	0.44
SKM06	$(CAT)_{13}$	2	200-220	0.000	0.142	1.000	0.271	0.15
SKM07	$(AGA)_7$	3	150-180	0.256	0.473	0.458	0.660	0.67
SKM08	$(CT)_{26}$	2	296-320	0.250	0.334	0.327	0.517	0.29
SKM09	$(CT)_{16}$	2	180-200	0.171	0.382	0.551	0.570	0.22
SKM10	$(CAT)_{11}$	2	200-220	0.840	0.399	0.539	0.589	0.40
Average		2.3		0.163	0.336	0.515	0.524	0.34
Std. Dev.				0.099	0.129		0.155	

Number of allele (N_A); Observed Heterozygosity (Ho); Nei's average gene diversity (Nei); fixation index (Fis); Shannon's informative index (I); polymorphic information content (PIC).

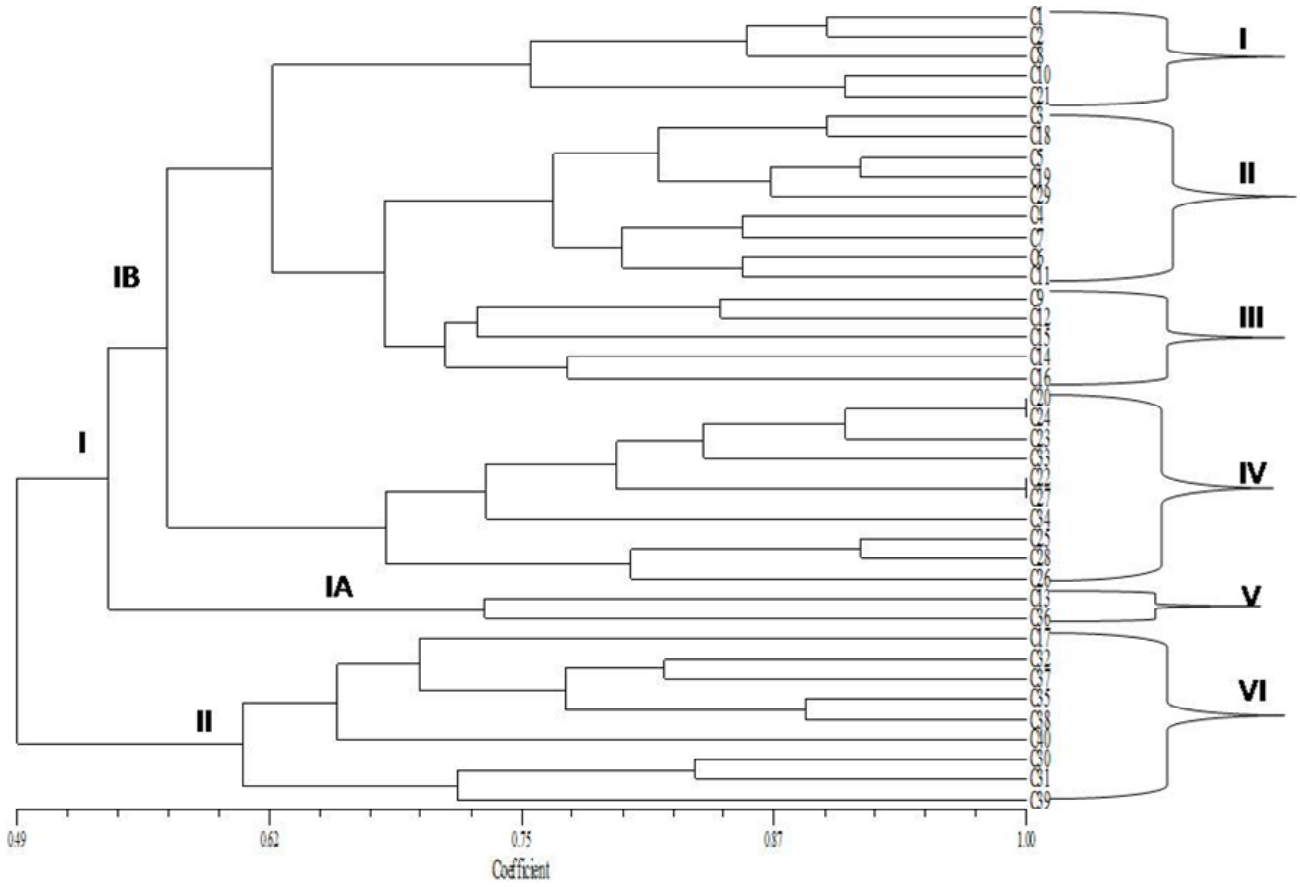

Figure 2. Dendrogram showing the relationship among 40 sugarcane genotype based on 10 SSR primer pairs.

comprising six clusters (Figure 2). The first major group consisted of two subgroup IA and IB with 31 genotypes. The subgroup IA was further subdivided into five clusters, first comprising five genotypes (C1, C2, C8, C10 and C21) of common origin with the only exception being C21. The Second cluster comprised of nine genotypes

(C3, C18, C5, C19, C29, C4, C7, C6 and C11), third cluster consisted of five genotypes (C9, C12, C15, C14 and C16), fourth cluster included maximum number of genotypes (C20, C24, C23, C33, C22, C27, C34, C25, C28, and C26) and the fifth cluster represented only two genotypes (C13 and C36), respectively. The clusters II and III included in Subgroup IB exhibited maximum similarity of 69% and showed 63% similarity with cluster I of same subgroup. Clusters I, II and III showed 55 and 50% similarity with clusters IV and V of subgroup IA, respectively. The group II included a single cluster with remaining 9 genotypes (C17, C32, C37, C35, C38, C40, C30, C31 and C39). Out of nine genotypes included in this group, five were parental genotypes. The single cluster included in this group exhibited minimum similarity of 49% with the clusters included in group I.

DISCUSSION

The identification of varieties based on molecular markers may be important to establish distinctness, uniformity and stability of protected cultivars (Swapna et al., 2010). Among the various molecular markers, SSR markers have evolved as a boon to breeders as they have revolutionized the crop breeding, by their higher efficiency and uses in assessment of genetic variability, characterization of germplasm, estimation of genetic distance between population, inbreeds and breeding material, detection of monogenic and quantitative trait (Tommasini et al., 2002; Hoxha et al., 2004; Gupta et al., 2012; Yepuri, 2013). Microsatellite variability is widely used to infer levels of genetic diversity in natural population; also its mutation rate is very high.

In the present study, SSR markers were developed from microsatellite library derived from DNA of inter specific hybrid (ISH 100). These genomic SSR markers were further used to study the genetic diversity and genetic relationship among 40 genotypes of sugarcane including their parents. Ten (10) out of 26 SSR primers were found polymorphic when screened. These polymorphic SSR primers were used for genetic analysis of sugarcane cultivars. The highest PIC value obtained was 0.67 for SKM07 and lowest as 0.15 for SKM06. Microsatellite markers have revealed high PIC values in other studies of sugarcane (Cordeiro et al., 2003; Pinto et al., 2006; Oliveira et al., 2009; Chen et al., 2009) which suggests the suitability of SSR markers for the diversity analysis. In present study, we have obtained a broad range of PIC values, indicative of the presence of unique alleles in some accession which facilitates their differentiation from another. The average number of alleles obtained per locus was 2.3, which closely resembles the previous reports from foxtail millet (Gupta et al. 2012) in which the number of alleles per locus was 2.2, sorghum (Brown et al. 1996) the number of alleles per locus was 2.3, for ground nut it was 2.3 (Gautami et al. 2009). Genetic diversity is commonly measured by genetic distance or genetic similarity, both of which imply that there are either differences or similarities at the genetic level (Weir, 1989). Selection of appropriate genetic distance involved in any study requires extremely careful consideration of the evolutionary history of the populations involved and the specific goals of the study. The gene diversity is a measure of genetic distance ranged from 0.129 to 0.473 in the present investigation with an average of 0.336 which indicates significant distance amongst the alleles using SSR markers, which is supported by previous reports on various molecular markers from sugarcane (Cordeiro et al., 2003; Pinto et al., 2004; Pan, 2006; Zhang et al., 2008; Chen et al., 2009; Pan, 2010).

Genetic similarity coefficient was carefully observed for deducing relationships between the studied varieties. The similarity coefficient 1.0 was observed in C20 and C24, C22 and C27 having same parents. The similarity coefficient 0.90 was observed between C1 and C8, C23 and C24, C20 and C23 genotypes exhibiting their similar lineages, while C1 and C40 genotype exhibit the lowest similarity 0.13 which reflects differences in lineage involved. The molecular results obtained in present study are represented and which agree with the possible evolutionary course of sugarcane genotypes. The UPGMA cluster analysis of 40 genotypes in the present study produced meaningful grouping based on pedigree or geographical origin of the accessions. The grouping pattern of 40 genotypes are based on their genetic similarity pattern which showed that sugarcane genotypes from same geographical regions tend to cluster together which may be the result of similar evolutionary relationship. The five out of six parental genotype, C35, C37, C38, C39 and C40 were clustered together representing their possible convergent evolution. Genotypes lying on similar clan were representatives of northwest origin (in Indian context) while one parental genotype, C36 which formed cluster in another group represented another region (Southern) origin. The grouping according to the place of origin is supported by previous findings (Zhang et al., 2004; Chen et al., 2009; Singh et al., 2010). Sugarcane cultivars are aneupolyploid hybrids that have the most complex genomes of any crop plant with chromosome number in excess of 100 (D'Hont et al., 1996). Interestingly, clones derived from the parental genotypes represented distant relation with their parents, may be due to the complex genetic makeup of sugarcane genome. The genotypes of same parental origin tend to remain closer as depicted from the dendrogram. The similarity Jaccard's coefficient values among 40 genotypes ranged from 0.13 to 1.00 with the highest value of 1.00 exhibited between genotypes C20 and C24, C22 and C27. The lowest similarity coefficient 0.13 was depicted in between genotype C1 and C40, showing their distant homology.

Thus in the present study, SSR markers was found to be the useful tool for genetic diversity analysis in sugar-

cane genotypes. These SSR markers can further be utilized to facilitate marker assisted selection as well as genetic analysis of other sugarcane cultivars and wild grasses related to sugarcane. The genetic relationship among various genotypes depicted from this study can be used to select the better genotypes as germplasm in breeding programmes.

Conflict of Interests

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

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