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RESEARCH ARTICLE

Development of Microsatellite (SSRs) markers and Evaluation of Genetic variability within Sugarcane commercial varieties (*Saccharum* spp. hybrids)

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Abstract

Sugarcane microsatellites markers were developed from ESTs and utilized for evaluation of genetic diversity of elite sub-tropical Indian commercial cultivars. Publically available EST database (dbEST/GenBank) offers a valuable resource for developing EST-SSRs markers. ESTs sequences, related to sucrose metabolism and yield traits were harvested from NCBI database and used to microsatellites (SSRs) identification by online SSR search tools. Among the identified SSRs types, tri-nucleotide repeats were frequently abundant (41.0%) followed by tetra-nucleotide repeats (22.4%), di-nucleotide repeats (19.1%), penta-nucleotide repeats (12.0%) and hexa-tri-nucleotide repeats (5.5%). Among 48 primer pairs, 29 (60.0%) were found to be polymorphic, 11(23.0%) monomorphic and 8 (17.0%) un-amplified at any temperature (Ta). Polymorphic markers were used to test the genetic diversity within a panel of 26 Indian sub-tropical sugarcane elite varieties. Most of the EST-SSRs markers were found to be informative and effective to revealing genetic variability within the sugarcane varieties of diverse pedigree. Thus, the developed EST-SSRs markers would be used as potential molecular tools in genetic mapping, phylogenetic analysis and in marker assisted selection (MAS) strategies in sugarcane.

INTRODUCTION

Sugarcane (*Saccharum* spp. hybrids) is an important agricultural commodity, provides the global (>70%) sugar and >30% ethanol production in tropical and subtropical countries (Singh *et al*., 2011). It belongs to the genus *Saccharum* of the family *Poaceae* in the tribe *Andropogoneae* composed of hybrids derived from *Saccharum officinarum* (Noble clones; 2n=80), and *S. spontaneum* (Roach, 1972). It is a genetically complex crop showing unstable and irregular chromosomal behavior (Singh *et al*., 2011). The complex genetic makeup established by high poly-aneuploidy, variable number of chromosomes, irregular meiotic division, factors contributing to making sugarcane a genetically complex and challenging crop for genetic studies (Grivet *et al*., 2002). Low fertility, variable environmental interactions and complex genetic background have hampered sugarcane varietal advancement and genetic evaluations (Singh *et al*., 2013a). Several molecular marker systems have been developed and used in genetic studies and taxonomic complexity of sugarcane (Parida *et al*., 2010).

Microsatellite or simple sequence repeats (SSRs) markers have several desirable genetic attributes like; high frequency, multi-allelic nature, hyper variability, co-dominant inheritance, high reproducibility and chromosome specific location etc. (Parida *et al*., 2009; Singh *et al*., 2011). These important characteristics makes SSRs as

markers of choice for the estimation of genetic variability (Singh *et al*., 2013b), DNA fingerprinting (Pan *et al*., 2010), linkage map construction (Singh *et al*., 2013a), population and conservation genetics studies (Powell *et al* 1995) etc. The publically available EST gene pool at National Centre for Biotechnology Information (NCBI) databse offers a valuable and cost effective means to develop SSRs makers which termed as EST-SSRs (Parida *et al*., 2010). EST-SSRs markers reveal genetic variability in expressed region of the plant genome and shows functional genetic variability within the different plant genomes (Oliveira *et al*., 2009). These markers have preferred owing to their cost effective development, represent real-function genes, putative function can be assigned and these show considerable crosstransferability across species and related genera (Varshney *et al*., 2005).

Initially, Cordeiro *et al*. (2001) was developed microsatellite (SSRs) markers for sugarcane from genomic library constructed at home. Later on Parida *et al*. (2009) have developed SSR markers from the unigene sequences. Further, SSR markers first time were developed from the EST sequences of *Saccharum* species (Pinto *et al*., 2004; Oliveira *et al*., 2009). In sugarcane, the level of genetic variability has been determined by exploiting various molecular marker techniques viz; restriction fragment length polymorphism (RFLP; Besse *et al*., 1997), randomly amplified polymorphic DNA (RAPD; Selvi *et al*., 2008), amplified fragment length polymorphism (AFLP; Selvi *et al*., 2006) and sugarcane microsatellites (SSR; Cordeiro *et al*. 2000; Singh *et al*., 2011). Microsatellite based genetic evaluation have been accomplished in many cereal crops (Castillo *et al*., 2008) legume plants (Sethy *et al*., 2006), oil seed plants (Luu *et al*., 2008), vegetable crops as well as in tree genomes (Gasic *et al*., 2009) and have proved as a strong molecular tool for genetic analysis. The present study was carried out to mine the available EST sequences of sugarcane for the identification of microsatellites (SSRs) and to examine genetic diversity among Indian sugarcane varieties.

Materials and methods

Plant material

In present investigation two commercial sugarcane varieties were selected first one Co 86011, (bearing the genes for earliness and high sugar content with high yield) and second UP9530 (has the genes for wide range of tolerance to stress with low sugar content) for polymorphic survey of microsatellite markers. Twenty six sub-tropical Indian sugarcane varieties (cultivating in whole north Indian sub-tropical zone) were used to evaluate the genetic variabilty with potential SSRs markers derived from the functional genomic sequences (Table1).

Table1. Description about twenty six Indian commercial varieties with their pedigree information used for genetic analysis by using EST-SSR markers

Sl. No.	Variety name	Pedigree/Parentage	Sl. No.	Variety name	Pedigree/Parentage
1.	CoS 08279	CoLK8102×Co89003	14.	CoSe 01424**	BO91×Co453
2	CoSe 92423**	BO91×Co453	15.	CoSe 01235**	CoS8119×Co62198
3.	CoS 95255*	Co1148×Co62198	16.	CoS 8436*	MS 68/47×Co1148
4.	CoS 96269*	BO108×Co1148	17.	CoSe 03234	BO91 PCGC
5.	CoS 96275**	CoS8119×Co62198	18.	CoS 03251*	Co1158×C062198
6.	CoSe 95422**	BO91×Co453	19.	CoS 08272	CoSe92423 GC
7.	CoS 98231*	CoS7927×Co775	20.	CoS 88230	Co1148×Co775
8.	CoS 98259*	BO108×Co1158	21.	CoS 767	Co419×Co313
9.	CoSe 01434	Co88039×Co775	22.	CoS 99259	CoS0767CG
10.	CoS 8432*	MS68/47×Co1148	23.	CoS 07250*	CoS8436×Co775
11.	CoS 96436	BO19×CoC62158	24.	UP 05125	GRL28×CoSe 92423
12.	UP 0097	Se1444/91×Se1854/91	25.	UP 9530	Se1084/86×Se122/85
13.	CoS 96268*	Co1158×Co62198	26.	CoJ 64	Co976×Co617

Note; **Cultivars generated from common male & female parentage and * cultivars having only single parentage common.

Identification of microsatellite (SSRs) in ESTs database and primer development

Expressed sequence tags (EST) were retrieved from NCBI (National Center for Biotechnology Information; http://www.ncbi.nlm.nih.gov) database and mined for the presence of simple sequence repeats (SSRs) stretches of 2-6 nucleotides using online search tool batchprimer3 (http:// probes.pw.usda.gov.batchprimer3/). The criteria for the SSRs search were repeat stretches having a minimum of 6 repeat units for di-nucleotide (DNRs), 5 for tri-nucleotide

(TNRs), 4 units for tetra-nucleotide (TtNRs), and a minimum 3 repeat units for penta-nucleotide (PNRs) and hexa-nucleotide repeats (HNRs). The threshold criterions for primer design were considered of 20-26 oligo-nucleotide length, melting temperature (Tm) of 50-60 ^{0}C and GC content were set on 45-65%. Primer pairs (forward and reverse) were designed in a batch module manner complementary of the flanking regions of the SSRs repeat sequences (Cordeiro *et al.*, 2001; Parida *et al*., 2009).

Functional characterization

To take information about the functionality of the identified SSRs markers putative function were identified by basic local alignment search tool (BLASTn) analysis at the National Centre for Biotechnology Information (NCBI), with the threshold of 10 for the expect value (*E*-value). Gene ontology was recorded for the SSRs-ESTs in terms of the cellular process, molecular functions and cellular components categories. All the putative functions of EST-SSR markers are explained in figure2.

DNA extraction and PCR amplification

Extraction of DNA from newly growing immature fresh leaves of all accessions was carried out using CTAB method (Delloporta *et al.*1983) with minor modifications (Singh *et al.*, 2011). Bulk segregation analysis for EST-SSRs primers polymorphism testing has accomplished with 48 primer pairs. All the information about the primer sequence, annealing temperature (Ta), PIC value and polymorphic status is given in Table2. PCR amplification of microsatellite regions were carried out in 10 µl volume containing 10-25 ng of template DNA, 1.0 pmol of each forward and reverse primers, 10 mM of dNTPs, 0.5U/µl of Taq DNA polymerase (M/S Bangalore Genei ™, India), 1.0 µl of 10× PCR buffer with 2.5 mM of $MgCl_2$. The PCR reactions were performed in a thermal cycler (MJ Research) as follows thermal profile: 94^0 C for 5 min, followed by 36 cycles of 94^{0}C for 1 min, appropriate annealing temperature (50-60 ^{0}C) for 1 min, extension step at 72^0 C for 2 min and a final extension step at 72^0 C for 7 min.

Table 2: PCR amplified details about the Ta, number of bands, polymorphism and PIC values of the ES-SSR markers in sugarcane

Sl. No.	Primer Id	Annealing Temp. Ta (^{0}C)	Total band	Polymorphic band	Polymorphic Status	PIC Value
1	SMS1	50	4	3	P	0.77
2	SMS2	53	3	-	M	-
3	SMS3	52	4	3	P	0.77
4	SMS4	50	7	4	P	0.25
5	SMS5	50	2	-	M	-
6	SMS6	50	2	-	M	-
7	SMS7	50	-	-	F	-
8	SMS8	52	3	-	M	
9	SMS9	52	9	7	P	0.72
10	SMS10	55	2	2	P	1.00
11	SMS11	52	9	5	P	0.20
12	SMS12	52	-	-	F	-
13	SMS13	55	2	-	M	-
14	SMS14	55	2	-	M	-
15	SMS15	53	12	4	P	0.34
16	SMS16	53	-	-	F	-
17	SMS17	53	6	1	P	0.17
18	SMS18	53	6	3	P	0.50
19	SMS19	53	-	-	F	-
20	SMS20	53	2	-	M	-
21	SMS21	53	2	-	M	-
22	SMS22	53	-	-	F	-
23.	SMS23	53	6	3	P	0.50
24.	SMS24	53	8	4	P	0.50
25	SMS25	52	6	3	P	0.50

26	SMS26	52	2	-	M	-
27	SMS27	52	15	7	P	0.47
28	SMS28	52	5	3	P	0.60
29	SMS29	53	7	6	P	0.86
30	SMS30	53	13	9	P	0.70
31	SMS31	53	14	9	P	0.67
32	SMS32	53	3	-	M	-
33	SMS33	53	5	3	P	0.60
34	SMS34	53	7	6	P	0.86
35	SMS35	53	2	-	M	-
36	SMS36	53	-	-	F	-
37	SMS37	50	8	2	P	0.25
38	SMS38	53	-	-	F	-
39	SMS39	50	2	1	P	0.50
40	SMS40	55	1	-	M	-
41	SMS41	50	7	5	P	0.60
42	SMS42	50	5	1	P	0.20
43	SMS43	50	1	1	P	1.00
44	SMS44	50	2	-	M	-
45	SMS45	53	18	10	P	0.56
46	SMS46	53	1	-	M	1.00
47	SMS47	53	1	-	M	-
48	SMS48	53	5	3	P	0.60
		Average	**5.3**	**4.1**		**0.58**

Agarose gel electrophoresis

Total 48 EST-SSR primer pairs were used to bulk segregation analysis for polymorphism survey in high sugar (HR brix%) and low sugar lines derived from cross (UP 9530 × Co 86011). All the details about the primer's amplification pattern are given in Table2. The amplified products were resolved on 7.5% natural polyacrylamide gel electrophoresis in 0.5× TBE buffer and run for 3 to 4 hrs at 120 volts. Amplified bands were visualized after staining with 0.5µg/ml ethidium bromide fluorescent dye. Gel photographs were captured by UV light in GelDoc system (Alpha Innotech).

Data analysis

Bands were scored for the presence (1) or absence (0) of each single band in a binary data matrix. This binary data was used to calculate the Jaccard's Similarity coefficient (JS) by using module of the software FreeTree (Hampl *et al.*, 2001). Genetic distance was calculated for each pair of accessions as D = 1 - JS. Clustering was based on a similarity matrix using a heuristic and well resolved algorithm un-weighted pair group method with arithmetic average (UPGMA), with freeware program FreeTree (Hampl *et al.*, 2001). Most universal re-sampling technique bootstrapping was carried out to estimate confidence levels of inferred relationships. Subsamples, consisting of different number of polymorphic bands, were generated by re-sampling 1000 times, with replacement, to estimate GS between every two pairs of genotypes for each subsample. Tree View, drawing software was used for interactive visualization of the dendrogram (Page RDM, 1996). The Polymorphism Information Content (PIC) values were computed using the formula of Milbourne *et al.*, (1997) as follow.

$$\left[PIC = 1 - \sum_{J=1}^{N} P_{IJ}^{2} \right]$$

Where *Pij* is the frequency of the *j*th allele for marker *i* and summation extends over *n* alleles. Results of prescreening were used to classify primer pairs as polymorphic, monomorphic or not amplified.

Results and Discussion

EST mining for simple sequence repeats (SSRs) markers

Only 4.20% ESTs sequences were found to be containing microsatellite repeats (SSRs) with on average 1 SSRs per 23.80 ESTs. Among identified SSRs, tri-nucleotide repeats the most abundant (41.0%) type of SSRs class,

followed by 22.4% tetra-nucleotide repeats, 19.1% di-nucleotide repeats, 12.0% penta-nucleotide repeats and only 5.5% hexa- nucleotide repeats were identified (Fig.1). Simple sequence repeats (SSRs) markers were developed from the genomic sequences of sugarcane and more or less similar results were reported in a earlier study (Cordeiro *et al*., 2001; Oliveira *et al*., 2009). It is noteworthy that the results of the SSRs identification and characterization are depends upon the search criterion of the parameters and microsatellite search software tools (Parida *et al*., 2010; Singh *et al*., 2013c).

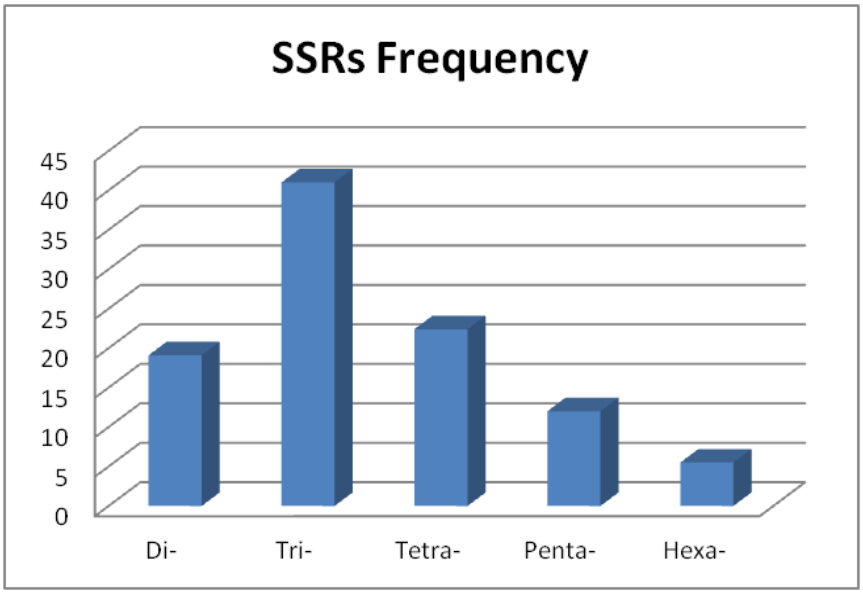

Fig.-1. Frequency distribution of the various SSRs motifs indentified in *Saccharum* ESTs.

Eighty four SSR primer pairs from sucrose metabolism related SSR containing ESTs (10, sucrose phosphate synthase III; 10 sucrose phosphate synthase II; 10 sucrose synthase; 6 Starch synthase; 6 pyruvate orthophosphate dikinase; 6 soluble acid invertase; 36 other enzymes) were designed and tested *In silico* for secondary structure formation (primer dimer and hairpins). Our results are with in accordance of the previous findings of SSRs development from EST database (Parida *et al*., 2006; Singh *et al*., 2013c). Total 48 EST-SSRs primers were used in the present study to analyze genetic diversity among the sugarcane varieties.

Functional characterization of SSRs markers

The identified simple sequence repeats (SSRs) were analyzed for their functional role in sugarcane physiology by using BlastX search. They belongs to the various sugar metabolic pathways and stress physiology related enzymes equivalents like sucrose synthase, sucrose phosphate synthase II (SPSII), pyruvate orthophosphate dikinase (PODK), transcription factors, superoxide dismutase (Mn-SOD), cytochrome b/f complexes and GTP binding proteins etc. (Fig.2). Many similar or different molecular functions of the SSRs containing ESTs and genes equivalents have been characterized by earlier microsatellite studies in cereals (Castilo *et al*., 2008) and sugarcane (Parida *et al*., 2009).

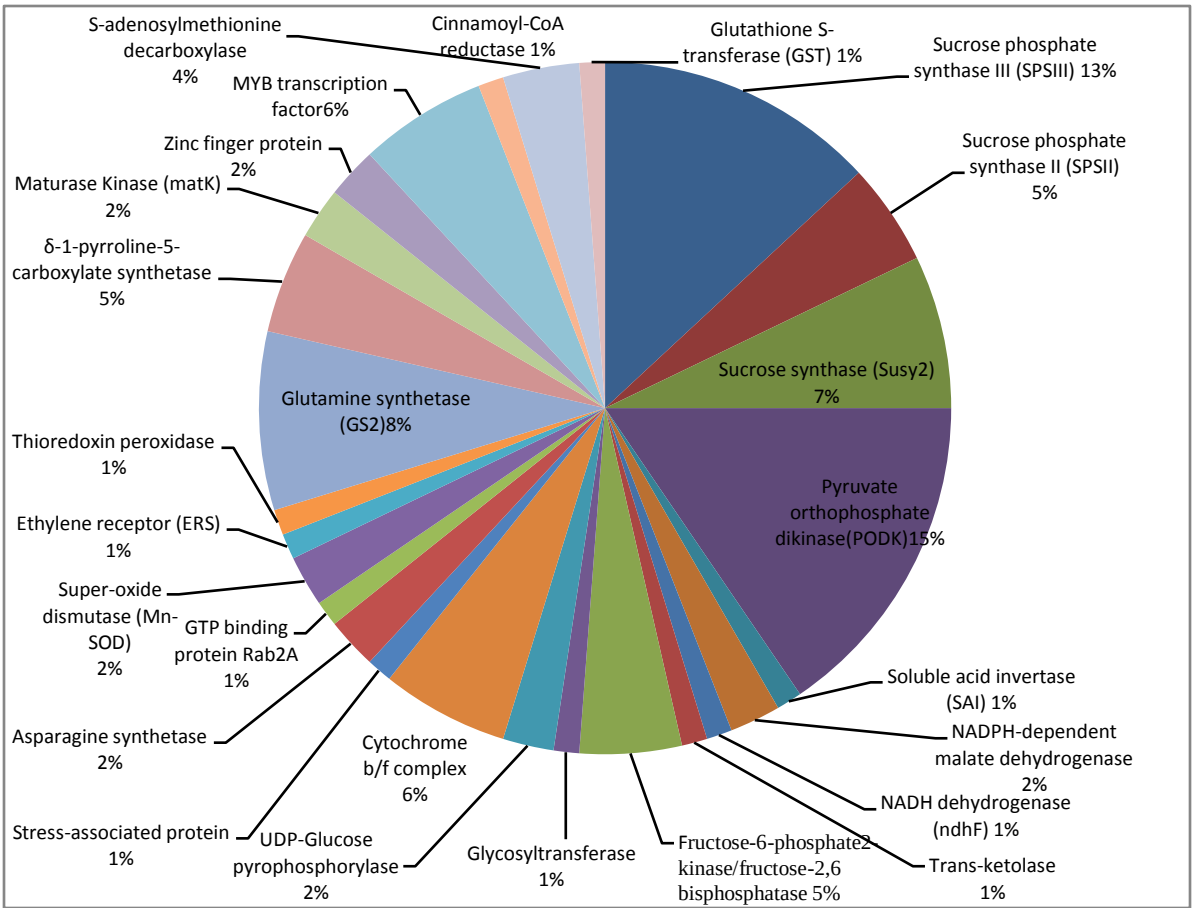

Fig.-2. Functional characterization of identified EST-SSRs markers on the basis of significant matched sequences by BlastX analysis.

Polymorphism of EST-SSRs markers in sugarcane

A profuse and distinctive PCR amplified DNA profile was generated by the EST-SSRs markers in all the commercial varieties of sugarcane, indicating a potential, polymorphic and robust marker system (Fig.3). On an average 5.3 DNA bands were produced by ESR-SSRs primer pairs at their respective annealing temperature (Ta). These developed microsatellite (SSRs) markers were found to be functional and predictable molecular (DNA) tools to evaluate the genetic variability, genome mapping and marker assisted selection of desirable agronomic traits in sugarcane. Similar SSRs markers based DNA profiling in sugarcane genotypes was reported in earlier studies (Selvi et al., 2003; Oliveira et al., 2009).

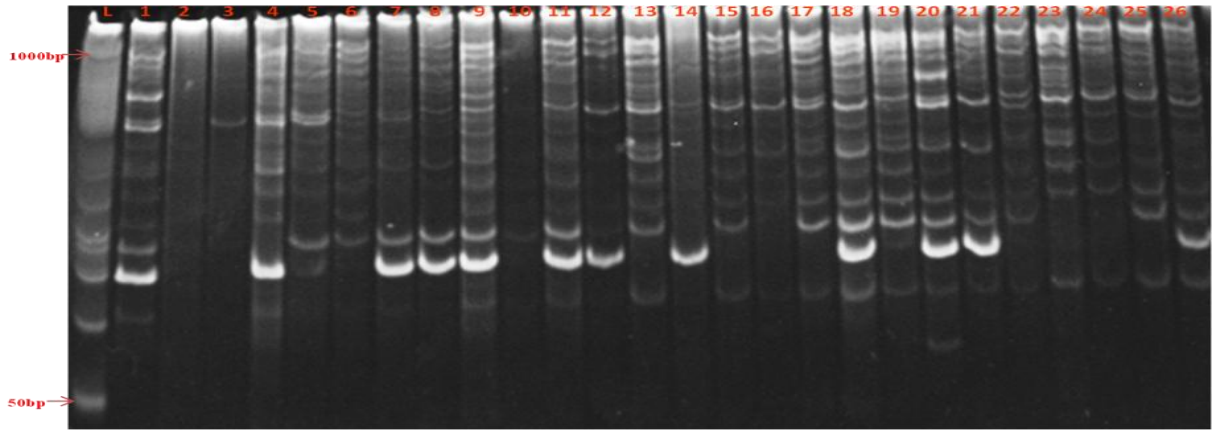

Fig.-3. PCR amplified DNA fingerprints of 26 commercial varieties generated by SSRs markers. Lane L; 50bp ladder, 1-CoS 08279, 2-CoSe 92423, 3-CoS 95255, 4-CoS 96269, 5-CoS 96275, 6-CoSe 95422, 7-CoS 98231, 8-CoS 98259, 9-CoSe 01434, 10-CoS 8432, 11-CoSe 96436, 12-UP 0097, 13-CoS 96268, 14-CoSe 01424, 15-CoSe 01235, 16-CoS 8436, 17-CoSe 03234, 18 CoS 03251, 19-UP 05125, 20-CoS 08272, 21-CoS 88230, 22-CoS 767, 23-CoS 99259, 24-CoS 07250, 25-UP 9530, 26-CoJ 64

Polymorphic Information Content (PIC) and Evaluation of Genetic diversity

The PIC is the measure of the potential of a molecular marker to discriminate between the two or more closely related species/genotypes. The range of PIC values was found to broad from 0.20 to 1.00, indicating a good discriminating power of the developed EST-SSRs markers. A variable range of PIC values were reported in many earlier SSRs based genetic studies in sugarcane and related crops (Cordeiro *et al*., 2001; Singh *at al*., 2011). The developed EST-SSR markers were tested in analysis of genetic diversity between sugarcane elite varieties of diverse pedigree derived from diverse progenitors (Table2). The used varieties are most popular in sub-tropical parts of the India. It can be attributed that EST-SSRs markers reveals the genetic variations in coding region of the genome and revealed a broad range of pair-wise genetic similarity among twenty six sugarcane accessions.

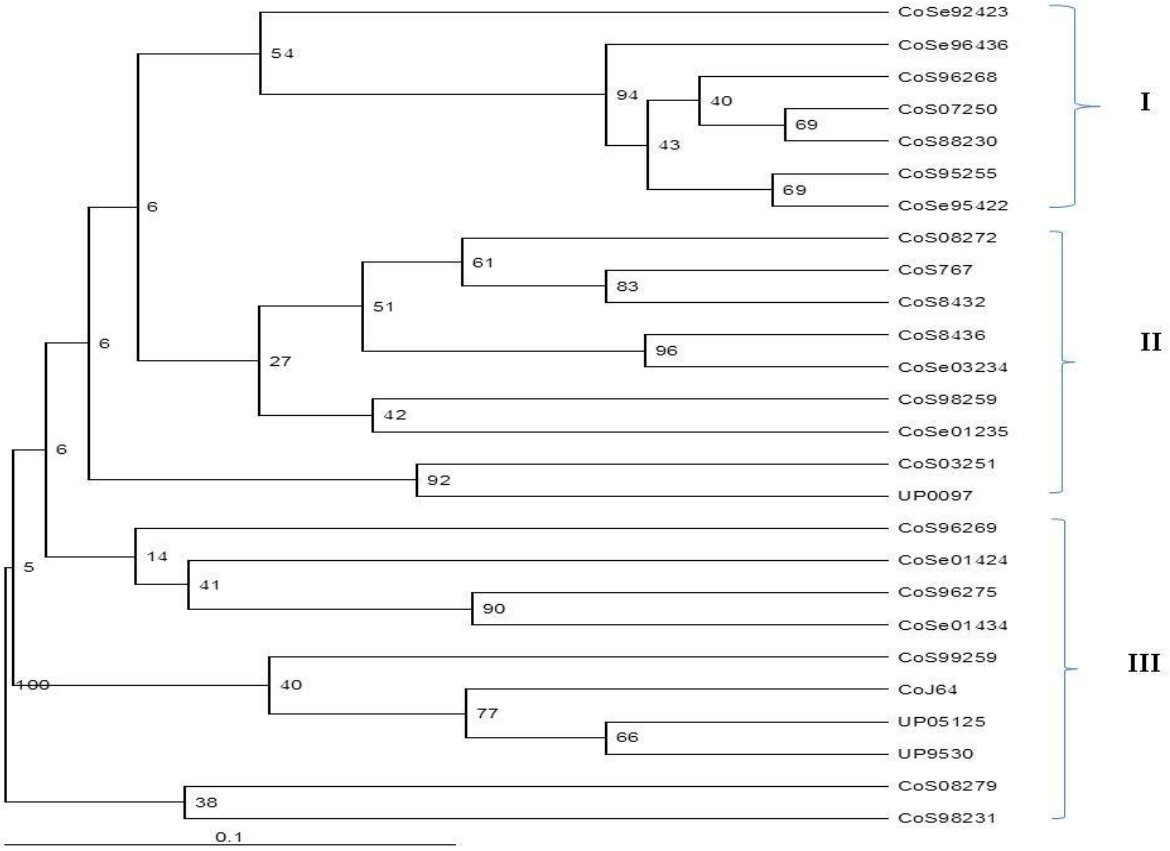

Fig.-4. Dendrogram showing genetic relationships among 26 sub-tropical sugarcane Indian varieties constructed by UPGMA clustering algorithm based on the Jacard's genetic similarity matrix with 48 SSR markers. The bootstrap values are indicated at the nodes in each cluster.

To analyze the genetic diversity a dendrogram was constructed using UPGMA method by Tree View software tool. In dendrogram analysis all the 26 sugarcane varieties were grouped in three distinct clusters I, II and III (Fig.4). The genetic similarity among the different groups of the dendrogram was found to be on the pedigree records of the varieties. The genetic similarity was ranged from 47.0% to 91.0% among commercial varieties of sugarcane. The broad range of genetic similarity was observed within the commercial varieties probably due to the polyploidy and heterozygous nature (Singh *et al*., 2013b). The extent of genetic diversity within the hybrid varieties was found to be at lower level. The all the varieties were developed by more or less similar breeding programmes and similar

parentage and hence showed very low genetic diversity. However, the assessed genetic diversity within the panel of 26 sugarcane commercial varieties was not in accordance with pedigree records, even though the study is informative.

Conclusion

The SSR markers were developed from the publically available ESTs sequences at NCBI website. These cost effective EST-SSRs markers were used to test genetic diversity analysis within the sugarcane varieties and found to be potential and informative in sugarcane. Thus the developed robust molecular markers will enriched the repertoire of sugarcane markers that could be effectively used in various genetic analysis of sugarcane and related cereal crops.

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