Electronic Journal of Plant Breeding, 3(1): 621-628 (Mar 2012)
ISSN 0975-928X

Research Article

Genetic profiling of sugarcane genotypes using Inter Simple Sequence Repeat (ISSR) markers

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(Received: 01 Nov 2011; Accepted: 22 Feb 2012)

Abstract:
Study of genetic diversity is an important factor in sugarcane breeding programs to identify genetically diverse parents for developing hybrid cultivars with improved cane and sugar yield. Genetic profiling studies of 17 sugarcane genotypes were carried out by using 27 ISSR markers having di, tri and tetra nucleotide repeat motifs. Out of the 252 amplicons amplified by 27 ISSR primers, 212 were polymorphic (84.13%) with an average of 9.3 alleles per locus. The polymorphic information content (PIC) value ranged from 0.19 (UBC 808, 843) to 0.36 (UBC876) primers with poly 'AG', 'CT' motifs and poly '(GATA) (GACA)' motifs respectively with an average of 0.27. The primer UBC 825 had a highest resolving power value 9.8 among the 27 primers. Genetic similarity indices by Jaccard's similarity coefficient ranged from 0.26 to 0.72 indicating a high level of genetic diversity and significant correlation found with biometrical data of sugarcane genotype. Cluster analysis by UPGMA method revealed similarity coefficient of 0.49 which was mainly attributed to inter specific diversity. Thus, ISSR markers can be used as a potential marker system for genetic profiling of sugarcane genotypes.

Key words: Sugarcane, ISSR marker, Polymorphic Information Content, Resolving power, Genetic diversity

Introduction

Sugarcane is one of the major source of sugar, plays a vital role in the economy of many countries through the sugars and ethanol production. Sugarcane (*Saccharum spp.* hybrid) is a C4 grass belongs to genus *Saccharum* of the family *Poaceae* with complex polyploidy genome. Present day modern cultivars are inter specific hybrids having genome 2n=100-300 and are believed to have originated from complex hybridization event (nobelization) estimated about 70-80% are from *S. officinarum* L. (2n=80), 10-20% of *S. spontaneum* L. (2n=40-128) (Sreenivasan et al, 1987; D'Hont *et al*., 1996). Several other species including *S. robustum*, *S. barberi* Jesw. and *S. Sinense* Roxb. have been involved to lesser extent in development of modern sugarcane varieties (Irvine, 1999). Sugarcane has characteristic ability to accumulate high sucrose concentration of approximately 650 mM in storage parenchyma, such high concentration and of usable compound encourage to do study on sugarcane genome for identification of diverse parents and genes associated with the sucrose accumulation in sink tissue of sugarcane. Currently, plant scientists working in crop improvement program encountered slow research progress due to its very narrow genetic base (Jackson 2005). Molecular marker technology has great potential to contribute to the genetic improvement of sugarcane by identifying novel genetic variation, improving the heritability related to the sucrose contributing trait (McIntyre *et al*., 2001).

Genetic profiling by using DNA-based molecular marker technique is an important tool to study genetic diversity in sugarcane breeding programs to identify genetically diverse parents for developing hybrid cultivars with improved cane and sugar yields. DNA-based molecular markers help to dissect the genome because of its specificity, robustness, required less DNA and it does not affected by environmental conditions during developmental stages of plant. There are numerous PCR based molecular marker techniques such as RAPD (Random amplified polymorphic DNA) (Kawar *et al*., 2009), RFLP (Restriction Fragments Length Polymorphism), AFLP (Amplified Fragment Length Polymorphism) (Selvi *et al*., 2005), SSR (Simple Sequence Repeat) or microsatellite (Maccheroni *et al*., 2009), STMS (Sequence Tag Micro Satellite) (Singh *et al*., 2005), ISSR (Inter Simple Sequence Repeat) (Shrivastava and Gupta 2008), TRAP (Target Region Amplified Polymorphism) (Alwala *et al*., 2006) has been used in explaining genetic diversity among different accessions of sugarcane.

Microsatellites or simple sequence repeat (SSR) marker amplified tandemely di, tri and tetra nucleotide repeats that are widely distributed on genome. A modification of SSR-based marker system *i.e.* ISSR (inter simple sequence repeat) analysis which target the flanking region of identical SSR in inverse direction. ISSR analysis is simpler than any other marker system because it generates highly reproducible and distinct results which helps in the differentiation of distinct sugarcane genotypes.

In the present study, ISSR markers were used to assess genetic profiling of cultivars with high, moderate and low sucrose sugarcanes and some wild-type sugarcane subjected to amplify and evaluate genetic diversity by using ISSR markers in comparison with biometrical data obtained from field .

Material and methods

The material consisted of 17 sugarcane genotypes including 5 wildtype and 12 *Saccharum spp*. hybrids of subtropical India available at research farm of VSI, Pune (Table 1). A total 27 ISSR primers with major motifs of poly 'AG', poly 'AC', poly 'GT' was used for the study (Table 2). Genomic DNA was isolated from freshly harvested leaves of each sugarcane genotypes by using CTAB method (Aljanabi *et al.* 1999). Quality and quantity of DNA were estimated by UV-visible spectrophotometer and also visually by ethidium bromide staining to 0.8% (w/v) agarose gel. DNA was diluted to uniform concentration of 10ng/ul. PCR reaction was performed in 96 well plate thermal cycler (Eppendorf AG 22331, Mastercycler, Germany) with reaction volume of 15 µl containing 50 ng of template DNA, 0.2 uM primer (University of British Columbia, Vancouver, Canada), 200 uM each dNTP, 1x PCR buffer with 10 mM Tris-Cl (pH 8.3), 50 mM KCl, 2.0 mM $MgCl_2$ and 1U of Taq polymerase (Sigma-Aldrich, USA). PCR amplification conditions were set as initial denaturation at 94^0C for 6 min followed by 30 cycles of denaturation at 94^0C for 50 sec, annealing at 50^0C for 50 sec, extension at 72^0C for 90 sec, and final extension at 72^0C for 7 min. The amplified products were mixed with gel loading dye, resolved on 3% (w/v) agarose gel having ethidium bromide using 1X Tris borate EDTA (TBE) buffer under room temp at constant voltage 100V. The gels were documented under ultraviolet light using gel documentation unit (UVITech).

Data Analysis: Amplified products with reproducible and distinct bands were scored as 1 or 0 depending on its presence and absence for data analysis. Allelic diversity at a given locus was measured by PIC for polymorphic band of primer and was calculated as $1-p^2-q^2$, where, p is presence of band frequency and q is absence of band frequency (Mondal *et al*., 2009). The ability of primer to distinguish between genotypes was assessed by calculating their resolving power (Rp) as $Rp = \sum l_b$. Where, l_b is band informativeness, $l_b = 1- (2 \times |0.5-p_i|)$, where p_i is the proportion of genotype containing the band i^{th} (Prevost and Wilkinson, 1999). The genetic association among genotypes was evaluated using Jaccard's similarity coefficient. All stastical analysis was done by using the software NTSYS-pc version 2.02 (Rohlf, 2000).

Results and Discussion

Polymorphism within sugarcane: The use of ISSR-PCR markers for the amplification of sugarcane genotypes showed high level of polymorphism. Genetic profiling studies of seventeen sugarcane genotypes were carried out by using twenty seven ISSR markers having di, tri and tetra nucleotide repeat motifs. The Total Number of Allele scored (TNA), Number of Polymorphic Alleles (NPA), Polymorphic Information Content (PIC) and Resolving power (Rp) obtained per ISSR-PCR markers were shown in Table 2. In all, total 252 alleles amplified by 27 ISSR markers, 212 were polymorphic (84.13%) with an average of 9.3 alleles per locus. Number of allele scored for locus by the marker ranged from 6 to 14 alleles amplified by UBC828 and UBC834 primer with di nucleotide repeat motifs of TG and AG respectively. The Polymorphic Information Content (PIC) value ranged from 0.19 (UBC808 and 843) to 0.36 (UBC876) primers with di nucleotide repeat motifs of 'AG' and tetra nucleotide repeat motifs '(GATA) (GACA)' respectively with an average value of 0.27. The primer UBC 825 exhibited highest resolving power (Rp) value 9.8 among twenty seven primers. Genetic similarity indices were estimated by Jaccard's similarity coefficient which ranged from 26% to 72% that indicated more informativeness about genotype discrimination and genetic diversity study in sugarcane genotypes.

Unique and rare loci: Among the 27 primers amplified in 17 sugarcane genotypes, two unique loci were obtained with primer 13 and 15 that differentiate the wild-type *Erianthus ciliaris* from the rest of genotypes. However, two rare bands with low band frequency of 510bp and 700bp were amplified by the primer 23 which was unanchored tri nucleotide (ATG) repeat primer able to distinguish Co 94012 and Co 85004 high sucrose genotypes while rest of the primers did not amplify rare bands.

Genetic diversity and clustering by ISSR markers**:**

The binary data were analyzed for the determination of genetic similarity (GS) and cluster analysis within

seventeen sugarcane genotypes. Genetic similarity indices were estimated by Jaccard's similarity coefficient. The similarity value varied in - between ranged from 26 % to 72 % with a mean value of 49 %. The range of diversity was more among the wild type sugarcane genotypes such a Malabar, Hemja, IJ-76-417, *Erianthus ciliaris* and Naringa. It showed that all 17 sugarcane genotypes could be clearly differentiated by UPGMA dendrogram and most of the wild relatives formed different grouping patterns in clusters. A dendrogram with cophenetic value of 0.97 was generated and genotypes were grouped into 3 main clusters with 3 sub clusters (fig.2). The number of genotypes varied from 2 (cluster II and III) to 4 (sub cluster i, ii and iii). Cluster I had thirteen sugarcane genotypes, it was a large cluster found in the dendrogram, all genotypes were derived as *Saccharum officinarum* hybrids. Cluster I differentiated into three subclusters shown in dendrogram (fig. 2). Subcluster (i) had four genotypes Co 94012, Co 91010, VSI 435 and VSI 434 with an average of 62% similarity coefficients. Pedigree data of the genotypes showed the closeness among themselves (Table1). Subcluster (ii) had four genotypes CoVSI 5-86, CoVSI 48-188, MS 68/47 and CoM 7125 in which CoVSI 5-86 and CoVSI 48-188 had highest similarity of 72% found. The average similarity for the sub cluster (ii) was found to be 64%. Subcluster (iii) had four genotypes CoVSI 9805, Co 85004, Co M9908 and Co 62175 and found with 60% of average genetic similarity with no similar pedigree data for the genotypes.

Cluster II had two sugarcane genotypes, Hemja and IJ-76-417 which belongs to parentage of *Saccharum barberi* and wild type *Saccharum robustum* respectively. GS was found to be 44% which was less than *Saccharum officinarum* hybrid of cluster I. Also cluster III formed with the two genotypes *Narenga* and *Erianthus ciliaris* derived from wild type sugarcane *Narenga* and *Erianthus* with 29% GS and it was the lowest GS value among the grouped pairs of seventeen genotypes. Association between the inter species and wild types sugarcane genotypes are shown in two and three dimensional figures.

The cane yield and yield contributing characters along with Hand Refractometer brix % data were depicted in Table 3. Clones from three different generas i.e. *Sachharum*, *Erianthus*, *Narenga* were undertaken for the present evaluation. The *Saccharum* includes species of *S. officinarum*, *S.barberi* and *S. robustum* along with some Saccharum species hybrid derivatives. *Erianthus ciliaris* (424.00) '000/ha. *Narenga prophyocoma* showed very high number of millable cane but less single cane weight (0.56 kg) and brix (6.55%), whereas, *Narenga* is grass type habit with lowest expected cane yield of 24.00 t/ha. The another accession IJ 76-416 from *S. robustum* group of wild species recorded 108.00/ha with low brix (9.80%), whereas cultivated *S. offficinarum* recorded with moderate brix (18.54%) and high NMC (144.00) '000/ha. Indigenous cane of India *S.barberi*, hemja recorded a very high number of millable canes (320.00) '000/ha and low single cane weight (0.13 kg) and brix (13.80%) respectively.

The Saccharum species hybrid viz. Co 94012, Co91010, VSI 435, VSI 434, CoVSI 9805, Co 85004, CoVSI 5-86, CoVSI 48-188, MS 68/47, CoM 7125, CoM 9908 and Co 62175 recorded high single cane weight and HR brix % than the *Erianthus* and *Narenga*.

In the present study the amplified DNA profiles generated by ISSR-PCR were significantly scored from independent gels for making identical data sets. Obtained results of twenty-seven ISSR-PCR markers showed polymorphism percentage ranging from 56 to 100% among seventeen sugarcane genotypes which are nearly greater than the range of 43-100% polymorphism found (Shrivastava and Gupta, 2008) in forty-two genotypes generated by ten ISSR-PCR markers. This may be because of the number of genotypes and availability of target sites in genome for primer motifs binding. Polyploid plants have increased heterozygosity and also harbor higher level of genetic diversity than was anticipated that may also beneficial for biochemical estimations. Comparison of allele per locus generated by ISSR-PCR marker from nuclear to mitochondrial and chloroplast genome analysis, it is clear that more allele per locus are produced by nuclear than mitochondrial and chloroplast genome of sugarcane (Virupakshi and Naik, 2008) that had happened because of large genome and heterozygocity in sugarcane. Genetic diversity in different accessions of sugarcane have been performed in most of the country because of its distribution in different geographic region by using various DNA based molecular markers such as RAPD, SSR, ISSR, STMS, TRAP and AFLP. Polymorphic allele per locus generated by ISSR are more than RAPD, SSR and STMS markers (Creste *et al*., 2010; Babu *et al*., 2010; Filho *et al.*, 2010; Kawar *et al*., 2009) while TRAP and AFLP marker generates greater no of polymorphic alleles than ISSR markers (Alwala, 2006). The mean value of GS was 49% indicating that the sugarcane genotypes used in the study were not much divergent. One of the fact behind the obtained result was the selection of genotypes,

among seventeen sugarcane genotypes, twelve belongs to the *Saccharum spp*. hybrids which show highest genetic similarity. Another possibility may be due to the use of limited number of markers for dissecting large genome size of sugarcane (2500-4000 Mb) (Babu *et al*., 2010). GS value clearly differentiated all genotypes from 26-72%, which contrasts with the value of 68-97% obtained by Dice similarity coefficient in Indian sugarcane (Shrivastava and Gupta, 2008). Most of the markers show 100% GS and it is unable to differentiate genotypes of more pedigree similarity, since the consistency of genetic similarity depends upon number and localization of markers in the genome (Lima *et al*., 2002) as well as the number of accessions used for genetic diversity study. Polymorphic information contents significantly measures the informativeness of ISSR-PCR towards the genetic diversity study (Maccheroni *et al*., 2009; Filho et al. 2010; Mondal *et al*., 2009) other than that presence of specific motifs provides the evidences of tolerance and yield like traits (Reddy *et al*., 2009). AG repeat motifs obtained with lowest PIC value (0.19) and GATA, GACA repeat motifs highest PIC value (0.36) can be correlated with the tolerance and yield of sugarcane. It has been proved that the AG motifs are frequently present and differentiates the flood-tolerant varieties of rice from drought and salt tolerance and it also exhibits photosensitive property (Reddy et al. 2009) while tetra nucleotide repeats are not efficiently correlated with tolerance and yield because of its less abundance in sugarcane genome. Estimated Rp value revealed a large variation (1.9 to 9.8) among the primers (Table 2). Higher PIC and Rp values indicated more informativeness about the genotype discrimination and genetic diversity studies (Mondal *et al*., 2009). Therefore, the ISSR markers are the most efficient marker system because of their capacity to reveal several informative bands in a single amplification (Fernandez *et al*., 2002).

A dendrogram with cophenetic value of 0.97 was generated by the ISSR data matrix agrees better association within the seventeen sugarcane genotypes (Fig. 1) and said to indicate a strong goodness of fit for dendrogram (Rohlf, 2000). Based upon the parentage, genotypes were grouped into 3 main clusters with 3 sub clusters. The range of diversity was more among the wild type sugarcane genotypes such a Malabar, Hemja, IJ-76-417, *Erianthus ciliaris* and Naringa. Cluster I formed largest group of sugarcane genotypes which are derived from *Saccharum spp.* hybrid except Malabar. Genotypes in sub-cluster (i) and sub-cluster (iii) possess a high sucrose yielding character and most of the genotypes show parental similarity. Three sub-clusters are present in the cluster I and it can be positively correlated with sucrose yielding trait. Sub-cluster (i) and (iii) genotypes are contributed to high sucrose varieties and their brix values 18-22% determined. It has been observed that most of the genotypes from sub-cluster (i) and (iii) have same pedigree records, Co 94012, VSI 434 and VSI 435 which are derived as somaclones of CoC 671 variety which is known for its higher stalk diameter, juice quality and brix (Ahmed and Obeid, 2010). VSI 434 and VSI 435 varieties were developed through plant tissue culture while CoVSI 9805 was developed through poly-cross (PC) of Co 8371 by breeding program of Vasantdada Sugar Institute (VSI) Pune, India. Sub-cluster (ii) genotypes had low brix values 12-16 % determined (Table 3) and pedigree data of MS 68/47 and CoM 7125 had common male parent Co 775 but it does not show any significant correlation in their brix value. These high and low sugar yielding sub-clusters help to find out the distinct polymorphism and it may be applicable to development of SCAR marker (Villancourt *et al*., 2008) as well as it could help from phylogenetic analysis to validation of marker identified in sugarcane genotypes contributing to sucrose yielding trait. Cluster II had two sugarcane genotypes, Hemja and IJ-76-417 which belongs to the *Saccharum barberi* and wild type of *Saccharum robustum* respectively. Brown *et al*., (2007) had analyzed clonal germplasm from one fifty six accessions of five *Saccharum* species and six cultivated commercial clones, they found that most of the clones showed association between clones of *Saccharum barberi* and *Saccharum robustum* on three dimensional plot. Cluster III formed with the two genotypes *Narenga* and *Erianthus ciliaris* derived from wild type sugarcane *Narenga* and *Erianthus* which shows the lowest GS value and diverse in three dimensional plot among the grouped pairs of seventeen genotypes indicating the extent of genetic divergence from *Saccharum officinarum*.

Attempts were made to represent the relationship between selected different seventeen genotypes of Saccharum related genera at molecular and biometrical level. The *S. officinarum* related group which showed moderate to high sucrose comes in cluster I. whereas, Saccharum species related to *S.robostum* and *S. barberi* grouped in separate cluster at molecular level i.e. cluster II. Similar type of finding was observed in the Saccharum related genera with very low brix value in *Erianthus* and *Narenga* in cluster III. Hence the yield and quality data of those selected genotypes and ISSR based genetic profiling were useful in planning the crossing program as well as to prove polymorphic markers and its significant use in mapping population

identification. In our view, crossing of genotypes belonging to high and low sugar yielding trait from the same group as studied in this research work can lead to confirmation of polymorphism contributing for sugar accumulation trait. This genetic diversity data would support for the gene isolation and gene expression studies in most divergent sugarcane genotypes.

Acknowledgment

The authors are grateful to the Director General and Director, Agricultural Science and Technology, Vasantdada Sugar Institute (VSI), Pune for their support during research work. We are also thankful to Dr. R. S. Hapase and Dr. J. M Repale, Plant Breeding section for providing sugarcane genotype leaf samples.

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Table1. List of genotypes and their respective parentage

Sr. No.	Genotypes	Parentage
1	Malabar	*Saccharum officinarum*
2	Hemja	*Saccharum barberi*
3	IJ-76-417	*Saccharum robustum*
4	*E. ciliaris*	*Erianthus*
5	*Narenga propyrocoma*	*Narenga*
6	Co 94012	Somaclones of CoC 671
7	Co 91010	Co 312 ×Co 775
8	VSI 435	Somaclones of CoC 671
9	VSI 434	Somaclones of CoC 671
10	CoVSI 9805	Co 8371PC
11	Co 85004	Co 6304×Co 740
12	CoVSI 5-86	Co 87002×CoH 70
13	CoVSI 48-188	CoC 8001GC
14	MS 68/47	Co798×Co 775
15	CoM 7125	Co740×Co 775
16	CoM 9908	Co740×CoC 671
17	Co 62175	Co 951×Co 419

Table2. Sequences and number of fragments amplified by the 27 ISSR primers

Sr. No.	ISSR Primer	Sequence 5'→3'	Total number of alleles scored	Number of Polymorphic Alleles	PIC	Rp
1	UBC808	(AG)8C	11	10	0.19	4
2	UBC818	(CA)8G	9	5	0.25	3.3
3	UBC819	(GT)8A	7	7	0.21	4.7
4	UBC822	(TC)8A	8	7	0.24	7.1
5	UBC823	(TC)8C	8	7	0.26	5.1
6	UBC824	(TC)8G	6	5	0.2	4.4
7	UBC825	(AC)8T	11	9	0.32	9.8
8	UBC826	(AC)8C	10	7	0.35	6.9
9	UBC827	(AC)8G	11	7	0.23	3.3
10	UBC828	(TG)8A	6	5	0.27	3.9
11	UBC834	(AG)8YT	14	12	0.23	4.2
12	UBC835	(AG)8YC	13	11	0.35	6.9
13	UBC836	(AG)8YA	11	11	0.27	4
14	UBC843	(CT)8RA	9	9	0.19	2.2
15	UBC844	(CT)RC	9	8	0.24	2.6
16	UBC846	(CA)8RT	11	9	0.21	5.1
17	UBC847	(CA)8RC	7	4	0.25	2.1
18	UBC849	(GT)8YA	9	8	0.2	1.9
19	UBC850	(GT)8YC	10	8	0.22	2.4
20	UBC851	(GT)8YG	8	6	0.28	4.6
21	UBC857	(AC)8YG	8	7	0.24	2
22	UBC859	(TG)8RC	9	8	0.22	2.5
23	UBC864	(ATG)6	10	9	0.21	2.2
24	UBC866	(CTC)6	8	7	0.3	3.2
25	UBC868	(GAA)6	13	11	0.24	8.8
26	UBC876	(GATA)2(GACA)2	7	7	0.36	4
27	UBC886	HBH (AG)7	9	8	0.22	2.6
	Total		252	212		
	Average		9.3	7.9	0.25	4.2

Note: R= (A, G); Y=(C, T); B= (C, G, T); and H= (A, C, T).

Table 3. Average mean values for cane yield and yield contributing characters with brix % recorded for different genotypes

Genotypes no.	Expected Cane Yield (t/h)	NMC 1000/ha	Millable height (cm)	No. of internodes	Cane diameter (cm)	Single cane weight (kg)	HR brix % (12 month)
1	95.04	114.00	182.65	17.00	2.15	0.66	18.54
2	41.60	320.00	55.00	14.50	1.20	0.13	13.80
3	60.48	108.00	235.54	23.50	2.15	0.56	9.80
4	80.56	424.00	290.54	18.00	1.15	0.19	6.55
5	24.00	160.00	102.23	17.00	0.50	0.15	4.60
6	80.42	67.58	202.55	20.00	2.83	1.19	22.80
7	101.80	82.77	194.65	18.00	2.03	1.23	21.65
8	76.50	74.28	202.60	21.00	2.42	1.03	23.00
9	79.80	70.00	196.43	19.00	2.70	1.14	24.20
10	106.25	61.42	208.16	21.00	3.12	1.72	22.28
11	101.47	105.70	222.11	17.66	2.30	0.96	23.60
12	124.80	80.00	195.43	18.90	2.70	1.56	16.80
13	141.39	74.42	202.18	19.00	2.87	1.90	12.20
14	127.83	65.22	208.23	19.00	3.30	1.96	16.20
15	58.82	82.85	197.71	20.50	2.70	0.71	21.40
16	54.51	75.71	195.43	18.00	1.81	0.72	22.00
17	110.55	61.42	220.56	21.33	2.83	1.80	18.90

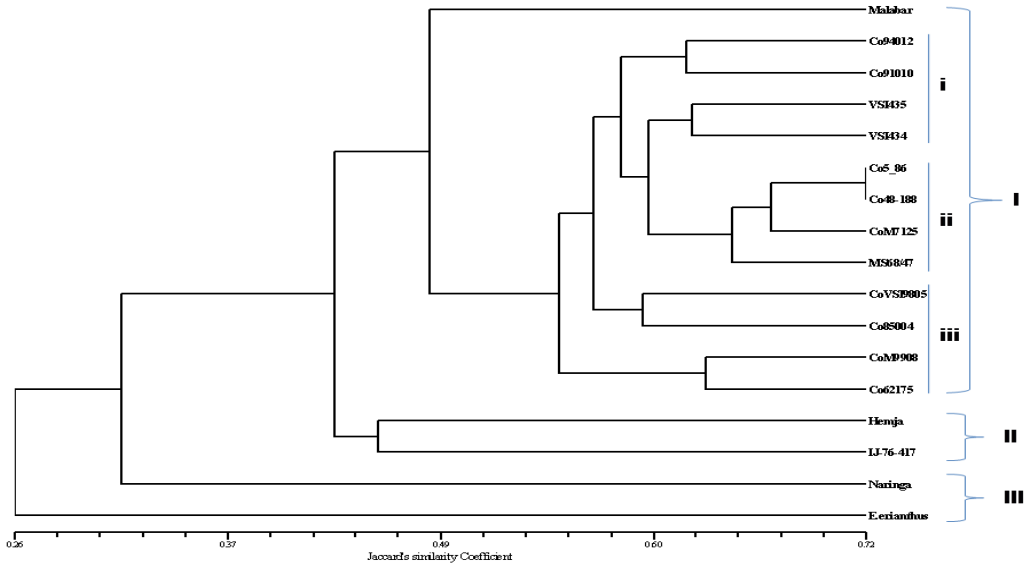

Fig. 1. Dendrogram representing the relationship between 17 sugarcane genotypes based on UPGMA cluster analysis of 27 ISSR primers using Jaccard's similarity coefficients