Journal of Sugarcane Research (2020) **10**, 140-151
https://doi.org/10.37580/JSR.2020.2.10.140-151

RESEARCH ARTICLE

Molecular identification and genetic diversity analysis of sugarcane clones by SSR markers

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(Received 16 September 2020; accepted 16 March 2021)

Abstract
Sugarcane is one of the important crops with high heterozygosity and phenotypic polymorphism. Varietal identification and characterization is one of the important aspects in any breeding programme. Forty sugarcane clones from Pre -zonal varietal trial (PZVT) conducted at Ugar, North Karnataka were characterized through Simple sequence repeats (SSR) markers using a set of 15 sugarcane specific primer pairs which amplified a total of 164 alleles with an average of 10.93 alleles per pair. Primer NKS 33 was highly polymorphic and produced more than 15 polymorphic alleles and was unique in all the clones studied. Six primers i.e., NKS 2, NKS 6, NKS 7, NKS 40, NKS 42 and NKS 11 were moderately polymorphic by producing 10 to 13 alleles. Percentage of polymorphic bands ranged from 75.0 % (NKS 3) to 100.0 % (NKS 40 and NKS 42). Primers viz., SMC 1039 GC, mSSCIR 54, NKS 2, NKS 7, NKS 9, NKS 33, NKS 40, NKS 42 and NKS 11 were highly informative and generated above 85.0 % of polymorphic bands. Among the primers used, SMC 1039 GC, NKS 33, NKS 42 and NKS 43 produced the maximum number of unique markers in different clones and hence the combined application of these primers will be useful in unambiguous varietal identification. The cluster analysis based on the genetic similarity matrix grouped the 40 clones into two major clusters CI and CII. The largest cluster CII contained the maximum of 38 clones. Cluster C II was further sub-grouped into IIa, IIb, IIc and IId and each sub-cluster comprised 7, 11, 6 and 14 clones respectively. The unique DNA markers and the genetically diverse combinations identified in the present study will enhance the exploitation of genetic diversity present in the clones in breeding programmes and promising twenty eight entries for yield and quality.

Keywords: Sugarcane; Molecular markers; SSR; Clones

Introduction

Sugarcane (*Saccharum sp*) is an important crop with high photosynthetic efficiency, biomass production and high phenotypic polymorphism. Genetic improvement in sugarcane through conventional breeding methods had effectively improved the sugar productivity for past few decades (Jackson 2005; Edme et al. 2005).The objective of sugarcane breeding programme is to select superior clones with high sugar content, yield and high juice purity. The narrow genetic variability in the breeding material (D'Hont et al. 1995; Tew 2003) necessitates the sugarcane breeders to improve the genetic base of cultivars through base broadening programs (Burner and Legendre 1993). A diverse cafeteria of varieties is very important for profitable sugarcane cultivation.

Genetic variability is the central dogma for any breeding programme and. In general, morphological descriptors are used for the assessment of genetic diversity of germplasm resources, varieties or cultivars and intellectual property protection of breeders (Cordeiro et al. 2003). Sugarcane varieties are identified based on phenotypic traits following the DUS testing guidelines. These morphological characters are highly influenced by environmental factors and it is also time-consuming and requires large areas of land and subjective decisions (Cooke 1995). Molecular marker analysis without any influence of

environmental factors is considered as an efficient alternative measure for genetic diversity studies. Hemaprabha et al. (2012, 2013) also suggested the use of molecular markers in generating molecular profiles for sugarcane cultivars.

Among the markers, SSR are considered as one of the most efficient markers for plant breeding due to large quantity, low dosage, co-dominant, reliability and ability to generate large number of alleles (Jiang 2013). In sugarcane SSR markers are widely used to study the genetic diversity of *Saccharum* species and related genera (Cordeiro et al. 2003; Cai et al. 2005; Ukoskit et al. 2012; Silva et al. 2012; Singh et al. 2019). SSR markers have also been important in fingerprinting of sugarcane varieties ((Hemaprabha et al. 2006), genetic mapping (Suman et al. 2011), identification of hybrid progeny from interspecific crosses (Pan et al. 2006) and in construction of sugarcane molecular identity database (Pan 2016). Among the molecular marker techniques, UPOV (UPOV-BMT, 2002) recommends SSR and SNP markers combined with morphological characters as efficient tools in varietal identification. Harvey and Botha (1995) reported that molecular characterization with the use of decamer microsatellite and telomere sequences are highly beneficial in identifying specific sequences for different cultivars. The present study designed to assess the genetic diversity among 40 sugarcane cultivars identified through PZVT testing at Ugar (U), North Karnataka and in varietal testing at ICAR-SBI Coimbatore, for the unambiguous identification of the clones and to use them in marker assisted selection.

Materials and Methods

During the year 2009-12, 100 clones of PZVT 2009 series were evaluated in RBD at Ugar with a plot size of 4 rows of 6 m length against four standards viz., CoC 671, Co 85004, Co 94008 and Co 86032. At ICAR - Sugarcane Breeding Institute, Coimbatore, 100 clones were evaluated along with the same set of standards in RBD with two replications. Standard package of practices were followed. Data on yield traits *viz*., cane thickness, cane height, number of millable canes, single cane weight and juice traits *viz.*, brix %, sucrose % and CCS % at 10 and 12 months of age were recorded.

Out of 100, 40 superior sugarcane clones identified from PZVT testing in Ugar and varietal testing at Coimbatore were used for molecular characterization in the present study (Table 1). Fifteen sugarcane specific SSR primer pairs were selected from Genbank database (www.nrcpb.org/STMS.html) for molecular characterization as presented in Table 2.

Total genomic DNA was extracted from leaf tissue by CTAB method and DNA concentrations were quantified using Nanodrop DNA/RNA quantifier (Doyle and Doyle, 1987). The forward and reverse primers of 15 sugarcane SSR markers were synthesized by Sigma Technol., Inc. (Coralville, IA). Polymerase chain reaction were performed in Eppendorf Thermal cycler PTC 100 with a 10 µl total reaction volume containing 10 ng of template DNA, 1 µM of each primer, 10 mM Tris–HCl (pH 8.3), 2mM of dNTPs, 1.75 mM $MgCl_2$ and 0.5 µ Taq polymerase. PCR cyclic conditions were followed as 5 min at 95°C; 35 cycles of 40 sec at 94°C, 45 sec at the annealing temperature, 45 sec at 72°C; and 72°C for 10 min. Amplified PCR products were separated in 7.5 % non-denaturing polyacrylamide gel using 1X TBE buffer and silver stained. The gel was observed in a Gel Documentation System (Alpha Innotech) and polymorphic bands were scored. The absence / presence of each marker was scored as 0 / 1 respectively and analyzed using NTSYSv2.0 software The SSR marker data was used to estimate similarity matrices and then to generate dendrogram to study genetic diversity of clones.

Table 1. Clones utilized for genetic diversity study

Characters	Clone	Parentage
High Yielding clones (100 -184 t/ ha cane yield)	Co 10023	Co 92024 GC
	Co 10027	Co 92024 GC
	Co 10031	Co 92024 GC
	Co 10033	Co 98008 x CoA 7602
	U09027	ISH 1 x CoV 92102
	U09133	Co 92024 GC
	U09018	CoV 89101 x Co 97015
	U09035	Co 85002 x CoM 0261
	U09110	Co 86010 x Co 86011
	U09081	Co 86010 x Co 86011
	U09073	Co 92024 GC
	U09009	Co 8371 x CoH 104
	U09076	Co 92024 GC
	U09078	Co 92024 GC
	U09077	Co 92024 GC
	U09098	Co 92024 GC
	U09102a	CoV 89101 x Co 97015
	U09068	Co 8371 x Co 86011
	U09100	Co 92024 GC
High yield and good quality clones (100-120 t/ha cane yield and 19.0 -20.0 % sucrose at 360 days)	Co 10032	Co 92024 GC
	U09030	CoV 89101 x Co 97015
	U09032	ISH 1 x CoV 92102
	U09022	ISH 1 x CoV 92102
	U09183	Co 740 GC
	U09019	ISH 1 x CoV 92102
	U09196	Co 89003 GC
	U09006	Co 98008 x CoA 7602
	U09132	Co 7329 GC
	U09178	Co 2000-10 GC
	U09102	Co 92024 GC
	U09059	Co 8371 x CoH 104
	U09003	87A380 x CoV 92102
	U09001	Co 98008 x CoA 7602

Table 2 contd...,

Good quality clones (19.0 -21.0 % sucrose at 360 days)	U09024	ISH 1 x CoV 92102
	U09075	Co 92024 GC
	U09053	Co 88006 x CoSe 92423
	U09031	ISH 1 x CoV 92102
	U09052	87A380 x CoV 92102
	U09069	Co 92008 GC
	U09028	ISH 1 x CoV 92102

U- Ugar

Results and Discussion

Phenotypic variability of genotypes

All the 40 clones exhibited significant differences and large amount of variability among them for quality (brix % and sucrose %) and yield traits (cane thickness, cane height, single cane weight, number of millable canes and cane yield). Among the checks CoC 671 and Co 86032 recorded a mean sucrose of 20.18 % and 19.44% respectively at 360 days. The clones U 09075, U 09032 recorded sucrose of 21.26 % followed by U 09196 (20.45 %) and U09052 (20.27 %). CoC 671 and Co 86032 recorded cane yield of 115.38 t/ha and 124.24 t/ha respectively. The sparse flowering clone U09027 recorded yield of 163.88 t/ha followed by U09098 with 158.79 t/ha yield. Eight clones viz., U09024, U09003, U09030, U09028, U 09031, U090183, U09059 and U09178 which were non / shy flowering recorded cane yield on par with Co 86032 and also combined quality. Twenty eight elite clones (out of which five Co canes viz., Co 10023, Co 10027, Co 10031, Co 10032 and Co 10033 showed good performance for yield, quality and with good field stand at Ugar and selected for further utilization.

In the early group the clone U09031 recorded the maximum sucrose of 20.20 %. U09077 recorded the maximum cane yield of 158.08 t/ha and 20.06 t/ha commercial sugar yield followed by U09102 with sugar yield of 18.85 t/ha and 142.08 t/ha cane yield in comparison with CoC 671. In the mid-late group four clones viz., U09032 , U09059, U090196 and U09030 were superior for quality in comparison with Co 86032(19.51%) at 360 days. The entry U09098 was the best with cane yield 155.09 t/ha. The best clone was U09032 with 21.70 t/ha for sugar yield and 21.26 % sucrose. Among the 28 non flowering types, nine clones were promising for yield and quality. Among them U09027 was high yielding type with 163.88 t/ha cane yield followed by U09078 (150.0 t/ha).

Among the clones U09075, U09029, U09028, U09031 and U09052 were on par for yield and quality parameters, both at Ugar and Coimbatore locations. The clones U09019, U09022 and U09032 combined both yield and quality and U09075, U09032, U09196, U09075 and U09052 were high quality types. The clones U09027, U09024, U09003, U09030, U09028, U09031, U090183, U09059 U09078 and U09098 were non flowering / shy flowering clones with high cane yield and quality. These clones were further characterized for their molecular variation. Coefficient of correlation studies of yield components with the cane yield showed that cane thickness, cane height, number of internodes and millable cane were positive and highly significant.

Table 2. Sugarcane specific SSR primers used for molecular characterization

SSR Marker	EST/ Genomic	Repeat motif	Forward primer sequence (5' to 3') Reverse primer sequence (5' to 3')	Annealing Tm (°C)	Number of Polymorphic bands	Allele size (bp)	Percentage of polymorphic markers
SMC 1039 GC	Genomic	(TG)17	AGGTGAGAGTTCCTGGCTTTC-CA TGTGCTGGCAAGCCCCTACTT		7	653-1194	87.5
mSSCIR 54	Genomic		CGAAGGACCAGTTGAAAG CGAAGGACCAGTTGA AAG	48.1	8	192-1310	88.8
NKS 1	EST	(GAA)6	TGGCATGTGTCATAGCCAAT CCCCAACTGGGACTTTTACA	58	8	221-909	80
NKS 2	EST	(GA)13	GCTGTCCCGTTCCAAGTTAC GCGACCGGATTATGATGATT	58	12	188-484	85.7
NKS 3	EST	(TGC)5	CGTGTTCCTCTTCAACAACG TGCTTCGCTATATATGGGTTCA	58	6	244-365	75
NKS 6	Genomic	(TG)32	TCCAAATTGCCTGTTGTTTTC CTTACACATGCACAGGCACA	58	11	143-485	84.6
NKS 7	Genomic	(CGG)9	TTACAGCCTGGAGCTCGTTT CGAAGCCTCTCCTCTCCTC	58	12	185-386	92.3
NKS 8	Genomic	(CGG)6	GTGACAGCGGCTTGTTCAG TTAAACACGCAGCCATTCAG	58	4	175-435	80
NKS 9	Genomic	(CGC)6	CTTTCAGTGGCCATCTCCAT GAATGCGCAGGGATAGGATA	58	9	179-429	90
NKS 17	Genomic	(AG)24	GCTCGCCATGAATAGAAAGG ACCGAGGTAGGAGGGAGTGT	57.1	7	223-457	77.7
NKS 33	Genomic	(TGT)6	ACAGGAGCGCTTGGAGATTA GAGCAGAAGGGCTAGAAGCA	57	17	125-815	94.4
NKS 40	Genomic	(TG)36	GATGGAGGCTTTGCAATGAT GCATGTCCCACTGAACTGAA	55	12	103-638	92.3
NKS 42	Genomic	(TG)35	ACCGATTGTTCAGTGGGAAG AACCTAGCAATTTACAA-GAGAATTAGA	57	11	129-380	91.67
NKS 43	Genomic	(TG)20	CTGATGGGAGGTTGAAGGAA ATAAGCACCAAAAGCGTGGT	57	9	166-806	81.8
NKS 11	Genomic	(CT)17	CACCACTCACATCCACTTGC TATGGAGAGATGCTGCTGCT	57.1	10	133-327	90.9

It was observed that progenies involving Co 92024 as female parent produced high proportion of non-flowering clones in comparison with the parents Co 86010, ISH 1 and CoV 92102.

Polymorphism potential of SSR markers

In the present study, fifteen sugarcane specific SSR markers and EST sequences identified from Genbank databases, (www.nrcpb.org/STMS.html) and developed from enriched sugarcane libraries (Parida et al. 2009) were utilized to understand the genetic diversity in forty sugarcane clones and to identify unique DNA markers. Genotypic characterization of forty clones (Table 1) using fifteen microsatellite markers (Table 2) revealed highly polymorphic profiles and also generated unique marker for most of the clones. The 15 SSR primer pairs amplified a total of 164 alleles with an average of 10.93 alleles per primer pair (Table 2). Of the 164 alleles, 145 alleles were polymorphic and the number of polymorphic alleles generated by single primer pair ranged from 4 (NKS 8) to 17 (NKS 33). Primer NKS 33 was highly polymorphic and produced more than 15 polymorphic alleles. The other six markers *i.e*., NKS 2, NKS 6, NKS 7, NKS 40, NKS 42 and NKS 11 were moderately polymorphic by producing 10 to 13 alleles. The remaining eight markers, namely, SMC 1039 GC, mSSCIR 54, NKS 1, NKS 3, NKS 8, NKS 9, NKS 17 and NKS 43 were less polymorphic by producing less than 10 alleles.

Percentage of polymorphic bands ranged from 75.0 % (NKS 3) to 100.0 % (NKS 40 and NKS 42). Primers *viz*., SMC 1039 GC, mSSCIR 54, NKS 2, NKS 7, NKS 9, NKS 33, NKS 40, NKS 42 and NKS 11 were highly informative and generated above 85.0 % of polymorphic bands (Table 2). Molecular characterization by Jannoo et al. (2001) in 96 sugarcane cultivars revealed that two primer pairs showed high level of heterozygosity. Hemaprabha et al. 2013 reported that six among 23 primer pairs (NKS 23) generated maximum polymorphism of above 80 % with more number of unique bands. Similarly Sharma et al. 2014 observed that ten among the 26 SSR primer pairs were highly polymorphic. The level of polymorphism observed in these sugarcane clones were high and the above findings explain that application of appropriate combination of SSR primer pairs will be highly efficient in discrimination of varieties. DNA fragment size ranged from 103 bp (NKS 40) to 1310 bp (SMC 1039 GC). The size of maximum bands ranged between 170 bp to 450 bp. The primers *viz*., NKS 11, NKS 42, NKS 7 and NKS 3 produced smaller fragments and the maximum size observed is below 400 bp; NKS 43, NKS 1, SMC 1039 GC and mSSCIR 54 produced the largest fragments of above 800 bp.

Identification of unique or variety specific DNA markers

The unique SSR markers identified for different clones are given in Table 3. Molecular characterization of forty clones with fifteen SSR primers generated nineteen unique DNA markers for seventeen clones. Among the primers used, SMC 1039 GC, NKS 33, NKS 42 and NKS 43 produced the maximum number of unique markers in different clones and hence the combined application of these primers will be useful in unambiguous varietal identification and maintaining identity / purity of the clones. Ali et al. (2017) reported 20 new alleles of 21 SSR primers identified through capillary electrophoresis in combination with alleles of PAGE detection system as a beneficial working tool for molecular identification of sugarcane varieties and diversity studies. The molecular profiles of Ugar clones with unique DNA markers are presented in Figure 1 (a-f). Primers, NKS 1, NKS 2, NKS 6 , NKS 7 amplified single unique markers in U09019 (Figure 1.a), U09075 (Figure 1.b), U09018

Table 3. Unique SSR markers identified in Ugar clones

Clone Name	SSR markers	Presence of band (bp)	absence of band (bp)
U09073	SMC 1039 GC, NKS 33	SMC 1039 GC_{890}, NKS 33_{724}	-
U09006	SMC 1039 GC	SMC 1039 GC_{1194}	-
U09069	NKS 1NKS 43	NKS 1_{909}, NKS 43_{155}	-
Co 10033	NKS 7	NKS 7_{366}	-
U09001	NKS 17	-	NKS 17_{223}
U09098	NKS 42 SMC 1039 GC	NKS 42_{196}, SMC 1039 GC_{833}	-
U09100	NKS 43	NKS 43_{176}	-
U09003	NKS 42	NKS 42_{380}	-
U09059	SMC 1039 GC	SMC 1039 GC_{1022}	-
U09019	NKS 1	NKS 1_{572}	-
U09075	NKS 2	NKS 2_{486}	-
U09018	NKS 7	NKS 7_{331}	-
U09081	NKS 6	NNKS 6_{442}	-
U09022	NKS 42	NKS 42_{187}	-
U09110	NKS 33	NKS 33_{138}	-
U09196	NKS 33	NKS 33_{166}	-
U09102	NKS 33	NKS 33_{500}	-

Gel polymorphism	
Monomorphic bands	1
Polymorphic (without unique)	6
Unique bands	1
Polymorphic (with unique)	7
Total number of bands	8
Polymorphism (%)	87.50 %
Mean of band frequency	0.475

(Figure 1.c) and U09081 (Figure 1.d) respectively. Primers *viz*., NKS 42 and SMC 1039 GC also efficiently generated unique markers for three different clones each viz., U09098, U09003 and U09022 and U U09006, U09059 and U09098 respectively (Table 3).

Unique DNA fingerprints developed are highly effective in identification of phenotypically most similar varieties, somaclones, mutants and in phylogeny analysis (Hemaprabha et al. 2012; Karpagam and Alarmelu 2016) and the efficiency of primer NKS 42 with identification of distinct markers for discrimination of three different sugarcane cultivars was reported. The clones U09100 and U09022 had unique markers which was generated by NKS 43 and NKS 42 (Figure 1.e and 1.f) respectively. Among the 15 primers, NKS 33 produced the maximum of four unique DNA bands in clones *viz*., U09110 (Figure 2.a), U09073 (Figure 2.b), U09196 (Figure 2.c) and U09102 (Figure 2d). The clones *viz*., U09073, U09069 and U09098 had unique identification bands amplified by two different primer pairs (Table 3) and this

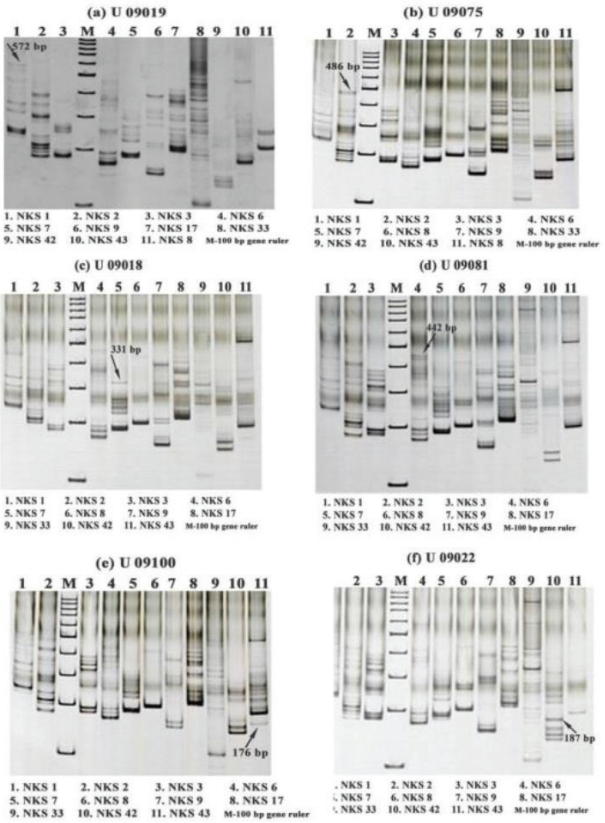

Figure 1. DNA fingerprints of clones

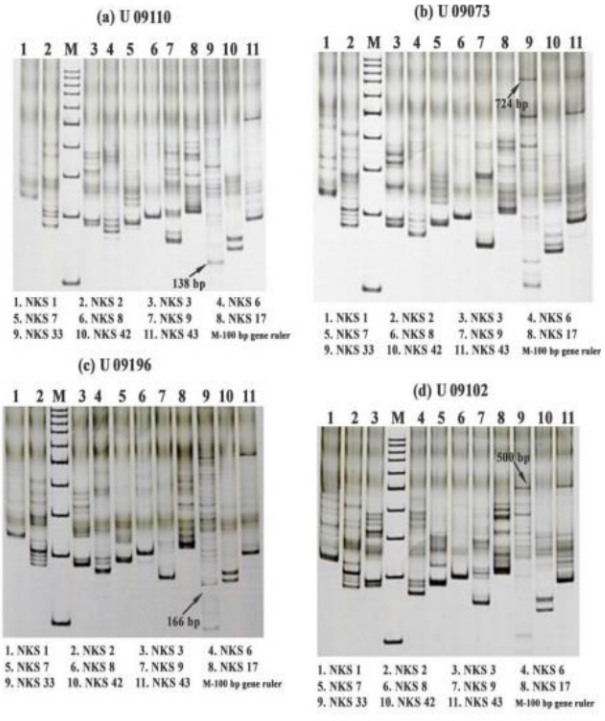

Figure 2. DNA fingerprints of clones

explains the high genetic variation present among the clones.

Genetic similarity and cluster analysis

The pair-wise genetic similarity coefficients (Jaccard's similarity coefficient) of forty Ugar clones ranged from 0.56 to 0.83 with a mean of 0.69 among the 40 clones. The cluster analysis based on the genetic similarity matrix grouped the 40 clones into two major clusters C I and CII (Fig. 3). The largest cluster C II contains the maximum of 38 clones. Cluster C II was further sub-grouped into IIa, IIb, IIc and IId and each sub-cluster consist of 7, 11, 6 and 14 clones respectively. Cluster I consists of only two clones *viz*., U09001 and Co 10033 (Fig.3).

The present study also indicated that the parentage of varieties did not contribute significantly to the clustering pattern which was in accordance with the earlier findings of Nair et al. (2002). Genotypes *viz*., U09078, U09098, U09133, U09176, U09077, U09102a, U09100, U09102, Co 10023, Co 10032 and Co 10031 though had common parentage (Co 92024 GC) they were placed in different clusters due to its complex genetic makeup of genome (D'Hont et al.1996). Sugarcane hybrids are complex aneuploids and hence clones derived from same parental genotypes will represent distant relationship with their parents. It is also found that few varieties of same parental origin tend to cluster together and clones of cluster I namely U09001 and Co 10033 is with a common parentage (Co 98008 x CoA 9602). These clones were high yielding types with moderate quality and resistance to red rot. The clones U09075 and U09073 with the highest similarity of 0.83 had the same parentage (Co 92024 GC) and were grouped under single sub-cluster and similar trend of grouping was also observed with the varieties U09028 and U09031 with a common parentage of ISH 1 x CoV 92102 which revealed higher degree of genetic

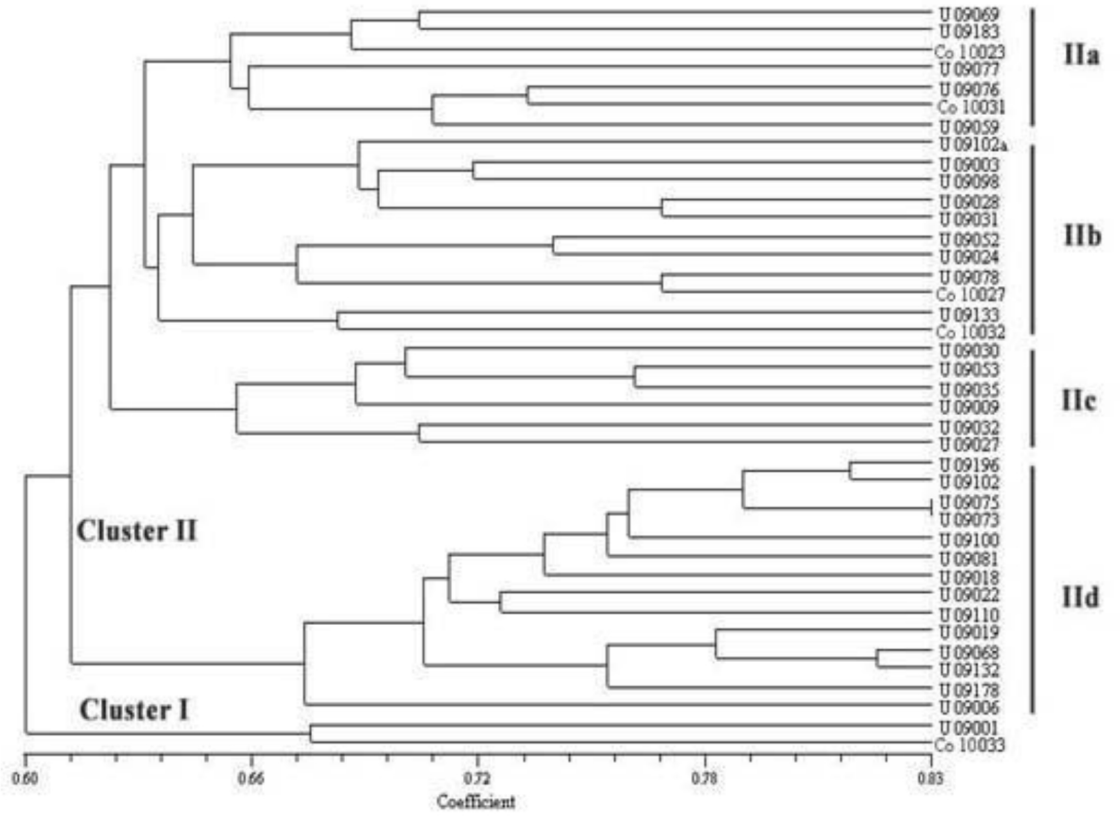

Figure 3. Dendrogram based on Jaccard similarity coefficient following the UPGMA clustering method

relatedness. Clones *viz*., U090178, U09183 and U09196 identified from the cross Co 2000 -10 GC were found to be totally divergent with least similarity and placed under different clusters. These clones had high phenotypic polymorphism which represented by yield traits and suggested as diverse parents for breeding programs.

The clones Co 10033, U09183, U09133, U09077, Co 10032, Co 10023 and U09052 and U09059, U09009, U09052 U09001, U09077 U09032, U09076 U09001 and U09183 with genetic similarity ≤ 0.65 and crosses Co 98008 x CoA 9602 , Co 86002 x CoM 0261, ISH 1 x CoV 92102, 87A 380 x CoV 92102 and Co 8371 x CoH 104 can be further exploited in breeding to identify transgressive segregants.

Sixteen cross combinations with least genetic similarity ≤ 0.65 were identified in the present study**.** Nine pairs viz., Co 10033 and U09183, Co 10032 and Co 10023, U09052 and U09059, U09009 and U09052, U09001 and U09077, U09032 and U09076, U09001 and U09183, Co 10033 and U09027 and U09035 and U09077 were found with genetic similarity ≤ 0.65. Largest degree of genetic similarity was found between U09075 and U09073 (0.83), U09068 and U09132 (0.81), U09196 and U09102 (0.81). The unique DNA markers and the genetically diverse combinations identified in the present study will also enhance the exploitation of genetic diversity on molecular basis. DNA markers identified can be used for identification of clones, maintain genetic purity and to maintain genetic diversity in the breeding population. Clustering pattern and genetic relationships obtained through SSR

markers had enabled to identify the diverse cultivars and parental genetic base to ensure high variability for future exploitation in hybridization programmes.

Conclusion

Choice of parents is very important and proper identification of donors is the basic work of breeding. Clones with high genetic differences can improve the variation level of the gene pool. The information on genetic similarity of the clones generated will be helpful in identifying and combining the best parents and to generate recombinants with high heterosis. SSR fingerprinting is an effective tool to identify sugarcane clones and the DNA fingerprints serves as reference for the selection of Ugar clones, maintain its genetic purity and also in protection of the material.

Identification of varieties based on molecular markers is thus important to establish the distinct characters and unique profile of genotypes and the study has thrown light on identification of unique markers specific to clones developed from PZVT testing under N.Karnataka conditions which can be used in identification and also in maintaining the purity of the clones. The clones *viz*., U09073, U09069 and U09098 had unique identification bands amplified by two different primer pairs which indicated high genetic variation among them and they were grouped in different clusters. These identified clones recorded high yield as well quality and combined resistance to red rot and smut and tolerance to white wooly aphid and can be effectively used in hybridization. The distinct clusters and clones identified can be further exploited in breeding programmes to harness the heterotic vigour and create new genetic variability.

Acknowledgement

The authors express gratitude to Dr. Bakshi Ram, Director, ICAR- Sugarcane Breeding Institute, Dr.N.Vijayan Nair (Former Director, Sugarcane Breeding Institute), Dr. M. N. Premachandran and Dr. G. Hemaprabha, Division of Crop Improvement for providing necessary facilities to carry out this research work. The technical support rendered by Shri.M.Muthuraj, Shri P. Periyasamy and Dr.N.Thiraviam and facilities provided by the management and staff of USW, North Karnataka in conduct of the field trials is acknowledged.

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