Mining and characterization of genic-microsatellites associated with sucrose synthesis and their utility in population structure analysis in sugarcane

R. Vigneshwari* and R. M. Shanthi

ICAR-Sugarcane Breeding Institute, Coimbatore–641007, Tamil Nadu, India.
*E-Mail: vignesh182019@gmail.com

Abstract
Sucrose content is a highly valued trait in sugarcane breeding that is regulated by several genes. Tracking of multiple genes involved in the expression of a complex trait in a highly heterozygous crop like sugarcane during the breeding programme is a huge challenge. Trait tagging through expressed sequence tags (ESTs) derived molecular markers has been considered as a powerful tool because they are encoded by functionally relevant elements of the genome and directly linked with the target traits. Functional markers related to sucrose content are very limited in sugarcane, which hindered the genetic improvement of this trait. Seventy-three sucrose rich and fifteen low sucrose clones chosen from twelve full-sib families were used for the study on the development and characterization of genic microsatellites associated with sucrose synthesis. A total of 186 alleles were obtained, with an average of 6.20 alleles per microsatellite marker. Polymorphism Information Content (PIC) values were ranged from 0.31 to 0.83, with a mean value of 0.61. The average values of gene diversity (GD) and resolving power (RP) were 0.60 and 5.14, respectively. The present study identified genic SSR markers for four major enzymes viz., CA076844 (FK), CA155160 (SREBF), CA163325 (SREBF), TA38405-4547 (PFK), and CA135596 (DFPP) which could be useful to identify sucrose rich clones in the initial testing stages of the selection programme in sugarcane. Analysis of molecular variance (AMOVA) showed 74 per cent of the total variation within population and 26 per cent among population. The EST-SSR primers showed a high allelic polymorphism, as demonstrated by high genetic diversity estimates ($I = 1.17, H_o = 0.78$). The low mean values of fixation index ($F_{ST} = 0.078$) and the high estimates of gene flow ($N_m = 4.166$) indicates the low level of population differentiation and maximum genetic exchange between two populations. Based on SSR marker data, eighty-eight clonal accessions were classified broadly into high and low sugar population which corresponded to the molecular diversity of the sucrose specific alleles through structure analysis and principal coordinate analysis.

Key words
Sugarcane, sucrose content, genic-microsatellites, AMOVA, population structure

INTRODUCTION
Sucrose is the major end product of photosynthesis in source tissues and an important component of yield in sugarcane. Sucrose metabolic pathway enzymes, such as invertases, sucrose phosphate synthase (SPS) and sucrose synthase (SS), hexokinase (HK), and phospho-fructokinase (PFK) are differentially expressed in leaves and stalks among different genotypes, developmental stages, and cellular locations (Zhu et al., 1997). Improving sucrose yield in sugarcane can be accomplished by down/up regulation of the enzymes that are associated with sucrose biosynthesis (Chandra et al., 2012). However, QTL mapping and marker assisted breeding in sugarcane have been challenging because of their complex interspecific aneuploidy genome that comprises of 100 to 130 chromosomes (Hoarau et al., 2006). Identifying functional markers closely associated with sucrose content are well
suited for this task since they derived from gene-rich areas of genome. Microsatellite markers are considered unique and efficient compared to other marker systems because of their high degree of polymorphism, multi-allelic character, co-dominant inheritance and less expensive with the availability of public databases (Agarwal et al., 2008).

Polymorphism in the sugar specific alleles of first clonal hybrids is likely to provide vital information for sugarcane breeders to choose best parents to cross and select better progenies to evaluate in developing new cultivars. Lvena Lavanya and Hemaprabha (2012) adopted a MAS approach using a mapping population involving the high sucrose parent (CoC 671) and a low sucrose parent (BC 6) to identify STMS markers associated with sucrose content. Marker-trait associations of two STMS markers viz., NKS45\textsubscript{140} and NKS46\textsubscript{200} for sucrose content were reported in high and low sucrose commercial clones. In another study by Shanthis et al. (2016), SSR markers developed for three major enzymes involved in sucrose metabolism viz., neutral invertase (TA31929-4547\textsubscript{55,19}), pyrophosphate-dependent phosphofructokinase (TA32256-4547\textsubscript{46,53}) and cell wall invertase (AF050129\textsubscript{52}) was reported in a set of high sucrose Co canes used as parental breeding stocks in sugarcane breeding programme. In view of the above, the present study was conducted to i) develop novel genic microsatellite markers from sugarcane transcript sequences linked to sucrose synthesis ii) evaluate the polymorphic potential of these SSR markers iii) estimate the utility of these recently developed polymorphic markers through genetic structure analysis.

**MATERIALS AND METHODS**
Using the FLIP software (Brossard, 1997) available through the Organelle Genome Megasequencing Project (OGMP), microsatellite sequences containing di, tri, and tetra repeat motifs were mined in 157,190 DNA sequences deposited in Cotton Microsatellite Database (CMD) (http://www.cottonssr.org). The results showed a large number of microsatellite repeats in the mined ESTs, resulting in 16,129 SSR repeat motifs. Tri-nucleotides accounted for 53.00 per cent, followed by di-nucleotides (45.00%) and tetra-nucleotides (2.00%) (Fig. 1a). Open reading frames (ORFs) had the most microsatellite motifs (71.88%), followed by 5'UTRs (15.66%), 3'UTRs (8.24%), and introns (4.22%). There were 187 different SSR motifs found, 76 of them occurring at least four times and remaining repeats present from two to five times. On the basis of distribution frequency, top twenty repeat motifs in the coding region of genes closely linked with sucrose content were identified (Fig. 1b). Primer3 software (Rozen and Skaletsky, 1999) was used to design suitable primers from the flanking sequences, based on the following optimum criteria: primer length of 20 nucleotides (nt) (18-26 nt), melting temperature of 50°C (45-55°C), and G/C content of 50 per cent. EST-SSR details such as repeat motifs, location, primer sequence with annealing temperature (Tm) and size of alleles amplified are presented in Table 1.

Hybridization was carried out involving nineteen Indian commercial Co cane varieties (sucrose % ranged between 18.00–21.00) and the seedling progenies were raised from the fluff (hybrid sugarcane seeds) collected during 2015 flowering season. After initial screening in ground nursery (120 seedlings/cross) for H.R.brix (%), 195 genotypes performing above cross average were advanced to the first clonal selection stage in 2016-17. These selections were laid out in a randomized block design with two replications along with five checks (Co 85004, CoC 671, Co 86032, Co 99004 and Co 0403) to evaluate juice quality parameters. From this, a representative set of clones numbering 88 for sucrose per cent with different ranges (recording above 19.0% sucrose and more than 85.0% purity were categorized as high sucrose and with less than 17.0 % sucrose and below 80% purity as low sucrose) were selected and served as the mapping population for progeny survey with genic-microsatellites linked with sucrose synthesis. Juice analysis was conducted at 360 days of crop age using Sucrolyser (Anton Paar) and the following parameters viz., brix (%) sucrose (%) purity (%) and CCS (%) were recorded using five millable stalks per genotype. The list of HS and LS clones and their parentage with sucrose (at 360 days of crop age) are presented in Table 2.

The CTAB protocol was used to isolate genomic DNA from shoot apical tissue (Murray and Thompson, 1980). Spectrophotometer (ND-1000) at absorbance 260/280 nm was used to check the concentration and consistency of extracted DNA and diluted to 25 ng/μl. In a total volume of 10 μL containing 50 ng of genomic DNA, 0.33 mM dNTPs, 2.5 mM MgCl\textsubscript{2}, 0.5U of Taq DNA polymerase and 0.15 μM of primer PCR reactions were performed. PCR amplifications were carried out in a Bio-Rad thermal cycler using the following cycle profile: 95°C for 2 minutes, then 35 cycles of 94°C for 1 minute, primer melting temperature for 40 seconds, then 72°C for 40 seconds, then 72°C for 7 minutes. Silver staining was used to visualise PCR products on an eight per cent polyacrylamide gel electrophoresis. A 100 bp ladder was used to estimate band size (GeNet). Each primer’s loci were assigned using polymorphic bands, which were scored as present (1) or absent (0).

SSR amplicons were manually scored and translated into a binary data matrix, with 1 indicating the presence of alleles and 0 indicating the absence of alleles. The PIC depicts the usefulness of loci and their ability to detect genotype variations, while gene diversity (GD) refers to the likelihood that two randomly selected alleles from the population are different, was calculated using Power Marker v3.25 (Liu and Muse, 2005). The resolving power of the marker indicates the potential of SSR markers to discriminate the accessions was computed as
RP = \sum I_b, where I_b explains allele information, which can be calculated by using the formula \( I_b = 1 - (2 \times (0.5 - P)) \). P value is the proportion of the total 88 genotypes containing the allele (Prevost and Wilkinson, 1999). Using GenAIEx v. 6.503 (Peakall and Smouse 2012), allele scores of polymorphic primers were compiled to estimate AMOVA. GenAIEx v. 6.503 was also used to calculate genetic diversity estimates such as the number of different alleles (N_a), the number of effective alleles (N_e), observed (H_o) and expected heterozygosity (H_e) and Shannon’s information index (I). The population structure of the genotypes was determined using a Bayesian model-based approach with Structure software ver. 2.2 (Pritchard et al., 2000). Five independent runs were used to evaluate population clusters (K), with K values varying from 1 to 10. Each run was given a 50,000 burn-in-time and run duration of 100,000 Markov Chain Monte Carlo (MCMC) generations. The genotypes were divided into sub-clusters based on their maximum likelihood values (L_KLD). The Delta K (\( \Delta K \)) approach was used to reassure the sub-population using Structure Harvester (http://taylor0.biology.ucla.edu/structureHarvester/). The molecular diversity produced by the EST-SSR markers for each genotype was visualised using principal coordinate analysis (PCoA) using PAST software ver. 4.03 (Hammer et al., 2001).

Table 1. Newly developed and characterized EST-SSR markers for major sucrose metabolic pathway enzymes

| No. | SSR loci | Enzyme | Repeat motif | Location | Forward | Reverse | Ta °C | Number of alleles | Size (bp) |
|-----|----------|--------|--------------|----------|---------|---------|-------|-------------------|-----------|
| 1   | TA31929-4547 N1I | (GG_A) | ORF | AAATTTGGTGAGACCCAGAAG | GGCTGAGCTGCTGCTGCTG | 53 | 9 | 139 - 303 |
| 2   | TA31932-4547 N12 | (GG_A) | ORF | AAATTTGGTGAGACCCAGAAG | GGAGCAGCTCCCTGAG | 56 | 5 | 133 - 335 |
| 3   | CA268640-2 SA1 1 | (TT_C) | ORF | AATGGTTCAGAGATGGCGAC | TATTTGAGAACGACAGAG | 53 | 4 | 136 - 204 |
| 4   | CA268640-2 SA1 2 | (TT_C) | ORF | AATGGTTCAGAGATGGCGAC | TATTTGAGAACGACAGAG | 53 | 4 | 136 - 204 |
| 5   | SA16124 SA1 3 | (ATT_C) | ORF | GTTCTCAGACCTTGTGTGT | CTTTGCGCAATTGTTTGGG | 56 | 5 | 235 - 425 |
| 6   | SA16123 SA1 4 | (ATT_C) | ORF | GTTCTCAGACCTTGTGTGT | CTTTGCGCAATTGTTTGGG | 56 | 5 | 235 - 425 |
| 7   | AF050129 CW1 | (TT_C) | ORF | CGGTTTCTTTGAGGCCGGA | CTTGGATGAGCTGCTT | 51 | 4 | 153 - 224 |
| 8   | AF050129 CW2 | (TT_C) | ORF | CGGTTTCTTTGAGGCCGGA | CTTGGATGAGCTGCTT | 51 | 4 | 153 - 224 |
| 9   | TA32256-4547 PPKF 1 | (GG_C) | ORF | CTTGCTTCTCCTAATGGGCTT | GTATCCGTAGTCGCCCTCCT | 58 | 7 | 296 - 525 |
| 10  | CA172223 PPKF 2 | (AGG_A) | 5'UTR | AGCTTCTTCAAGCTG TGC | CTATTTGAGAACGACAGAG | 56 | 5 | 130 - 374 |
| 11  | CA139117 PPKF 3 | (GG_C) | ORF | CAAGAGAGATGAACGCCGAC | GACTGCTCCCAAGAGAAAC | 56 | 5 | 295 - 525 |
| 12  | TA38405-4547 PPKF 4 | (C_AC) | ORF | ATGCACTGCTTCTCTGCTGAT | CTGATCCGTAGTCGCCCTCCT | 58 | 8 | 137 - 443 |
| 13  | CA118742 PPKF5 | (AGG_A) | 5'UTR | CGGTTTCTTTGAGGCCGGA | CTTGGATGAGCTGCTT | 53 | 3 | 170 - 385 |
| 14  | CA155160 SREBF1 (GT_S) | ORF | ATCCTATCCTACACCGGCAC | CTTCTATCCTACACCGGCAC | 59 | 12 | 127 - 557 |
| 15  | CA163325 SREBF2 (GT_S) | ORF | CGTGGTGGCAATCGCACACT | CTTCTATCCTACACCGGCAC | 53 | 11 | 115 - 412 |
| 16  | CA082782 SREBF3 (GT_S) | ORF | CGTGGTGGCAATCGCACACT | CTTCTATCCTACACCGGCAC | 56 | 12 | 165 - 225 |
| 17  | CA246373 FK 1 | (CT_S) | ORF | CCCTTCTTCTCTCTCTCTCT | GCCAATCTACACACACGCTC | 56 | 11 | 127 - 593 |
| 18  | CA076844 FK 2 | (CT_S) | 5'UTR | GTTCTCTTCTCTCTCTCT | AACTACCAAACACGCTCTC | 56 | 5 | 126 - 296 |
| 19  | TA32931-4547 PFK | (AT_S) | 3'UTR | AACTCAGAAAGAGACGCTGT | CTCTAACCACGACCAACC | 60 | 4 | 127 - 187 |
| 20  | CA201178 SPS 1 | (CG_S) | ORF | CTTCCCAAGAGATGCC | CAGCTGCTCCAGTGGTTC | 50 | 6 | 135 - 499 |
| 21  | SP22998 SPS 2 | (GG_C) | ORF | AAGGTGATTTGGCCTTGTTG | TTACCACAACCAATCTCTCTC | 56 | 5 | 140 - 245 |
| 22  | SS22299 FBP1 | (GG_C) | ORF | AAAAACTGGGATGGGGCCTC | GACTTCTTCTACACCGGCAC | 60 | 6 | 258 - 330 |
| 23  | CA069535 FBP2 | (GG_C) | ORF | ATCCTAACAGATGGCCACACT | TCTTACGCTGCTGCTGCTGC | 58 | 6 | 122 - 246 |
| 24  | CA135596 DFPP 1 | (CG_A) | ORF | AAGCTACGACCAAAACACAT | GAGGATGAGCTGGTGAAGT | 60 | 10 | 117 - 349 |
| 25  | CA096869 DFPP 2 | (CG_A) | ORF | GAACCGAAGAAGAGGACGAGGAGG | CCCTCAGGACATGTGACGTA | 56 | 6 | 145 - 259 |
| 26  | CA173915 DFPP 3 | (GA_S) | 3'UTR | ACTGCCGTCGAAAGGTCGC | GGAGGATGTCGCTGACGTC | 58 | 4 | 155 - 268 |
| 27  | SS22297 SS1 | (ATT_S) | ORF | ATGTTAATTCTCCGAAAGTGG | CCTGCCGATTTCTGGAAATG | 51 | 5 | 257 - 582 |
| 28  | SS22296 SS2 | (GTT_S) | ORF | CTTGCTCTACCTTCTGCTG | GATTGCTGCTGCTGACG | 56 | 6 | 213 - 356 |
| 29  | SS02040 SS3 | (ATT_S) | 3'UTR | CACGGAGATGATGATGGTTAATGACCGGACACAGAACAGGAGGATG | CTGACGAGATGATGATGGTTAATGACCGGACACAGAACAGGAGGATG | 54 | 8 | 157 - 320 |
| 30  | TA23722-4547 SS4 | (GTT_S) | 5'UTR | GGCTTGCTGGTCTACCATCCT | ACCAACATCCACACACGAAA | 56 | 4 | 225 - 442 |

6-phospho-1-fructokinase (PFK), fructokinase-1 (FK), pyrophosphate-dependent phosphofocto-1-kinase (PPFK), alkaline/neural invertase (NI), Soluble acid invertase (SAI), D-fructose-6-phosphate 1-phosphotransferase (DFPP), Sucrose-phosphate synthase (SPS), sucrose-responsive element binding factor (SREBF), Sucrose synthase (SS)

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Fig. 1. Frequency of repeat motifs and distribution of EST derived microsatellites related to sucrose metabolic pathway enzymes. a) Frequency of major SSR motifs b) Top twenty SSR repeat motifs in sucrose related ESTs
## RESULTS AND DISCUSSION

Improvements in sucrose content were reported in SI (Sucrose Isomerase) transgenic sugarcane clones under greenhouse conditions, but not in field grown conditions (Basnayake et al., 2012). Sucrose accumulation is a multi-enzyme mediated complex trait, therefore, developing sugarcane clones with altered enzyme activity to improve sucrose yield has been more challenging (Moore et al., 1997). The results on SSR polymorphism, informativeness and its utility in genetic structure analysis corresponding to nine major enzymes of sucrose metabolism are discussed in this paper.

Sucrose is cleaved into UDG-glucose and fructose for further sugar metabolism by sucrose synthase or invertases. Fructokinases (FK) have a high affinity and substrate specificity for phosphorylating free fructose. Higher expression of FK genes reported earlier in matured...
culm tissues than in younger culm tissues implies the significance of this enzyme in sucrose accumulation (Chen et al., 2017). They also reported FK gene family (SsFRK1–SsFRK7) with seven genes in Saccharum spp. through transcriptome analysis. In this study, two primers CA246373 and CA076844 were designed for the FK1 and FK2 genes. FK1 was found to be highly polymorphic and amplified 11 SSR alleles, of which two alleles of 166 bp and 187 bp size were observed in all high sucrose genotypes otherwise absent in all low sucrose genotypes.

SREBF is a DNA binding protein and functions as repressor in the presence of high sucrose levels. Grierson et al. (1994) reported two sucrose responsive elements (SUREs) in the potato patatin promoter region. For the SREB factor, we designed three EST-SSR primers: CA155160, CA163325, and CA082782. Of these, CA155160 and CA163325 had the highest number of amplicons, with 127 bp -557 bp and 115 bp - 412 bp, respectively. CA155160 amplified a 295 bp fragment; while CA163325 amplified two SSR alleles 191 bp and 213 bp in high sugared genotypes but not in low sugared genotypes.

Pyrophosphate-dependent phosphofructokinase (PFP) is one of the key enzymes involved in the phosphorylation of fructose. PFP activity and sucrose levels were found to be negatively correlated in both commercial types and in a segregating F1 sugarcane population (Whittaker and Botha, 1999). Groenewald and Botha (2001) correlated low level of PFP activity with an increase of sucrose accumulation in transgenic sugarcane clones. Four microsatellite primers for SSR motifs found in the ORF and 3'UTR regions of PFK genes were developed. Using the primer TA38405-4547, a SSR amplicon with a size of 232 bp possibly associated with high sucrose content was established. Similarly, Merwe et al. (2010) reported a negative association between DFPP (D-fructose-6-phosphate 1-phosphotransferase) activity and sucrose concentration in immature internodes of transgenic sugarcane. For repeat motifs found in the ORF and 3'UTR regions of PFK genes were designed. The primer CA135596, which was designed to detect SSR repeat motifs on the ORF, was found to be successful in discrimination of high and low sucrose genotypes. Two SSR alleles of 178 bp and 225 bp in size were specific to high sucrose genotypes and absent in the low sucrose progenies.

As part of the present study, attempts were made to validate the recently identified markers among thirty high and eleven low sucrose commercial clones in order to enhance their applicability in identifying sucrose rich clones during early phases of selection in a sugarcane breeding programme. The analysis showed that seven transcript markers namely, CA076844137 (FK), CA155160295 (SREBF), CA163325912213 (SREBF), TA38405-4547232 (PFK) and CA135596178, 225 (DFPP) was specifically present in all the high sugared clones, while absent in the low sucrose genotypes. Thus, transcript markers developed in this present study can be used in the screening of a large seedling and clonal population in the early selection stages and also expected to improve marker-trait association of the QTLs linked with sucrose trait. Polymorphism of high and low sucrose commercial genotypes using TA38405- 4547 and CA135596 are presented in Fig. 2.

Compiled allele scores of thirty transcript SSRs detected a total of 186 alleles, of these 135 alleles (72.58%) were polymorphic. The number of amplicons per locus ranged from four to thirteen, with an average of 6.20 alleles per SSR locus. The allele size ranged from 115 bp to 582 bp. From 30 SSR markers screened, 15 markers which exhibited clear and repeatable polymorphic bands were selected for developing sucrose specific markers. Allelic diversity estimates on 15 polymorphic SSR markers are presented in Table 3. The PIC values ranged from 0.31 to 0.83, with an average of 0.61 per marker locus. The average PIC value in this investigation was similar to the previous reports of 0.87 in Saccharum hybrids and 0.86 in S. spontaneum (Ali et al., 2019). Ten microsatellite markers were found to be highly informative (PIC > 0.5), while five SSR loci moderately informative (0.5 < PIC > 0.25) as described by Botstein et al. (1980). The probability that two randomly selected alleles from the population are different is measured by gene diversity (GD). The GD ranged from 0.44 (SS22299) to 0.76 (CA163325), with a mean value of 0.60. The distribution frequency of alleles within genotypes determines the loci’s resolving power (RP). The RP ranged from 2.65 (CA172223) to 9.14 (CA163325), with a mean value of 5.14. Kalwade et al. (2012) reported a mean value of 4.20 utilizing 17 sugarcane genotypes and 27 ISSR markers, which is similar to the observation made in the present study. The number of alleles and effective alleles were in the range of 4 to 13 and 2.52 (CA268640-2) to 7.05 (CA155160) respectively. Expected heterozygosity (H_e) ranged from 0.42 (CA172223) to 0.85 (CA163325), with an average of 0.66. Further, three SSR loci namely TA38405-4547, CA163325, and CA135596 produced high value of observed heterozygosity (H_o) of 1.00. Shannon’s information index (I) ranged from 0.53 (SS22299) to 2.07 (CA155160). An average Shannon’s index (I) was calculated as 1.17, which indicated the high polymorphic potential of transcript SSRs in this study.

The average value of F_st was 0.078 and the AMOVA results revealed that it was mostly related to within population variation (74%) rather than variation among population (26%). (Table 4). Gene flow (vN_m) is used to describe the transfer of genetic variation between populations, high value of gene flow indicates the greater genetic exchange and vice versa. High gene flow estimate (4.166) obtained in this study did show an evidence for maximum genetic exchange in the studied population. In a previous investigation, which involved 139 accessions of the genus...
Fig. 2. a) Polymorphism among the high and low sucrose commercial clones/varieties using TA38405_4547 b) Marker pattern of CA135596 SSR loci among the high and low sugar clones.
Table 3. Genetic diversity estimates of fifteen polymorphic microsatellite markers

| SSR loci        | PIC | GD   | RP | N_a | N_e | H_o  | H_e  | I    |
|-----------------|-----|------|----|-----|-----|------|------|------|
| TA31932-4547    | 0.61| 0.53 | 3.26| 5   | 3.82| 0.90 | 0.73 | 1.40 |
| CA268640-2      | 0.43| 0.50 | 3.19| 4   | 2.52| 0.64 | 0.49 | 0.66 |
| CA172223        | 0.47| 0.55 | 2.65| 6   | 4.33| 0.68 | 0.42 | 0.58 |
| TA38405-4547    | 0.83| 0.74 | 8.48| 7   | 4.26| 1.00 | 0.75 | 1.53 |
| CA155160        | 0.79| 0.71 | 6.94| 13  | 7.05| 0.86 | 0.84 | 2.07 |
| CA163325        | 0.81| 0.76 | 9.14| 9   | 6.80| 1.00 | 0.85 | 2.02 |
| CA082782        | 0.69| 0.63 | 5.30| 6   | 4.89| 0.76 | 0.62 | 0.79 |
| CA246373        | 0.72| 0.74 | 7.85| 11  | 6.23| 1.00 | 0.83 | 1.92 |
| CA076844        | 0.69| 0.61 | 4.86| 5   | 3.87| 0.82 | 0.74 | 1.43 |
| TA32931-4547    | 0.42| 0.51 | 3.11| 4   | 3.82| 0.69 | 0.53 | 0.71 |
| CA135596        | 0.78| 0.70 | 8.69| 10  | 6.71| 1.00 | 0.84 | 1.99 |
| CA201178        | 0.51| 0.48 | 2.69| 6   | 4.50| 0.59 | 0.53 | 0.56 |
| SP22298         | 0.44| 0.49 | 3.19| 5   | 3.20| 0.59 | 0.47 | 0.61 |
| SS22299         | 0.31| 0.44 | 3.15| 4   | 2.56| 0.54 | 0.51 | 0.53 |
| CA069535        | 0.68| 0.62 | 4.66| 6   | 3.86| 0.71 | 0.76 | 0.87 |
| Mean            | 0.61| 0.60 | 5.14| 6.33| 4.56| 0.78 | 0.66 | 1.17 |

MAF – Major allele frequency, PIC – Polymorphic information content, GD – Gene diversity, RP – Resolving power, N_a – Number of alleles, N_e – Number of effective alleles, H_o – Observed heterozygosity, H_e – Expected heterozygosity, I - Shannon’s information index

Table 4. Analysis of molecular variance (AMOVA) for 88 sugarcane clones based on fifteen polymorphic SSR markers

| Source of variation | DF  | Sum of squares | Mean sum of squares | Estimated variance | Percentage of variation |
|---------------------|-----|----------------|---------------------|-------------------|------------------------|
| Between             | 1   | 236.345        | 236.345             | 3.517             | 26                     |
| Within              | 86  | 1006.311       | 11.701              | 28.015            | 74                     |
| Total               | 87  | 1242.656       |                     | 31.532            | 100                    |
| Fixation Index F_{ST} | 0.078 |                 |                     |                   |                        |
| Gene Flow           | Nm  | 4.166          |                     |                   |                        |

Saccharum, allied genera of Poaceae, and sugarcane cultivars, 83 per cent of the total variation was attributed to variation within the population while variation among population accounted for the remaining 17 per cent and this observation demonstrated greater exchange of gene pool across regions of origin (Singh et al., 2020).

Compiled allele scores of fifteen polymorphic SSR markers were used for population structure analysis. Bayesian model-based structure analysis, divides clones into various groups which corresponded to allelic heterogeneity in sucrose-specific genes. When we examined the distribution of genotypes from \( K = 1 \) to \( K = 10 \) using the model established by Evanno et al. (2005), the \( \Delta K \) value was relatively high when \( K = 3 \). (Fig.3a). The sub-population I (red group) had 17.04 per cent of membership proportion (15 out of the 88 genotypes) and consisted of the low sucrose genotypes. The sub-population II (green group) and III (blue group) contained the high sucrose genotypes with membership proportion of 71.59% (63 genotypes out of 88 genotypes) and 11.36 per cent (10 genotypes out of 88 genotypes), respectively. The genotypes with 0.8 per cent or more membership proportion was considered as pure and other genotypes considered as admixture, all three sub-populations I, II and III possessed lower proportion of admixture 13.33, 6.25 and 10.00 per cent (Fig.3b). Population structure analysis showed that the genotypes with high sucrose content belonged to the sub-population II and III, while most of the genotypes with the low sucrose content belonged to the sub-population I because of the extensive similarity in their allelic combination in the each estimated sub-population. In a similar study, 61 polymorphic SSR markers grouped 139 clones of the genus Saccharum and allied genera of Poaceous into three distinct sub-populations, but the sub-grouping had no significant association with the geographic origin of the region (Singh et al., 2020).
Fig. 3a. The relationship between number of $K$ and $\Delta K$.

DeltaK = mean([L*(K)]) / sd(L(K))

Fig. 3b. Assignment of 88 sugarcane genotypes into three sub-population (I, II and III) using structure. Each bar represents one genotype, the Y axis representing the proportion of each genotype genome assigned into three sub-population and the numbers at the X axis representing clones as listed in Table 2.
The results on principal coordinate analysis (PCoA) are presented in Fig. 4. A clear separation was observed between the high and low sucrose sugarcane genotypes. The first three axes explained 60.02 per cent of total variation, while the first and second coordinates each explained 48.20 per cent of total variation. The eighty-eight sugarcane genotypes were divided into two groups: the high and low sucrose (HS and LS). This demonstrates that the two populations (HS and LS) are distinct, when it comes to the molecular diversity of genes regulating sucrose metabolism as determined by the study.

Eleven distinct groups were reported in an earlier study involving 79 accessions from five species of Saccharum, six accessions from Erianthus arundinaceus and 30 Saccharum spp. hybrids using 21 polymorphic SSR primers (Ali et al., 2019).

The present study identified novel polymorphic SSR markers for four key enzymes associated with sucrose synthesis namely CA076844187 (FK), CA155160295 (SREBF), CA163325191,213 (SREBF), TA38405-4547232 (PPFK), and CA135596178, 225 (DFPP) would be a valuable tool in the marker assisted selection programme (MAS) for genetic improvement of sucrose trait. The transcript SSRs with high polymorphic potential, high value of effective number of alleles and observed heterozygosity developed in this study will be useful in the germplasm characterization and genetic diversity studies across Saccharum spp. The results on PCoA and structure analysis indicated a substantial association between sub-grouping with that of sucrose content, such genetic heterogeneity is directly correlated with allelic diversity in the QTLs influencing sucrose content, a property that could be beneficial in sugarcane breeding programmes.

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