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Enriched Mini-Genomic Library for the Development and Characterization of Simple Sequence Repeat (SSR) Markers in Sugarcane (*Saccharum* sp. Hybrids)

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Enriched mini-genomic libraries of sugarcane variety VMC 87-599 were constructed using AatII and PstI restriction enzymes. A total of 517 sequences were obtained from the combined libraries. BLASTn homology revealed that majority (49%) of the sequences had no significant similarity whereas 22% had similarity with *Saccharum officinarum*, 12% with both *Sorghum bicolor* and *Zea mays*, 2% with *Setaria italica*, 1% with *Oryza sativa*, and 2% with other grass species. Also, 3.6% of the sequences had similar gene identity that is organellar and 12.7% nuclear. Of the total sequences, 54 (4.64%) had SSRs and 63 primers were developed with 11 primers mined from genic sequences. SSR repeat motifs were predominantly tetranucleotide (51%) and trinucleotide (30%). The primer sets were used to screen 20 sugarcane accessions of which 46 primers were amplified fragments. A total of 326 alleles were detected with the mean value of 7 alleles per locus. The average PIC value of the 46 optimized primers ranged from 0 to 0.94 with a mean value of 0.73. Cluster analysis of genotypic data of 20 accessions from 46 primers revealed 5 clusters at a dissimilarity coefficient of 0.5. SSR markers designed from the enriched library. These are highly informative and can be utilized for sugarcane genetics and breeding.

Key Words: sugarcane, genomic mini-library, SSR, primer design, methylation-sensitive restriction enzymes

Abbreviations: BLAST - basic local alignment search tool, BLASTn – nucleotide-nucleotide BLAST, NCBI – National Center for Biology Information, PIC – polymorphism information content, PCR – polymerase chain reaction, SSR – simple sequence repeat

INTRODUCTION

Sugarcane is a member of the family *Poaceae* and is one of the main sources of sugar and biofuel in the Philippines. It provides a significant source of livelihood through farming, processing, and trading activities, hence, it is considered as one of the important industrial crops (Altoveros and Borromeo 2007; Fernandez and Nuthall 2009; Aragon et al. 2013). In 2015, a total volume of 25,029,000 metric tons were produced and planted in 421,000 ha over the country where production was valued at 42,413.9 million pesos (Philippine Statistics Authority 2015). The Philippines exported 150,000.48 metric tons of centrifugal sugar accounting to 107.14 million USD (Philippine Statistics Authority 2014). In bioethanol production, sugarcane and molasses are used wherein a ton of sugarcane yields roughly 60 liters of bioethanol. In 2016, bioethanol production reached 230 million liters amounting to 230 milion pesos; 50,000 metric tons were used as feedstock for bioethanol (USDA Foreign Agricultural Service 2017).

Sugarcane is a polyploid hybrid from the cross between *Saccharum officinarum* and *Saccharum spontaneum*. Production of disease-resistant and high sucrose-producing varieties is challenging due to the highly complex genome of sugarcane. In addition, most of the regions in the country do not favor its flowering. Hybridization of this complex crop is a time-consuming process, hence, the need for molecular-assisted selection of parental material. Molecular markers are currently used in plant breeding to shorten the breeding process as

well as to select individuals with desired traits. Several methods are used to identify molecular markers in different crops. One of these techniques is through genome filtration using methylation-sensitive restriction enzyme (PstI, AatII, etc.). It involves ligation of digested genomic DNA using methyl-sensitive restriction enzymes to plasmid vector and insertion of transformed pDNA into *Escherichia coli* competent cells (Whitelaw et al. 2003; Palmer et al. 2003). Libraries generated using the methyl filtration technique are sources of molecular markers that are linked to putative gene-containing DNA fragments. Among molecular markers, microsatellites or simple sequence repeat (SSR) markers have gained considerable importance in their application to plant genetics and breeding, particularly in the assessment of genetic variation among crop genotypes. Desirable attributes of microsatellite markers include hypervariability, multiallelic nature, codominant inheritance, reproducibility, relative abundance, extensive genome coverage (including organellar genomes), chromosome-specific location, amenability to automation and high throughput genotyping (Parida et al. 2009). In addition, they have high mutation rate and potentially the most informative molecular marker with the advantage of easy and low-cost detection by polymerase chain reaction (Hoshino et al. 2012). Development of SSR primers that are sugarcane-based through genome enrichment approach is of primary importance. In particular, this marker system was employed in genetic fingerprinting with subsequent applications in crop biology, such as taxonomy and phylogeny, diversity analysis, hybridity testing, gene mapping, molecular breeding and somaclonal variation (Romero et al. 2009).

In this study, a genomic mini-library of sugarcane variety VMC 87-599 was constructed using methyl-sensitive restriction enzymes to identify SSR markers. Specifically, the study screened the designed primers across 20 Philippine sugarcane varieties with a known reaction to downy mildew to identify the level of polymorphism of SSRs.

MATERIALS AND METHODS

Plant Material and Genomic DNA Isolation

VMC 87-599, a sugarcane variety with high sucrose content and downy mildew resistance, was used for the construction of an enriched microsatellite mini-library. High quality and quantity DNA was isolated using modified cetyltrimethylammonium bromide (CTAB) DNA extraction (Doyle and Doyle 1990).

Construction of Enriched Genomic Mini-library

A genomic mini-library of VMC 87-599 was constructed by genome filtering using methylation-sensitive restriction enzymes (PstI and AatII) as described by Fellers (2008) with some modifications. Sugarcane genomic DNA was digested using 10 units of each restriction enzyme. Ligation mixtures were enriched by polymerase chain reaction (PCR) using a universal primer and amplified fragments were ligated to ampicillin-resistant plasmid vector (Promega pGEM T-easy). After ligation, clones were transformed into E. coli competent cells (Promega JM109 strain) by heat-shock method. Transformed cells were grown on lysogeny broth (LB) agar plates with ampicillin and X-galactose. Transformed cells (white colonies) were subcultured in LB broth overnight. Plasmid DNA containing sugarcane DNA fragments were isolated and were sequenced using vector-based primers. Low quality base reads, vector sequence, adapters and primers were trimmed from the sequences using CLC Genomics Workbench 7.5 (Qiagen).

Characterization of the Library

Trimmed sequences were characterized through homology search against non-redundant nucleotide collection database optimized for highly similar sequences (megablast) using the nucleotide-nucleotide BLAST (BLASTn) tool of the National Center for Biology Information (NCBI) with default algorithm parameters. BLAST hit, size (base pair), query cover, percent identity and possible gene identity (for genic sequences) were obtained.

Primer Design and Evaluation

The genomic library sequences were mined for the presence of SSRs and primers were designed using BatchPrimer3 (You et al. 2008) with default logarithmic parameters. Selection of SSR primers were based on annealing temperature (T_a), GC content, primer size and lack of secondary structures. Twenty Philippine sugarcane varieties were used to investigate the level of polymorphism of the designed primers including the original genotype (VMC 87-599) from where the library was constructed as a positive control. Prior to screening, primer sets were optimized to identify the optimal PCR condition that yielded expected fragment size. Gradient PCR was performed to determine the best temperature that will amplify target motifs in the genome. Moreover, concentrations of PCR components were adjusted accordingly for specific and efficient amplification. Best conditions that gave consistent results were considered in the successive evaluation.

Table 1. Designed microsatellite primers from enriched mini-genomic libraries of sugarcane variety VMC 87-599.

Code	SSR Motif	Orientation	Primers (5' to 3')	T_M °	Size (bp)
Sach 1	$(TCGA)_3$	Forward	ATCCAAATATCCAATCAATCC	55.36	170
		Reverse	TCTCGTGTCATGTGTACTTGT	54.01	
Sach 2	$(CT)_{19}$	Forward	GTACAACTTTTGCTTCCTGTG	55.14	146
		Reverse	GGTACTCAGACCCTTGAATTT	54.9	
Sach 3	$(CAAA)_3$	Forward	TCGTAATCTCGGTCTACTTTTT	55.03	150
		Reverse	AAATTTTGGGGTGATCTAAAC	54.87	
Sach 4	$(TTC)_4$	Forward	ATGATTGATGAGACGAGATGA	55.56	202
		Reverse	AAGCACATAAACTGAACCGTA	55.11	
Sach 5	$(GAA)_4$	Forward	GAAACTTTCGCTCTACTGCAT	56.43	154
		Reverse	AACACCTGAGCTTCAGACAG	55.53	
Sach 6	$(AATA)_4$	Forward	GTAAAATGTTTTTCCCCCTTA	55.05	150
		Reverse	TGTGTCAAAAGATTTGATGTG	54.57	
Sach 7	$(AGAAA)_3$	Forward	CTTTCCCTGCAAGATTTTC	54.99	119
		Reverse	ATGATCACAGTCTGAGAAAGG	54.29	
Sach 8	$(CAGG)_3$	Forward	TCGTAGACTGCGTACAACCA	57.93	101
		Reverse	CGCGTAGACATAGCAACTAAC	55.45	
Sach 9	$(AAG)_4$	Forward	GAATTTCTACTCCACCCATCT	54.82	146
		Reverse	TTAGCTTTCTTTTGTCCCTTT	55.14	
Sach 10	$(AAAG)_6$	Forward	AAAGAGGGAATGACTTATCGT	54.6	149
		Reverse	ATTAGTGCAGTTCAAACTTGG	54.66	
Sach 11	$(TGC)_4$	Forward	CGCGGTGAGAAGAATAGATA	55.67	157
		Reverse	GGCATGCTACAGTCTACTCC	54.96	
Sach 12	$(CCAG)_3$	Forward	ATATAAAAACCACTCCCGAAC	54.9	138
		Reverse	GTTACCGGTGGTGATGAG	54.53	
Sach 13	$(CATG)_3$	Forward	AAATGTGAATTCGTAGTGACG	55.34	172
		Reverse	TCTCAAAGA-GAAGTGATCAACA	55.16	
Sach 14	$(ACTG)_3$	Forward	GGTGCTGATTTGTTATGAGAG	54.97	151
		Reverse	GAACATACTCCAGCGTTCAT	55.23	
Sach 15	$(CTC)_4$	Forward	ATATGTCAACCGAGTGTTTTG	55.18	234
		Reverse	CTGAGTTGGAGAAGAAA-GAGG	55.84	
Sach 16	$(AGGC)_3$	Forward	CCTCTTTCTTCTCCAACTCAG	55.84	168
		Reverse	GCTGCCATCTTCTTTAACTC	55.33	
Sach 17	$(GGAA)_3$	Forward	GTGTTGGAATTCATA-CAAAATG	54.71	149
		Reverse	GCATTTGACCAAGACATTTAC	54.82	

Table 1. Designed microsatellite primers from enriched mini-genomic libraries of sugarcane variety VMC 87-599.

Code	SSR Motif	Orientation	Primers (5' to 3')	T_M°	Size (bp)
Sach 18	$(CACG)_4$	Forward	TCGTTAGAACTTGCTTTTTGT	54.57	147
		Reverse	GAGATAGGCTCAATCTTGAAA	53.86	
Sach 19	$(CATG)_3$	Forward	AGCCTATCTCCTATGGCTATG	55.31	157
		Reverse	TCGTAGACTGCGTACATGAG	54.96	
Sach 20	$(AATA)_3$	Forward	TAAAATGTTGTTCCAACCATC	55.08	153
		Reverse	TCTGCTCATTTTGTTTGTTCT	55.18	
Sach 21	$(GAGGAC)_3$	Forward	GGAAGTAGAGGTGGTTCTTGT	54.96	150
		Reverse	CTGTTGTCGCTATCGTAATG	54.49	
Sach 22	$(GCA)_5$	Forward	GGCGACTAACTCTATCAACAA	54.74	156
		Reverse	TAGCAGCAAAGAGATGAATGT	55.3	
Sach 23	$(TATT)_3$	Forward	ATTTCACTGAACACGTATGCT	54.91	140
		Reverse	TTGTTTAGCTTCAAAATGGAC	54.73	
Sach 24	$(TGTA)_3$	Forward	AAGATGGTCACACTGACAAAG	55.2	165
		Reverse	AGTGATTCTGCTCGTAGACTG	54.77	
Sach 25	$(ATT)_5$	Forward	TATGAAAAGGCAGGCATACTA	55.28	143
		Reverse	GTCAACTTGTCAGAACCATGT	55.02	
Sach 26	$(CATG)_3$	Forward	GCACGTAAATCAAGTGAA-TAAA	54.8	148
		Reverse	CCACACAACTGATGAAGAGAT	55.11	
Sach 27	$(TTCTTT)_3$	Forward	TAGAAAGACACTTGGAGATGC	54.67	151
		Reverse	GGTAACGGGTTTGGAATATAA	55.7	
Sach 28	$(TTC)_4$	Forward	TTGGCAGACACCTTCTTG	55.63	159
		Reverse	GGTCAGAGCAATTTTCAACC	57.23	
Sach 29	$(TGCA)_3$	Forward	CACATGAGTTCATCCTTGAAT	55.05	162
		Reverse	ATGCACCGTACCACACATA	55.66	
Sach 30	$(GTT)_4$	Forward	CATGTCCATGTGTCAAGAA	53.35	151
		Reverse	GAAACAGCTCTGTATCCAAAA	54.7	
Sach 31	$(AGA)_4$	Forward	ACGTACGCTGTACATGACTTC	55.44	127
		Reverse	TAACAGAAAAGCAGGAACAAG	54.96	
Sach 32	$(AT)_6$	Forward	AGACTGCGTACAACGTCTATC	54.65	146
		Reverse	GGTCAGATTTAGGGTCAAAAA	55.87	
Sach 33	$(CTG)_4$	Forward	ATGTATGTCATTGTCGTGGTT	55.29	150
		Reverse	TGCTCGTAGACTGCGTACAA	58.24	
Sach 34	$(TGC)_4$	Forward	ATGAATTCGGCAATCTTGT	55.57	172
		Reverse	ATGATCACAGTCTGAGAAAGG	54.29	
Sach 35	$(CAG)_7$	Forward	GGTGGAGGACGAGATATTCTA	55.83	145
		Reverse	TGCTCGTAGACTGCGTACAT	57.11	

Table 1. Designed microsatellite primers from enriched mini-genomic libraries of sugarcane variety VMC 87-599.

Code	SSR Motif	Orientation	Primers (5' to 3')	T_M°	Size (bp)
Sach 36	$(TTGT)_3$	Forward	CCTTACTTCATGCTTCTTTGA	54.88	173
		Reverse	TACATGCAGAGAGGAAGAGAC	54.64	
Sach 37	$(AT)_6$	Forward	GCATTAGTCAGAGCATGAAAC	55.12	122
		Reverse	CTTGCTGATATAAGTGGCATC	55.1	
Sach 38	$(AAG)_4$	Forward	GAATTTCTACTCCACCCATCT	54.82	146
		Reverse	TTAGCTTTCTTTTGTCCCTTT	55.14	
Sach 39	$(TGT)_4$	Forward	TTGGGTCTGCATGCTACT	55.09	118
		Reverse	ATGCGTAGACCTAGCAACAG	55.69	
Sach 40	$(TTTC)_4$	Forward	CAAGCTCTTCCTCTCTGTTTT	55.58	143
		Reverse	CATGCAGACCCAACAGTAG	55.03	
Sach 41	$(GCCG)_3$	Forward	ATAATTAGCGGAGCACACTTT	55.83	154
		Reverse	GTGGTGAGCCTCCTCTTC	55.6	
Sach 42	$(TGGA)_3$	Forward	GAGGTTTGACTCATGGATACC	56	169
		Reverse	GAAATTTTTGCTCGTTTCC	55	
Sach 43	$(AACA)_3$	Forward	GCTTTGAATACCAGCACATAG	55.18	159
		Reverse	ATGTACGTTGTGCTCTTCCTA	55.09	
Sach 44	$(CCA)_5$	Forward	ACATTTTTCAGCTTTGTTCAC	54.61	129
		Reverse	ACCTTTCAGACCATCTGTTTC	55.79	
Sach 45	$(TGGA)_3$	Forward	CACGCACATCATGTTCATAC	55.82	138
		Reverse	GTACCTGGCCATCTCCTAC	54.51	
Sach 46	$(TTTC)_3$	Forward	GTCTCAATGCTCTGCTCTG	54.44	160
		Reverse	TCCCATCGTTGTACAGATTAC	55.1	
Sach 47	$(TTCT)_3$	Forward	CTGCATTTCTTTCTTTCTTTG	55.62	144
		Reverse	TCCCATCGTTGTACAGATTAC	55.1	
Sach 48	$(CTGCTC)_3$	Forward	GCTCGTAGACTGCGTACAA	55.11	139
		Reverse	CATCTGCAGAGGATCTCG	55.14	
Sach 49	$(ATA)_4$	Forward	CCCCTAAAAACTTAGGTCTGA	55.25	153
		Reverse	CGTTAATTTGTTAGTGCCTGT	54.72	
Sach 50	$(ATGA)_3$	Forward	TTTCTCGATGAAGGAATATGA	54.96	146
		Reverse	GCCTCTTTTCCTTGTTTATGT	55.23	
Sach 51	$(AG)_6$	Forward	GAGAGTGAAGTACCAGCAGTC	54.1	134
		Reverse	TGCGCATCCTACTACTACTTT	54.55	
Sach 52	$(TTGC)_3$	Forward	GTCACCTTTCTGACTTCACTG	54.89	127
		Reverse	TTCTAGCAGAATCCTCAAAAG	54.13	

Table 1. Designed microsatellite primers from enriched mini-genomic libraries of sugarcane variety VMC 87-599.

Code	SSR Motif	Orientation	Primers (5' to 3')	T_M°	Size (bp)
Sach 53	$(CGAA)_3$	Forward	TACCAAATACAAACGAAATGC	55.44	152
		Reverse	TAAGCTGAAACAAGCTGAAAC	55.11	
Sach 54	$(GTGCGC)_4$	Forward	CAAGTACAGCGTCGTCAGT	54.82	136
		Reverse	GGACAAACAAACAGAGAACAG	54.92	
Sach 55	$(AAAAAG)_3$	Forward	CAAGCTATGGTCTTTCTTTGA	54.88	166
		Reverse	CTAGAACTTGATGCCAAGTCA	55.64	
Sach 56	$(AAAAAG)_3$	Forward	AGGTAACGGGTTTGGAATA	54.6	146
		Reverse	AGACACTTGGAGATGCTCTTA	54.24	
Sach 57	$(GAAG)_3$	Forward	TGAAAATGGGAAAACAGACTA	54.97	150
		Reverse	AGTAGTGCTGGTGAACAGGTA	54.99	
Sach 58	$(CTA)_4$	Forward	CTATATCGCGTCTTCTAGCC	54.53	156
		Reverse	ATGAAACCCGAAAAGTCTG	55.19	
Sach 59	$(GAC)_4$	Forward	CGTAGACTGCGTACATCGT	54.8	193
		Reverse	AGATGATGATGAGGTCAAGTG	55.03	
Sach 60	$(AATT)_4$	Forward	GATCTCATCCTTGAATTTTCC	55.27	137
		Reverse	TTCCTCATTAATCATGGACTG	55.16	
Sach 61	$(ACG)_4$	Forward	CTCGTACGTGTCACCGATA	55.53	136
		Reverse	CCGCATTTATAATACGTAAGC	54.52	
Sach 62	$(CAGG)_7$	Forward	GTAGACTGCGTACAACGTCA	54.86	182
		Reverse	CCTGTATCGGTGACACGTA	55.36	
Sach 63	$(AT)_6$	Forward	CAAGAGCTGGAGATAAGTTGA	54.85	173
		Reverse	CTCGTAGACTGCGTACAACC	56.03	

PCR was carried out in a SpeedCycler (Analytikjena) with a final mix of 10 µL containing 100 ng/µL DNA, 1X PCR buffer, 1.5 mM $MgCl_2$, 0.20 mM dNTPs, 0.50 mM of each forward and reverse primers and 1 U/µL *Taq* polymerase (Invitrogen). PCR cycling consisted of initial denaturation at 94 °C for 5 min, followed by 35 cycles of denaturation at 94 °C for 10 s, annealing at 54.6–56.7 (depending on the SSR primer used) for 10 s, extension at 72 °C for 20 s, with a final extension at 72 °C for 5 min. Amplified fragments were resolved in 8% polyacrylamide gel which was run at 100V for 4 h in 1X TBE using ClearPAGE™ (C.B.S. Scientific Co., Del Mar, CA). The gel was stained with Gel Red dye and was visualized using GenoSens 1510 Gel Documentation System (Clinx Science Instruments Co., Ltd.).

SSR Characterization

The polymorphic marker of each sample was identified through clear and unambiguous bands. A numeric value of 1 was assigned for the presence of a band and 0 for absence. Allelic diversity at a given locus for a pool of genome was measured using Polymorphism Information Content (PIC). PIC values can be determined using the formula:

$$\text{PIC} = 1 - \sum_{i=1}^{h} p_i^2$$

where p_i is the frequency of the i^{th} allele out of the total number of (n) alleles at an SSR locus.

Clustering Analysis

The genetic relationship among sugarcane varieties was visualized through clustering analysis using R statistical language and environment (R Core Team 2013) based on the genetic dissimilarity values of sugarcane varieties.

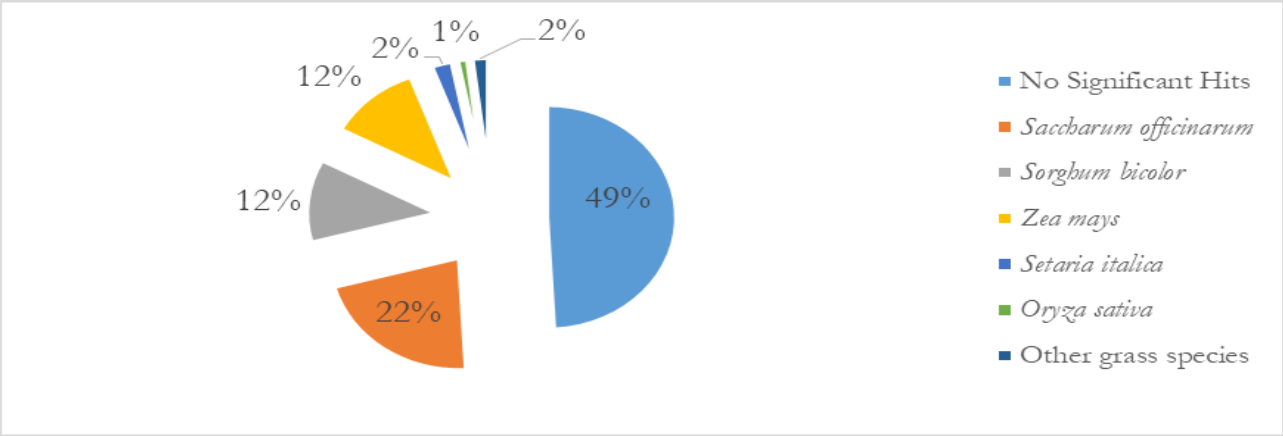

Fig. 1. Pie chart showing species distribution of BLASTn hits of sequences from AatII and PstI libraries.

RESULTS AND DISCUSSION

Genomic Mini-library Characterization

Genomic libraries of sugarcane (VMC 87-599) were constructed using methylation filtration technique to target gene-rich regions of the genome. Only a few sequences derived from this method have similarity to the database with half of the total fragments resulting in no significant alignment with known genes (Fig. 1). Twenty-two percent of the sequences aligned to *S. officinarum* with the highest number of match (105) to the sequences of *S. officinarum* cultivar R570 clone, the most used sugarcane parent in several crosses in most breeding programs of Reunion Island, Mauritius and Guadaloupe (Asnaghi et al. 2000).

Also, the libraries contained 3.6% organellar sequences; 1.9% of these are chloroplastic and 1.7% mitochondrial. Nuclear gene sequences comprised 12.7% of the total sequences and 6.9% were hypothetical/ uncharacterized proteins. The study of Fellers (2008) also presented the same trend using the hexaploid wheat cultivar, Chinese Spring, wherein organellar hits were relatively lower. This trend was also observed in corn libraries for both enzymes but higher organellar hits were observed in tobacco with an AatII library compared with a PstI library.

Primer Design and Characterization

In total, 517 quality clones from the two libraries (AatII and PstI) were obtained after sequencing and trimming. Of these sequences, 54 (4.64%) had SSRs and 63 (Table 1) primers were designed; among these, 32 (51%) were tetranucleotide, 19 (30%) trinucleotide, 7 (11%) are hexanucleotide repeats, 4 (6%) dinucleotide, and 1 (2%) were pentanucleotide repeats. Predominantly, tetranucleotide repeats of SSRs mined were AT-rich with numerous GC-rich content while trinucleotide repeats were predominantly GC-rich. The higher frequency of trinucleotide SSR repeat motifs usually corresponds to protein coding regions which are typically found in the coding region of genes (Toth et al. 2000) due to selection pressure against mutation that alter the reading frame

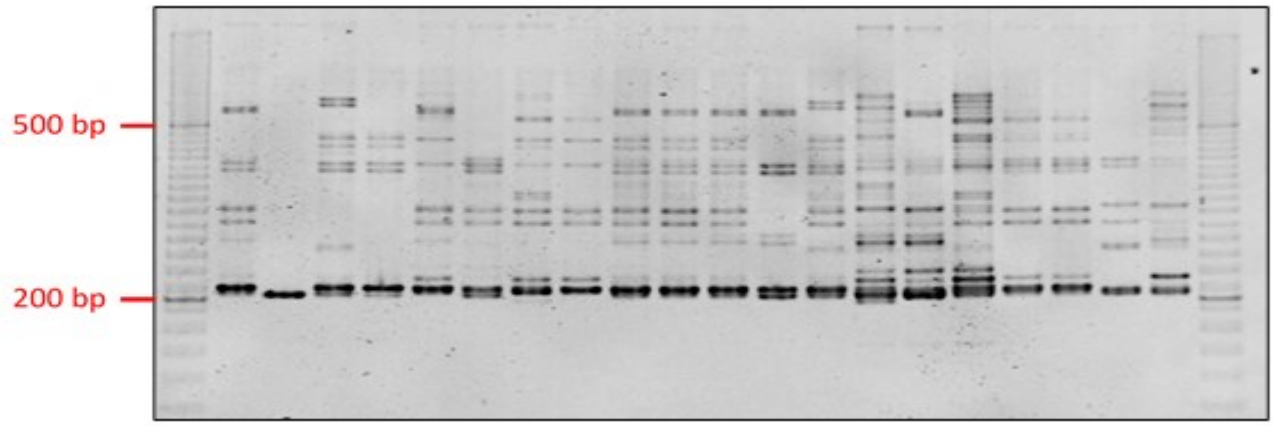

Fig. 2. Representative gels showing banding patterns generated using Sach 6 in 8% polyacrylamide gel.

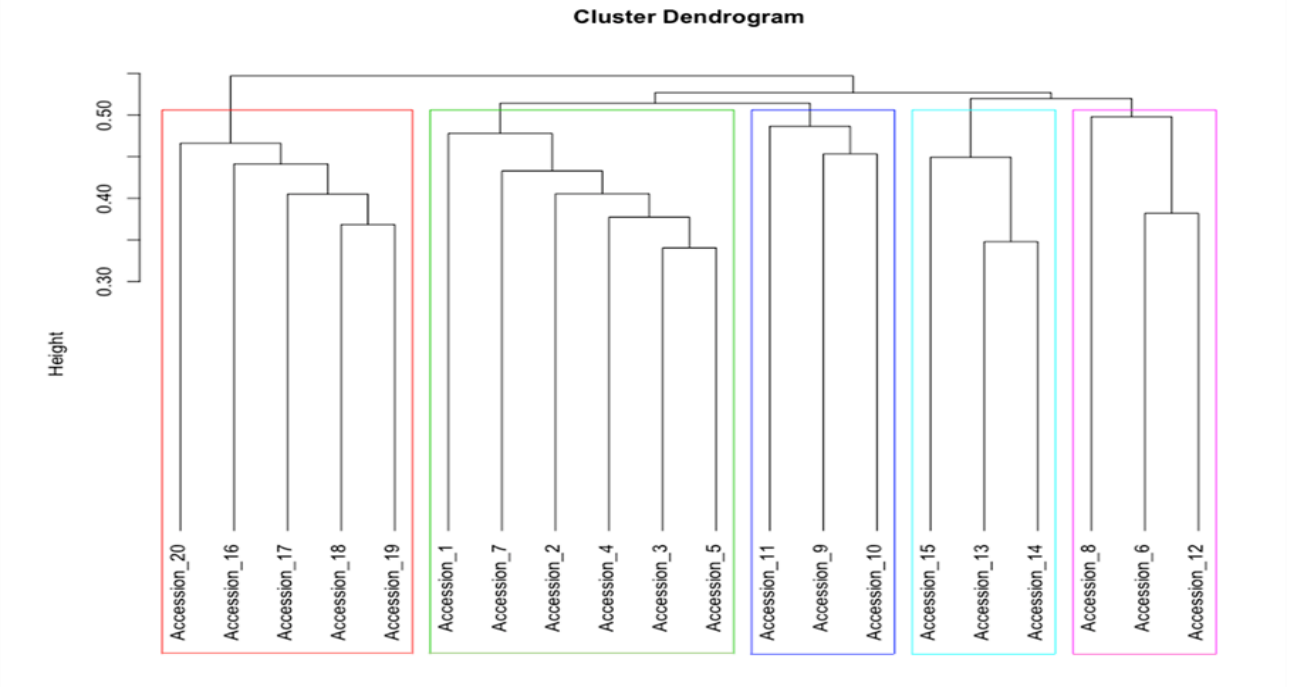

Fig. 3. Cluster dendrogram of 20 sugarcane varieties showing five clusters at 0.5 dissimilarity coefficient.

(Xu et al. 2013) and selection for certain stretches of amino acids (Morgante et al. 2002).

Primer sets were screened across 20 Philippine sugarcane accessions. Forty-six primers amplified fragments and showed polymorphism at 46 loci. Example of amplification is shown in Figure 2. The polymorphism observed in SSRs is due to the result of differences in the number of repeat motif caused by polymerase strand slippage during DNA replication (template and nascent strand mismatched) or by recombination errors (unequal crossing over, and gene conversion). New alleles at SSR loci will be formed if these mutations are not corrected by the DNA mismatch repair system. The presence of different alleles at a given SSR locus is attributed to the highly informative character of SSR markers compared with other molecular markers (Viera et al. 2016).

It was observed that the higher the number of amplified fragments of a primer, the higher its PIC value. Also, primers that have longer repeat motifs (≥ 20 nucleotides) were highly polymorphic. Specifically, Sach 6 (27 nucleotides) and Sach 12 (24 nucleotides) were highly polymorphic with PIC values of 0.93 and 0.94, respectively. This trend was also noticed in rice-based developed SSR markers using publicly available rice sequences database and sugarcane-based markers from an enriched microsatellite library (Temnykh et al. 2001; James et al. 2012). This study revealed that for all types of repeat motifs, the polymorphic SSRs had a longer length compared with the non-polymorphic SSRs. Cordeiro et al. (2001) noted that SSRs derived from enriched genomic libraries are less transferable to other genera such as Erianthus and Sorghum because of their presence in more conserved transcribed regions of the genome and are expected to be less polymorphic within the species they were derived from but this study revealed that SSRs derived from genomic libraries still show higher polymorphism and were able to discriminate different genotypes.

Cluster Analysis

The 46 optimized primers were used to assess the genetic relationships among 20 selected sugarcane accessions. Optimized primers revealed moderately high degree of polymorphism (average PIC of 0.67) and were able to discriminate sugarcane accessions. Employing R, a language and environment for statistical computing and graphics for clustering analysis revealed four clusters at a dissimilarity coefficient of 0.5 (Fig. 3). Clusters 1 and 2 include five and six accessions, respectively, while clusters 2, 3 and 5 include three accessions. Accessions that grouped together are genetically similar and distantly related with the accessions of the other group. The results of the clustering can be applied in parental

Table 2. Simple sequence repeat (SSR) primers screened across 20 sugarcane accessions.

Primer	Repeat Type	Annealing Temperature	Expected Band Size	No. of Alleles	Polymorphism Information Content (PIC)	Polymorphism
Sach1	(TCGA)3	54.6	170	13	0.91	Y
Sach2	(CT)19	55.3	146	15	0.88	Y
Sach3	(CAAA)3	54.7	150	5	0.48	Y
Sach4	(TTC)4	55.3	202	8	0.73	Y
Sach5	(GAA)4	55.3	154	7	0.77	Y
Sach6	(AATA)4	55	150	27	0.93	Y
Sach7	(AGAAA)3	55.5	119	9	0.82	Y
Sach8	(CAGG)3	54.7	101	4	0.33	Y
Sach9	(AAG)4	54	146	7	0.63	Y
Sach10	(AAAG)6	-	149	-	-	N
Sach11	(TGC)4	-	157	-	-	N
Sach12	(CCAG)3	57	138	23	0.94	Y
Sach13	(CATG)3	52	172	-	-	N
Sach14	(ACTG)3	52.1	151	12	0.87	Y
Sach15	(CTC)4	55	234	7	0.99	Y
Sach16	(AGGC)3	50.8	168	4	0.62	Y
Sach17	(GGAA)3	-	149	-	-	Y
Sach18	(CACG)4	-	147	-	-	Y
Sach19	(CATG)3	54.7	157	6	0.83	Y
Sach20	(AATA)3	52.1	153	13	0.9	Y
Sach21	(GAGGAC)3	55	150	10	0.88	Y
Sach22	(GCA)5	56.4	156	7	0.78	Y
Sach23	(TATT)3	55.4	140	8	0.72	Y
Sach24	(TGTA)3	-	165	-	-	N
Sach25	(ATT)5	55.3	143	4	0.6	Y
Sach26	(CATG)3	56	148	4	0.68	Y
Sach27	(TTCTTT)3	54.8	151	1	0	N
Sach28	(TTC)4	55.2	159	7	0.75	Y
Sach29	(TGCA)3	55.3	162	10	0.85	Y
Sach30	(GTT)4	-	151	-	-	N
Sach31	(AGA)4	-	127	-	-	N
Sach32	(AT)6	54.6	146	1	0.58	Y
Sach33	(CTG)4	56.5	150	3	0.54	Y
Sach34	(TGC)4	56.7	172	4	0.55	Y
Sach35	(CAG)7	-	145	-	-	
Sach36	(TTGT)3	55.1	173	6	0.66	Y

Table 2. Simple sequence repeat (SSR) primers screened across 20 sugarcane accessions.

Primer	Repeat Type	Annealing Temperature	Expected Band Size	No. of Alleles	Polymorphism Information Content (PIC)	Polymorphism
Sach37	(AT)6	54.7	122	10	0.82	Y
Sach38	(AAG)4	55	146	5	0.63	Y
Sach39	(TGT)4	55.5	118	2	0.36	N
Sach40	(TTTC)4	55.5	143	9	0.82	Y
Sach41	(GCCG)3	56	154	5	0.72	Y
Sach42	(TGGA)3	55.5	169	3	0.57	Y
Sach43	(AACA)3	55	159	3	0.25	N
Sach44	(CCA)5	-	129	-	-	N
Sach45	(TGGA)3	55.5	138	4	0.67	Y
Sach46	(TTTC)3	54.5	160	5	0.74	Y
Sach47	(TTCT)3	-	144	-	-	N
Sach48	(CTGCTC)3	55	139	14	0.88	Y
Sach49	(ATA)4	55	153	5	0.69	Y
Sach50	(ATGA)3	55	146	3	0.38	N
Sach51	(AG)6	54.5	134	8	0.86	Y
Sach52	(TTGC)3	54.5	127	2	0.44	N
Sach53	(CGAA)3	-	152	-	-	N
Sach54	(GTGCGC)4	55	136	10	0.85	Y
Sach55	(AAAAAG)3	55.5	166	3	0.39	N
Sach56	(AAAAAG)3	54.5	146	3	0.66	Y
Sach57	(GAAG)3	55	150	7	0.8	Y
Sach58	(CTA)4	-	156	-	-	N
Sach59	(GAC)4	-	193	-	-	N
Sach60	(AATT)4	-	137	-	-	N
Sach61	(ACG)4	-	136	-	-	N
Sach62	(CAGG)7	-	182	-	-	N
Sach63	(AT)6	-	173	-	-	N
Mean				7	0.67	

selection for hybrid breeding in which parentals are selected from different clusters. The result of the clustering analysis suggests that newly developed SSRs employed in genotyping could discriminate 20 sugarcane accessions. Forty-six primers successfully discriminated 20 sugarcane accessions implying that these markers were utilizable in varietal genotyping. Several studies employed highly polymorphic markers in various practical sugarcane breeding applications including germplasm evaluation (genetic diversity assessment) (Cordeiro et al. 2003; Fu et al. 2016), variety identity testing (Liu et al. 2011), and cross-transferable studies (Cordeiro et al. 2001; Singh et al. 2013).

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