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SHORT COMMUNICATION

Development of Microsatellite Markers from Enriched Genomic Library of ISH 100 (*Saccharum* hybrid) for Genetic Variability of Sugarcane

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Microsatellite primers were developed from the interspecific hybrid ISH 100 of sugarcane to study patterns of population genetic structure across the species. About 26 SSR primers were screened, out of which 13 exhibited polymorphism (50%). These 13 SSR primers were assessed on 8 individuals sampled from one population. The number of alleles per locus ranged from 2 to 6 and observed heterozygosity ranged from 0.16 to 0.60. The genetic diversity among the population depicted from this study can be used for genetic diversity analysis of sugarcane.

Key Words: ISH 100, Microsatellite, Population genetic structure, Sugarcane

Sugarcane is a monocotyledonous crop plant that is cultivated in the tropical and sub-tropical regions of the world, primarily for its ability to store high concentration of sucrose in the internodes of the stem. In 2010, Food and Agricultural Organization of the United Nations estimated that sugarcane is cultivated in about 23.8 m ha, in more than 90 countries with a worldwide harvest of 1.69 billion tons. Modern sugarcane varieties cultivated for sugar production are complex interspecific hybrids (*Saccharum* spp.) that have arisen through intensive selective breeding of species within the genus *Saccharum*, primarily involving crosses between the species *S. officinarum* L. and S. *spontaneum* L. (Cox *et al*., 2000). Commercial hybrid cultivars of sugarcane descended from interspecific hybridization between *S. officinarum* and *S. spontaneum* (Banumathi, 2010). ISH 100 is an interbreed of Co7202 {CoC671 × (*S. officinarum* (57NG110) × *S. robustum*)}. Genetic make-up of ISH 100 makes it an ideal candidate for phytogeographic and population genetic diversity analysis. This hybrid has good representation of different genomes. In sugarcane a number of microsatellite markers are available but the density of microsatellite and other markers in available microsatellite maps is still not adequate. Compared to other crops, sugarcane breeding programs have historically been slow in progress because of the low efficiency and technical difficulties in crossing and selection processes (Chen *et al*., 2009). Therefore it is important to develop large number of useful polymorphic microsatellite markers (Singh *et al*., 2013). Thus, in the present study microsatellite markers were developed from ISH 100 and were tested for polymorphism, on parents of mapping population.

Genomic DNA of ISH 100 was extracted from fresh leaves, using CTAB method (Hoisington *et al*., 1994) and was digested using *Eco*R1. The recombinant plasmids were produced by ligating restriction fragments from *Saccharum* DNA into the *Hin*d III site of pUC19 plasmid. The fragments were enriched for microsatellite motifs CA, GA, ATG and TAG prior to ligation. Ligated products were introduced into *E. coli* strain DH5α (Electro Max J, Invitrogen) by electroporation. 2 µl of ligation mix was used for each of the libraries. After transformation and recovery incubation in SOC broth (Invitrogen), glycerol was added to a level of 20% of the final volume. Libraries were stored at approximately -70^{o}C. To isolate colonies for sequencing, cells from the glycerol stock were spread on X-gal/IPTG/ampicillin-LB agar plates. The capture protocol sometimes results in inserts that were less than 300 bp long. These smaller inserts were less likely to have sequences flanking a microsatellite that were long enough for primer design. Additionally, non-recombinants did not develop blue precipitate fully, and it was possible to select two colonies simultaneously if they were not well separated on the plate. Sterilized toothpicks were used to transfer white

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colonies from the spread stock plates on X-gal/IPTG/ ampicillin LB plate. The plate was incubated overnight, and colonies were selected from this plate. Some colonies have subsequently developed faint blue pigmentation; that generally contained recombinant plasmid with an insert of proper size.

The DNA fragments were cloned into the *Hin*d III (AAGCTT) cut site of the pUC19 plasmid. To obtain desired sequences Primer A (forward): 5'- AGG AAA CAG CTA TGA CCA TG -3'. Primer B (reverse): 5'- ACG ACG TTG TAA AAC GAC GG -3'were found to be effective. Typical annealing temperature for these primers was 57^{o}C; they also worked well at 58^{o}C. The clones from which sequences obtained were identified, were prepared for overnight culturing and subsequent mini-prepping. Cultures were grown overnight at 37^{o}C in a 96-deep well plates using 2X LB broth. Purification of plasmid DNA was accomplished using Millipore MultiScreen MAFB NOB Plates. DNA sequencing was accomplished using Applied Biosystems's Big Dye Terminator v3.1 Sequencing Standard Kit, followed by electrophoresis on an Applied BioSystems Model 3730 DNA Sequencer.

Once appropriate microsatellites were found, PCR primers were designed from the unique flanking regions using Designer PCR, version 1.03 (Research Genetics, Inc.). To test the primer, PCR reaction comprised : initial denaturation at 94^{o}C for 3 minutes, denaturation at 94^{o}C for 40 seconds, annealing at 55-57^{o}C for 40 seconds, extension at 72^{o}C for 30 seconds and final extension at 72^{o}C for 4 minutes. Initially products were separated on 3% agarose gels stained with ethidium bromide to check amplification.

Twenty six primer pairs (Table 1) were found to consistently amplify PCR products of expected size and were selected for fragment analysis. These 26 loci were amplified as above and products were visualized using an Applied Biosystems Avegene. Of the 26 loci checked, one failed to produce consistent results and 12 were monomorphic across the samples. The remaining 13 loci were used to assay 8 genotypes of sugarcane. SSR markers were scored on the basis of their band size, either present (1) or absent (0) for each SSR loci. Polymorphic information content (PIC) value was estimated with Power Marker v3.23 (Liu *et al*., 2005). The calculations of population genetic parameters, including alleles, observed and expected heterozygosity between the two populations, were estimated with the program POPGENE1.32 (Tech, 2000). The number of alleles per locus ranged from 2 to 6 with an average of 3.69 (Table 2). The expected and observed heterozygosity ranged from 0.40 to 0.83 and from 0.16 to 0.60, respectively (Table 2).

Thus, in the present investigation 13 SSR markers were isolated and characterized. The markers identified from ISH 100 can be used for mapping population, gene tagging and genetic diversity analysis. The ISH 100 was developed from a cross of S. *officinarum* and

Table 1. Characteristics of 13 microsatellite loci developed from inter-specific hybrid (ISH 100) of sugarcane

S.No	Primer	Forward sequence	Reverse sequence	Fragment size (bp)	Repeats motif	Annealing temp. (Ta) ^{o}C	Gene bank ID
1	A8	TATGGAGAGAGCAACCTATCA	GACGGAAGATTGGGATTC	220-610	(CA)25	56.9	28374217
2	A12A	TCAAAGTGGCTACAGAATAGGT	CAGCAAGGTTCCAAGTACC	170-310	(CA)15	56.7	28374214
3	A119	CCTATCGAATTGTGCTACTC	GCATGTGTATTGTGTTAGAGAA	118-180	(CA)16	54.8	28374210
4	A132	GGCCTTCGATTAACCGAT	ACAGGACGCTGCTTCTTG	194-310	(GT)17	57.7	28374215
5	B105	GGTGGCTAACAGACAGGG	TTGCTGCCGAGAGTCATA	118-500	(CT)16	56.9	28374220
6	B107	TTATCCCTTTCGTTCAGTAGAG	ATTTTGCGTAGGGTCTGAG	281-390	(CT)26	57.3	28374222
7	B112	TTATTTGTCCAACCTGCTTCTG	CATGGATGCTTTTGCGTTAG	118-190	(CT)22	56.9	28374225
8	B119	CACCCAGCAGTTATTGGA	CAGCAATCAAGTGTTCACTG	194-380	(CA)7,(CT)11	56.6	28374228
9	B126	ACCACCACCACTTTGTCTT	GGATTGCTAAAGCATTGGT	281-400	(CT)25	57.3	28374233
10	C1	ACCACCACCACTTTGTCTT	CGTGAGAAGGTAGGGAAACA	118-194	(ATC)13	56.7	28374240
11	C9	CCAAACCACATTGTAGCAG	CTTCTTGTCATCATCACTTGAG	194-234	(GAA)7	56.6	28374252
12	C123	GCGCCTATTTAATACCAGA	CTTTCCCTATACCCATGATAG	194-281	(ATC)12	56	28374247
13	C130	AAGGGAAGAGCAGGAGAG	CGGGAGGTCAAAATGTTA	234-281	(ATG)13	56.8	28374248

Table 2. Genetic properties of the newly developed 13 microsatellites of inter-specific hybrid (ISH 100) of sugarcane

Primer	Number of alleles	Expected heterozygosity (H_O)	Observed heterozygosity (H_E)	Inbreeding coefficient (F_{IS})	Polymorphism information content (PIC)
A8	5	0.19	0.8	0.56	0.33
A12A	5	0.18	0.81	0.35	0.44
A119	3	0.41	0.58	0.73	0.39
A132	4	0.45	0.54	0.13	0.30
B105	4	0.36	0.63	0.4	0.38
B107	2	0.46	0.53	-0.42	0.32
B112	4	0.27	0.72	0.26	0.42
B119	6	0.16	0.83	0.37	0.40
B126	3	0.3	0.69	0.39	0.43
C1	3	0.35	0.64	0.45	0.43
C9	2	0.6	0.4	1	0.37
C123	4	0.23	0.76	0.17	0.51
C130	3	0.37	0.62	0.81	0.39

S. spontaneum and the F_1 of this cross was again crossed with two of the commercial hybrids having representation of different species of sugarcane. The ISH 100 was studied extensively in our laboratory at molecular cytogenetic level using genomic *in situ* hybridization technique (unpublished data) and we have observed that this hybrid has the chromosomes of different species of sugarcane as *S. spontaneum, S. officinarum, S. robustum* and *S. sinense*. The hybrid has a good representation of different genomes and therefore, the markers developed from ISH 100 may be utilized across different species of sugarcane for genetic diversity analysis.

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