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Molecular Characterization of Six Sugarcane Varieties Using Microsatellite Markers

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ABSTRACT

The experiment was carried out at the Biotechnology Laboratory, Bangladesh Sugarcane Research Institute (BSRI), Ishurdi, Pabna, Bangladesh in the cropping season 2006-2007. Six BSRI released sugarcane varieties (Isd 16, Isd 20, Isd 28, Isd 29, Isd 30 and Isd 31) and three microsatellite primers (SMC119CG, SMC319CG and SMC334BS) were used for the investigation. All the three microsatellite primer pairs amplified a total number of 34 bands from the six varieties through 8.0% polyacrylamide gel electrophoresis. The total number of bands scored and number of polymorphic bands varied from 7 to 15 and 7 to 11 respectively. The same percentage (100.0%) of polymorphism was recorded from the primer pair SMC119CG which produced the lowest number of total bands(7) followed by primer pair SMC334BS(75.0%) and SMC319CG(73.3%). The sizes of the amplified bands in the six varieties ranged from 80 to 200 bp. The most polymorphic marker was SMC119CG. DNA polymorphism based on microsatellite markers showed the higher linkage distance of the sugarcane varieties Isd 16 and Isd 20 from other varieties investigated. Results of this investigation construct the genetic fingerprinting and molecular characterization of six sugarcane varieties based on microsatellite markers which coincided with field performances of these varieties.

Key words: Sugarcane, molecular characterization, microsatellite marker

INTRODUCTION

Characterization of sugarcane germplasm as well as released varieties based on agronomical and morphological traits are being practiced since initiation of sugarcane breeding. Phenotypic differences may also reveal genetic differences. Theoretically, phenotypic diversity should approximate genetic diversity. The number of genes involved in the control of phenotypic traits increases as the number of phenotypic traits being evaluated increases. Consequently, it improves the utility of phenotypic diversity in predicting genotypic diversity. Genetic relationships among varieties and populations can be measured by similarity of any number of phenotypic characters. Differences between the characters are assumed to reflect the genetic divergence of the genotypes. However, characterization of varieties based on agronomic and morphological traits is subjective, labour intensive and can be influenced by genotype and environment interactions. Molecular markers are valuable tools in plant breeding programme as well as for evolutionary and conservation studies. They are almost unlimited in number and are not

influenced by environment. Microsatellite markers also known as Simple Sequence Repeats (SSRs) are molecular markers based on tandem repeats of short (2-6bp) DNA sequences (Litt and Lutty, 1989). These DNA sequences are highly polymorphic even among closely related varieties due to mutation causing variation in the number of repeating units (Saghai-Maroof *et al.*, 1994). Microsatellites can be analyzed by a rapid, technically simple and inexpensive polymerase chain reaction (PCR) based assay that requires only small DNA quantities. Through PCR, different alleles at a locus can be detected by using conserved DNA sequences flanking the microsattelite as primer. Microsattelite markers are co-dominant and can be transmitted in simple Mendilian segregation. Lastly microsatellits are abundant and uniformly distributed in plant genomes (Lagererantz *et al.*, 1993; Wang *et al.*, 1994; Akkaya *et al.*, 1995). At Bangladesh Sugarcane Research Institute, germplasm characterization based on agronomic and morphological traits has been initiated. Varieties are being released based on agronomic and morphological traits. Molecular characterization using RAPD markers have been started recently. Therefore, this investigation was undertaken to characterize selected six sugarcane varieties through fingerprinting using microsatellite markers.

MATERIALS AND METHODS

The experiment was carried out at the Biotechnology Laboratory, Bangladesh Sugarcane Research Institute (BSRI), Ishurdi, Pabna, Bangladesh in the cropping season 2006-2007.

Plant materials and sample collection: Six BSRI released sugarcane varieties (Isd 16, Isd 20, Isd 28, Isd 29, Isd 30 and Isd 31) were used for DNA isolation. The fresh tops from the 8 month old field grown sugarcane were collected and outer leaf sheaths were removed leaving inner spindle to get meristem cylinder. Then the meristem cylinder (spindle base) was cut down into small pieces (about 5 mm long with 3 mm dia) with sterile scissors and weighed out the required amount (0.2g) using a fine balance. Care was taken during sampling from injury of meristem cylinder after removing of outer leaf sheaths.

DNA isolation: A modified method of Aljanbi *et al.* (1999) were used to isolate total genomic DNA. Cut pieces of meristem cylinder (0.2g) was taken in small mortar (dia 6.5cm and 3.5cm depth) homogenized with pestle in 800 μl of extraction buffer (200mM Tris HCl, pH 8.0; 50mM EDTA.H_2O, pH 8.0; 2.2M NaCl; 2% CTAB; 0.06% Sodium Sulfite) until finely shredded within 40-50 second. Total grinded sample was taken into 2 ml eppendorf tube, then added 150μl of each 5% SDS, 10% PVP, 20% CTAB were mixed well quickly and incubated at 65 °C at water bath for 40 minutes. During incubation 3-4 times inversion was done. After incubation the samples were cooled to room temperature and equal volume of Phenol t Chloroform t Isoamyl Alcohol (25 t 24 t 1) was added, mixed well by inversion and centrifuged at 10,000 rpm at room temperature for 30 minutes. Then aqueous phase (about 800 μl) was recovered and transferred to a fresh ice-cold 2ml eppendorf tube and an equal volume of ice-cold isopropanol was added followed by 120μl of 6M NaCl. The samples were incubated at -20 °C for at least 1hr and centrifuged at 10,000 rpm at room temperature for 20 minutes. The upper layer of solution was discarded

carefully by using adjustable micropipette and 70% ice-cold ethanol of about 2.5 times of the solution was added. The same was again centrifuged at 10,000 rpm at room temperature for 10 minutes. After pellet formation the solution was discarded from the tube carefully so that the DNA pellet remains constant and undisturbed. Then 70% ethanol was added up to the slant of the tube. The ethanol was discarded from the tube carefully so that the DNA pellet remains constant and undisturbed. The DNA pellet was dried for at least 30 minutes by putting the tubes upside down on a filter paper. Then the DNA pellet was re-suspended in 50μl of TE buffer (10mM Tris HCl, pH 8.0; 1mM EDTA.H_2O pH 8.0) and stored at -20°C for subsequent use.

Primers used: Three selected sugarcane microsatellite primers (markers) developed by International Sugarcane Microsatellits Consortium were used to amplify simple sequence repeats of genomic DNA from six sugarcane varieties. These primers are SMC119CG, SMC319CG and SMC334BS (Table 1). Primers were evaluated on the basis of intensity or resolution of bands, repeatability of markers and consistency within individual and potential to differentiate varieties (polymorphism).

Table 1. Parameters of primers sequences of three sugarcane microsatellite primers from the International Sugarcane Microsatellite Consortium.

Primer Code	Sequence ($5^{/}$-$3^{/}$)	G+C Content (%)
SMC119CG	Forward: -TTC ATC TCT AGC CTA CCC CAA-	47.61
	Reverse: -AGC AGC CAT TTA CCC AGG A-	52.63
SMC319CG	Forward: -CCT TTC ATC CAC CGA GGA CAA-	52.38
	Reverse: -GGT TCA CCG AAG CAA GAG AAC-	52.38
SMC334BS	Forward: -CAA TTC TGA CCG TGC AAA GAT-	45.85
	Reverse: -CGA TGA GCT TGA TTG CGA ATG-	47.61

PCR amplification and electrophoresis: PCR amplification was done in an oil-free thermal cycler (Master Cycler Gradient, Eppendorf) following the PCR profile of 94 ºC for 3 minutes (initial denaturation) followed by 25 cycles of 45 seconds denaturation at 94 ºC, 30 second annealing at 57 ºC and elongation or extension at 73 ºC for 30 seconds. After the last cycle, a final step of 3 minutes at 73 ºC was added to allow complete extension of all amplified fragments. After completion of cycling programme, reactions were held at 4 ºC. The PCR reactions were performed on each DNA sample in a 10μl reaction mixture containing 1.0μl of 10x Ampli Taq polymerase buffer (PCR buffer), 0.3μl of 50mM $MgCl_2$, 0.8μl of 2.5mM DNTPs, 1.0μl each of Primer Forward and Reverse from 2.0μM stock, 0.2μl of 5U/μl Ampli Taq DNA polymerase (Bangalore Genei Pvt. Ltd., India), 3.0μl of 5ng/μl genomic DNA and a suitable amount (2.7μl) of sterile deionized water. After amplification 2.0μl loading dye was added to the amplification product and stored at 4ºC for separation using polyacrylamide gel electrophoresis. In each well 1.5μl of PCR product of each DNA

sample for each primer was loaded in 8% polyacrylamide gel (acrylamide and biacrylamide of SRL, India). Electrophoresis was performed at 50V for 2.5 hours. DNA ladder 100bp (Bangalore Genei Pvt. Ltd., India) was run alongside the reactions. The gel after electrophoresis was silver stained and dried at room temperature. The DNA bands were observed on white light box and photographed by digital camera.

Data analysis: All distinct bands or fragments were thereby given identification numbers according to their position on the gel and scored visually on the basis of their presence (1) or absence (0), separately for each variety and each primer. The scores obtained using all primers were then combined to create a single data matrix. This was used for estimating linkage distance (D) among the varieties using computer programme, Statistica.

RESULTS AND DISCUSSION

All the three microsatellite primer pairs used amplified a total of 34 bands from the six varieties in 8.0% polyacrylamide gel electrophoresis (PAGE). One of the representative electrophoregram is shown in Figure 1. The total number of bands scored and number of polymorphic bands varied from 7 to 15 and 7 to 11 respectively (Table 2). The primer pair SMC319CG identified the highest number of bands(15) followed by SMC334BS(12). The lowest number of bands(7) scored from the primer pair SMNC119CG. The highest number of polymorphic bands(11) was scored from the primer pair SMC319CG followed by SMC334BS(9) and SMC119CG(7). However, the same percentage(100.0%) of polymorphism was recorded from the primer pair SMC119CG which produced the lowest number of total bands(7) followed by primer pair SMC334BS(75.0%) and SMC319CG(73.3%). The sizes of the amplified bands in the six varieties ranged from 80 to 200 bp (Table 2). Microsatellite primer pair SMC119CG revealed band sizes that ranged from 89bp to 124bp; from 150bp to 200bp for locus SMC319CG and band sizes ranging from 80bp to 184bp for SMC334BS.

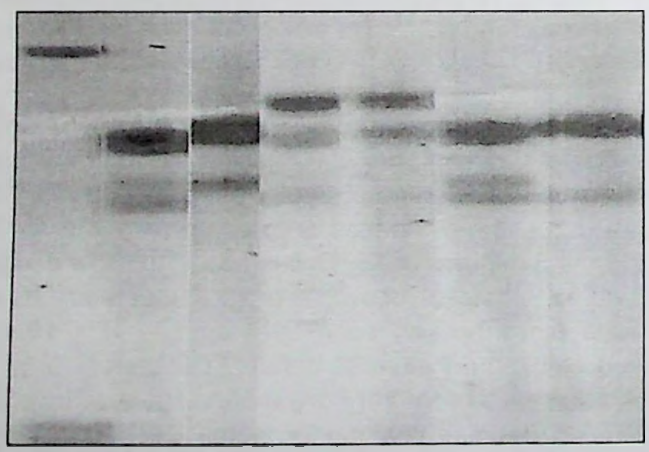

Figure 1. DNA Fingerprinting of BSRI released six varieties of sugarcane based on SSR primer pair SMC319CG through PAGE (M= marker 100bp ladder, Lane 1= Isd 16, Lane 2= Isd 20, Lane 3= Isd 28, Lane 4= Isd 29, Lane 5= Isd 30, Lane 6= Isd 31).

Table 2. Microsatellite primers with corresponding bands scored and their size range together with polymorphic bands in six sugarcane varieties.

Primer codes	Size ranges (bp)	Total number of bands scored	Number of polymorphic bands	Polymorphism (%)
SMC119CG	89-124	7	7	100.0
SMC319CG	150-200	15	11	73.3
SMC334BS	80-184	12	9	75.0
Total bands scored		38	27	-

The data of linkage distances between varieties were computed and combined data for the three primers and six varieties are shown in the Table 3. Linkage distances of variety Isd 16 from varieties Isd 20, Isd 28, Isd 29, Isd30 and Isd 31 were 14.0, 11.0, 12.0, 12.0 and 13.0; of variety Isd 20 from varieties Isd 16, Isd 28, Isd 29, Isd 30 and Isd 31 were 14.0, 9.0, 12.0, 10.0 and 13.0; of variety Isd 28 from varieties Isd 16, Isd 20, Isd 29, Isd 30 and Isd 31 were 11.0, 9.0, 7.0, 7.0 and 10.0; of variety Isd 29 from varieties Isd 16, Isd 20, Isd 28, Isd 30 and Isd 31 were 12.0, 12.0, 7.0, 10.0 and 11.0; of variety Isd 30 from varieties Isd 16, Isd 20, Isd 28, Isd 29 and Isd 31 were 12.0, 10.0, 7.0, 10.0 and 9.0; of variety Isd 31 from varieties Isd 16, Isd 20, Isd 28, Isd 29 and Isd 30 were 13.0, 13.0, 10.0, 11.0 and 9.0 respectively. The highest linkage distance (14.0) was recorded between the varieties Isd 16 and Isd 20 from other varieties investigated. The similar linkage distance (12.0) was recorded between variety pairs Isd 16 and Isd 29, Isd 16 and Isd 30, Isd 20 and Isd 29. The lowest linkage distance (7.0) was observed between variety pairs Isd 28 and Isd 29, Isd 28 and Isd 30. The average linkage distances of varieties Isd 16, Isd 20, Isd 28, Isd 29, Isd 30 and Isd 31 from others varieties were 10.33, 9.67, 7.33, 8.67, 8.00 and 9.33 respectively.

Table 3. Summary of linkage distances for different pairs of sugarcane varieties.

Variety	Isd 16	Isd 20	Isd 28	Isd 29	Isd 30	Isd 31	Average
Isd 16	0.0	14.0	11.0	12.0	12.0	13.0	10.33
Isd 20	14.0	0.0	9.0	12.0	10.0	13.0	9.67
Isd 28	11.0	9.0	0.0	7.0	7.0	10.0	7.33
Isd 29	12.0	12.0	7.0	0.0	10.0	11.0	8.67
Isd 30	12.0	10.0	7.0	10.0	0.0	9.0	8.00
Isd 31	13.0	13.0	10.0	11.0	9.0	0.0	9.33

The variety Isd 16 is one of the best and sustainable variety among the release varieties of BSRI. Molecular characterization based on DNA polymorphism of six varieties showed the highest average linkage distance of variety Isd 16 from other varieties investigated. The result agreed with the findings of Shahnawaz (2006). He also analyzed DNA polymorphism of four varieties/accessions using RAPD markers and found that the

linkage distance of variety Isd 16 was the highest from other varieties. Another variety Isd 20 having the superior performances against biotic and abiotic stress lies in higher distances from other varieties. This result also coincided with field performances of varieties.

Results of this investigation construct the genetic fingerprinting and molecular characterization of six sugarcane varieties based on microsatellite markers which coincided with field performances of these varieties.

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