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Genetic Relationship in Ten Sugarcane Varieties of Bangladesh and Thailand Based on SSR Markers

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ABSTRACT

The experiment was carried out at the Biotechnology Laboratory, Bangladesh Sugarcane Research Institute (BSRI), Ishurdi, Pabna, Bangladesh. Five BSRI released sugarcane varieties (Isd 24, Isd 28, Isd 29, Isd 30 and Isd 31), five Thai varieties (K84200, K764, K8865, K8887 and K8892) and nine microsatellite primers (SMC687 CG, SMC384BS, SMC119CG, SMC336BS, MSSCIR8, SMC703BS, MSSCIR74, SMC 766BS and SMC18SA) were used for the investigation. Polymorphic information content of microsatellite markers ranged from 0.44 to 0.90. The most polymorphic marker was MSSCIR74 with polymorphic information content of 0.90. The highest linkage distance (28.0) was recorded between the varieties Isd 31 and K8887 from other varieties investigated. The lowest linkage distance (9.0) was observed between variety K8892 and K887. Genetic relationships among the varieties showed that at the average linkage distance of 23.5 varieties were grouped into two major clusters. One variety of Bangladesh included in the one cluster while others included in other cluster. Variety Isd 24 of Bangladesh separated from the other varieties of both countries. The variety Isd 24 is one of the varieties having chewing quality with superior performances against the abiotic (drought, flood and water-logging) stress than the other varieties of Bangladesh. Results of this investigation indicate the wide genetic distances between the sugarcane varieties of Bangladesh and Thailand.

Key words: Sugarcane, genetic relationship, SSR marker.

INTRODUCTION

Genetic diversity of germplasm resources plays a very important role in any crop improvement program and determines the potential for long-term genetic gain. The amount of genetic diversity present in the genetic base depends in part on the number and diversity of the original ancestors involved in the creation of a germplasm pool. Modern varieties of sugarcane have narrow genetic base. 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. Simple Sequence Repeats (SSRs) also known as microsatellite markers are molecular markers based on

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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). The SSRs 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 SSR as primer. The SSR markers are co-dominant and can be transmitted in simple Mendilian segregation. Lastly, the SSRs 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, varieties are being released based on agronomic and morphological traits. Molecular characterization using RAPD and SSR markers has been initiated. Therefore, this investigation was undertaken to analyse the genetic relationship in ten sugarcane varieties of Bangladesh and Thailand based on SSR 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: Five BSRI released sugarcane varieties (Isd 24, Isd 28, Isd 29, Isd 30 and Isd 31), five Thai varieties (K84200, K764, K8865, K8887 and K8892) were used. 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) and homogenized with pestle in 800 µl of extraction buffer (200 mM Tris HCl, pH 8.0; 50 mM EDTA.H_2O, pH 8.0; 2.2 M 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 150 µl 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 120 µl 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 50 µl of TE buffer (10 mM Tris HCl, pH 8.0; 1 mM EDTA.H_2O, pH 8.0) and stored at -20°C for subsequent use.

Primers used: Nine selected sugarcane microsatellite primers (markers) developed by International Sugarcane Microsatellits Consortium were used to amplify simple sequence repeats of genomic DNA from ten sugarcane varieties. These primers are SMC687CS, SMC334BS, SMC119CG, SMC336BS, SMSSCIR8, SMC703BS, MSSCIR74, SMC766BS and SMC18SA (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).

PCR amplification and electrophoresis: The PCR amplification was done in an oil-free thermal cycler (Master Cycler Gradient, Eppendorf) following the PCR profile of 94 °C for 3 minutes (initial denaturation) followed by 35 cycles of 30 seconds denaturation at 94 °C, 45 second annealing at 55 °C and elongation or extension at 72 °C for 1 minute. After the last cycle, a final step of 7 minutes at 72 °C was added to allow complete extension of all amplified fragments. After completion of cycling programme, reactions were held at 4 °C. 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), 2.5 µl of Primer Forward and Reverse from 1.0µM stock, 1.0 µl of 2.5mM DNTPs, 0.2µl of 5U/µl Ampli Taq DNA polymerase (Bangalore Genei Pvt. Ltd., India), 2.0µl of 25 ng/µl genomic DNA and a suitable amount (0.8 µl) of sterile deionized water. After amplification 2.5µl of 5x loading dye was added to the amplification product and stored at 4°C for separation using polyacrylamide gel electrophoresis. In each well 3.0µl 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 distances among the varieties using computer programme, Statistica.

RESULTS AND DISCUSSION

The values of pair-wise genetic distances between varieties were ranged from 9.0 to 28.0 (Table 2). The highest linkage distance (28.0) was recorded between the varieties Isd 31 and K8887 from other varieties investigated. The linkage distance (10.0) was recorded between variety pairs Isd 31 and K8892, K764 and K8865, K8892 and K8865. The same linkage distance (12.0) was recorded between variety pairs Isd 31and Isd 29, K887and K8865. The distinct linkage distance (20.0) was recorded between variety pairs Isd 28 and Isd 31, Isd 28 and K8865, Isd 29 and K84200, Isd 28 and Isd 30, Isd 30 and

K764, lsd 30 and K8887, lsd 30 and K8892, lsd31 and K84200, lsd 29 and K84200, lsd 30 and K8865. The lowest linkage distance (9.0) was observed between variety K8892 and K8887. Diverse cultivars based on their mean Euclidian distance values can be utilized as parents in the hybridization program. Integrating available information on their good combining ability with other cultivars to the phenotypic distance data, as a criterion in parental selection, ensures a higher chance of generating better performing hybrids. Thus, cross combinations between genetically related cultivars can be avoided. Crosses between genetically distant sugarcane cultivars should produce higher variances for quantitatively inherited traits in segregating populations.

All the nine SSR primer pairs generated multiple fragments among the eight sugarcane varieties. Genetic diversity or polymorphism information content (PIC) per primer pair ranged from 0.44 to 0.90 with a mean of 0.81 for all loci (Table 3). The primer pairs MSSCIR74 showed the highest PIC value (0.90) and SMC687CS showed the lowest PIC value (0.44). The PIC values are dependent on the genetic diversity of the varieties under study. A high proportion of closely related genotypes would have the effect of lowering the PIC values (Garland *et al.*, 1999). Mean PIC value among the eight varieties was 0.81 indicating a high level of variability present in the varieties based on the nine SSR primers. The comparable results were reported by Cordeiro *et al.* (2000) on sugarcane SSRs. The 91 polymorphic markers showed the PIC values that ranged from 0.48 to 0.80 and a mean PIC value of 0.72 on the genotypes tested. The results of the present investigation are in agreement with findings of Cordeiro and co-workers (2000).

Genetic relationships among the varieties revealed that at the average distance of 23.5 showed two major clusters (C_1 and C_2) presented in the Figure 1. One variety of Bangladesh included in the cluster C_1 while others included in cluster C_2. At the linkage distance of 20.1 the cluster C_2 produced sub-cluster SC_1 and SC_2. Sub-cluster SC_2 included 3 varieties (lsd 29, lsd 30 and lsd 31) of Bangladesh. Sub-cluster SC_1 included all Thai varieties except one variety (lsd 28) separated at the linkage distance of 16.0. The major cluster C_1 separated the variety lsd 24 from the other varieties of both the countries. The variety lsd 24 is one of the variety having chewing quality among the released varieties of BSRI (Paul *et al.*, 2005). Variety lsd 24 was shown to be outliers in the dendrogram and distantly related with the rest of the cultivars based on their genetic distances. The variety lsd 24 having the superior performances against the abiotic (drought, flood and water-logging) stress than the other varieties of Bangladesh (Paul *et al.*, 2005). The presented dendrogram demonstrates clearly the ability of the nine SSR markers to detect a large amount of genetic variation in ten sugarcane varieties of Bangladesh and Thailand. Cluster analysis by UPGMA of the ten varieties based on the nine SSR primers grouped variety lsd 24 into one major cluster from major and sub-clusters. The varieties were clustered based on their mean genetic distances. Identical and closely related cultivars were clustered together. In choosing parental clones for crossing work, selection criteria should be based on genetic distances between cultivars and information on their relationships in cluster analysis. Crossing cultivars based only on their genetic distances limits the number of parents and reduces the level of genetic variability in the population derived from them. Cross combinations involving parents that are distantly related and coming from different clusters are more expected to produce heterotic offspring.

Results of this investigation indicate the wide genetic distances between the sugarcane varieties of Bangladesh and Thailand.

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Table 1. Sequence of nine sugarcane microsatellite primers used for PCR amplification of DNA in ten sugarcane varieties of Bangladesh and Thiland.

Sl. no.	Primer Code	Sequence (5′-3′)
1.	SMC687CS	Forward: -AGC CAT GCA GGC AGG CAT- Reverse: -CGC ACA ATC TGC AAG TGC ATC A-
2.	SMC334BS	Forward: -CCT TTC ATC CAC CGA GGA CAA- Reverse: -GGT TCA CCG AAG CAA GAG AAC-
3.	SMC119CG	Forward: -TTC ATC TCT AGC CTA CCC CAA- Reverse: -AGC AGC CAT TTA CCC AGG A-
4.	SMC336BS	Forward: - ATT CTA GTG CCA ATC CAT CTC A- Reverse: - CAT GCC AAC TTC CAA ACA GAC-
5.	MSSCIR8	Forward: -ACC AGC ACA CGG AAA CGG AAC- Reverse: -ATG CGC AGG GAT AGG ATA GGA G-
6.	SMC703BS	Forward: -GCC TTT CTC CAA ACC AAT TAG T- Reverse: -GTT GTT TAT GGA ATG GTG AGG A-
7.	MSSCIR74	Forward: -GCG CAA GCC ACA CTG AGA- Reverse: -ACG CAA CGC AAA ACA ACG-
8.	SMC766BS	Forward: -TTA CTC GGC TGG GTT TTG TTC - Reverse. -TAA GAA TCG TTC GCT CCA GC -
9.	SMC18SA	Forward: - ATT CGG CTC GAC CTC GGG AT- Reverse: -AGT CGA AAG GTA GCG TGG TGT TAC-

Table 2. Summary of linkage distances for different pairs of ten sugarcane varieties of Bangladesh and Thailand.

Variety	Isd 24	Isd 28	Isd 29	Isd 30	Isd 31	K 84200	K 764	K 8865	K 8887	K 8892	Average
Isd 24	0.0	18.0	25.0	24.0	27.0	21.0	18.0	24.0	26.0	23.0	20.0
Isd 28	18.0	0.0	17.0	20.0	21.0	15.0	24.0	20.0	14.0	19.0	16.8
Isd 29	25.0	17.0	0.0	13.0	12.0	20.0	23.0	19.0	19.0	16.0	16.4
Isd 30	24.0	20.0	13.0	0.0	17.0	17.0	20.0	20.0	20.0	15.0	16.6
Isd 31	27.0	21.0	12.0	17.0	0.0	20.0	27.0	27.0	23.0	10.0	18.4
K 84200	21.0	15.0	20.0	17.0	20.0	0.0	19.0	19.0	13.0	16.0	16.0
K 764	18.0	24.0	23.0	20.0	27.0	19.0	0.0	10.0	20.0	17.0	17.8
K 8865	24.0	20.0	19.0	20.0	27.0	19.0	10.0	0.0	12.0	13.0	16.4
K 8887	26.0	14.0	19.0	20.0	28.0	13.0	20.0	12.0	0.0	9.0	16.1
K 8892	23.0	19.0	16.0	15.0	10.0	16.0	17.0	10.0	9.0	0.0	13.5

Table 3. Polymorphism Information Content (PIC) values of nine microsatellite primers across ten sugarcane varieties of Bangladesh and Thailand.

Sl. No.	Primer codes	Polymorphism Information Content (PIC) values
01	SMC687CS	0.44
02	SMC334BS	0.87
03	SMC119CG	0.82
04	SMC336BS	0.84
05	MSSCIR8	0.84
06	SMC703BS	0.86
07	MSSCIR74	0.90
08	SMC766BS	0.86
09	SMC18SA	0.86
	Mean	0.81

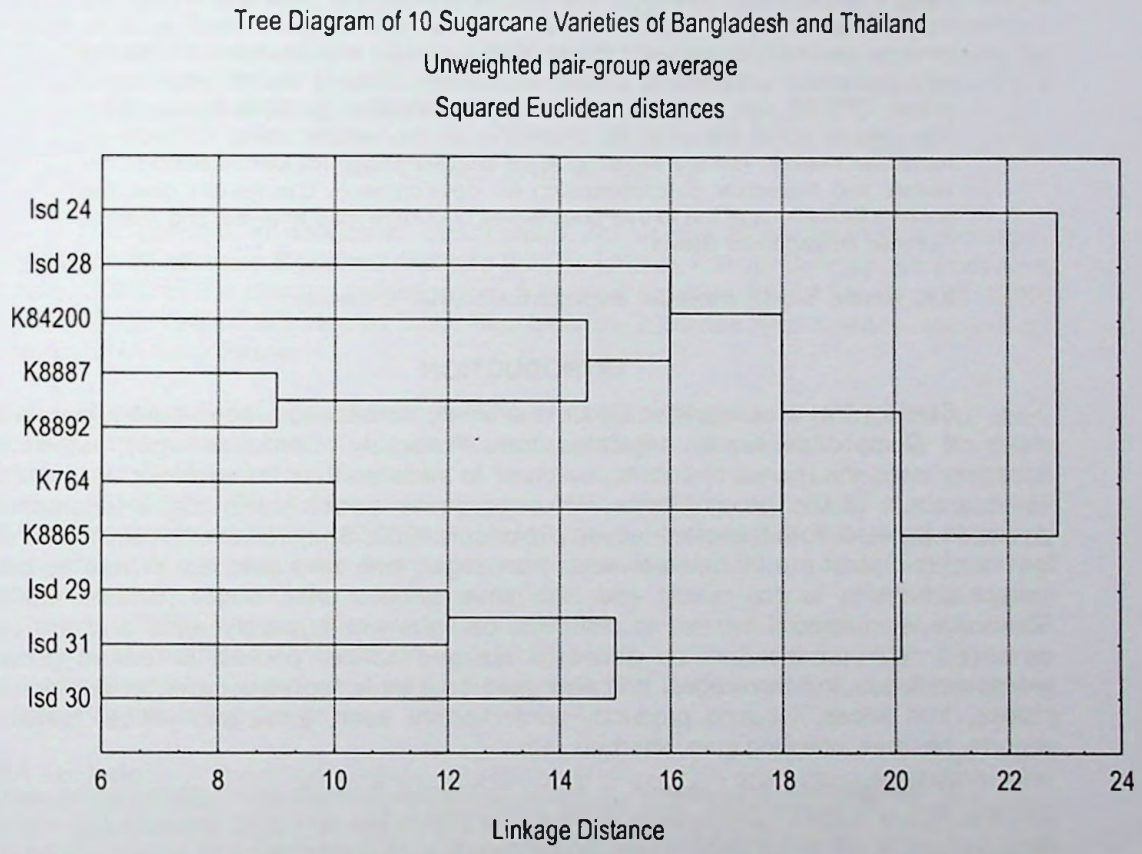

Figure 1. Cluster analysis by unweighted pair group method of arithmetic means (UPGMA of five sugarcane varieties of Bangladesh and five sugarcane varieties of Thailand based on nine SSR markers.