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Genetic Diversity Analysis and DNA Fingerprinting of Ten Sugarcane Germplasm Using SSR Markers

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ABSTRACT

The investigation was conducted for genetic diversity analysis and DNA fingerprinting of ten sugarcane germplasm viz., K84-200, K76-4, K88-65, K8887, K8892, PS851, PS862, PS863, PS86-10029 and PS81-362 using four Simple Sequence Repeats (SSR) primers such as SMC687CS, SMC334BS, SMC119CG and SMC766BS. The four microsatellite primer pairs amplified a total number of 102 bands from ten germplasm visualized through 8.0% polyacrylamide gel electrophoresis. The sizes of the amplified bands obtained from ten germplasm ranged from 80 to 184bp. The highest number of allele per locus (4.85) and number of genotypes per locus (4.85) were obtained from the primer SMC766BS followed by SMC687CS (4.75), SMC119CG (2.17) and SMC334BS (1.64) respectively. Genetic diversity or polymorphism information content (PIC) per primer pair ranged from 0.85 to 0.90 with a mean of 0.87 for all loci across the germplasm evaluated. The primer pairs SMC334BS showed the highest PIC value 0.90 followed by SMC119CG (0.88), SMC766BS (0.88) and SMC687CS (0.85). The most polymorphic SSR marker was associated with the highest number of bands detected. The primer pairs SMC334BS were the most polymorphic marker for ten germplasm with PIC values of 0.90. The number of germplasm with unique banding patterns distinguished by the four SSR markers ranged from 20% to 100%. The four SSR markers were able to discriminate 42.5% of all the germplasm evaluated with unique banding patterns. The highest linkage distance was recorded from the germplasm K88-65, K8892, PS862 and PS863compare with other germplasm investigated. Genetic relationships among the germplasm at the average distance of 11.8 separated the germplasm K88-65 from the others. Germplasm K88-65 and PS863 were shown to be outliers and distantly related with the rest of the germplasm. The four primers were able to identify and classify ten sugarcane germplasm indicating genetic differences among them.

Key words: Germplasm, fingerprinting, SSR markers, genetic diversity, sugarcane

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INTRODUCTION

Sugarcane is one of the major sugar producing crops of the world. It is major cash-cum-industrial crop in tropical and subtropical regions of the world and important export product in many developing countries (Heinz *et al.*, 1987). It provides cheap food in the form of "Sugar" and "Goor (Zaggary)" that lend itself for the production of energy and many byproducts, all of which are of great economic value to both the developing and developed countries. Sugarcane is the second most important cash crop, especially north and southern part of Bangladesh.

Sugarcane breeding programs throughout the world have limited genetic diversity (Berding and Roach, 1987). Modern sugarcane varieties (*Saccharum* spp., 2n=100-130) derived from the interspecific crosses between the sugar-producing species *Saccharum officinarum* (2n=80) and wild relatives mainly *S. spontaneum* (2n=40-128) involved only a few parental clones (Arceneaux, 1965; Price, 1965). Data from various studies on chloroplasts (cpDNA) DNA and mitochondria (mtDNA) DNA in the clones of *Saccharum* spp. and hybrids indicated a narrow cytoplasmic genome for sugarcane (Mangelsdorf, 1983; D'Hont *et al.*, 1994; Al-Janabi *et al.*, 1994). Such a narrow genetic base has failed to regenerate new higher yielding varieties. Therefore, there is a need to introduce new genetic resources in the sugarcane breeding program (Chang, 1996). Genetic base broadening and enhancement of germplasm are major concerns in variety development. Potential for maximum gains in the breeding program are only realized when a broad genetic base is present. Thus, it is needed that DNA fingerprinting of sugarcane germplasm should be done as short-term strategies to determine the level of genetic diversity in the collection. The information gained can be used in subsequent activities to broaden the genetic base of the sugarcane germplasm.

Molecular markers are valuable tools in plant breeding programs as well as for evolutionary and conservation studies. They are accurate, abundant and less affected by the environment. Simple sequence repeats (SSRs) also known as mircrosatellites markers based on tandem repeats of short (2-6 bp) DNA sequences (Litt and Lutty, 1989). These DNA sequences are highly polymorphic even among closely related cultivars due to mutation causing variation in the number of repeating units (Saghai-Maroof *et al.*, 1994). SSRs can be analyzed by a rapid, technically simple and inexpensive polymerase chain reaction (PCR) based assay that requires only small quantities of DNA. Through PCR, different alleles at a locus can be detected by using conserved DNA sequences flanking the SSR as primers. SSR markers are co-dominant and can be transmitted in simple Mendelian segregation. Lastly, SSRs are abundant and uniformly distributed in plant genomes (Lagererantz *et al.*, 1993; Wang *et al.*, 1994, Akkaya *et al.*, 1995).

In Bangladesh, characterization of sugarcane germplasm based on agronomic and morphological traits is being practiced. DNA fingerprinting using RAPD markers has been initiated by Shahnawaz, 2006. This study was undertaken to identify germplasm using DNA fingerprinting and determine the genetic diversity of ten sugarcane germplasm of BSRI germplasm bank using microsatellite markers. Microsatellite is important particularly for germplasm identification, germplasm selection, hybridization, evaluation

and conservation core germplasm collection. The study was undertaken to identify DNA fingerprints of sugarcane germplasm and determine the genetic diversity and relationship among the germplasm by clusters analysis.

MATERIALS AND METHODS

The experiment was carried out at the Biotechnology Laboratory, Bangladesh Sugarcrop Research Institute (BSRI), Ishurdi-6620, Pabna, Bangladesh.

Plant materials

Ten Sugarcane germplasm viz.,K84-200, K76-4, K88-65, K8887, K8892, PS851, PS862, PS863, PS86-10029 and PS81-362 collected from BSRI germplasm bank were used as plant materials for DNA isolation.

Sample collection

Meristem cylinder of sugarcane was used as sample. Top of the field grown 8 month old sugarcane was cut and placed vertically in a bucket containing. Then it was taken in the laboratory. The outer leaf sheaths were removed, leaving inner spindle. Then the spindle base was cut down into small pieces with sterile scissors and 0.2g was taken for DNA isolation.

DNA isolation

In the present investigation, Modified Method of Al-janbi (1999) reported by Hossain (2006), mini-prep method adopted from Shahnawaz (2006) has been combined and used to isolate total genomic DNA from sugarcane. Isolated DNA was stored at -20^0C for future use.

Primers used

Four selected sugarcane microsatellite primers developed by International Sugarcane Microsatellites Consortium were used to amplify Simple Sequence Repeats of genomic DNA of ten sugarcane germplasm (Muyco, 2002). The primers were SMC687CS, SMC334BS, SMC119CG and SMC766BS (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

PCR amplification was done in an oil-free thermal cycler (Genius, Techne, Cambridge Limited) following the PCR profile of 94°C for 5 minutes (initial denaturation) followed by 35 cycles of 1 minutes denaturation at 94°C, 1 minutes annealing at 55°C and extension at 72°C for 2 minutes. After the last cycle, a final step of 7 minutes at 72°C was added to allow the complete extension of all amplified fragments. After completion of cycling programme, the reactions were held at 4°C. PCR reactions were performed on each DNA sample in a 10µl reaction mixture containing 1.0µl of 10 x (Ampli polymerase PCR buffer), 1µl of 2.5mM dNTPs, 2.25µl each of forward and reverse primer from 1.0µM working solution, 0.2µl of 5U/µl Ampli DNA polymerase (Bangalore Genei Pvt. Ltd., India), 3.0µl of 25ng/µl genomic DNA and a suitable amount (0.3µl) of sterile de-ionized distilled water.

After amplification, 2.0µl loading dye was added to the PCR amplified product and for separation using polyacrylamide gel electrophoresis. In each well, 3.0µl of PCR amplified product of each DNA sample for each primer was loaded in 8% polyacrylamide gel (acrylamide and biacrylamide of SRL, India). Electrophoresis was performed at 50 Volts (5-8V/cm) for 2.5 hours. DNA ladder pBR322HaeIII (Bangalore Genei Pvt. Ltd., India) for primer pairs SMC687CS, SMC334BS, SMC119CG and SMC766BS was run along sides the reactions. After electrophoresis, the gel was silver stained and dried at room temperature. DNA bands were observed on white light box and photographed by digital camera.

SSR data analysis

Percentage of polymorphic loci (*p*)

$P = (k/n) \times 100\%$, where k is the number of polymorphic loci and n is the total number of loci investigated.

Average number of alleles per locus (*A*)

$A = \Sigma Ai/n$, where Ai is the number of alleles at the ith locus and n is the total number of loci investigated.

Average number of alleles per polymorphic loci (*Ap*)

$Ap=\Sigma Api/n_p$, where Api is the number of alleles at a certain polymorphic locus and n_p is the total number of polymorphic loci investigated.

Average number of genotypes per locus (*G*)

$G=\Sigma g/n$, where g is the number of genotypes at a certain locus and n is the number of loci investigated.

Gene diversity (polymorphic information content)

According to Weir (1990) gene diversity = $1 - \Sigma P^2_{ij}$, where P^2_{ij} is the frequency of the jth pattern for the marker i and is summed across n patterns. Anderson et al., (1993) suggest that gene diversity is the same as the polymorphism information content.

Cluster analysis and dendrogram construction

Following electrophoresis, the size of amplification products were estimated by comparing the migration of each amplified fragment with that of a known size fragments of molecular weight marker: 100bp DNA ladder and pBR322HaeIII. All distinct bands or fragments (SSR marker) 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 individual germplasm and each primer. The scores obtained using all primers in the SSR analysis were then combined to create a single data matrix. This was used for estimating linkage distance (D) and constructing a UPGMA (Unweighted Pair Group Method of Arithmetic Means) Dendrogram among the varieties using computer program "Statistica". Linkage distances were computed from frequencies of polymorphic markers to estimate genetic relationship between the studied ten sugarcane germplasm using the Unweighted Pair-Group Method of Arithmetic Means (UPGMA) (Sneath and Sokal, 1973). The Dendrogram was constructed using the "Statistica" computer package.

RESULTS AND DISCUSSION

DNA fingerprinting followed by genetic diversity was assessed in the investigation of ten sugarcane germplasm (K84-200, K76-4, K88-65, K8887, K8892, PS851, PS862, PS863, PS86-10029 and PS81-362) using four SSR primers (SMC687CS, SMC334BS, SMC119CG and SMC766BS).

The sizes of the amplified bands in the ten sugarcane germplasm ranged from 80 to 184bp (Table 2). SSR primer pair SMC687CS revealed band sizes that ranged from 90bp to 172bp; from 80bp to 184bp for primer SMC334BS; from 89bp to 124bp for primer SMC119CG and band sizes ranging from 82bp to 166bp for SMC766BS. However, the primer pair SMC119CG identified band sizes that ranged from 102bp to 152bp (Muyco, 2002). This was perhaps due to the sample differences from this investigation. Yang *et al.* (1994) pointed out that the range in allele sizes can be influenced by the large number of samples screened.

All the four SSR primer pairs amplified a total of 102 bands from the ten germplasm of sugarcane after PCR amplification and 8% polyacrylamide gel electrophoresis. For each primer pairs, the number of bands varied from 19 to 34 (Figure 1 and 2). The primer pair SMC766BS amplified the highest number of bands (34) followed by SMC119CG (26), SMC334BS (23) and SMC687CS (19). Taylor and Cordeiro (2000) showed that the SSR primer pairs SMC36BUQ and SMC334BS produced identical fingerprints. The highest number of bands (3.40) per variety was amplified from the primer pair SMC766BS followed by SMC119CG (2.60), SMC334BS (2.30) and the lowest number of bands per variety was recorded from the primer SMC687CS (1.90). Due to the polyploidy nature of sugarcane, the SSR markers revealed multiple bands per locus. At South Africa Sugar Association Experiment Station (SASEX), Natal, South Africa work on the application of 35 sugarcane microsatellites identified from 1 to 18 alleles per marker across four varieties (Bester, 2000) (Table 2).

The average number of allele per locus and average number of genotypes per locus for four primers were identical. The highest number of allele per locus and number of genotypes per locus (4.85) was obtained from the primer SMC766BS followed by SMC687CS (4.75), SMC119CG (2.17) and SMC334BS (1.64) respectively. The average number of allele per polymorphic locus was recorded the highest (19.0) from primer SMC687CS followed by primer SMC766BS (17.0), SMC119CG (8.67) and the lowest (2.55) was recorded from primer SMC334BS (Table 3).

Genetic diversity or polymorphism information content (PIC) per primer pair ranged from 0.85 to 0.90 with a mean of 0.87 for all loci across the ten germplasm evaluated. The Primer pairs SMC334BS showed the highest PIC value (0.90) followed by SMC119CG (0.88), SMC766BS (0.88) and the lowest PIC value was 0.85 obtained from the primer pairs SMC687CS (Table 3).

The most polymorphic SSR marker was associated with the highest number of bands detected. The primer pairs SMC334BS was the most polymorphic marker across the ten germplasm with PIC value of 0.90 (Table 3). The PIC values are dependent on

the genetic diversity of the germplasm under study. A high proportion of closely related genotypes would have the effect of lowering the PIC values (Garland *et al.*, 1999).

The number of germplasm with unique banding patterns distinguished by the four SSR markers ranged from 20% to 100%. The most polymorphic markers were the most discriminant (SMC334BS) and discriminated 100% of the germplasm evaluated. The four SSR markers were able to discriminate 42% of all the germplasm evaluated with unique banding patterns (Table 2).

The highest linkage distance (16.0) was recorded between the germplasm pairs K88-65 and PS862, K88-65 and PS863, K889 and PS863 than other germplasm investigated. The linkage distance (7.0) was recorded between germplasm pairs K84-200 and K76-4, PS86-10029 and PS81-362. The same linkage distance (8.0) was recorded between germplasm pairs PS851 and K8887, PS851 and K8892, PS851 and PS863. The identical linkage distance (9.0) was recorded between germplasm pairs PS851 and PS81-362. The germplasm pairs K84-200 and K8887, K84-200 and K8892, K84-200 and PS86-10029, K88-65 and K8887, PS851 and PS862, PS851 and PS86-10029, PS862 and PS86-10029, PS863 and PS-10029 showed similar linkage distance (10.0). The linkage distance (11.0) was recorded between germplasm pairs K84-200 and PS81-362, K76-4 and K88-65, K76-4 and K8887, K76-4 and K8892, K76-4 and PS862, K8887 and PS81-362, PS862 and PS81-362, PS863 and PS81-362. The germplasm pairs K84-200 and K88-65, K84-200 and PS851, K84-200 and PS862, K8865 and K8892, K8887 and PS862, K8892 and PS862, PS862 and PS863 showed linkage distance (12.0). The linkage distance (13.0) was recorded between germplasm pairs K76-4 and PS851, K76-4 and PS863, K76-4 and PS86-10029, K8865 and PS81-362, K8892 and PS81-362. The linkage distance (14.0) was recorded between germplasm pairs K84-200 and PS863, K76-4 and PS81-362, K88-65 and PS851, K88-65 and PS86-10029, K8887 and PS863, K8887 and PS86-10029, K8892 and PS86-10029. The lowest linkage distance (4.0) was observed between germplasm pairs K8887 and K8892 (Table 4).

Genetic relationships among the germplasm at the average distance of 12.5 showed two major clusters (C_1 and C_2). At the linkage distance of 11.8 the major cluster C_1 produced sub-cluster SC_1 and SC_2. Sub-cluster SC_2 produced sub-cluster SC_3 and SC_4 at the linkage distance of 11.2. At the linkage distance of 11.0 the major cluster C_2 produced sub-cluster SC_5 and SC_6. Sub-cluster SC_6 produced sub-cluster SC_7 and SC_8 at same linkage distance 10.5. At the linkage distance (8.0) dividing Sub-cluster SC_3 produced sub-cluster SC_9 and SC_{10}. Similarly, sub-cluster SC_4 produced sub-cluster SC_{11} and SC_{12} (7.0) and sub-cluster SC_8 produced SC_{13} and SC_{14} (7.0). Finally, sub-cluster SC_{10} divided into two sub-cluster of germplasm K8887 and K8892 at the linkage distance of 4.0. The sub-cluster SC_1 separated the germplasm K88-65 from the other germplasm. The results of DNA polymorphism of the ten germplasm bear the genetic diversity of germplasm K88-65 from other germlasms investigated. Germplasm K88-65 and PS863 were shown to be outliers in the dendrogram and distantly related with the rest of the germplasm based on their genetic distances. The germplasm PS863 lies in the sub-cluster SC_5 and separated from the other germplasm at the linkage distance of 11.0 (Figure 3).

Results of the present investigation revealed that the four SSR primers were able to identify and classify ten sugarcane germplasm indicating genetic differences among germplasm. Therefore, DNA fingerprinting and diversity analysis of all the germplasm as well as entire germplasm collection should be done in order to determine their genetic relationships among them.

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

Primer Code	Sequence	G+C Content (%)
SMC687CS	Forward: -AGC CAT GCA GGC AGG CAT-	61.11
	Reverse: -CGC ACA ATC TGC AAG TGC ATC A-	50.00
SMC334BS	Forward: -CAA TTC TGA CCG TGC AAA GAT-	42.85
	Reverse: -CGA TGA GCT TGA TTG CGA ATG-	47.61
SMC119CG	Forward: -TTC ATC TCT AGC CTA CCC CAA-	47.61
	Reverse: -AGC AGC CAT TTA CCC AGG A-	52.63
SMC766BS	Forward: -TTA CTC GGC TGG GTT TTG TTC-	47.61
	Reverse: -TAA GAA TCG TTC GCT CCA GC-	50.00

Table 2. Microsatellite primers with corresponding bands scored, their size range, number of polymorphic bands, polymorphism and number of band per germplasm together with germplasm distinguished in ten sugarcane germplasm

Primer codes	Size ranges (bp)	Total number of bands scored	Number of polymorphic bands	Polymorphism (%)	Number of band per germplasm	Germplasm distinguished (%)
SMC687CS	90-172	19	1	5.26	1.90	20.00
SMC334BS	80-184	23	9	39.13	2.30	100.00
SMC119CG	89-124	26	3	11.54	2.60	30.00
SMC766BS	82-166	34	2	5.88	3.40	20.00
Total	-	102	15	-	-	170
Average	-	25.5	3.75	-	-	42.5

Table 3. Microsatellite primers with corresponding average number of Alleles per locus, average number of alleles per polymorphic locus, average number of genotypes per locus together with Polymorphism Information Content (PIC)

Primer codes	Average number of alleles per locus	Average number of alleles per polymorphic locus	Average number of genotypes per locus	PIC (Polymorphism Information Content)	Mean PIC
SMC687CS	4.75	19.00	4.75	0.85	0.87
SMC334BS	1.64	2.55	1.64	0.90	
SMC119CG	2.17	8.67	2.17	0.88	
SMC766BS	4.85	17.00	4.85	0.88	

Table 4. Summary of linkage distances for different pairs of sugarcane germplasm

Germplasm	K84-200	K76-4	K88-65	K8887	K8892	PS851	PS862	PS863	PS86-10029	PS81-362
K84-200	0.0	7.0	12.0	10.0	10.0	12.0	12.0	14.0	10.0	11.0
K76-4	7.0	0.0	11.0	11.0	11.0	13.0	11.0	13.0	13.0	14.0
K88-65	12.0	11.0	0.0	10.0	12.0	14.0	16.0	16.0	14.0	13.0
K8887	10.0	11.0	10.0	0.0	4.0	8.0	12.0	14.0	14.0	11.0
K8892	10.0	11.0	12.0	4.0	0.0	8.0	12.0	16.0	14.0	13.0
PS851	12.0	13.0	14.0	8.0	8.0	0.0	10.0	8.0	10.0	9.0
PS862	12.0	11.0	16.0	12.0	12.0	10.0	0.0	12.0	10.0	11.0
PS863	14.0	13.0	16.0	14.0	16.0	8.0	12.0	0.0	10.0	11.0
PS86-10029	10.0	13.0	14.0	14.0	14.0	10.0	10.0	10.0	0.0	7.0
PS81-362	11.0	14.0	13.0	11.0	13.0	9.0	11.0	11.0	7.0	0.0

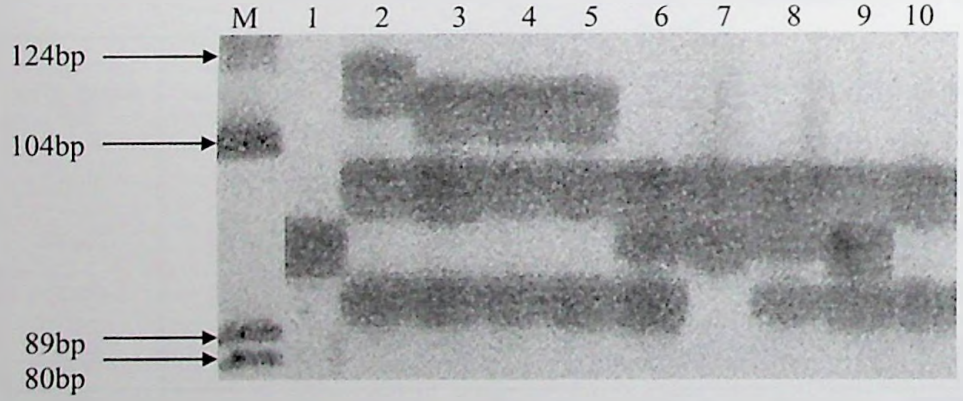

Figure 1. DNA Fingerprinting of BSRI ten germplasm of sugarcane based on SSR primer pair SMC119CG through PAGE (M = marker pBR322HaeIII, Lane 1 = K84-200, Lane 2 = K76-4, Lane 3 = K88-65, Lane 4 = K8887, Lane 5 = K8892, Lane 6 = PS851, Lane 7 = PS862, Lane 8= PS863, Lane 9 = PS86¬-10029 and Lane 10 = PS81-362)

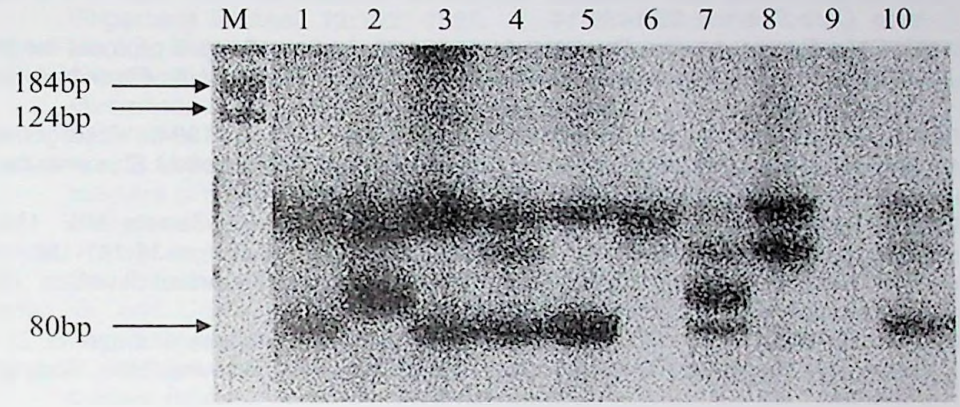

Figure 2. DNA Fingerprinting of BSRI ten germplasm of sugarcane based on SSR primer pair SMC334BS through PAGE (M = marker pBR322HaeIII, Lane 1 = K84-200, Lane 2 = K76-4, Lane 3 = K88-65, Lane 4 = K8887, Lane 5 = K8892, Lane 6 = PS851, Lane 7 = PS862, Lane 8= PS863, Lane 9 = PS86¬-10029 and Lane 10 = PS81-362)

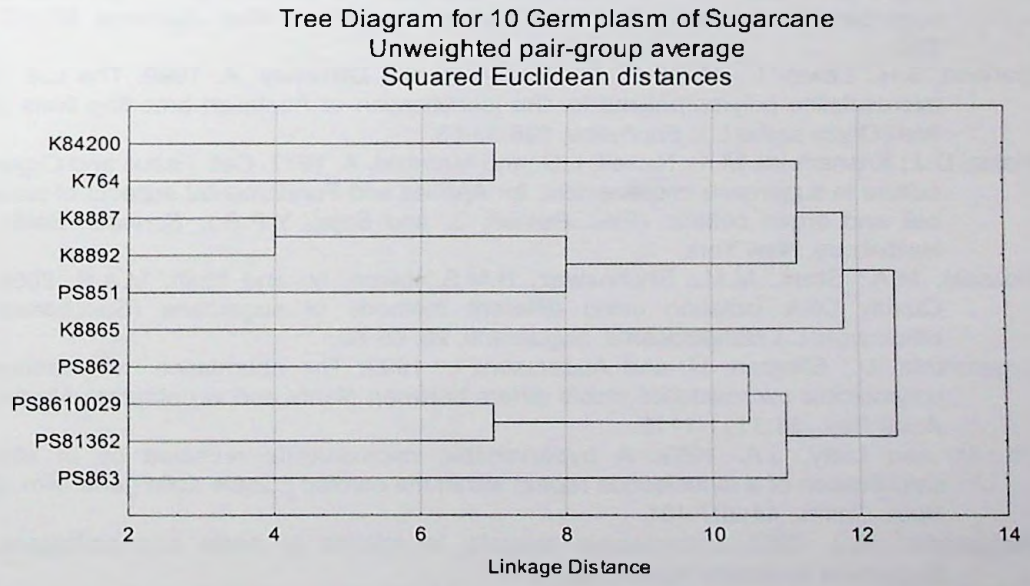

Figure 3. Cluster analysis by unweighted pair group method of arithmetic means (UPGMA) of BSRI ten sugarcane germplasm based on four SSR markers

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