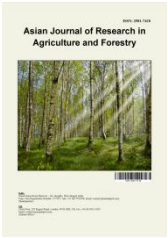

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Assessment of Genetic Diversity and Fingerprinting of Sugarcane Varieties Using Simple Sequence Repeat (SSR) Markers

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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ABSTRACT

Simple sequence repeat (SSR) fingerprinting was chosen because of its high polymorphism, which enables precise analysis of genetic diversity, for the assessment of genetic variety among several sugarcane varieties. This method focuses on certain areas of the sugarcane genome and offers

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important insights into the distinctive genetic patterns and relationships between the many sugarcane types under study. To increase sugarcane production and resilience, crop development plans, breeding programmes, and conservation activities must be guided by the data obtained by SSR fingerprinting. This study aimed to assess the genetic diversity and fingerprinting of eight sugarcane varieties (Isd 16, Isd 20, Isd 21, Isd 24, Isd 28, Isd 29, Isd 30, and Isd 31) using four SSR primers. A simple and efficient method for DNA isolation was employed, and the primers successfully amplified a total of 59 bands from the eight varieties. The results showed that the marker SMC687CS exhibited the highest number of alleles and genotypes per locus, followed by SMC336BS and SMC334BS, while SMC119CG showed the lowest. The highest PIC value was obtained from the marker SMC687CS, indicating its high level of polymorphism. The four SSR markers were able to distinguish 56.25% of all the cultivars evaluated, with the highest linkage distance recorded between the varieties Isd 24 and Isd 28. This study identified and classified the eight sugarcane varieties, indicating genetic differences among them that coincide with their field performances.

Keywords: SSR; sugarcane; fingerprinting; variability.

1. INTRODUCTION

Sugarcane is derived from the Sanskrit word “Sarkara” meaning sugar in the first edition of Species Plantarum [1]. “Sugarcane is a highly efficient crop that converts sunlight into biochemical energy and is the second largest commercial crop after cotton, providing sustainable economic growth and food security in tropical and subtropical regions worldwide” [2,3,4]. “The crop is mainly used as a feedstock for the sugar industry, which contributes 70-80% of global sugar production” [5,6]. “However, the leftover cellulosic biomass from sugar production, such as bagasse, is now being used for electricity cogeneration and second-generation bioethanol production, among other industrial applications” [7,8]. With the growing demand for sugar and green fuel, plant breeding efforts are being challenged to meet the needs of an increasing population and changing lifestyles.

“Sugarcane belongs to the Saccharum genus, which includes six inter-breeding species and is closely related to Sorghum and other grasses” [9]. “Modern sugarcane cultivars are genetically complex, polyploid, and frequently aneuploid with a giant genome size” [10]. “They are derived from crossing noble and wild cane, and a breeding process called 'nobilization' is used to recover constructive alleles for sucrose accumulation” [9]. However, a narrow genetic base and high linkage disequilibrium are common in modern sugarcane cultivars due to the limited clones used in the initial hybridization programs. The crop is challenging due to its multi-specific origin, high ploidy levels, unstable genetic constitution, and huge genome size.

Genetic diversity of germplasm resources plays a vital role in crop improvement programs and determines the potential for long-term genetic gain [11]. Thus, DNA fingerprinting of sugarcane varieties is needed to broaden the genetic base of the sugarcane varieties [12]. Simple sequence repeats (SSRs) are molecular markers based on tandem repeats of short DNA sequences and are highly polymorphic even among closely related cultivars [13]. These markers can be analyzed by a rapid, technically simple, and inexpensive polymerase chain reaction (PCR) based assay that requires only small quantities of DNA [14]. This study aimed to use microsatellite markers to conduct DNA fingerprinting of sugarcane varieties and determine their genetic diversity and relationship through cluster analysis.

2. MATERIALS AND METHODS

The study was conducted at the DNA Laboratory of the Biotechnology Division at the Bangladesh Sugarcane Research Institute (BSRI) in Ishurdi, Pabna, Bangladesh.

2.1 Plant Materials

In this study, eight different sugarcane varieties (Isd 16, Isd 20, Isd 21, Isd 24, Isd 28, Isd 29, Isd 30, and Isd 31) that were released by BSRI were selected as the plant materials for DNA isolation.

2.2 Collection of Samples

Sugarcane plants that were 8 months old and grown in the field were cut from the top and placed in a bucket of water to remain fresh. The plants were then transported to the laboratory, where the outer leaf sheaths were removed,

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

Primer Code	Sequence (5/-3/)	G+C Content (%)
SMC687CS	Forward: -AGCCATGCAGGCAGGCAT-	61.11
	Reverse: -CGCACAATCTGCAAGTGCATCA-	50.00
SMC334BS	Forward: -CAATTCTGACCGTGCAAAGAT-	42.85
	Reverse: -CGATGAGCTTGATTGCGAATG-	47.61
SMC119CG	Forward: -TTCATCTCTAGCCTACCCCAA	47.61
	Reverse: -AGCAGCCATTTACCCAGGA-	52.63
SMC336BS	Forward: -ATTCTAGTGCCAATCCATCTCA-	40.90
	Reverse: -CATGCCAACTTCCAAACAGAC -	47.61

leaving the inner spindle. The spindle base was then cut into small pieces of approximately 1.0cm using sterile scissors, and a required amount of 0.2g was weighed using a fine balance.

2.3 Isolation of Genomic DNA

The total genomic DNA from sugarcane was isolated using a modified method of Aljanabi et al. [15], as reported by Hossain et al. [16]. In addition, the mini-prep method adopted by Shahnawaz [17] was also combined with the modified method.

2.4 Primer Used

Four sugarcane microsatellite primers (markers) from the International Sugarcane Microsatellites Consortium were selected to amplify Simple Sequence Repeats of genomic DNA from eight sugarcane varieties. The primers used were SMC687CS, SMC334BS, SMC119CG and SMC336BS (Table 1). Evaluation of the primers was based on the intensity or resolution of bands, repeatability of markers, consistency within individuals and potential to differentiate varieties (polymorphism).

2.5 PCR Amplification and Electrophoresis

PCR amplification was performed using an oil-free thermal cycler, following a PCR protocol that included initial denaturation at 94ºC for 5 minutes, 35 cycles of denaturation at 94ºC for 1 minute, annealing at 55ºC for 1 minute, and elongation or extension at 72ºC for 2 minutes. A final extension step of 7 minutes at 72ºC was added to ensure complete extension of all amplified fragments. Each DNA sample was amplified using a 10 µl reaction mixture containing the necessary components. After amplification, loading dye was added, and the PCR products were separated using polyacrylamide gel electrophoresis. Electrophoresis was performed at 50 Volts for 2.5 hours, and DNA bands were visualized using silver staining and photographed using a digital camera. A DNA ladder was run alongside the reactions.

2.6 SSR Data Analysis and Dendrogram Construction

After gel electrophoresis, the size of each amplification product was estimated by comparing its migration with known molecular weight markers (100bp DNA ladder). Each distinct band or fragment on the gel was assigned an identification number based on its position and scored visually as present (1) or absent (0) for each individual and primer used. The scores for all primers were then combined to create a data matrix. The data matrix was used to estimate linkage distance (D) and construct a UPGMA (Unweighted Pair Group Method of Arithmetic Means) dendrogram among populations using the computer program Statistica.

Genetic-similarity values, defined as the fraction of shared bands between the SSR profiles of any two individuals on the same gel, were calculated manually from the SSR primer of the same molecular weight on the data matrix. Linkage distances were computed from the frequencies of polymorphic markers to estimate the genetic relationship between the four aromatic sugarcane cultivars studied, using the unweighted pair-group method of arithmetic means (UPGMA). The dendrogram was constructed using the Statistica computer package.

3. RESULTS AND DISCUSSION

DNA fingerprinting of 8 sugarcane varieties using SSR markers (SMC687CS, SMC334BS,

SMC119CG and SMC336BS) showed maximum high-intensity bands with no smearing.

3.1 Band Size

The sizes of the amplified bands in the eight sugarcane varieties ranged from 80 to 200 bp (Table 2). SSR primer pair SMC687CS revealed band sizes that ranged from 90 bp to 172 bp, from 80 bp to 184 bp for primer SMC334BS, from 89 bp to 124 bp for primer SMC119CG and from 86 bp to 158 bp for primer SMC336BS. However, the primer pair SMC119CG identified band sizes that ranged from 104 bp to 135 bp [18]. This was perhaps due to the sample differences from this investigation. Wang et al. [19] pointed out that the range in allele sizes can be influenced by the large number of samples screened.

3.2 Number of Bands and Polymorphism revealed by SSR Markers

The study used four SSR primer pairs to amplify DNA from eight varieties of sugarcane, resulting in a total of 59 bands. The number of bands per primer pair ranged from 9 to 18, with SMC336BS amplifying the highest number. Due to sugarcane's polyploidy, multiple bands per locus were observed [20]. Previous studies found varying numbers of alleles per primer pair across different sugarcane varieties [21,22,23]. The marker SMC334BS was able to distinguish all varieties, while SMC119CG distinguished the fewest. SMC687CS had the highest number of alleles per locus, while SMC119CG had the lowest. SMC336BS did not have any polymorphic loci.

All four SSR primer pairs generated multiple fragments among eight sugarcane varieties. The PIC values ranged from 0.67 to 0.94, with SMC687CS showing the highest value and SMC119CG the lowest. The most polymorphic marker was associated with the highest number of bands detected [24]. The mean PIC value was 0.82, indicating a high level of variability among the varieties based on the three SSR primers. Results were comparable to previous studies, suggesting that SSR markers could be useful for DNA fingerprinting and varietal identification in sugarcane [25,21,26].

3.3 Number of Varieties Distinguished

The study found that four SSR markers were highly polymorphic and able to distinguish between 56.25% of the sugarcane varieties evaluated, with the most polymorphic marker (SMC334BS) able to discriminate 100% of the varieties. The ability to discriminate genotypes using SSR markers depends on the assumption that alleles of one marker are not linked to alleles of other markers [27]. Previous research has shown that a single primer pair can discriminate many sugarcane varieties and a minimum of two primers can provide similar results as five primers [28,29].

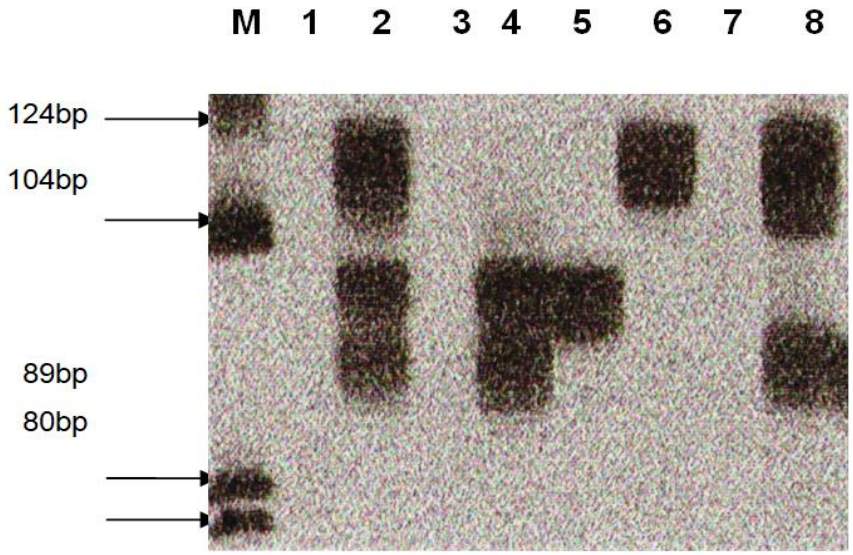

Fig. 1. DNA Fingerprinting of BSRI released 8 varieties of sugarcane-based on SSR primer pair SMC119CG through PAGE (M = marker pBR322HaeIII, Lane 1 = Isd 16, Lane 2 = Isd 20, Lane 3 = Isd 28, Lane 4 = Isd 29, Lane 5 = Isd 30 and Lane 6 = Isd 31)

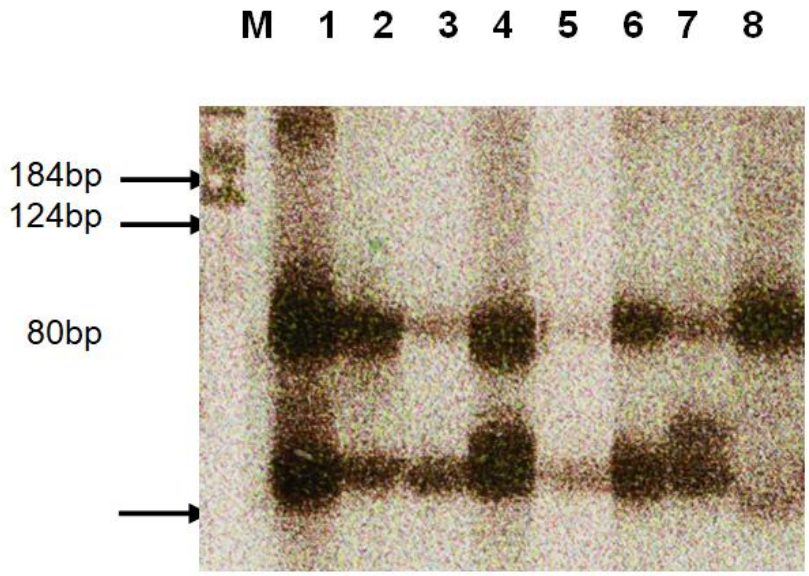

Fig. 2. DNA Fingerprinting of BSRI released 8 varieties of sugarcane-based on SSR primer pair SMC334BS through PAGE (M = marker pBR322HaeIII, Lane 1 = Isd 16, Lane 2 = Isd 20, Lane 3 = Isd 28, Lane 4 = Isd 29, Lane 5 = Isd 30 and Lane 6 = Isd 31)

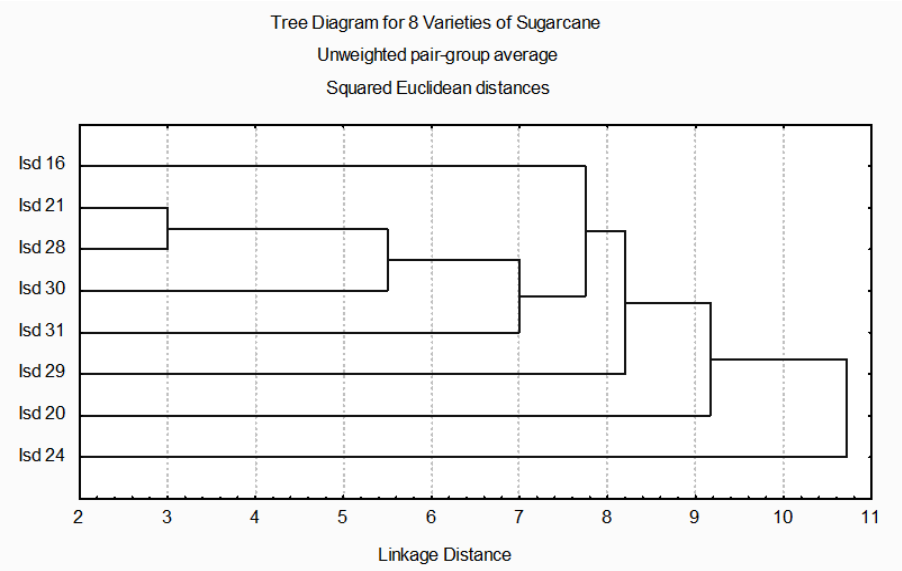

Fig. 3. Cluster analysis by unweighted pair group method of arithmetic means (UPGMA) of BSRI released 8 sugarcane varieties based on four SSR markers

3.4 Genetic Distances

Genetic distances between sugarcane varieties were analyzed using computer software Statistica and the results ranged from 3.0 to 13.0. The highest genetic distance was recorded between the varieties Isd 24 and Isd 28, while the lowest was observed between Isd 21 and Isd 28. To maximize diversity in the core collection, it's recommended to include genotypes with maximum contribution to the total diversity and exclude duplicates or closely related cultivars. Parent selection for cross combinations should be based on distance estimates and available information on good combining ability [30]. Crosses between genetically distant sugarcane cultivars are expected to result in higher variances for quantitatively inherited traits in the segregating populations [31].

Table 2. Microsatellite primers with corresponding bands scored, their size range, number of polymorphic bands polymorphism number of band per variety together with variety distinguished in eight sugarcane varieties

Primer codes	Size ranges (bp)	Total number of bands scored	Number of polymorphic bands	Polymor-phism (%)	Number of bands per variety	Variety distinguished (%)
SMC687 CS	90-172	15	1	6.66	1.88	25
SMC334 BS	80-184	17	10	58.82	2.125	100
SMC119 CG	80-124	9	9	100	1.125	62.5
SMC336 BS	86-158	18	-		2.25	37.5
Total		**59**	**20**	**165.48**	**7.38**	**225**
Average		**14.75**	**5**	**41.37**	**1.85**	**56.25**

Table 3. Microsatellite primers withthe 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)
SMC687CS	5.00	15	5.00	0.94
SMC334BS	1.42	1.70	1.42	0.84
SMC119CG	1.00	1	1.00	0.67
SMC336BS	4.50	-	4.50	0.81
Average	2.98	4.43	2.98	0.82

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

Variety	Isd 16	Isd 20	Isd 21	Isd 24	Isd 28	Isd 29	Isd 30	Isd 31
Isd 16	0	9	7	9	10	8	6	8
Isd 20	9	0	8	12	11	9	9	9
Isd 21	7	8	0	10	3	9	5	7
Isd 24	9	12	10	0	13	11	9	11
Isd 28	10	11	3	13	0	10	6	8
Isd 29	8	9	9	11	10	0	6	8
Isd 30	6	9	5	9	6	6	0	6
Isd 31	8	9	7	11	8	8	6	0

3.5 Cluster Analysis

In this study, the genetic relationships among eight sugarcane varieties were analyzed using molecular markers. The results showed two major clusters, C_1 and C_2, with sub-clusters further dividing at lower linkage distances. Variety Isd-24, which has chewing quality, was separated from the other varieties in major cluster C_1. Additionally, variety Isd-20, which has superior performance against abiotic stresses, was an outlier and distantly related to the other varieties. The level of genetic diversity among the varieties was found to be reduced, which may slow progress in selection. Plant breeders need to consider genetic distance and cluster analysis when selecting parents for breeding programs [32]. Selecting diverse parents that are distantly related and from different clusters can increase the likelihood of producing heterotic offspring [33].

Overall, the results demonstrate the ability of molecular markers to detect genetic variation in sugarcane varieties. By using these markers, plant breeders can make informed decisions about selecting parents for breeding programs, ultimately leading to the development of more productive and stress-resistant sugarcane varieties.

4. CONCLUSION

In conclusion, DNA fingerprinting using four SSR primers was effective in assessing genetic diversity among eight sugarcane varieties. The primers successfully identified and classified the varieties, revealing genetic differences that correspond to their field performances. The most polymorphic marker was SMC334BS, which was able to discriminate all the cultivars evaluated. The results suggest that DNA fingerprinting and molecular characterization should be conducted for the entire germplasm collection to determine their genetic relationships.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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