DETECTION OF GENETIC VARIABILITY AMONG SUGARCANE SOMACLONES USING SSR MARKER

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ABSTRACT

The experiment was conducted for detection of genetic variability among ten sugarcane somaclones including TC2-10(16), TC3-10(16), TC5-10(16), TC8-10(16), TC10-10(16), TC1-10(20) PEG, TC3-10(20) PEG, TC7-10(20) PEG, TC16-10(20) PEG and TC23-10(20) PEG along with their parent varieties (Isd 16 and Isd 20) using two Microsatellite (SSR) markers UGSM 565 and UGSM 567. The sizes of amplified bands in ten sugarcane somaclones of two varieties Isd 16 and Isd 20 ranged from 80 to 650bp. Two SSR markers amplified a total of 58 bands. SSR markers UGSM 565 and UGSM 567 amplified 25 and 33 number of bands, respectively. The highest number of bands (2.75) per variety was amplified from UGSM 567 marker and lowest number of bands (2.08) per variety were recorded from UGSM 565 marker. Genetic diversity or polymorphic information content (PIC) for UGSM 565 was 0.64 and for UGSM 567 it was 0.69 with a mean of 0.67 for all loci across ten somaclones and their parent varieties evaluated. The highest linkage distance 6.0 and lowest linkage distance 1.0 were recorded among ten somaclones and two parent varieties. Genetic relationships based on UGSM 565 among ten somaclones along with their two parent varieties at the average distance of 4.95 showed two major clusters. The somaclones TC23-10(20) PEG and TC10-10(16) were separated from other investigated somaclones and varieties. On the other hand, based on UGSM 567 genetic relationships among ten somaclones along with their two parent varieties at the average distance of 5.5 showed two major clusters and somaclones TC23-10(20) PEG and TC10-10(16) were separated from other somaclones and their parent varieries respectively. The results revealed that two SSR markers were able to detect/identify and classify ten sugarcane somaclones along with their parent varieties indicating genetic differences among somaclones and their parent varieties.

Keywords: *Saccharum officinarum, SSR markers, Genetic variability*

INTRODUCTION

Sugarcane (*Saccharum* spp.) is a genetically complex crop of major economic importance in tropical and sub-tropical countries (Khan *et al*, 2004). Traditional tools for sugarcane breeders to identify different varieties rely on anatomical and morphological characters (Skinner, 1972). The morphological characters like stalk wax, leaf sheath wax, leaf sheath margin, leaf sheath hair (pubescence), dewlap appearance, stalk color, auricle size and color, and other distinguishing characteristics, are generally used by sugarcane breeders in variety development (LaBorde, Legendre, Bischoff, Gravois, & Robert, 2008). For development of improved varieties, genotypic studies of sugarcane is required. To ensure correct variety identity and its genetic pedigree, a procedure for accurate identification using molecular data is urgently needed (Y.-B. Pan, Cordeiro, Richards, & Henry, 2003; Y Pan, Miller, Schnell, Richard Jr, & Wei, 2003; YB Pan, Scheffler, & Richard Jr, 2007). Rapid advances in the field of molecular biology and its allied sciences made the use of molecular markers a

routine practice providing plant breeders a precise tool in analyzing genetic diversity for plant improvement (Andersen & Lübberstedt, 2003). The molecular markers are of many types e.g. RFLPs, TRAPs, RAPDs, SNPs, simple sequence repeats (SSRs) and AFLPs. Simple sequence repeats (SSR) markers reveal polymorphisms due to variation in lengths of microsatellites at specific individual loci. Microsatellites or simple sequence repeats (SSRs) are stretches of DNA, consisting of tandemly repeated short units of 1-6 base pairs in length. They are ubiquitous in eukaryotic genomes and can be analyzed through PCR (Polymorphic Chain Reactions) technology.

In Bangladesh, germplasm characterization is mainly based on agronomic and morphological traits. So far, all varieties have been released in country on the basis of agronomic and morphological traits. Obtaining accurate estimates of genetic diversity among germplasm resources might be helpful in sugarcane breeding. Knowledge of genetic diversity and relationships among breeding genome, their polymorphic nature, co-dominance and materials might have a significant impact on crop improvement. Recently, genetic diversity and molecular characterization study have been initiated using RAPD markers. The SSR marker is important particularly for variety identification by DNA fingerprinting, variety selection for breeding purpose, hybridization evaluation and conservation of their diverse gene pool. Considering the fact, this investigation has been conducted for detection of genetic variability of sugarcane somaclones using microsatellite markers with the objectives of (1) detection of genetic variability of sugarcane somaclones along with their parent varieties using microsatellite markers; (2) DNA fingerprinting of sugarcane somaclones and their parent; and (3) determining genetic diversity and relationship among somaclones based on cluster analysis.

MATERIALS AND METHOD

The experiment was carried out in the Biotechnology Division of Bangladesh Sugarcane Research Institute (BSRI), Ishurdi, Pabna, Bangladesh. The following materials and methods were considered to conduct the experiment:

Plant materials and sample collection

Five somaclones of BSRI released sugarcane of variety Isd 16 [TC2-10(16), TC3-10(16), TC5-10(16), TC8-10(16) and TC10-10(16)] and five somaclones of variety Isd 20 [TC1-10(20)PEG, TC3-10(20)PEG, TC7-10(20)PEG, TC16-10(20)PEG and TC16-10(20)PEG], developed by DMSO (Dimethyl sulfoxide) along with PEG (Polyethylene Glycol), were used as plant materials for DNA isolation. The fresh tops from the 8 month old field grown sugarcane were collected and outerleaf sheaths were removed leaving inner spindle to get meristem cylinder. Then meristem cylinder (spindle base) was cut into small pieces (about 1.0cm) with sterile scissors and the required weight (0.2 g) was taken using a fine balance.

DNA isolation

A modified method of (Aljanabi *et al.*, 1999) was used to isolate total genomic DNA. Cut pieces of meristem cylinder (about 5mm long with 3mm dia) weighing 0.2g were taken in a small mortar (dia 6.5cm and 3.5cm depth) and homogenized with pestle in 800µL of extraction buffer (200mM Tris HCl, pH 8.0; 50mM EDTA.H2O, pH 8.0; 2.2M NaCl; 2% CTAB; 0.06% Sodium Sulfite) until finely shredded within 40-50 second. The grinded sample was taken into a 2mL eppendorf tube to which was added 150µL of each 5% SDS, 10% PVP, 20% CTAB. It was mixed well and incubated at 65°C in a water bath for 40 minutes. During incubation 3-4 times inversion was done. After incubation samples were cooled at room temperature and equal volume of Phenol Chloroform Isoamyl Alcohol (25 : 24 : 1) was added mixed well by inversion and centrifuged at 10,000rpm at room temperature for 30 minutes. Then aqueous phase (about 800µL) was recovered and transferred to a fresh ice-cold 2mL eppendorf tube and an

equal volume of ice-cold isopropanol was added followed by 150µL of 5M NaCl. The samples were incubated at -20°C for at least 1h and centrifuged at 10,000rpm at room temperature for 20 minutes. The upper layer of solution was discarded carefully by using an adjustable micropipette and 70% ice-cold ethanol about 2.5 times of the solution was added. It was centrifuged again 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 to the slant of the tube. The ethanol was discarded from the tube carefully so that the DNA pellet remained constant and undisturbed. DNA pellet was dried for at least 30 minutes putting the tubes upside down on a filter paper. 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 lateral use.

Primers used

Two selected sugarcane microsatellite primers (markers) developed by International Sugarcane Microsatellites Consortium, NSW, Australia were used to amplify Simple Sequence Repeats of genomic DNA in six sugarcane varieties. The primers were UGSM 565 and UGSM 567 (Table 1). The primers were evaluated on the basis of intensity/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 PCR profile of table-2 94ºC for 3 minutes (initial denaturation) followed by 35 cycles of 40 seconds dennaturation at 94ºC, 30 seconds annealing at 55ºC and elongation or extention at 72ºC for 1 minutes. After last cycle, a final step of 7 minutes at 72ºC was added to allow the complete extention of all amplified fragments. After completion of cycling program, reactions were held at 4ºC. PCR reactions were performed on each DNA sample in a 10µL reaction mixture containing 1.0µL of 10x Ampli Taq polymerase buffer (PCR buffer), 0.6µL of 25mM MgCl2, 1.0µL of 2.5mM DNTPs, 2.5µL each of Primer Forward and Reverse from 2.5µM stock, 0.2µL of 5U/µl Ampli Taq DNA polymerase (Bangalore Genei Pvt. Ltd., India), 2.0µL of 25ng/µL genomic DNA and a suitable amount (0.2µL) of sterile deionized water. After amplification, 2µl loading dye was added to the PCR amplified product and stored at 4ºC for separation using Agarose Gel Electrophoresis. In each well, 8.0µl of PCR amplified product of each DNA sample for each primers was loaded in 1% agarose gel. Electrophoresis was performed at 120V for 1.10 hours. The DNA ladder for primer pairs was run along the sides of the reactions. After electrophoresis, the DNA bands were observed and the data was analyzed. After electrophoresis, the gel was taken out carefully from the electrophoresis chamber and placed in Gel Documentation System (uvitec DBT-2000LS) for scoring of DNA bands. The DNA was observed as band and photographed using alpha view–Fluor Cham FC2 software Gel Documentation system.

SSR data analysis

SSR data were analyzed for Percentage of Polymorphic Loci (*P*), Average Number of Alleles per Locus (*A*), Average Number of Alleles per Polymorphic Loci (*Ap*), Average Number of Genotypes per Locus (*G*), and Gene Diversity-Polymorphic Information Content (PIC). Cluster analysis and Dendrograms were constructed following electrophoresis, and the size of amplification products was estimated by comparing themigration of each amplified fragment with that of a known size fragments of molecular weight marker: 80bp DNA ladder. All distinct bands or fragments (SSR marker) were thereby given identification number according to their position on gel and scored visually on the basis of their presence (1) or absence (0), separately for each individual variety and each primer. The scores obtained using all primers in SSR analysis were then combined to create a single data matrix. Linkage

distances were computed from frequencies of polymorphic markers to estimate genetic diversity and relationship between six sugarcane varieties using the Unweighted Pair-Group Method of Arithmetic Means (UPGMA) (Sneath & Sokal, 1973) using computer program "Statistica".

RESULTS AND DISCUSSION

DNA fingerprinting of ten sugarcane somaclones of two varieties was performed. For this purpose, two SSR primers were selected after test from initially selected five primers because these two primers were able to amplify bands in all used sugarcane somaclones and parent varieties. Initially five primers were selected for the investigation developed by International Sugarcane Microsatellite Consortium. Molecular characterization and genetic diversity analysis of 81 sugarcane varieties were also studied by Muyco (R.R, 2002) using six SSR primers. He selected these six primers from the list of 259 primers developed by the International Sugarcane Microsatellite Consortium based on observation. From this primer list and according to the selection of Muyco, the two primers (UGSM 565 and UGSM 567) were selected for this investigation.

SSR primers with corresponding bands scored, their size range, number of polymorphic bands, percentage of polymorphic bands and number of bands per variety in two sugarcane varieties and their respective somaclones are presented in table-2. The size of amplified bands in ten sugarcane somaclones and two parent varieties ranged from 80 to 650. The SSR primer pair UGSM 565 revealed the band sizes ranging from 80bp to 650bp while the primer pair UGSM 567 showed a range from 350bp to 650bp. This was perhaps due to the differences of the samples used in this investigation. The result was in conformity with the findings of Yang *et al*. (Yang, Maroof, Xu, Zhang, & Biyashev, 1994) who pointed out that the range in allele sizes can be influenced by different samples. The two SSR primer pairs amplified a total number of 58 bands from ten sugarcane somaclones and two parent varieties of sugarcane. Representative Electrophoregrams according to primer pairs UGSM 565 and UGSM 567 were shown in Figures 1 and 2, respectively For two primer pairs, the total number of bands produced varied from 25 to 33 (Table 2). The primer pair UGSM 567 amplified the highest number of bands (33) followed by UGSM 565 (25). Taylor and Cordeiro (Corderio *et al.,* 2000) showed that the SSR primer pairs UGSM 567 and UGSM 565 produced identical fingerprints which partially supports the present findings. The highest number of bands (2.75) per variety was amplified from the primer pair UGSM 567 followed by UGSM 565 (2.08). Due to the polyploidy nature of sugarcane, the SSR markers revealed multiple bands per locus. At South Africa Sugar Association Experiment Station (SASEX), Natal, application of 35 sugarcane microsatellites identified from 1 to 18 alleles per marker across four varieties. In Mauritius Sugar Industry Research Institute (MSIRI), Mauritius, number of alleles generated per primer pair ranged from 9 to 20 using 5 primer pairs on 96 sugarcane cultivars (Jannoo, Forget, & Dookun, 2001). At Center for Plant Conservation Genetics (CPCG), Southern Cross University (SCU) in NSW, Australia 3 to 12 alleles per primer pair were recorded across five sugarcane genotypes using 91 primer pairs (Corderio *et al*., 2000). These results supports present findings. In this investigation, highest percentage (16%) was recorded from the primer pair UGSM 565 which produced the lowest number of total bands (25) whereas lowest percentage (6.06%) of polymorphism was recorded from the primer pair UGSM 567 which produced the highest number of total bands (33). This variation in band formation and polymorphism may be due to variation of primer pairs. The two SSR markers UGSM 565 and UGSM 567 discriminated 17.33% and 22.92% of varieties and somaclones evaluated, respectively. This discrimination may be due to the differential response of the primer pairs with the somaclones and parent varieties.

Both the two SSR markers generated multiple fragments among ten sugarcane somaclones and two parent varieties. The respective genetic diversity or polymorphic information content (PIC) per marker of UGSM 565 and UGSM 567 was recorded as 0.64 and 0.69 for all loci across the ten somaclones and two parent varieties evaluated (Table 3). The primer pair UGSM 565 showed PIC value 0.64 while the marker UGSM 567 produced PIC value 0.69. The most polymorphic SSR marker was associated with the highest number of polymorphic bands detected. The primer pair UGSM 565 was most polymorphic marker across ten somaclones and two sugarcane varieties with PIC value of 0.64. The PIC values are dependent on genetic diversity of somaclones and varieties under study. According to Garland *et al,* (Garland, Lewin, Abedinia, Henry, & Blakeney, 1999) a high proportion of closely related genotypes would have effect of lowering PIC values. In this study, mean PIC value among ten somaclones and two varieties was 0.67 indicating a high level of variability present in varieties and somaclones based on two SSR primers. Comparable results were also reported by Corderio *et al,* (Corderio *et al*., 2000) in sugarcane where they used the SSR primers.

Genetic relationships based on UGSM 565 among ten somaclones along with their two parent varieties at the average distance of 4.95 showed two major clusters (C_1 and C_2) presented in Figure 3. At linkage distance of 4.00 the cluster C_2 produced sub-cluster SC_1 and SC_2. Finally, sub-cluster SC_2 produced sub-sub-cluster SSC_1 and SSC_2 at the linkage distance of 2.0. The major cluster C_1 separated the sugarcane somaclone TC23-10(20) PEG from the other somaclones and the parent varieries. On the other hand, the major cluster C_2 separated the somaclone TC10-10(16) from other somaclones and the parent varieties. Based on UGSM 567, genetic relationships among the ten somaclones along with their two parent varieties at the average distance of 5.5 showed two major clusters (C_1 and C_2) presented in Figure 4. At the linkage distance of 1.00, the cluster C_1 produced sub-cluster SC_1 and SC_2 and at the linkage distance 4.80, the C_2 produced sub-cluster SC_1 and SC_2. Finally, sub-cluster SC_2 of the cluster C_2 produced sub-sub-cluster SSC_1 and SSC_2 at the linkage distance of 1.0. The major cluster C_1 separated the sugarcane somaclone TC23-10(20) PEG from the other somaclones and the parent varieries. On the other hand, the major cluster C_2 separated the somaclone TC10-10(16) from other somaclones and the parent varieties. In both markers, results of DNA polymorphism of the ten somaclones and two sugarcane varieties prove that they bear the genetic diversity of somaclones TC23-10(20) PEG and TC10-10(16) from other investigated somaclones and varieties. Hence, it is clear from figure 3 and 4 that somaclones TC23-10(20) PEG and TC10-10(16) shown to be outliers in dendrogram and distantly related with rest of somaclones based on their genetic distances. The result was in partial agreement with findings of Shahnawaz (R.M.S, 2006) who analyzed DNA polymorphism of the four varieties/accessions and found that the variety Isd 16 was separated from the other varieties in a major cluster while the remaining three were in another cluster. He also used variety Isd 20 having superior performances against biotic and abiotic stresses than the other varieties. It lied in the sub-cluster SC_1 and was separated from other varieties at the linkage distance of 11.0.

CONCLUSION

The results of the present investigation revealed that two SSR primers were able to detect or identify and classify ten sugarcane somaclones along with their parent varieties indicating genetic differences among sugarcane somaclones and their parent varieties. Therefore, detection of genetic variability among sugarcane somaclones should be continued in order to determine their genetic distances and relationships among them using microsatellite markers.

Table-1 Parameters of primers sequence of two sugarcane microsatellite primers from the International Sugarcane Microsatellite Consortium, NSW, Australia

Primer Code	Sequence (5′-3′)	G+C Content (%)
UGSM 565	Forward: 5'-CAT AGC AAG CAC CAC CTC TC-3' Reverse: 5'-TCT TCT TCT CGT CCA CCC-3'	55.00 55.56
UGSM 567	Forward: 5'-CTT CAT ACG CCA CCT TCT C-3' Reverse: 5'-CAA ATG TTC ACT CGC ATC A-3'	52.63 42.10

Table-2 Microsatellite primers with corresponding bands scored, their size range, number of polymorphic bands, polymorphism and number of band per variety together with variety distinguished in two sugarcane varieties and ten somaclones

Primer Codes	Size ranges (bp)	Total number of band score	Number of polymor-phic bands	Polymor-phism (%)	Number of band per variety	Variety distinguished (%)
UGSM 565	80-650	25	4	16.00	2.08	17.33
UGSM 567	350-650	33	2	6.06	2.75	22.92
Total		58	6	-	-	-

Table-3 Microsatellite primers with Polymorphism Information Content (PIC).

Primer Codes	PIC(Polymorphism Information Content)
UGSM 565	0.64
UGSM 567	0.69

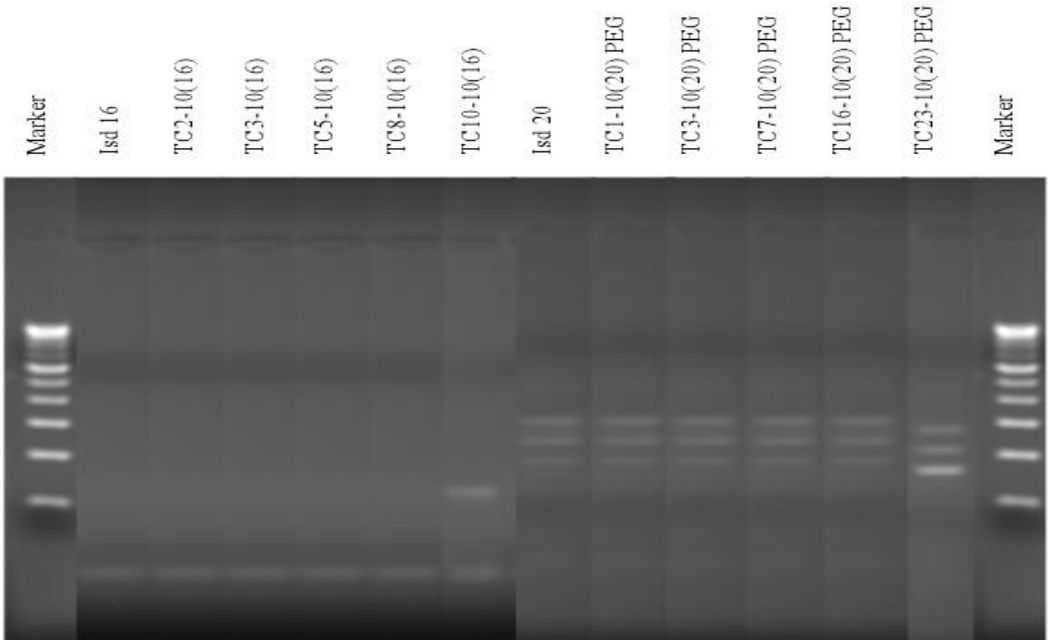

Figure-1 SSR profile of ten somaclones along with their two parent varieties based on Primer pair UGSM 565

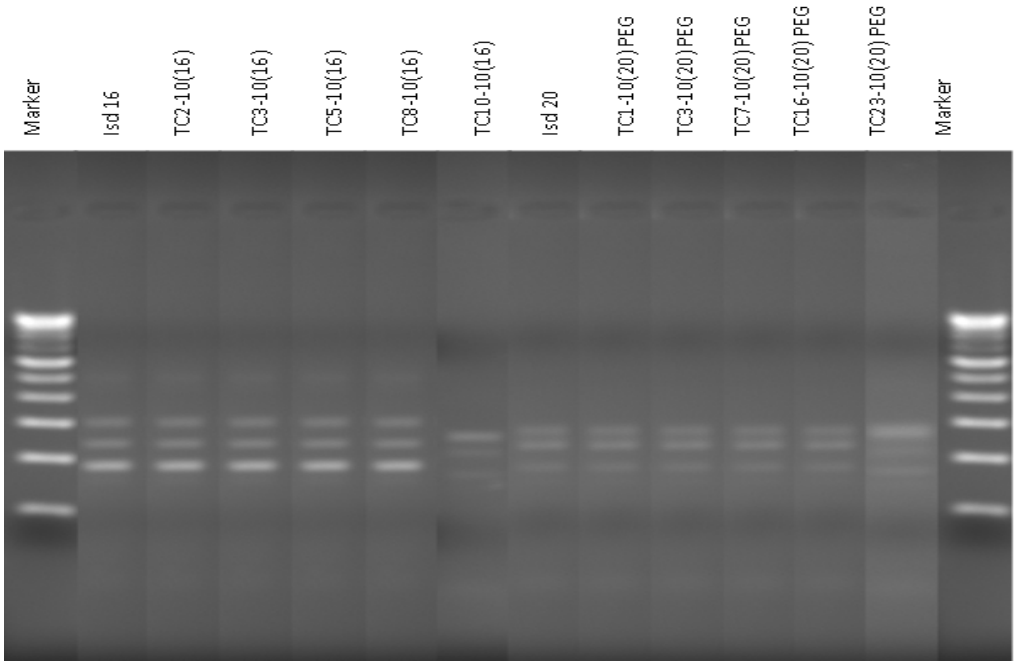

Fig. 2 **SSR profile of ten somaclones along with two parent varieties of sugarcane based on primer pair UGSM 567**

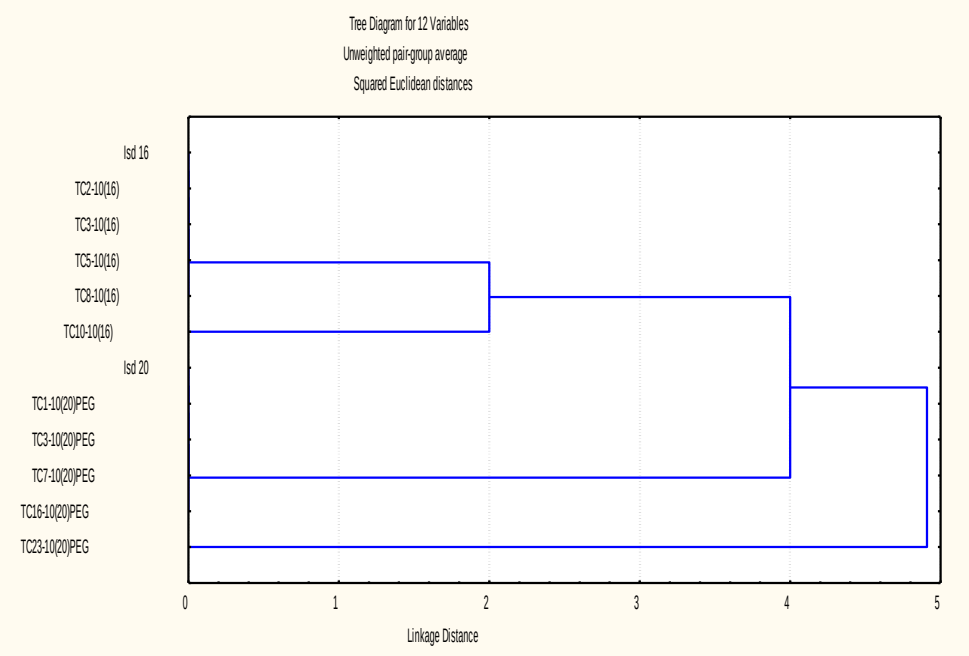

Fig.3 **Tree Diagram for ten sugarcane somaclones and two varieties based on Unweighted Pair-group average Squared Euclidean distances using primer pair UGSM 565**

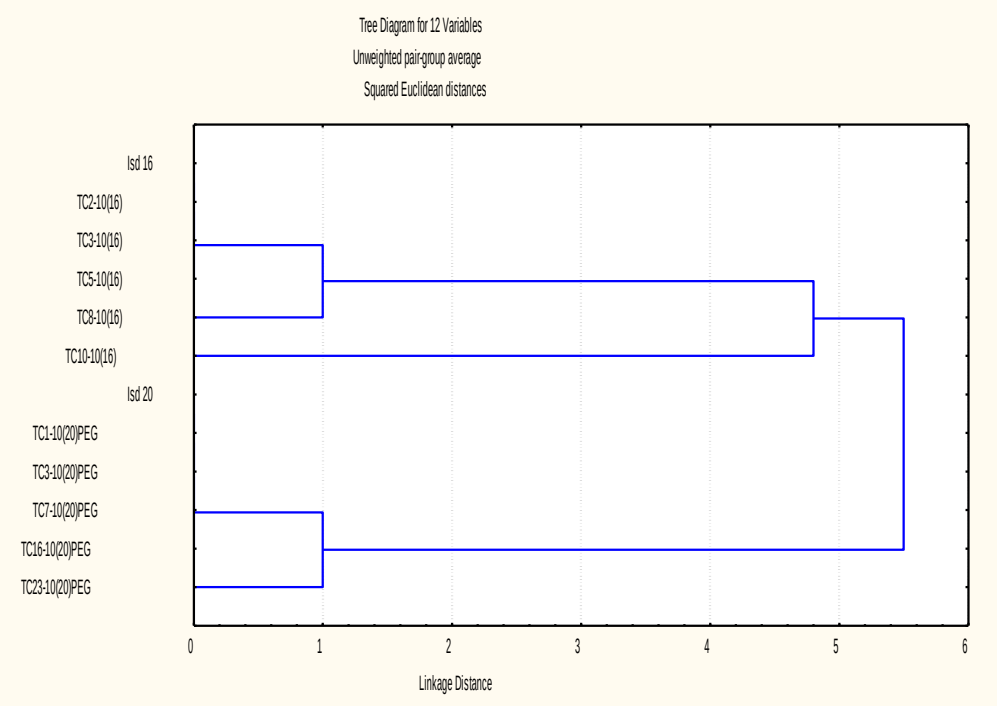

Fig.4 **Tree Diagram for ten sugarcane somaclones and two varieties based on Unweighted Pair-group average Squared Euclidean distances using primer pair UGSM 567**

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