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Research Article

Genetic diversity analysis of sugarcane (*Saccharum* sp.) clones using simple sequence repeat markers of sugarcane and rice

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Abstract

Molecular markers are powerful tools, which help in differentiating plant varieties at the DNA level and have been widely used for genetic diversity studies in a number of crop species'. Understanding the genetic diversity of available clones of *S. officinarum* and *S. spontaneum* will be helpful in breeding programs. In the present study, a set of 48 sugarcane clones from National Hybridization Garden, Sugarcane Breeding Institute, Coimbatore was subjected to genetic diversity analysis involving 40 SSR markers of sugarcane and rice. SSR analysis involving a set of 20 sugarcane and 20 rice primer pairs generated 147 and 114 markers with average polymorphism information content (PIC) value of 0.665 and 0.532 respectively. Cluster analysis using rice marker across 48 genotypes revealed two major clusters with ISH 23 remaining a deviant from the rest of the cultivars. Cluster analysis based on sugarcane SSR markers were found to be more or less similar to that based on rice SSR markers showing minor difference in grouping. Hence, similar results obtained for characterizing the sugarcane clones by using SSR markers from sugarcane and rice reveal that markers with better discriminating power from other cereals can bring out better characterization of sugarcane clones.

Key words : *Saccharum officinarum*, SSR markers, Polymorphism information content, Cluster analysis.

Introduction

Sugarcane is a tall growing monocotyledonous crop plant that is cultivated in the tropical and subtropical regions of the world primarily for its ability to store high concentrations of sucrose, or sugar, in the internodes of the stem. Modern sugarcane varieties that are cultivated for sugar production are complex interspecific hybrids *(Saccharum* spp.) that have arisen through intensive selective breeding of species within the *Saccharum* genus primarily involving crosses between the species Sa*ccharum officinarum* L. and *S. spontaneum* L. (Cox *et al.,* 2000). Commercial hybrid cultivars of sugarcane descended from interspecific hybridisation between *S. officinarum* and *S. spontaneum.*

S.officinarum or the 'noble cane' accumulates very high levels of sucrose in the stem but have poor disease resistance. Conversely *S. spontaneum* accumulates no sucrose but is a highly polymorphic species with much higher levels of disease resistance, adaptability and stress tolerance (Sreenivasan *et al,* 1987). The basic breeding concept involves the combination and improvement of vigor and disease resistance from *S. spontaneum* and high sucrose content from *S. officinarum.* Increasing sucrose content while maintaining disease resistance of commercial hybrid cultivars has been achieved through a number of back-crossing to several different cultivars of *S. officinarum* (Bull and Glasziou, 1979). Consequently, *S. spontaneum* genetic component is reduced in commercial hybrid cultivars. Of the chromosomes in these commercial hybrid cultivars, approximately 80 percent are derived from *S. officinarum* and 10 percent are from *S. spontaneum,* with remainder being chromosome from the two species produced by the natural process of synapsis during meiosis (D'Hont *et al.,* 1996).

Interspecific hybridisation between *S. officinarum* as the female parent and *S. spontaneum* as the male parent produce progeny that have a triploid chromosome number (2n + n = 100 to 130) (Sreenivasan *et al,* 1987; D'Hont *et al,* 1996). This phenomenon, thought to arise either through endoduplication or fusion of two nuclei during meiosis (D'Hont *et al,* 1996), facilitated breeding

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of modern sugarcane varieties as the '*officinarum'* qualities recovered more quickly in the hybrids, thus requiring fewer round of backcrossing to produce high sucrose varieties.

The genetic diversity analysis based on DNA fingerprinting is one of the approaches by which clear-cut decisions could be taken to select parents for hybridization and efficient management of germplasm collections (Warburton and Hoisington, 2001).

Cordeiro *et al.* (2001) used sugarcane SSR primer pairs in related genera like *Erianthus* and sorghum. The level of polymorphism in sugarcane cultivars detected by these SSR was found to be very low in sugarcane. However, a subset of these markers showed a significantly higher level of polymorphism when applied to progenitors and related genera (*Erianthus* and sorghum). In contrast, SSRs isolated from sugarcane genomic libraries amplify more readily, showing high levels of polymorphism within with a higher PIC value (0.72) but their cross transferability across related species or genera was found to be poor.

Cordeiro *et al.* (2003) determined the level of genetic diversity among the genera *Saccharum (S. spontaneum, S. officinarum, S. sinense),* old world *Erianthus* Micnx.. Ripidum, North American *E. giganteus (S. giganteum), Sorghum* and *Miscanthus* using microsatellite markers

Selvi *et al.* (2003) used a set 34 SSR primer pairs from maize and found repeatable amplifications for 14 in *Saccharum* species clones, commercial hybrids, and the related genera *Erianthus,* accounting for 41.17 per cent cross transferability. The present study aimed at determining the extent of genetic diversity across the sugarcane accessions using SSR markers having their origin from sugarcane and rice.

Materials and Methods

A set of 48 clones (19 tropical, 18 subtropical, 6 exotic varieties and 5 interspecific hybrids) obtained from National Hybridization Garden (NHG), Sugarcane Breeding Institute (SBI), Coimbatore formed the biological materials for this study.

DNA extraction

DNA was extracted from the leaf samples from each of the above clones following Cetyl Trimethyl Ammonium Bromide (CTAB) method as described by Honeycutt *et al.* (1992). DNA quality and quantity of each genotype was assessed by running the DNA in 0.8 per cent agarose gel with known standards. All the DNA samples were uniformly diluted to have a final concentration of 20ng/µl.

SSR analysis

A total of 40 SSR primer pairs (20 from sugarcane and 20 from rice obtained from National Research Centre on Plant Biotechnology, New Delhi and Research Genetics Inc. USA respectively) was used for PCR amplification of repeat sequences from the genomic DNA of each clone. The primer pairs used are given in Tables 1 and 2.

PCR reactions were performed using PTC 100 programmable thermal cycler from MJ Research Waltham Mass. The PCR reaction using sugarcane SSR primer pairs was carried out with the volume of 15µl containing 20 ng of genomic DNA, 20 µM of each forward and reverse primer, 1.25 mM dNTP's, l0x PCR buffer containing 10 mM Tris HC1 (pH 8.3), 50 mM KC1, 1.5 mM MgCl, 0.01 percent gelatin and 0.5 units of *Tag* DNA Polymerase.The PCR reaction using rice SSR primer pairs was carried out with the volume of I5µl containing 20ng of genomic DNA, 20 µM of forward and reverse primers, 1.25 mM dNTPs mixture containing dATP, dCTP, dGTP, dTTP's, l0xPCR buffer and 0.5 units of *Tag* DNA polymerase. Amplifications were done under conditions of 94°C for 5 minutes followed by 34 cycles of 94°C for 45 seconds, X°C for 45 seconds, 72°C for 2 minutes followed by extension at 72°C for 5 minutes and finally at 4°C. (X°C refers to the annealing temperature, which is specific for each of the primer pairs used).

PCR products were loaded on 5 percent denaturing polyacrylamide: bis acrylamide (38:2) gels and electrophoresed in IxTBE buffer (pH 8.3) at constant power (100Watts) for 1 hour using Sequi Gen® GT Nucleic acid electrophoresis cell from Biorad Laboratories and the products were resolved using silver staining procedure.

Data analysis

Only clear and unambiguous bands of SSR markers were scored. Markers were scored for the presence and absence of the corresponding band among the genotypes. The scores ‘1’ and '0' indicates the presence and absence of bands respectively. In case of binary coding a data matrix comprising of ' 1' and '0' was formed and the resultat data matrix was subjected to further analysis.

Polymorphism information content (PIC)

PIC values or expected heterozygosity scores for SSR (polyallelic) markers were calculated following the formula: $Hj = 1 - \sum pi^{2,}$ where p_i is the allele frequency for the *i*- th allele (Nei, 1973).

Cluster analyses

The marker data gathered were subjected to cluster analysis. Sequential Agglomerative Hierarchical Non-overlapping (SAHN) clustering was performed on squared Euclidean distance matrix and similarity matrix using Dice coefficient (Dice, 1945) for quantitative and binary data respectively utilizing the Unweighted Pair Group Method with Arithmetic Averages (UPGMA) method. Data analysis was done using NTSYSpc version *2.021* (Rohlf, 1998).

Comparison of similarity coefficient matrices

The correspondence between similarity coefficient matrices based on rice and sugarcane SSR markers was tested based on correlation analysis and Mantel (1967) matrix correspondence test. The Mantel matrix correspondence test was carried out using the; MXCOMP procedure in NTSYSpc version 2.02i (Rohlf, 1998)

Results and Dissusion

A total of 40 primer pairs, 20 each having the origin from sugarcane and rice were used to generate 114 and 147 SSR markers respectively. The average number of markers produced per primer pair was found to be higher with sugarcane primer pairs (7.35) than rice primer pairs (5.70) (Table 3.). The difference in the percentage of polymorphism observed with SSR markers generated by sugarcane and rice was not very wide. More than 90 percent of the markers generated remained polymorphic irrespective of the origin of primer pairs. Though SSR markers are considered as single locus, codominant and polyallelic in nature, the generation of multiple markers denied the power of extracting clear-cut allelic variation for each of the markers. Out of 40 primer pairs employed in generating markers nine primer pairs produced more than 10 markers per primer.

SSR markers are di-, tri-, tetra-nucleotide tandem repeats containing loci of eukaryotic genomes. It is demonstrated that these loci are very polymorphic due to changes in the number of repeating units among the individuals of populations. Each SSR locus can easily be amplified by using PCR knowing the DNA sequence flanking the repeat region specifically. The only limiting feature of the application of these markers is the need for prior sequence information for developing primers for locus-specific PCR amplification. This limitation is alleviated for the economically important species and the ones closely related, since primer sequences of the SSR DNA markers and the amplification conditions are available in the published reports. However, when the reported PCR amplification conditions were applied, not all the primer pairs produce specific markers, which are specific for the locus. Generation of complex banding patterns for SSR loci could be due to various reasons such as type of repeat, non-optimization of PCR conditions and the nature of genome.

Nelson *et al.* (1996) reported that the banding patterns of SSR markers varied widely depending on the repeat motif and anchor by testing SSR primer pairs from eastern white pine *(Pinus strobus)* on loblolly pine *(P. taedd).* Kaye *et al.* (1999) established several clones having the sequences containing GA, GT, ACC, GAA, CAA, ATT, GCC, CATA and GATA repeats from sugarcane cultivar R570. The resulting banding patterns of these markers ranged from simple, monomorphic patterns with few bands to highly complex multi-band "ladder-like" patterns. Dourar and Akkaya (2001) stressed the need for PCR optimization, especially with reference to the annealing temperature and Mg^{2+} ion concentrations to have locus specific markers by an experiment conducted in wheat using primer pairs *viz.* WMS30 and WMS46. Selvi *et al.* (2003) reported that the complex banding pattern for SSR markers in sugarcane could be due to its polyploid and heterozygous nature of the genome by using a set of 34 primer pairs from maize.

In the present study, most of the primer pairs produced complex banding patterns. Considering the ambiguity in the number of alleles per locus, all the markers generated were scored as dominant markers. The primer pairs derived from rice genome generated comparatively less number of markers (114) when compared to markers generated by the sugarcane derived primer pairs (147). Though the rice derived primer pairs were cross transferable in maize, the number of informative markers were less. The same trend was established by Selvi *et al.* (2003) by having 41.2 percent transferability of maize markers in their experiment conducted for proving the utility of SSR primer pairs developed for one species to amplify DNA of close relatives, especially in Poaceae. Not much difference was found in the PIC values of the SSR primer pairs derived from sugarcane (0.665) and rice (0.532).

Cluster analyses were carried out independently by using 147 markers produced by sugarcane derived primer pairs, 114 markers from rice derived primer pairs and 261 markers by combining the markers of sugarcane and rice derived primer pairs as Operational Taxonomic Units (OTUs).

The results of all the three cluster analyses revealed that clone ISH 23 was isolated from the rest of the 47 clones. Cluster analysis based on sugarcane derived SSR primer pairs resulted in the grouping of 47 clones into three subgroups (Fig.1) whereas the cluster analysis based on rice derived SSR primer pairs produced only two subgroups (Fig. 2) indicating better discriminating power of the markers generated by sugarcane derived SSR primer pairs. Cluster analysis based on the 261 markers of both sugarcane and rice primer pairs grouped the clones into three subgroups (Fig.3). However, the results did not reveal the grouping of the remaining clones into clones based on different geographical adaptations *viz.* tropical, subtropical, exotic varieties and interspecific hybrids.

Among the 47 clones, CP 44-101, an exotic variety and CoA 7602, a tropical variety had the similarity coefficient of more than 0.9. Almost the same trend was observed between the two clones *viz.* CoS 92263, a subtropical variety and Co 86011, a tropical variety when the markers of sugarcane derived primer pairs were used as OTUs for establishing the similarity. But these two pairs of clones were distanced from each other in other two analyses. The non-occurrence of grouping of clones into tropical, subtropical, exotic varieties and interspecific hybrids in all the three cluster analyses could be due to polyploidy and highly heterozygous nature of sugarcane genome.

The comparative analysis of similarity matrices of above analyses based on Mantel's (1967) t-test indicated existence of congruence between the dendrograms of SSR sugarcane *vs.* sugarcane +rice and dendrograms of SSR rice *vs.* sugarcane +rice. This congruence could be due to the SSR markers generated by rice derived primer pairs, which are comparatively less, polymorphic across the 48 clones when compared to SSR markers amplified by sugarcane derived primer pairs. But the use of rice derived primer pairs in sugarcane clearly established the possibility of cross-transferability of SSR markers across closely related species such as sugarcane and rice or across the cereals. This cross-transferability of SSR markers across close relatives was well established in mammals (Ammer *et al.,* 1992; Kondo *et al.,* 1993; Moore *et al..,* 1991) and in plants (Brown *et al.,* 1996; Hernandez *et al.,* 2001; Ishii and McCouch, 2000; Kresovich *et al,* 1995; Provan *et al.,* 1996). Moreover, cross species amplification of SSR markers of rice in maize and bamboo and of potato in tomato (Provan *et al.,* 1996) provided further evidence that SSR markers are cross-transferable not only between species but also between genera. Wang *et al.* (2005) reported the cross-transferability of SSR markers from major cereals like wheat, rice, maize and sorghum to minor grass species such as fingermillet, paspalum and Bermuda grass, especially for germplasm characterization and evaluation. However, the cross-transferability of SSR markers varied from species to species. Gupta *et al.* (2003) reported that the cross-transferability of SSR markers from wheat to barley was 55 percent while Saha *et al.* (2004) reported a cross-transferability of 57 percent from tall fescue to several grass species including wheat and rice. While evaluating the maize SSR markers for genetic diversity analysis and fingerprinting in sugarcane, Selvi *et al.* (2003) reported 41.17 percent cross transferability to *Saccharum* species clones, commercial hybrids, and the related genera *Erianthus.*

From the present study, it is very evident that the availability of primer pairs to generate locus specific SSR markers are enormous which .makes no-necessity of using random markers for DNA fingerprinting and genetic diversity analysis. The abundance, hyper-variability, co-dominance and high reproducibility of these SSR markers facilitate better discrimination of genotypes at sequence level. Moreover, the cross-transferable nature of these SSR markers can be exploited ignoring the need of developing markers for each and every species. But one must be careful in the selection of primer pairs before venturing into plant genotyping by characterizing the primer pairs for the production of markers with locus specificity, hyper-variability and less complex banding pattern. It is expected that using more primer pairs from sugarcane and other cereals *viz.* rice, maize, sorghum and wheat will bring out better characterization of sugarcane clones used in this study for establishing their genetic relationship and clone identification.

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Table 1. Total number of SSR markers, polymorphism percentage and average PIC value for SSR primer pairs derived from sugarcane

Primer	Total markers	Polymorphic markers	Polymorphism (%)	Average PIC value
NKS 1	6	6	100.0	0.627
NKS 2	12	12	100.0	0.721
NKS 3	3	3	100.0	0.641
NKS 5	5	5	100.0	0.924
NKS 6	8	8	100.0	0.513
NKS 7	6	6	100.0	0.344
NKS 8	6	6	100.0	0.734
NKS 9	7	7	100.0	0.333
NKS 11	11	11	100.0	0.484
NKS 12	8	8	100.0	0.660
NKS 14	8	8	100.0	0.694
NKS 15	13	13	100.0	0.776
NKS 16	13	13	100.0	0.590
NKS 17	5	5	100.0	0.705
NKS 20	8	8	100.0	0.785
NKS 21	4	4	100.0	0.599
NKS 22	8	7	87.5	0.677
NKS 23	11	11	100.0	0.853
NKS 24	3	3	100.0	0.794
NKS 25	3	3	100.0	0.857
TOTAL	**147**	**146**	**99.4**	**0.665**

Table 2. Total number of SSR markers, polymorphism percentage and average PIC value for SSR primer pairs derived from rice

Primer	Total markers	Polymorphic markers	Polymorphism (%)	Average PIC value
RM 5	2	0	0.0	0.000
RM 9	13	13	100.0	0.891
RM 18	2	2	100.0	0.582
RM 84	5	5	100.0	0.643
RM 201	2	2	100.0	0.783
RM 336	5	5	100.0	0.812
RM 1135	3	0	0.0	0.000
RM 1279	7	7	100.0	0.554
RM 2819	7	4	57.1	0.386
RM 3555	3	3	100.0	0.581
RM 3583	10	10	100.0	0.746
RM 3589	5	5	100.0	0.656
RM 5405	10	10	100.0	0.130
RM 5499	5	5	100.0	0.625
RM 5508	3	3	100.0	0.441
RM 6083	4	3	75.0	0.517
RM 6111	9	9	100.0	0.396
RM 6779	2	2	100.0	0.581
RM 7564	7	7	100.0	0.797
RM 8261	10	10	100.0	0.532
TOTAL	**114**	**105**	**92.1**	**0.532**

Table 3. Comparative statement of total number of SSR markers. polymorphism percentage and average PIC value for SSR primer pairs derived from sugarcane and rice

Marker Parameter	Sugarcane Primer pairs	Rice Primer Pairs
Number of primers used	20	20
Total number of markers	147	114
Average number of markers/primers	7.40	5.70
Number of monomorpgic markers	1	9
Number of polymorphic markers	146	105
Percentage polymorphism	99.40	92.10
Minimum PIC value	0.333	0.000
Maximum PIC value	0.924	0.891
Average PIC value	0.665	0.532

Table 4. Comparison of similarity matrices derived from various OYUS

Similarity matrices compared	Matrix Correlation : r	Mantel t-test : t	P Value
SSR sugarcane vs rice	0.7907	6.3754	1.0000
SSR sugarcane *vs* sugarcane + rice	0.9327	7.3924	1.0000
SSR rice vs sugarcane +rice	0.9578	7.4777	1.0000

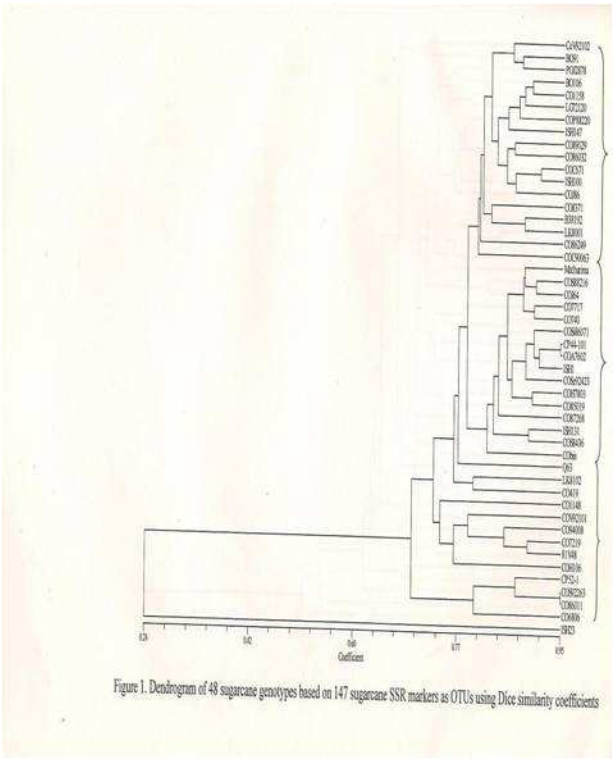

Figure 1. Dendrogram of 48 sugarcane genotypes based on 147 sugarcane SSR markers as OTUs using Dice similarity coefficients

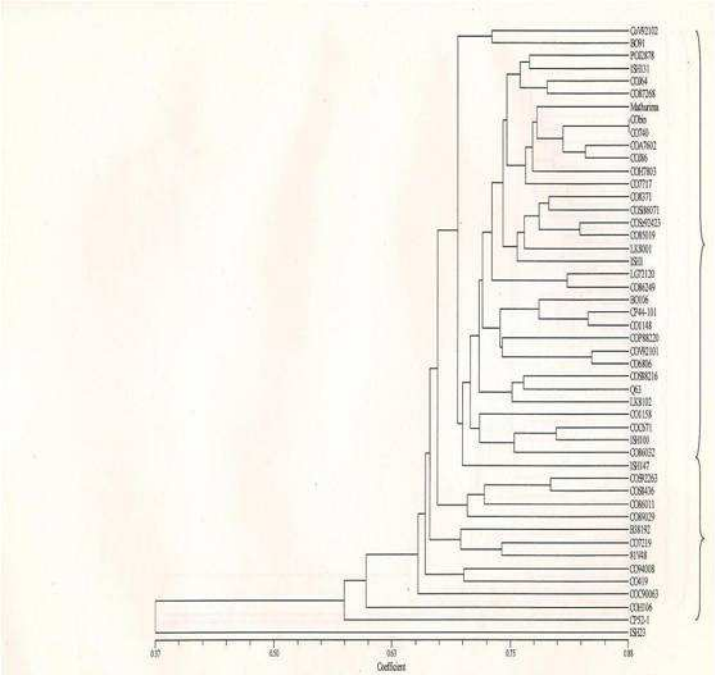

Figure 2. Dendrogram of 48 sugarcane genotypes based on 114 rice SSR markers as OTUs using Dice similarity coefficients

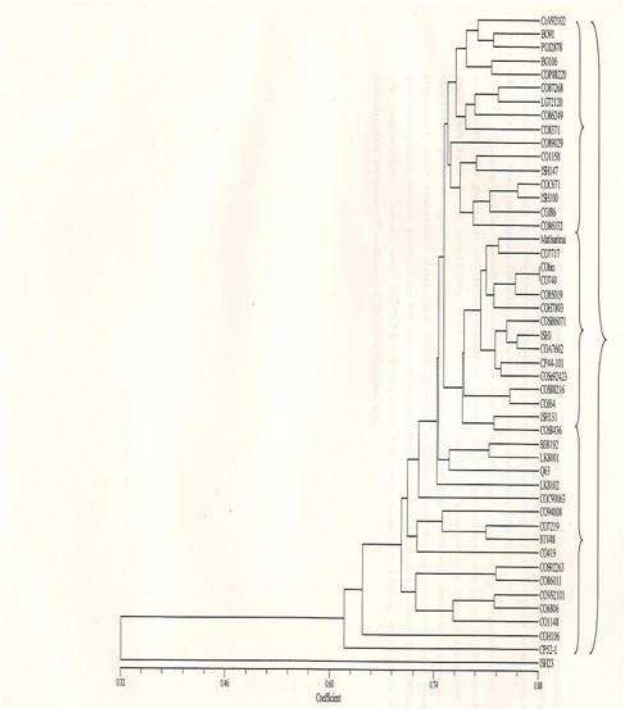

Figure 3. Dendrogram of 48 sugarcane genotypes based on 147 sugarcane + 114 rice SSR markers as OTUs using Dice similarity coefficients

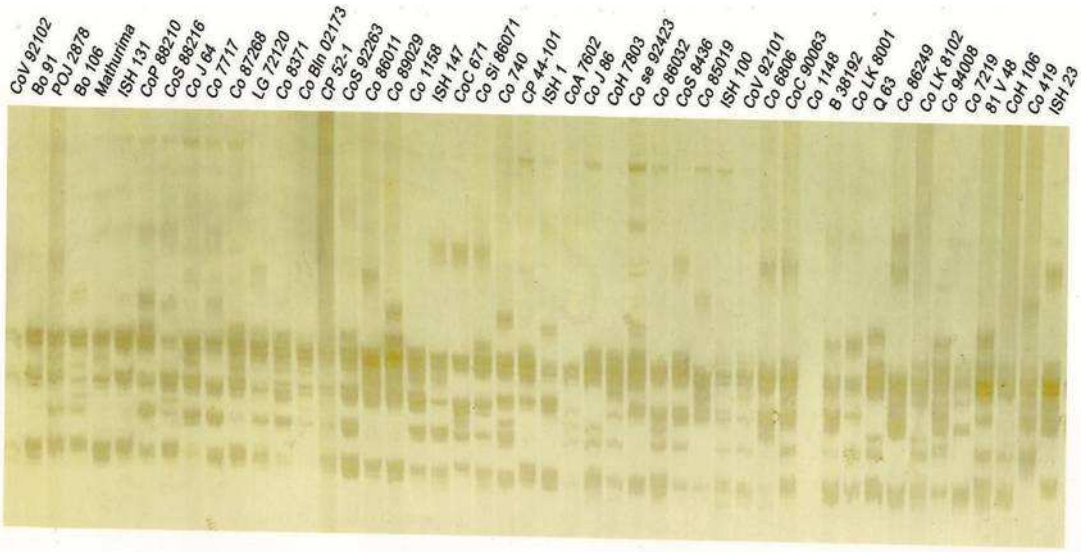

Plate 1. SSR marker profile of a section of 48 genotypes of sugarcane produced by the primer pair NKS 2

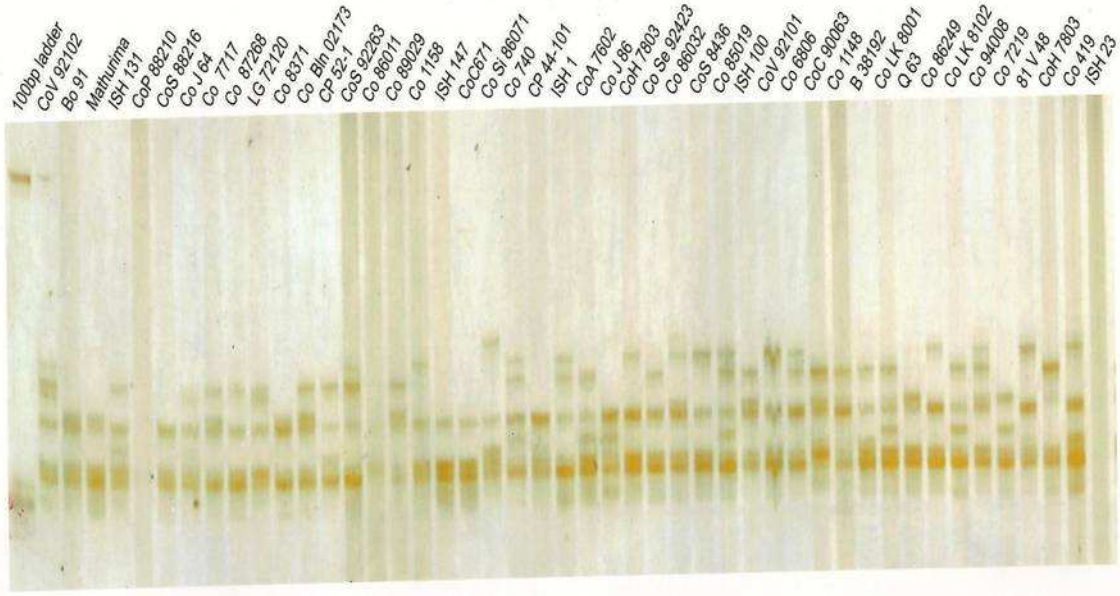

Plate 2. SSR marker profile of a section of 48 genotypes of sugarcane produced by the primer pair NKS 22

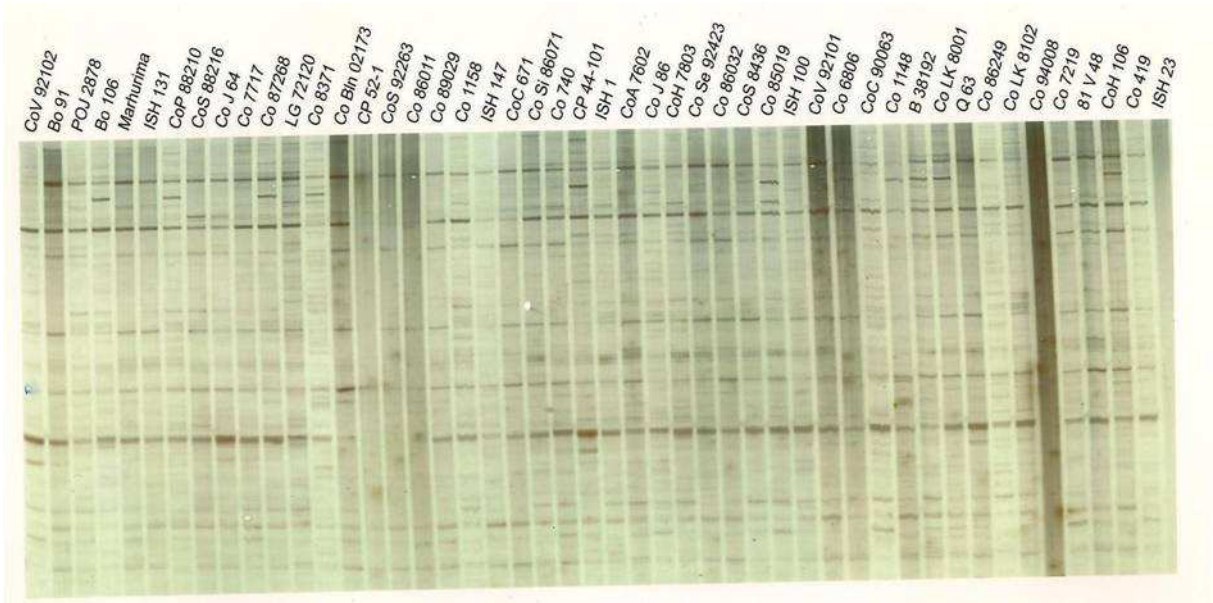

Plate 3. SSR marker profile of a section of 48 genotypes of sugarcane produced by the primer pair RM 1279

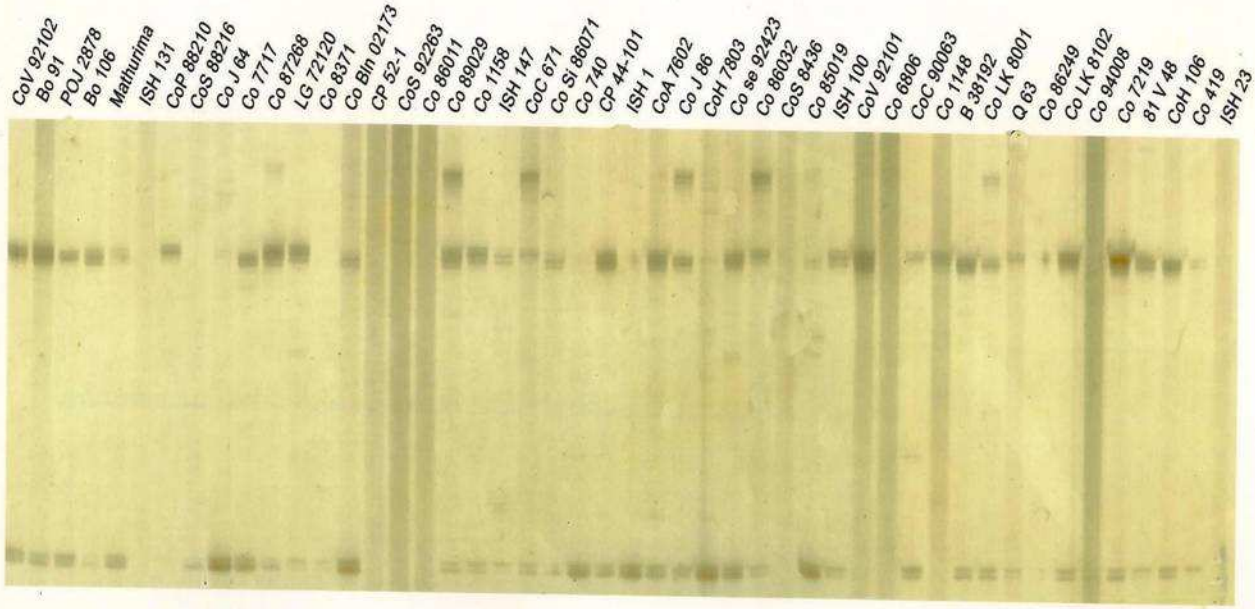

Plate 4. SSR marker profile of a section of 48 genotypes of sugarcane produced by the primer pair RM 3555