Genetic Polymorphism among Some Sugarcane Germplasm Collections as revealed by RAPD and ISSR analyses

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Abstract: Nine genotypes of sugarcane (*Saccharum* spp) namely G.T.54-9, G.84-47, POJ.28-78, Co.997, F.161, F.153, N.Co.310, G.74–96 and Phil.8013; which available at Sugar Crops Research Institute (SCRI) screened to detect the genetic polymorphism using RAPD and ISSR techniques. Based on RAPD data, the percentage of polymorphic amplified products ranged from 37.5-72.7%, the total number of the amplified RAPD produced by each primer varied from 8-11 amplified products. Unique DNA bands with different sizes were detected in particular genotypes, primer OP-A01produced two DNA bands displayed in the genotype G.T. 54-9 (258 bp and 700 bp). While primer OP-O10 produced two DNA bands, one band displayed in the genotype G.84-47 (924 bp) and one in G.T. 54-9 (1104 bp). Some of the primers produced polymorphic bands specific to a set of genotypes. These bands could be considered as genotype-specific bands. Based on ISSR data, the percentage of polymorphic amplified products ranged from 9.09 to 80%. The total number of the amplified RAPD produced by each primer varied from 10-12 amplified products. Unique DNA bands with different sizes were detected in particular genotypes. Primer 17899B produced two DNA bands for genotypes G.84-47 (972 bp) and F 161 (690 bp), while each primers 844B and HB 15 produced one band displayed in genotypes NCo310 (715 bp) and G.T. 54-9 (312 bp) respectively; primer HB 14 produced two bands, one of them displayed in genotype Co. 997. These bands could considered as genotype-specific bands. The overall similarity indices based on both techniques revealed that the highest similarity was 79%, between genotypes G.74-96 and Nco.310 followed by genotypes F.153 and F.161 (78%), while the lowest similarity was 50% between genotypes G.T.54-9 and POJ.28-78, followed by genotypes G.T.54-9 and F.153 (53%). This investigation highly recommended using of RAPD and ISSR systems in order to detect genetic polymorphism and genetic similarity in sugarcane genotypes.
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1. Introduction

The cultivated sugarcane (*Saccharum* spp. hybrids) is a genetically complex polyploidy grass because of its multi-species origin, resulting in chromosome mosaicism generally 2n=100-130 (D'Hont *et al*., 2001). The genetic base of the modern genotypes appears to be narrow and reflected in the slow progress in sugarcane improvement (Jackson, 2005). Using DNA-based molecular marker technique is an important tool to study genetic polymorphism and genetic diversity in sugarcane breeding programs to identify genetically diverse parents for developing hybrid cultivars with improved cane and sugar yields (D'Hont *et al*., 2001). DNA-based molecular markers help to dissect the genome because of its specificity, robustness, requires less DNA and it is not affected by environmental conditions during developmental stages of plant (D'Hont *et al*., 2001). There are numerous PCR based molecular marker techniques such as RAPD (Random amplified polymorphic DNA) (Williams *et al*., 1990) and ISSR (Inter Simple Sequence Repeat) (Shrivastava and Gupta, 2008) has been used in explaining genetic diversity among different genotypes of sugarcane. As compared to the earlier techniques, RAPD analysis employs single short primers with arbitrary sequence to generate genome specific fingerprints of multiple amplification products (Welsh & McClelland, 1990 and Williams *et al*., 1990). ISSRs are semi-arbitrary markers amplified by PCR using a single primer composed of a microsatellite repeated sequences (Shrivastava and Gupta, 2008). Such amplification does not require genome sequence information and leads to multi- locus and highly polymorphic patterns (Wolfe, 1998).

In sugarcane, RAPDs and ISSRs are used to assess levels and patterns of variation among genotypes, species as well as members of Saccharum complex and to identify putative markers linked to phenotypic traits (Srivatava and Gupta 2006; and Fahmy, 2008). Khaled (2010) used RAPD markers to obtain molecular profiles and determine the genetic diversity among sugarcane genotypes used in

breeding program of the Sugar Crops Research Institute (SCRI) in order to maximize cane and sugar yields. He mentioned that RAPD could provide an additional discriminatory power for genetic diversity and crossing of the working germplasm in the breeding program. Ali *et al*. (2013) used RAPD technique for characterize rust resistance in sugarcane. They found that three primers generated five specific loci in four sugarcane cultivars, which is a potential use of RAPD-PCR to identify *Saccharum* spp. hybrids and clones. Kalwade *et al*. (2012) studies 17 sugarcane genotypes using 27 ISSR. Out of the 252 amplicons amplified by 27 ISSR primers, 212 were polymorphic (84.13%).Cluster analysis by UPGMA method revealed similarity coefficient of 0.49 that mainly attributed to inter specific diversity. Khaled *et al*. (2011) evaluate 26 sugarcane clones for their sugar content and detect some markers associated with sugar content using RAPD and ISSR-based PCR techniques; they showed that 173 RAPD bands show 95.95% polymorphism revealed by nine primers, while 36 ISSR bands show 37.72% polymorphism revealed by seven primers in the studied sugarcane clones.

The present paper reports the results of study on the Genetic Polymorphism and genetic diversity among nine sugarcane genotypes from the sugarcane germplasm collection revealed by RAPD and ISSR.

2. Material and Methods

Nine sugarcane genotypes named POJ.28-78, Co.997, F.161, F.153, N.Co.310, G.74–96, Phil.8013, G.T.54-9 and G.84-47 (Table 1); which available at Sugar Crops Research Institute (SCRI), Giza, Egypt; were used as experimental material to detect the genetic polymorphism among the studied genotypes using RAPD and ISSR techniques.

Isolation of genomic DNA

Genomic DNA was isolated from sugarcane meristem cylinder using CTAB method described by Doyle *et al*. (1987) and modified by Khaled and Esh (2008). DNA quantification was done using spectrophotometric measurement of UV absorption at wave lengths 230, 260 and 280 nm and DNA was checked by using 1% agarose gel electrophoresis (AGE)/TBE. The DNA was diluted in TE buffer to a working concentration of ~10 ng/µL.

Table 1: Names, pedigrees and origins of the nine sugarcane genotypes.

Variety name	Pedigree			Source of seed
	Female		Male	
G.T. 54-9	NCO 310	X	F 37-925	Seed fuzz from Taiwan
G 84-47	NCO 310	X	?	Local seed fuzz
POJ.28-78	POJ2364	X	EK28	Official cross
F.161	F 146*	X	F 149*	Taiwan
Co.997	CO 683	X	P63-32	India
F.153	NCO 310	X	P 34-136	Taiwan
NCo.310	Co.421	X	Co.312	Crossed in India and selected in Natal (S.A.)
G.74-96	Open pollination			
Phil 8013	CAC 71-312	X	Phil 642227	Seed cutting from The Philippines

? Unknown parent
F 146 = NCO 310 x PT 43-52   F 149 = NCO 310 x PT 43-52

PCR Amplification and Gel Electrophoresis

RAPD-PCR analysis

Reaction conditions were optimized according to Sambrook *et al*. (1989). Seven arbitrary 10-mer primers (Operon Technologies, USA) were used (Table 2) for PCR amplification. Amplifications were done in 25 µL of reaction mixture containing 50 ng template DNA, 2.5 µL of 10× PCR buffer, 2.5 mM MgCl2, 2 mM dNTPs, 15 ng of primer and 1 Unit of Taq Polymerase supplied by Promega company. Amplification was performed using MJ 200CT thermo cycler (4.30 min at 92°C, 1 min at 35°C and 2 min at 72°C; followed by 44 cycles each of 1 min at 92°C, 1 min at 35°C and 2 min at 72°C, followed by one final extension cycle of 15 min at 72°C). The amplification products were separated in 1.4% Agarose and stained by ethidium bromide.

Inter simple sequence repeats (ISSRs)-PCR analysis

Five ISSR primers were used for the PCR amplification (Table 3). PCR Amplification was performed for 42 cycles using MJ 200CT thermo cycler. It was programmed as single denaturation step of 4 min at 94 °C, followed by a step cycle program for 40 cycles of denaturation at 94 °C for 1 min, annealing at 55 °C for 1 min and extension at 72 °C for 1 min and final extension at 72 °C for 10 min, then hold at 4°C. The amplification products were separated by in 1.4% Agarose with 1× TBE and stained by ethidium bromide.

Table 2: Codes and sequences for seven random primers used in RAPD-PCR analysis

Primer code	Sequence	Primer code	Sequence
OP-A01	5`-CAG GCC CTT C-3`	OP-B10	5`-CTG CTG GGA C-3`
OP-A04	5`-AAT CGG GCT G-3`	OP-O10	5`-TCA GAG CGC C-3`
OP-A07	5`-GAA ACG GGT G-3`	OP-O14	5`-AGC ATG GCT C-3`
OP-B07	5`-GGT GAC GCA G-3`		

Table 3: Codes and sequences of five primers used in ISSR-PCR analysis

Primer code	Sequence (5`→ 3`)	Primer code	Sequence (5`→ 3`)
17899B	(CA)6GG	HB14	(CTC)3GC
844B	(CT)8GC	HB15	(GTG)3GC
HB11	(GT)6GG		

Data Analysis

Each analysis was a consensus of two replicates run. The genetic similarity between genotypes assessed based on the Dice similarity coefficient and complemented with a UPGMA-based cluster analysis according to TotalLab software package v. 2009 supplied by Nonlinear Dynamics Co. The banding patterns obtained with the RAPD and ISSR primers were scored and converted to binary values of (1) and (0) for the presence and absence of bands, respectively. The binary matrix were analyzed using SPSS software package v.15 in order to develop the consensus tree for these genotypes and to estimate their similarity indices.

3. Results and Discussions

PCR Amplification and Gel Electrophoresis

Assessing variability and identification of available germplasm are essential components of crop improvement programs. Knowledge of the genetic distances among different genotypes is very useful for genetic improvement.

RAPD-PCR Amplification

The RAPD profiles of the amplification products of the seven random primers are shown in Figures 1, 2 and the number of bands generated by each primer is given in Table 4. The Seven primers used to screen the nine sugarcane genotypes generated a total number of 67 amplified DNA with an average of 9.57 bands/primer. Out of the total bands, 35 were polymorphic and the percentage of polymorphic amplified products ranged from 72.7% for primer OP-A04 to 37.5% for primer OP-O14. The total number of the amplified RAPD produced by each primer varied from a minimum number of 8 amplified products by primer OP-A07 and OP-O14 to a maximum of 11 amplified products by primer OP-A04 and OP-B07. The size of amplified bands also varied with different primers. Primer OP-O14 amplified the largest 1465 bp band, while primer OP-B07 amplified the band smallest size 75 bp.

Such a level of polymorphism was consistent with some reports based on RAPD marker. Sreevastava and Gupta (2006) listed that 998 RAPD bands showed 77.5% polymorphism in 42 varieties. Khaled *et al* .(2011) mentioned that 173 RAPD bands showed 95.95% polymorphism in 26 sugarcane clones and Ali *et al*. (2013) demonstrated that seven RAPD primers produced 21 detectable bands and showed 100% polymorphism among eight cultivars of sugarcane. In most of the varieties studied, not a single was specific to any individual variety.

In some of the genotypes studied, a single band was specific to an individual genotype. Unique DNA bands with different sizes were detected in particular genotypes, but not in the others. Primer OP-A01produced two DNA bands displayed in the genotype G.T. 54-9 (258 bp and 700 bp) (Fig. 1). While primer OP-O10 produced two DNA bands, one band displayed in the genotype G.84-47 (924 bp) and the other in G.T. 54-9 (1104 bp) (Fig. 2). These bands could be considered as genotype-specific bands (Fig.1).

Table 4: TAF, PF, P% and SB for sugarcane genotypes using RAPD-PCR.

Primer No.	Primer name	TAF	PF	P%
1	OP-A01	10	6	60
2	OP-A04	11	8	72.7
3	OP-A07	8	4	50
4	OP-B07	11	5	45.5
5	OP-B10	10	5	50
6	OP-O10	9	4	44.4
7	OP-O14	8	3	37.5
Total		**67**	**35**	**52.2**

TAF = total amplified bands, PF = polymorphic bands, P % = Polymorphism percentage

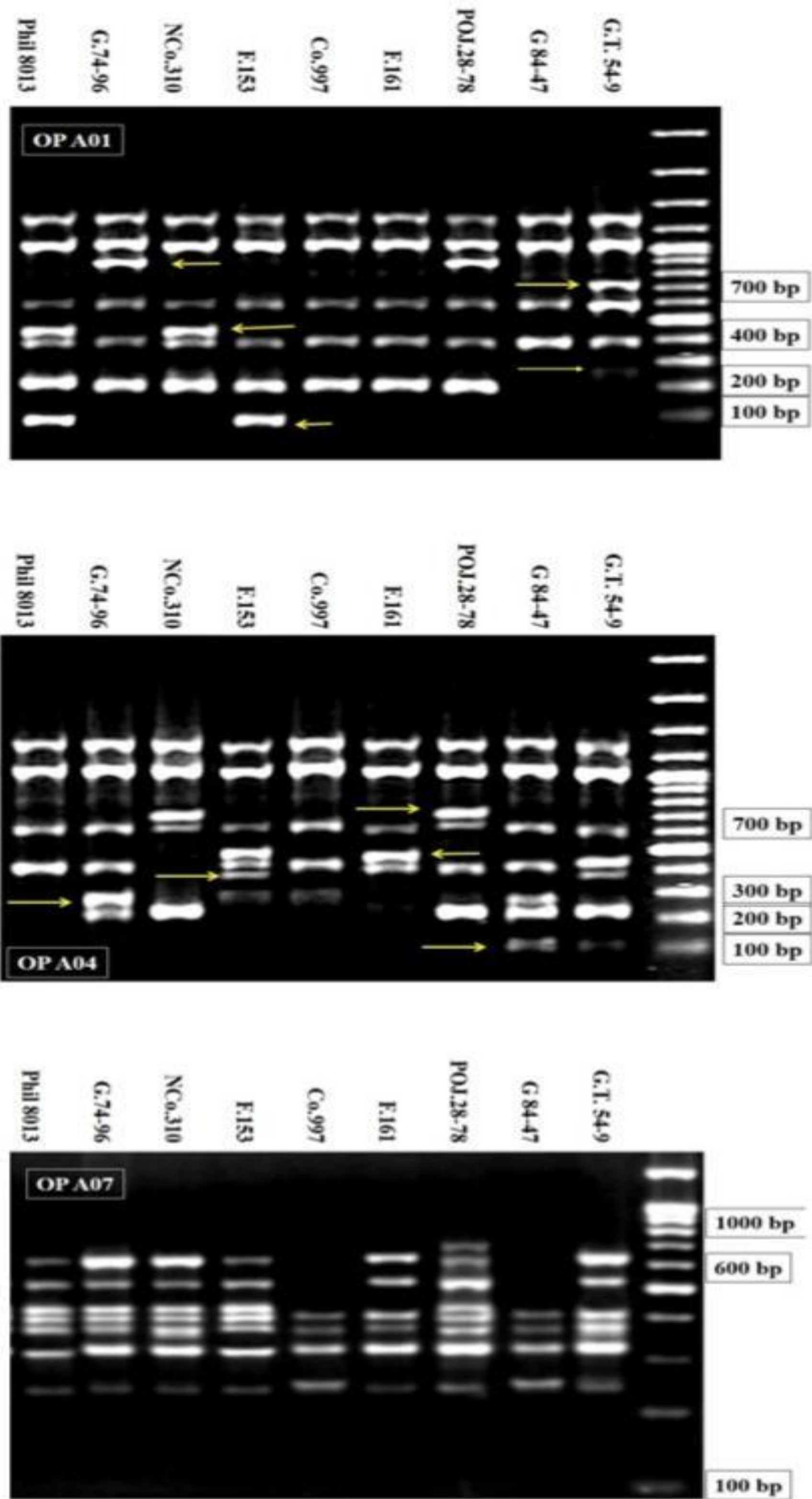

Figure 1: RAPD banding patterns of the nine sugarcane genotypes amplified with primers; OP-A01, OP-A04, OP-A07and 100-bp ladder.

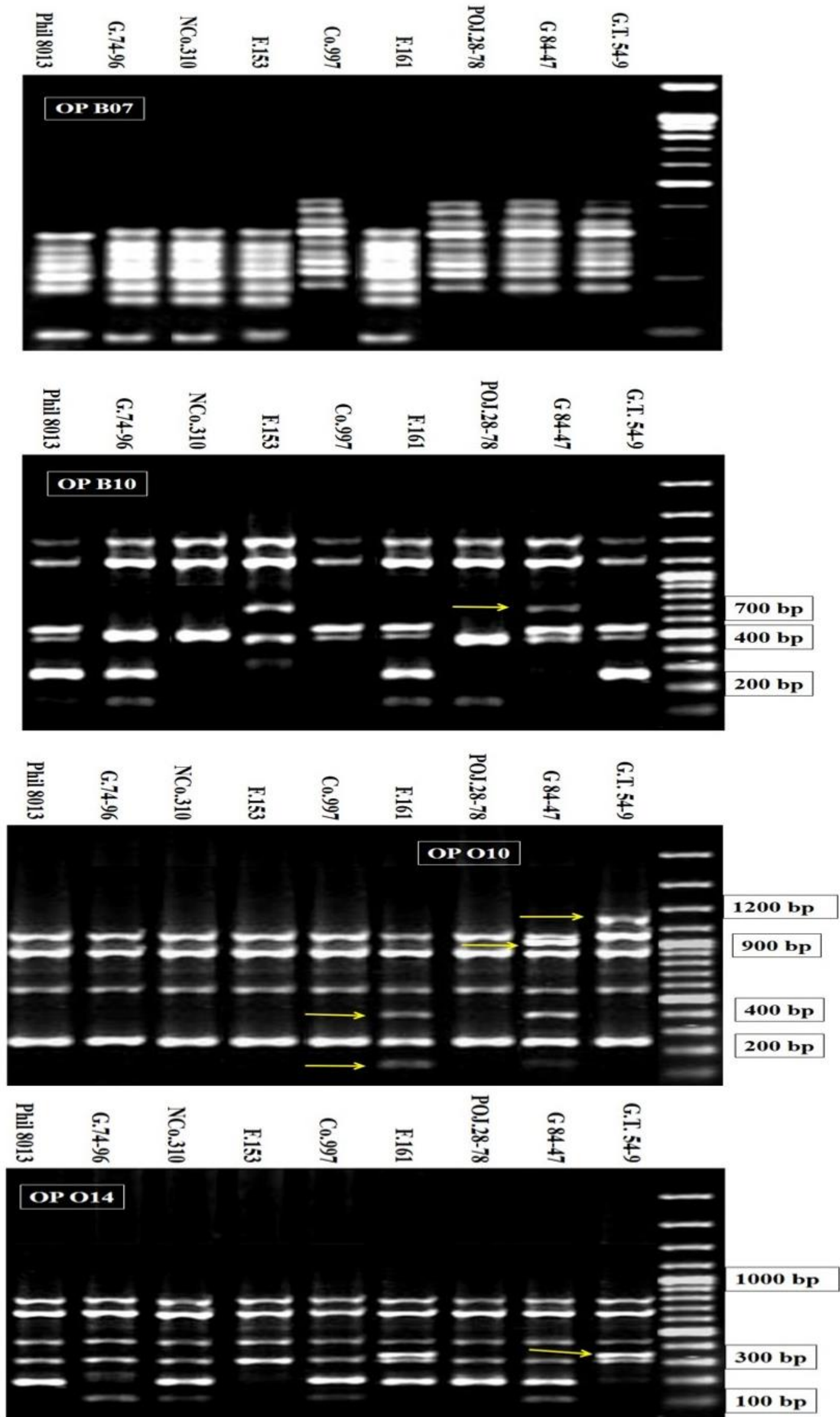

Figure 2: RAPD banding patterns of the nine sugarcane genotypes amplified with primers OP-B07, OP-B10, OP-O10, OP-O14 and 100-bp ladder.

Some of the primers produced polymorphic bands specific to a set of genotypes. Unique DNA bands with different sizes were detected in set of genotypes. Primer OP-A01produced three DNA bands displayed in three set of genotype; 109 bp (F.153 and Phil.8013), 452 bp (N.Co310 and Phil.8013) and 850 bp (POJ28-78 and G.74-96). Similarly, primer OP-A04 produced five DNA bands displayed in five set of genotypes, primer OP-O10 produced two bands. While primers OP-B10 and OP-O14 produced one band for each. (Figs.1 and 2).

These results confirmed the importance of using RAPD analysis for genotypic characterization, with specific bands giving informative bands that can discriminately distinguish all tested species.

Table 5: Primers that produced specific bands with respect to sugarcane genotypes

Primer name	Sequence (5`→3`)	Band size (bp)	Genotype Specific Band
OP-A01	CAGGCCCTTC	109	F.153, Phil.8013
		258	G.T. 54-9
		452	N.Co310, Phil.8013
		700	G.T.54-9
		850	POJ28-78, G.74-96
OP-A04	AATCGGGCTG	120	G.T.54-9, G.84-47
		284	G.84-47, G.74-96
		339	G.T.54-9, F.153
		652	F.161, F.153
		711	POJ28-78, N.Co.310
OP-B10	CTGCTGGGAC	764	G.84-47, F.153
OP-O10	TCAGAGCGCC	221	G.84-47, F.161
		461	G.84-47, F.161
		924	G.84-47
		1104	G.T.54-9
OP-O14	AGCATGGCTC	366	G.T.54-9, F.161

ISSR-PCR Amplification

The ISSR profiles of the amplification products of the five primers are shown in Fig. 3 and the numbers of bands generated by each primer is given in Table 6. Five primers were used to screen nine sugarcane genotypes resulted in a total number of 56 amplified DNA products that were generated across nine sugarcane genotypes with an average of 11.2 bands/primer. Out of the total bands, 22 were polymorphic and the percentage of polymorphic amplified products ranged from 80% for primer 17899B to 9.09% for primer HB 11. The total number of the amplified RAPD produced by each primer varied from a minimum number of 10 amplified products by primer 17899B to a maximum of 12 amplified products by primer 844B and HB 14. The size of amplified bands also varied with different primers. Primer 17899B amplified the largest 1122 bp band, while primer HB 11 amplified the band smallest size 183 bp band.

Such a level of polymorphism was consistent with some reports based on ISSR marker. Khaled *et al*. (2011) showed that 36 ISSR bands show 37.72% polymorphism in 26 sugarcane clones and **Sachin B. Kalwade** *et al*. (2012) studies 17 sugarcane genotypes using 27 ISSR. Out of the 252 amplicons amplified by 27 ISSR primers, 212 were polymorphic (84.13%)

In most of the genotypes studied, a single band was specific to an individual genotype. Unique DNA bands with different sizes were detected in particular genotypes, but not in the others. Primer 17899B produced two DNA bands, one band displayed in the genotype G.84-47 (972 bp) and the other in F 161 (690 bp) (Figs. 3 and Table 7). While primers 844B and HB 15 produced one band each displayed in genotypes NCo310 (715 bp) and G.T. 54-9 (312 bp) respectively; primer HB 14 produced two bands, one of them displayed in genotype Co. 997. These bands could considered as genotype-specific bands (Fig. 3 and Table 7).

Primer HB 14 produced polymorphic band specific (620 bp) to a set of genotype (F.153 and F. 161) (Fig. 3 and Table 7).

These results confirmed the importance of using ISSR analysis for genotypic characterization, results in specific information bands that can discriminately distinguish all tested species.

Genetic Similarity based on RAPD and ISSR

Based on overall combined class patterns of RAPD and ISSR, the overall similarity indices (Table 8) revealed that the highest similarity indices were 79% between genotypes; G.74-96 and Nco.310 followed by genotypes; F.153 and F.161 (78%), while the lowest similarity indices were 50% between

genotypes; G.T.54-9 and POJ.28-78, followed by genotypes; G.T.54-9 and F.153 (53%). Kalwade *et al*. (2012) studies 17 sugarcane genotypes Cluster analysis by UPGMA method revealed similarity coefficient of 0.49, which mainly attributed to inter specific diversity.

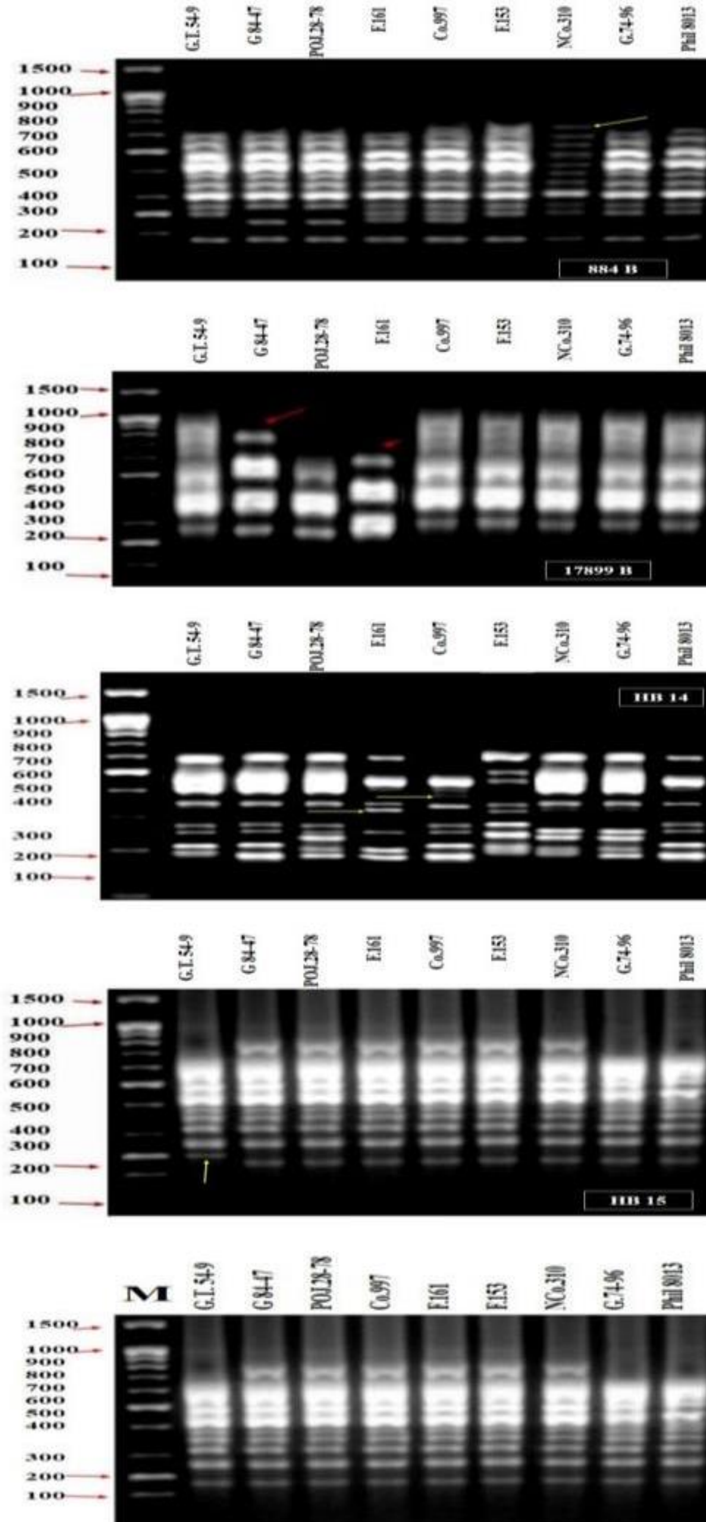

Figure 3: ISSR banding pattern of the nine sugarcane varieties amplified with ISSR primers; 844B, l7899B, HBI4 HB11, HBl5 and 100-bp ladder

Table 6: The total number of amplified polymorphic bands and polymorphism % displayed in the nine sugarcane genotypes using ISSR-PCR analyses

Primer No.	Primer name	TAF	PF	P%
1	844B	12	4	33.33
2	17899B	10	8	80
3	HB14	12	7	58.33
4	HB15	11	2	18.18
5	HB 11	11	1	9.09
Total		**56**	**22**	**39.29**

TAF = total amplified bands, PF = polymorphic bands, P % = Polymorphism percentage.

Table 7: Primers that produced specific bands with respect to sugarcane genotypes

Primer name	Sequence (5` →3`)	Band size (bp)	Specific to
844B	(CT)8GC	715	NCo310
17899B		972	G.84-47
		690	F.161
HB14	(CTC)3GC	620	F.161, F.153
		517	Co.997
HB15	(GTG)3GC	312	G.T.54-9

A dendrogram, representing the relationships among the nine genotypes, indicated that F. 153 was the most diverse among the studied sugarcane genotypes (Fig. 4). The dendrogram deduced from the combination of RAPD and ISSR systems separated the nine sugarcane genotypes into two main clusters, where genotype F.153 was placed in a separate cluster, while the remaining genotypes constituted the second cluster. The second cluster was subdivided further into two sub-clusters; the first one included genotype Co.997 only, while the rest of the varieties occurred in a separate sub-clusters. These results were in agreement with the pedigree information of these genotypes (Table 1), emphasizing the advantages of using RAPD and ISSR systems in obtaining high resolution profiles that discriminates among closely related and different genotypes, which agreed with the findings obtained by Fahmy *et al*. (2008).

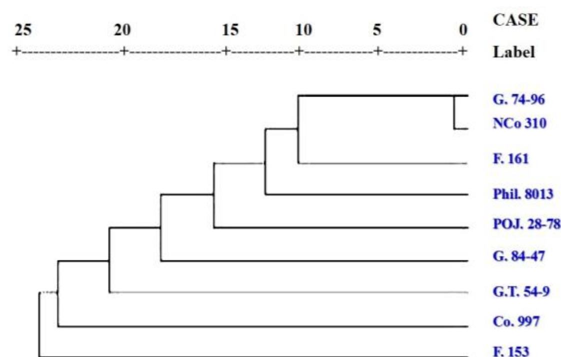

Figure 4: The genetic relationship among the nine sugarcane varieties based on the combined RAPD and ISSR analyses

Table 8: Similarity matrix among the nine sugarcane genotypes based on the total RAPD and ISSR analyses.

Variety	G.T.54-9	G.84-47	POJ.28-78	Co.997	F.161	F.153	N.Co.310	G.74–96
G.84-47	0.64							
POJ.28-78	0.50	0.59						
Co.997	0.58	0.57	0.57					
F.161	0.60	0.61	0.71	0.65				
F.153	0.53	0.56	0.67	0.64	0.78			
N.Co.310	0.67	0.69	0.57	0.61	0.67	0.70		
G.74–96	0.62	0.67	0.57	0.59	0.69	0.67	0.79	
Phil.8013	0.56	0.67	0.71	0.63	0.77	0.74	0.69	0.73

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