Egyptian Journal Of

GENETICS AND CYTOLOGY

INTERNATIONAL JOURNAL DEVOTED TO GENETICAL AND CYTOLOGICAL SCIENCES

Published by

THE EGYPTIAN SOCIETY OF GENETICS

Volume 37	January 2008	No. 1

DEVELOPMENT OF RAPD AND ISSR MARKERS FOR DROUGHT TOLERANCE IN SUGARCANE (*Saccharum officinarum* L.)

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Sugar amounts of cane represent 72% of the world production of crystallized sugar (FAO, 1999). In Egypt, sugarcane crop is one of the important industrial crops and it is the main source of sugar production. Moreover, it is widely used in Egypt for fresh juice consumption and molasses industry. Limitation of irrigation water led to minimizing the cultivated area of sugarcane, so, there is a very limited opportunity to increase it horizontally. Improving agricultural practices, in addition to developing new promising cultivars, became the possible ways of raising sugarcane production. Drought resistance is the ability of plant to resist all adverse conditions created by unfavorable phenomena related to water deficit (Clark *et al.*, 1986). It is well known that sugarcane water requirements are very high compared to our limited share in River Nile, so studying the effect of drought is a major limiting factor in the production of field crops. One effective approach is to develop sugarcane varieties tolerant to drought stress (Abdel-Tawab *et al.,* 1999). Biotechnology has been used as a tool to increase agricultural productivity in the context of sustainable agriculture (Tecson, 2002). Molecular markers have been used for studying

genetic diversity, cultivar identification and for marker-assisted selection (MAS) of major crops such as rice, maize, wheat and sugarcane. Moreover, Molecular markers such as RFLP, RAPD, ISSR and SSR have recently shown excellent potentiality to assist selection of quantitive trait loci (QTLs) associated with these markers (Stuber, 1992). The developing of RAPD approach (Williams *et al.,* 1990) has allowed simple, easy and less time-consuming genome analysis at DNA level compared with RFLP. The detection of molecular markers linked to many genes of interest has been widely achieved in this regard. RAPD-PCR as a simple and rapid procedure has gained a worldwide acceptance and application (Michelmore *et al.*, 1991; Paran *et al.*, 1991). Moreover, RAPD markers were detected for salt and drought stresses in sugarcane (Abdel-Tawab *et al.*, 2003a&b; Piperidies *et al.*, 2004), in wheat (Abdel-Tawab *et al.*, 2003c), in maize (Abdel-Tawab *et al.*, 2002) and in sorghum (Abdel-Tawab *et al.*, 1998). ISSRs are semi-arbitrary markers amplified by PCR using a single primer composed of a microsatellite repeated sequences. Such amplification does not require genome sequence information and leads to multi-locus and highly polymorphic patterns (Wolfe, 1998). Therefore, ISSRs have proven to be a powerful amplification-based fingerprinting technique. The aim of this study was to obtain molecular markers for drought tolerance in sugarcane and to assess the genetic relationships among some of its varieties.

MATERIALS AND METHODS

a. Materials

Nine sugarcane varieties (*Saccharum* spp.), (Table 1), were obtained from Sugar Crops Research Institute (ARC), Giza, Egypt.

b. Methods

1. Field experiment

The nine varieties were subjected to water stress experiment to evaluate their drought tolerance in field experiment under two treatments; control (irrigated every 15 days) and water stress (drought) treatment (irrigated every 30 days). The experiment was applied for three months, then the two irrigation regimes (control and treatment) were left until harvest to observe their effects on cane growth and yield according to Wagih *et al.* (2001 and 2003). The recorded yield-related traits were; stalk length, stalk diameter, stalk weight, leaf area and number of stalks/plant.

The collected data were statistically analyzed according to Snedecor and Cochran (1969). The differences among means were compared using the Least Significant Difference test (LSD).

2. Molecular genetic studies

2.1. DNA isolation

DNA was isolated from sugarcane seedlings (three weeks old) using CTAB mini-prep method (Saghai- Maroof *et al.,* 1984).

2.2. RAPD-PCR analysis

Reaction conditions were optimized according to Maniatis *et al.* (1982) and Sambrook *et al.* (1989). The amplification was performed for 42 cycles, using a Coy thermocycler programmed, as follows; initial denaturation at 94°C for 4 min, one cycle, denaturing at 94°C for 1 min, annealing at 36°C for1 min, extension at 72°C for 2 min (40 cycle) and final extension at 72°C for 10 minutes (one cycle). Then hold at 4°C (infinite).

The product was fractionated on agarose gel (1.2 %) in TAE buffer and was stained with 0.2g/ml ethidium bromide and 100bp ladder was used as a DNA marker. Eight random primers were used for RAPD- amplification, their names and sequences are presented in Table (2).

2.3. Inter simple sequence repeats (ISSRs)-PCR analysis

An alternative method to SSR, called ISSRs-PCR, has also been used to get molecular markers for water stress tolerance in sugarcane varieties as described by Ratnaparkhe *et al.* (1998). The five used primers are present in Table (3).

Amplification was performed for 42 cycles using a Coy thermocycler programmed as follows; initial denaturation at 94°C for 4 min, denaturing at 94°C for1 min, annealing at 55°C for 1 min and extension at 72°C for 2 min (40 cycle) and final extension at 72°C for 10 minutes (one cycle), then hold at 4°C. The product fractionated on agarose gel (1.2 %) in TAE buffer, stained with 0.2g/ml ethidium bromide and 100bp ladder was used as a DNA marker.

2.4. Genetic relationships

The banding patterns of the nine RAPD and five ISSR primers were scored and data were inserted in a computer as values of (1) and (0) for the presence and absence of bands, respectively. The data were analyzed using SPSS program in order to develop the consensus tree for these genotypes and to estimate their similarity indices.

RESULTS AND DISCUSSION

1. The effect of water stress on some yield-related traits

The nine sugarcane varieties were assessed for their water stress tolerance in the field experiment. Plants were irrigated primary during the formative growth phase (90-120 days after planting), then the irrigation regime was applied until harvest according to Wagih *et al.* (2003) as control; irrigated every 15 days and water stress (drought) treatment; irrigated every 30 days. At harvest, five yield-related traits, which are important in breeding programs (Harvey and Botha, 1995), were measured as shown in Table (4). Analysis of variance showed significant differences for these traits among the nine varieties under control

and drought treatments. The results (Table 4) indicated that varieties G.T.54-9, Co. 997 and F153 were the most tolerant and varieties F161, NCo.310 and Phil.8013 were the most sensitive ones.

2. Molecular markers

RAPD and ISSR-PCR techniques succeeded in developing some molecular markers for drought tolerance in the nine sugarcane varieties. Then, allowed a reliable identification of these varieties to be used in marker-assisted selection (MAS) programs.

2.1. RAPD-PCR analysis

The banding patterns of RAPD-fragments using the eight *10-mer* primers with the nine sugarcane varieties (Figs 1a-h) exhibited 59 amplified fragments; 48 of them were polymorphic (81.4%). The total number of amplified and polymorphic fragments generated with each primer is shown in Table (5). According to the data of the field experiment under water stress, the results indicated the presence of eight positive and two negative RAPD molecular markers for drought tolerance trait (Table 5). These results agreed with Oropeza *et al.* (1997) who confirmed that RAPD analysis is an efficient and reliable method to identify sugarcane varieties, and also with Abdel-Tawab *et al.* (2003a&b) and Piperidies *et al.* (2004) who detected molecular markers associated with drought and salt stress tolerance using RAPD-PCR analysis in sugarcane.

2.2. ISSR-PCR analysis

The banding patterns of the amplified fragments using the five ISSR primers are shown in Table (5) and Figs (2a-e). They gave 27 amplified fragments with 24 polymorphism (88.8%). ISSR-PCR analysis exhibited five positive and two negative molecular markers for drought tolerance. Ratnaparkhe *et al.* (1998) and Reddy *et al.* (2002) indicated that ISSR markers could be used as highly informative markers for genome mapping and gene tagging because the evolutionary rate of change within microsatellites is considerably higher more than many other types of DNA markers. In ISSR studies, the uses of such highly informative primers lower the cost, time and labors for diversity analysis (Reddy *et al.*, 2002). Fahmy *et al.* (2007) confirmed the useful of ISSR markers to detect the highly drought tolerance genotypes in rice.

2.3. Genetic relationships of the nine sugarcane varieties based on molecular markers

Variability and identification of the available germplasm are essential for varieties improvement. Knowledge of the genetic distances among the different varieties is very useful and successful for genetic improvement (Ceron and Angel, 2001). RAPD-PCR and ISSR-PCR methodologies have been used to measure the degree of difference/similarity among varieties and to calculate the genetic distance between these germplasms. The aforementioned results of RAPD and ISSR patterns were used to study the

genetic relationships of the nine sugar-cane genotypes; at first, on the level of each marker alone, then the combined of the two systems were applied.

RAPD-PCR amplification resolved varying degrees of polymorphisms between the sugarcane genotypes. A total of 59 fragments were resolved and were scored as (1) or (0) and the data was entered into the computer program SPSS to develop the similarity matrices (Table 6) and the dendrogram of the genetic distances (Fig. 3). The genetic similarity matrices based on all possible pairs of varieties ranged from nearly 51% to 87%. The highest genetic similarity indices were noted between varieties F.153 and F.161 (87%) followed by varieties F.153 and Phil.8013 (82%), while the lowest genetic similarity indices were noted between varieties G.T.54-9 and POJ.28-78 (51%) followed by varieties POJ.28-78 and Nco.310 (52%). These results suggested that there was a limited genetic diversity within these varieties, particularly within the genotypes currently grown commercially. As shown in Fig. (3), the dendrogram based on RAPDs similarity indices separated the nine sugarcane varieties into two main clusters. The first cluster was subdivided into two sub-clusters, the first sub-cluster included only variety G.T.54-9, while varieties G.84-47, G.74-96 and NCo.310 were placed in the second sub-cluster. The second cluster was subdivided further into two sub-clusters; the first sub-cluster included variety Co.997 only and the rest of varieties appeared in the second sub-cluster. Ubayaseua and Perera (1999) showed that RAPD analysis could be used effectively in germplasm identification of sugarcane and found that genetic diversity of the investigated accessions ranged from 0% to 69.23%.

The ISSR data showed that the genetic similarity indices ranged from 43% to 96.4% (Table 7 & Fig. 4). The closest relationship was recorded between varieties; G.74-96 and NCo.310 (96.4%) followed by varieties; F.161 and NCo.310 (86%). On the other hand, the lowest similarity indices were observed between varieties; F.153 and G.T.54-9 (43%) followed by varieties; POJ.28-78 and G.T.54-9 (57%). As shown in Fig. (4), the dendrogram based on ISSRs similarity indices separated the nine sugarcane varieties into three main clusters. The first cluster included variety G.T.54-9 only. Whereas, variety Co.997 was placed at the second cluster, while the third cluster was subdivided further into two sub-clusters. The first sub-cluster included two groups, one contained variety; POJ.28-78 and the second contained varieties; Phil.8013, F.153 and F161. The second sub-cluster included varieties; G.84-47, G.74-96 and NCo.310. The importance of ISSR patterns in obtaining the genetic relationships among different genotypes was confirmed by Abdel-Tawab *et al.* (2003a) in sugarcane and with Adawy *et al.* (2004) in date palm.

2.4. Genetic relationships of the nine sugarcane varieties overall combined class patterns

Based on overall combined class patterns of RAPD and ISSR, the overall similarity indices (Table 8) revealed that the highest similarity indices were 83% between varieties; G.74-96 and Nco.310 followed by varieties; F.153 and F.161 (82%), while the lowest similarity indices were 53% between varieties; G.T.54-9 and POJ.28-78, followed by varieties; G.T.54-9 and F.153 (56%). The dendrogram deduced from the combination of the two systems (Fig. 5) separated the nine sugarcane varieties into two main clusters, where variety F.153 was placed in a separate cluster, while the second cluster involved the rest of the varieties. The second cluster was subdivided further into two sub-clusters; the first sub-cluster included variety Co.997 only, while the rest of the varieties occurred in a separate sub-clusters. Abdel-Tawab *et al.* (2003a & b) found that similarity indices between some sugarcane genotypes ranged from 56.3% to 84% over all the DNA markers and Piperidies *et al.* (2004) stated that DNA markers are a powerful tool for varieties identification in sugarcane breeding and selecting programs.

The aforementioned results emphasized the advantages of using a combination of several molecular systems over the use of one system in order to obtain higher resolution to discriminate between different genotypes, which agreed with the findings obtained by Abdel-Tawab *et al.* (2003a&b) in sugarcane.

SUMMARY

Nine varieties of sugarcane (*Saccharum officinarum* L.) were screened for their water stress tolerance in field experiment. Analysis of variance showed significant differences for five yield-related traits among the nine varieties under control and drought treatments. The results indicated that varieties G.T.54-9, Co.997 and F153 were the most tolerant while varieties F161, NCo.310 and Phil.8013 were the most sensitive ones.

RAPD- and ISSR-PCR techniques were used in this study to detect some molecular markers associated with drought tolerance in sugarcane. RAPD-PCR results using eight random primers exhibited 59 amplified fragments; 48 of them were polymorphic (81.4%).The results indicated the presences of eight positive and two negative molecular markers for drought tolerance trait. ISSR-PCR results with five primers showed 27 amplified fragments; 24 of them were polymorphic (88.8%). ISSR analysis exhibited five positive and two negative molecular markers for drought tolerance trait. Each technique was used to detect the similarity indices and the dendrogram of varieties relationships. However, the overall similarity indices based on both techniques revealed that the highest similarity was 83% between varieties; G.74-96 and Nco.310 followed by varieties; F.153 and F.161 (82%), while the lowest similarity was 53% between

varieties; G.T.54-9 and POJ.28-78, followed by varieties; G.T.54-9 and F.153 (56%). The dendrogram resulting from the combination of the two systems separated the nine sugarcane varieties into two main clusters, where variety F.153 was placed in a separate cluster, while the second cluster involved the rest of the varieties. The second cluster was subdivided further into two sub-clusters. The first sub-cluster included variety Co.997 only, while the rest of the varieties occurred in a separate sub-cluster. The aforementioned results emphasized the advantages of using a combination of several molecular systems over the use of one system in order to obtain higher resolution to discriminate between different genotypes.

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Table (1): Code numbers, names and origins of the nine sugarcane varieties.

Code no.	Variety name	Origin
1	G.T.54-9	Giza-Taiwan
2	G.84-47	Egypt
3	POJ.28-78	Indonesia
4	Co.997	India
5	F.161	Taiwan
6	F.153	Taiwan
7	N.Co.310	India
8	G.74–96	Egypt
9	Phil. 80-13	Philippen

Table (2): RAPD primers code and their sequences.

Primer code	Sequence (5`→3`)	Primer code	Sequence (5`→3`)
OP-A01	CAGGCCCTTC	OP-B10	CTGCTGGGAC
OP-A04	AATCGGGCTG	OP-O10	TCAGAGCGCC
OP-A07	GAAACGGGTG	OP-O13	GTCAGAGTCC
OP-B07	GGTGACGCAG	OP-O14	AGCATGGCTC

Table (3): ISSR-primers code and their sequences.

Primer code	Sequence (5`→ 3`)	Primer code	Sequence (5`→ 3`)
17899B	$(CA)_6GG$	HB14	$(CTC)_3GC$
844B	$(CT)_8GC$	HB15	$(GTG)_3GC$
HB11	$(GT)_6GG$		

Table (4): Means of five yield-related traits under the effect of water stress (drought treatment) on the nine sugarcane varieties compared with the control.

Varieties	Treatment	Stalk length (cm)	Stalk diameter (cm)	Stalk weight (kg)	Leaf area $(cm)^2$	No. stalks $/m^2$
G.T.54-9	C	192.66	2.63	0.889	332.0	21.0
	D	180.33	2.36	0.411	199.6	38.0
	M	186.50	2.50	0.650	265.8	29.5
G.84-47	C	151.66	2.36	0.584	277.3	58.0
	D	133.33	2.23	0.376	253.6	38.0
	M	142.50	2.30	0.480	265.5	48.0
POJ. 28-78	C	170.00	3.36	0.745	449.6	23.0
	D	130.33	2.53	0.419	348.0	37.0
	M	150.00	2.90	0.582	398.8	25.0
Co. 997	C	180.00	2.56	0.769	391.0	19.0
	D	155.66	2.23	0.752	381.0	29.0
	M	167.83	2.39	0.761	386.0	24.0
F.161	C	139.00	2.06	0.553	362.3	22.0
	D	126.00	2.03	0.354	344.0	22.0
	M	132.50	2.00	0.453	353.2	22.0
F.153	C	189.00	2.83	0.723	476.0	19.0
	D	156.66	2.73	0.705	383.0	14.0
	M	172.83	2.70	0.714	429.5	16.5
G.74-96	C	172.00	2.46	0.827	286.0	22.0
	D	165.33	2.23	0.676	278.0	34.0
	M	168.83	2.30	0.752	283.5	28.0
NCo.310	C	210.00	2.60	0.747	438.0	26.0
	D	166.66	2.33	0.436	241.3	34.0
	M	188.33	2.40	0.591	339.0	30.0
Phil.8013	C	183.33	2.46	0.553	396.0	45.0
	D	173.33	2.30	0.238	303.3	38.0
	M	178.83	2.33	0.395	349.5	41.5
LSD at 5% level						
Treatments		4.27*	0.27*	0.29	36.56*	2.40*
Cultivars		24.40*	0.32	0.54	120.00	12.17*
C×T		34.50	0.45	0.77	169.90	17.90

C = control (irrigation every 15 days), D= drought treatment (irrigation every 30 days), M= mean.

Table (5): The total number of amplified and polymorphic fragments, polymorphism % and the specific markers for drought tolerance in the nine sugarcane varieties using both RAPD-PCR and ISSR-PCR analyses.

Primer No.	Primer name	TAF	PF	P%	SM
RAPD-PCR					
1	OP-A01	11	8	72.7%	(+2) & (-1)
2	OP-A04	7	6	85.7%	(+1)
3	OP-A07	7	6	85.7%	-
4	OP-B07	6	6	100%	(+1) &(-1)
5	OP-B10	7	6	85.7%	(+1)
7	OP-O10	6	6	100%	(+1)
8	OP-O13	7	5	71.4%	-
9	OP-O14	8	5	62.5%	(+2)
Total		59	48	81.4%	(+8) & (-2)
ISSR-PCR					
10	844B	5	5	100%	-
11	17899B	7	7	100%	(+1)
12	HB11	4	3	75.0%	(+1)
13	HB14	7	5	70.4%	(+1) & (-1)
14	HB15	4	4	100%	(+2) & (-1)
Total		27	24	88.8%	(+5) & (-2)

TAF = total amplified fragments, PF = polymorphic fragments,
P % = Polymorphism percentage, SM = Specific marker, (+) = Marker for drought-tolerance, (-) = Marker for drought sensitive.

Table (6): Similarity matrix among the nine sugarcane varieties based on RAPD-PCR analysis.

Variety	1	2	3	4	5	6	7	8
2	0.66							
3	0.51	0.58						
4	0.61	0.63	0.60					
5	0.58	0.61	0.73	0.63				
6	0.61	0.60	0.72	0.70	0.87			
7	0.67	0.72	0.54	0.64	0.66	0.76		
8	0.63	0.70	0.52	0.60	0.67	0.69	0.78	
9	0.58	0.69	0.75	0.67	0.81	0.82	0.70	0.75

Table (7): Similarity matrix among the nine sugarcane varieties based on ISSR-PCR analysis.

Variety	1	2	3	4	5	6	7	8
2	0.71							
3	0.57	0.71						
4	0.61	0.54	0.61					
5	0.71	0.71	0.79	0.82				
6	0.43	0.57	0.64	0.61	0.64			
7	0.75	0.75	0.75	0.64	0.82	0.68		
8	0.71	0.71	0.79	0.68	0.86	0.71	0.96	
9	0.61	0.75	0.75	0.64	0.82	0.68	0.79	0.82

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

Variety	1	2	3	4	5	6	7	8
2	0.67							
3	0.53	0.62						
4	0.61	0.60	0.60					
5	0.63	0.64	0.75	0.68				
6	0.56	0.59	0.70	0.67	0.82			
7	0.70	0.73	0.60	0.64	0.71	0.74		
8	0.65	0.71	0.60	0.62	0.73	0.70	0.83	
9	0.59	0.71	0.75	0.66	0.81	0.78	0.73	0.77

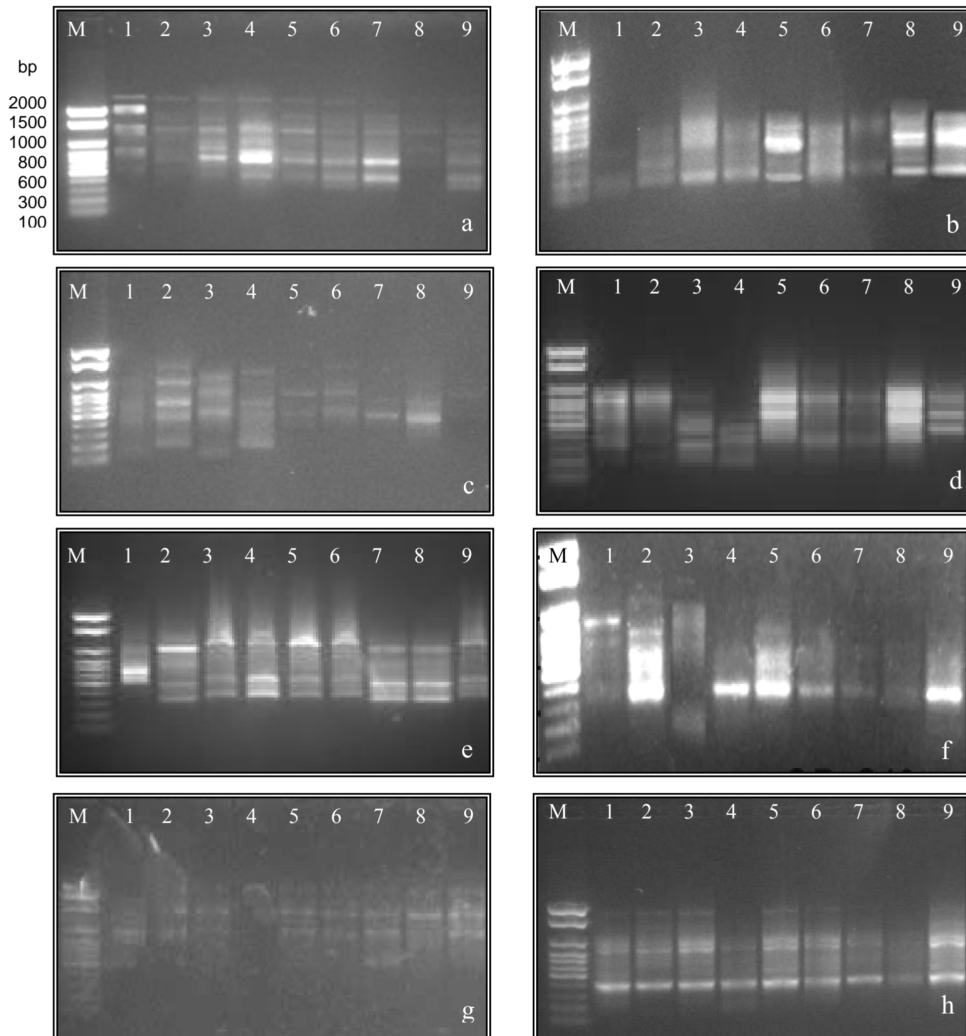

Fig. (1): RAPD banding patterns of the nine sugarcane varieties amplified with the eight *10-mer* random primers; OP-A01 (a), OP-A04 (b), OP-A07 (c), OP-B07 (d), OP-B10 (e), OP-O10 (f), OP-O13 (g) and OP-O14 (h); M = 100-bp ladder.

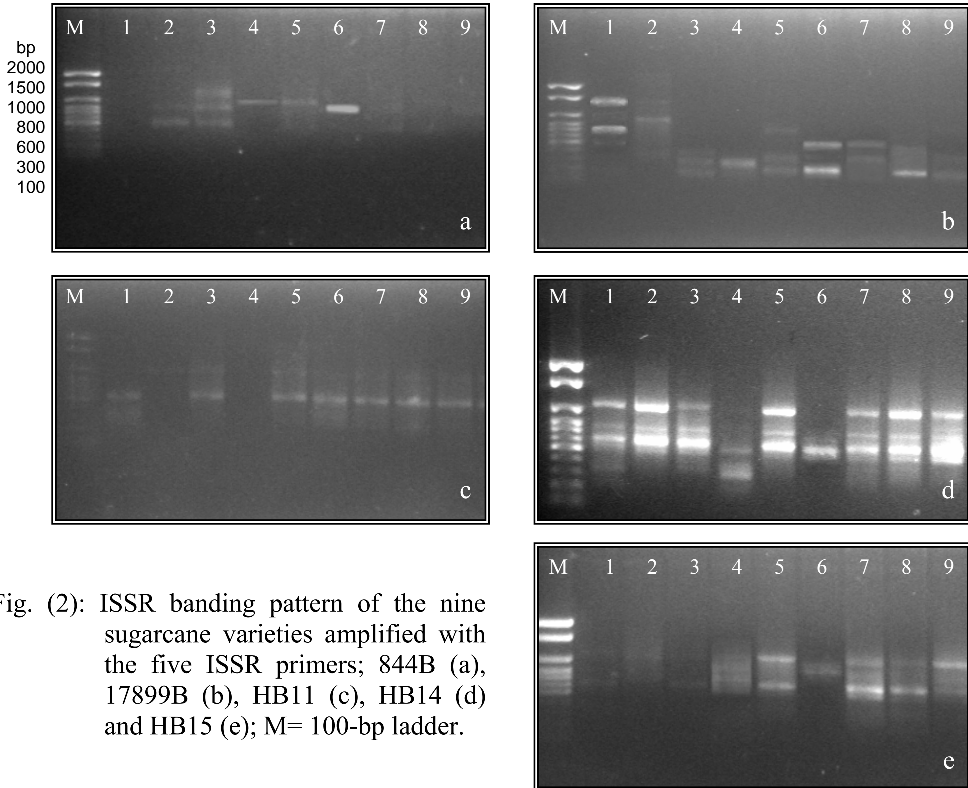

Fig. (2): ISSR banding pattern of the nine sugarcane varieties amplified with the five ISSR primers; 844B (a), 17899B (b), HB11 (c), HB14 (d) and HB15 (e); M= 100-bp ladder.

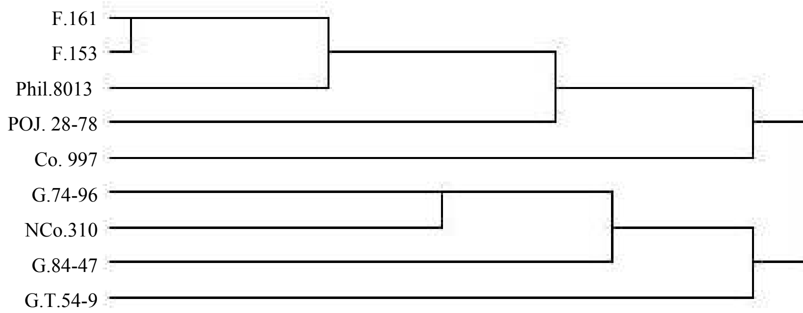

Fig. (3): The genetic relationships among the nine sugarcane varieties based on RAPD analysis.

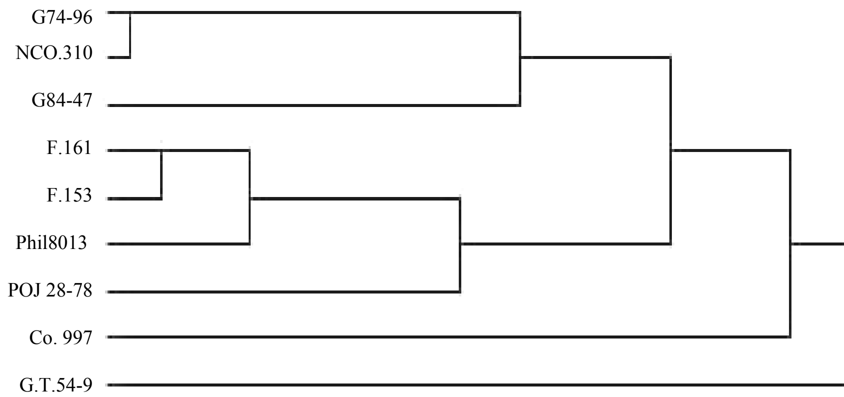

Fig. (4): The genetic relationships among the nine sugarcane varieties based on ISSR analysis.

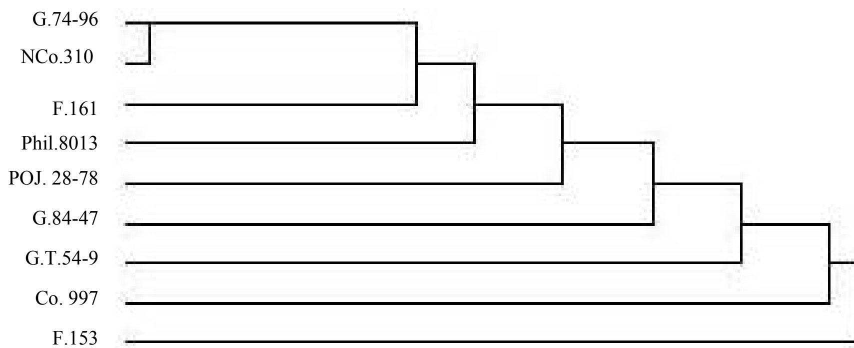

Fig. (5): The genetic relationship among the nine sugarcane varieties based on the combined RAPD and ISSR analyses.