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Molecular Genetic Characterizion of Some Promising Sugarcane Varieties under Smut Disease

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ABSTRACT

The aim of the present study was to genetic characterize some identified promising sugarcane cultivars resistant to smut fungus (*Ustilago scitaminea*) at an early stage of the breeding program. Ten cultivars were used in this study, including seven promising cultivars: 'G99-165', 'G95-19', 'G95-21', 'G98-28', 'G98-24', 'G84-47', 'G85-37', one susceptible cultivar 'NCo310', and two commercial cultivars, 'GT54-9' and 'PH8013'. The performance of the 10 cultivars that were artificially infected with a teliospore suspension was assessed under greenhouse conditions and the results revealed that nine cultivars were relatively resistant. Random amplified polymorphic DNA (RAPD) analysis using 20 10-mer primers showed that 182 of 233 total amplified fragments were polymorphic with 78.1% polymorphism. Nine-anchored inter simple sequence repeat (ISSR) primers revealed 102 polymorphic fragments with 69.9% mean polymorphism from a total of 146 amplified fragments under smut infection. Using four SSR markers, 15 polymorphic fragments with 88.2% mean polymorphism from 17 total amplified fragments were detected. No specific SSR markers were determined among the 10 sugarcane cultivars. A total of 29 and 9 specific fragments existed in the resistant cultivars and not in the susceptible cultivar 'NCo310' were detected for RAPD and ISSR, respectively.

Keywords: ISSR, RAPD, smut resistance, SSR

INTRODUCTION

Sugarcane (*Saccharum* spp.) is the most important sugar crop in the world. In Egypt, sugarcane is considered among the leading industrial crops. Therefore, the improvement of the sugar crop is one of the main objectives of Egyptian agricultural policy. Cultivated sugarcane cultivars have 80-140 chromosomes, comprising 8-18 copies of basic number, X= 8 or 10 (Hont *et al.* 1995; Irvine 1999). Plant disease resistance is a complex phenomenon involving a multitude of genes and several interconnected signaling pathways.

Resistance to fungal and bacterial pathogens often involves the induction of a hypersensitive response (HR) and the development of systemic acquired resistance (SAR) via the salicylic acid signaling pathway. HR involves localized expression of pathogenesis-related (PR) proteins and causes localized host cell death and callose deposition at the site of infection, thereby restricting fungal growth and limiting the spread of disease (Eckardt 2002).

Sugarcane smut inflicts a significant reduction in cane yield (30% of the crop) in the susceptible cultivars, while eradication of the disease is not feasible. Smut spores survive for long periods in soil or debris, therefore, careful removal of infected plants must be applied. Therefore, the disease is also managed by thermosanitization (burning) of infected fields after harvest and by use of healthy seed cane free from disease will help to prevent secondary spread of the disease (Gupta 2006).

DNA is a powerful diagnostic technique for identification of pathogens and other stresses in agriculture. Most DNA diagnostics are now based on the use of the polymerase chain reaction (PCR), a common research tool. It can be used to specifically amplify segments of DNA. Marker-assisted selection (MAS) has the potential to deploy favorable gene combinations for disease control. MAS is based on the concept that it is possible to infer the presence of a gene from the presence of a marker that is tightly linked to the gene (Lalitha 1999). Several studies used random amplified polymorphic DNA (RAPD) markers to identify the molecular markers linked to head smut resistance gene (*Shs*) in sorghum. For instance, Oh *et al.* (1994) used 326 RAPD markers to detect linkage analysis with *Shs* and analyses revealed one RAPD locus from primer OPG05 that was linked to the resistant gene. Procunier *et al*. (1997) identified molecular markers linked to a race T10 loose smut (*Ustilago tritici*, *U. segetum* var. *tritici*) resistance gene. One RAPD marker and another RFLP marker were located on opposite flanks of the resistance gene and were closely linked. These markers can be used for a faster and more reliable selection of T10 resistant plants than previous conventional loose smut ratings. Multiple regression analysis was used to identify putative markers associated with resistance to smut (*Ustilago scitaminea*) and to eldana (*Eldana saccharina*) (Barnes *et al*. 1997). Autrey *et al.* (1998) applied PCR to several viruses and fungi such as smut and *Pythium* spp. Two RAPD fragments were consistently present in *Sugarcane mosaic virus* (SCMV)-resistant plants and absent in susceptible plants while eight showed a reverse trend (Huckett and Botha 1995; Barnes *et al.* 1997; Zambrano *et al*. 2003). RAPD was also useful for diagnosing new diseases such as yellow leaf syndrome (Dookun *et al*. 1996; Autrey *et al.* 1998; Aljanabi *et al.* 2007). Molecular markers associated with rust (*Puccinia melanocephala*) were identified by RAPD (Dookun *et al*. 1996; Barnes and Botha 1998). RAPD markers were developed for ratoon stunting disease caused by (*Clavibacter xyli* subsp. *Xyli*), pachymetra (*Pachymetra chaunorhiza*) root rot and white leaf (Fegan *et al.* 1998).

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Table 1 The 10 sugarcane cultivars and their pedigrees.

Cultivar		Pedigree
Commercial	GT 54-9*	NCo 310 X F37-925
	PH8013*	CAC 71-312 X Ph 64-2227
Promising cultivars	G99-165	CP 76-130 X Q 76-1053
	G95-19	RB 72-2454 X ?
	G95-21	Sp 81-1763 X Sp 77-3024
	G98-28	C 34-33 X ?
	G98-24	C 34-33 X ?
	G84-47	CP 62-374 X CPs 61-39
	G85-37	NCo 310 X ?
Smut susceptible	NCo310*	Co 421 X Co 312

* = Imported cultivars, ? = Polycross

The objectives of the present study were to genetic characterize some promising sugarcane varieties under biotic stress resistance (smut disease) in comparison with commercial cultivars 'GT54-9' and 'PH8013' using RAPD, ISSR and microsatellites (SSR) analyses and to identify the promising cultivars resistant to smut disease in sugarcane at an early stage of the breeding program.

MATERIALS AND METHODS

Plant materials

Ten sugarcane cultivars belonging to different pedigrees (**Table 1**) were obtained from the Sugar Crop Research Institute (SCRI), Agriculture Research Center (ARC) in Giza, Egypt and were used in the study.

Isolation of sugarcane smut (*Ustilago scitaminea*)

Isolation of sugarcane smut was performed according to Bischoff and Gravois (2004), whereas smut whips were collected in the field and shaken vigorously to release the teliospores, which were dried over a desiccant. Before inoculation, the spores were tested for viability on 1% sucrose agar and stored over a desiccant at 21°C until used for inoculations of cuttings.

Inoculation and sugarcane planting

Inoculations were done by hypodermic injection technique according to Gillaspie *et al.* (1983). Sugarcane stalks were stripped of all leaves, cut into three-bud sets, then given a hot water treatment for 10 min at 52°C to stimulate growth. The sets were further cut into single bud pieces, dipped in 300 mg/l benomyl solution (systemic fungicide), and placed in wooden trays. Sufficient 0.25% sodium hypochlorite (NaOCl) solution was added to the trays to eliminate saprophytic fungi and souring of sugar exudates by bacteria and yeast. The trays were sealed with plastic warp, and then placed in a greenhouse maintained at 30°C. Shoots were inoculated when they were 8-12 cm long. The teliospores suspension contained about 5×10^4 viable spores/ml distilled water and was injected twice into each cutting at the base of the shoot (0.25 ml per injection) around the meristematic region. After inoculation, the stalk pieces were returned to the trays and allowed to incubate for two days at 30°C. After inoculation, two pieces were planted in a 30-cm clay pot filled halfway with a steam-pasteurized mixture of equal parts of sand, soil and peat and the pot was then filled with more of the mixture so that part of the shoot tip was left above the soil line. The pots were arranged in a randomized block design with four pieces in five pots and un-inoculated plants were used as controls. The percentage of smutted canes was recorded six months after planting.

DNA isolation, RAPD, ISSR and SSR amplification conditions

DNA was extracted using DNeasy plant Kit (Qiagen, USA) from young and fresh leaves of 10 sugarcane cultivars. RAPD, inter simple sequence repeats (ISSR) and simple sequence repeats - microsatellite (SSR) analyses were performed using 20 arbitrary 10-mer primers for RAPD (Metabion, Martinsried, Germany), nine ISSR primers and four pairs of SSR primers procured from Integrated DNA Technologies Inc. (San Diego, CA, USA), as shown in **Table 2**. RAPD, ISSR and SSR amplifications were performed according to Williams *et al.* (1990) for RAPD, Wang *et al.* (2002) for ISSR and Cordeiro *et al*. (2003) for SSR. Amplification reactions for RAPD, ISSR and SSR analyses were used in a final volume of 25 µl containing 10X PCR buffer, 25 mM $MgCl_2$, 2.5 mM dNTPs, 10 mM primer, 50 ng of template DNA and 5 U of *Taq* polymerase (promega, USA). Reactions were performed in a DNA thermocycler (MWG-Biotech Primus, USA).

Table 2 Names of 20 RAPD primers and 9 ISSR primers used in the study with their nucleotide sequences.

	No	Name	Sequence (5′ to 3′)
RAPD	1	OP-B12	CCTTGACGCA
	2	OP-C05	GATGACCGCC
	3	OP-C10	TGTCTCGGTG
	4	OP-C18	TGAGTGGGTG
	5	OP-C20	ACTTCGCCAC
	6	OP-D02	GGACCCAACC
	7	OP-D07	TTGGCACGGG
	8	OP-G05	CTGAGACGGA
	9	OP-I17	AGCCTGAGCC
	10	OP-L13	ACCGCCTGCT
	11	OP-L16	AGGTTGCAGG
	12	OP-L20	TGGTGGACCA
	13	OP-M01	GTTGGTGGCT
	14	OP-M17	TCAGTCCGGG
	15	OP-M20	AGGTCTTGGG
	16	OP-018	GTCTGTGCGG
	17	OP-O20	CAATCGCCGT
	18	OP-X06	AGGCATCGTG
	19	OP-Z01	TCTGTGCCAC
	20	OP-Z03	CAGCACCCCA
ISSR	1	B44	CTCTCTCTCTCTCTCTGC
	2	B98	CACACACACACAGT
	3	B99	CACACACACACAGG
	4	HB09	GTGTGTGTGTGTGG
	5	HB10	GAGAGAGAGAGACC
	6	HB11	GTGTGTGTGTGTCC
	7	HB12	CACCACCACGC
	8	HB13	GAGGAGGAGGC
	9	HB14	CTCCTCCTCGC
SSR	1	SMC222CG	F: TTTCACGAACACCCCACCTA
			r: AGGGACTAGCACACATTATTGTG
	2	SMC226CG	F: GAGGCTCAGAAGCTGGCAT
			r: ACCCTCTATTTCCGAGTTGGT
	3	SMC477CG	F: CCAACAACGAATTGTGCATGT
			r: CCTGGTTGGCTACCTGTCTTCA
	4	SMC863CG	F: CGGTCGCTGTTGCATTTAG
			r: TGGATCACTCAATCTCACTTCG

RAPD-PCR was performed as one cycle of 94°C for 5 min (denaturation), 45 cycles of {94°C for 1 min, 36°C for 90 sec and 72°C for 2 min (annealing)} and a final extension of 7 min at 72°C. ISSR amplification was performed with an initial denaturation of 5 min at 94°C, followed by 45 cycles of 94°C for 30 sec, annealing at 52°C for 45 sec, extension at 72°C for 2 min and a final extension at 72°C for 7 min. SSR amplification was performed with an initial denaturation of 5 min at 94°C, followed by 45 cycles of 94°C for 20 sec, annealing at 50 or 60°C for 35 sec, extension at 72°C for 45 sec and a final extension at 72°C for 3 min. PCR products were analyzed using 1.2% agarose gel electrophoresis and visualized with 10 µg/µl ethidium bromide staining. Gels were photographed and scanned with Bio-Rad video densitometer model 620. The sizes of the fragments were estimated based on a DNA ladder of 100 to 2000 bp (MBI, Fermentas).

Data analysis

RAPD, ISSR and SSR fragments were scored as present (+) or absent (-). The data was used for similarity-based analysis using the program MVSP (v. 3.1b) from www.kovcomp.com. Molecular

marker analyses were analyzed using Nei's genetic similarity index (Nei and Li 1979) based on the equation:

Similarity = 2Nab/(Na+ Nb), whereas Nab = number of scored amplified fragments with the same molecular size shared between a and b, Na and Nb = number of scored amplified fragments in a and b, respectively. A dendrogram was constructed based on the similarity matrix data by an unweighted pair group method with average (UPGMA) cluster analysis.

RESULTS AND DISCUSSION

Classification of smut resistant degrees for sugarcane cultivars

The reaction of the 10 sugarcane cultivars differed appreciably with respect to smut infection (**Fig. 1, Table 3**). The commercial cv. 'GT54-9' was represented by 24 treated plants, two of which were infected; the infection percentage was 9% equivalent to three degrees in a numerical rating model (**Table 3**), which implies that 'GT54-9' was a resistant cultivar. In the second commercial cv. 'PH8013', out of 21 plants treated, only one plant was infected; the reaction percentage was 5% equivalent to two degrees in a numerical rating model, which means that 'PH8013' was a resistant cultivar.

The other eight cultivars displayed different degrees of resistance to smut infection. For instance, 'G99-165' exhibited two infected plants; the infection percentage was 11.7% with four degrees in numerical rating model indicated that it is a resistant cultivar. The 19 plants of each of 'G95-19' and 'G98-24' recorded one infected plant with an infection percentage 5.5% (two degrees in the numerical rating model), which means that they are resistant to smut infection. 'G95-21' had one infected plant out of 23 under smut treatment, the infection percentage was 4.5% that means two degrees in the numerical rating model that indicated that 'G95-19' was a resistant cultivar.

The 28 plants of 'G98-28' showed one infected plant; the infection percentage was 5.8% with two degrees in the numerical rating model, which means that 'G98-28' is a resistant cultivar. The cultivar 'G84-47' exhibited one infected plant, while 23 plants were non-infected; the reaction percentage was 4.3% with two degrees in the numerical rating models that means that 'G84-47' is a resistant cultivar. The 20 infected plants of cultivar 'G85-37' recorded one infected plant, while 19 plants were non-infected; the infection percentage was 5.2% with two degrees in the numerical rating models that means that 'G98-24' is a resistant cultivar. However, the cultivar 'NCo310' exhibited seven infected plants out of 25; therefore, the infection percentage was 38.8% with seven degrees in the numerical rating model which means that 'NCo310' was the only highly susceptible cultivar. Cumulative smut disease incidence was calculated after 6 months of planting using numerical rates from 1 to 9 according to Satya Vir and Beniwal (1978).

Reactions of different sugarcane cultivars to artificial inoculation with *Ustilago scitaminea* are shown in **Table 3**. In most sugarcane cultivars, smut whips emerge out within 120 days after planting (Agnihotri 1990). Generally, susceptible cultivars show smut infection earlier (within 90 days) as compared to the resistant ones (Durairaj *et al.* 1972). Although several methods have been tried for infecting buds in seed pieces, the spore suspension method has been is widely used (Shukla *et al*. 1999).

Our results are in agreement with those of Abdu *et al.* (1990) who tested sugarcane varieties for susceptibility to culimicolous smut by the seed cutting dipping inoculation technique in telospore suspension. Cultivars were classified into four categories; resistant, moderately susceptible, susceptible and highly susceptible based on the infection percentage and none were highly resistant. Briceno *et al.* (2005) established an experiment to determine the performance of 20 sugarcane hybrids (*Saccharum* spp.) to smut disease (*U. scitaminea*). The 20 hybrids comprised 17 experimental clones, 16 of them belong to the Venezuelan series 1991 (V91) and one belongs to the Venezuelan series 1998 (V98), and three commercial clones (PR61-632, PR980 and V64-10). The percentage of stool infection was measured to

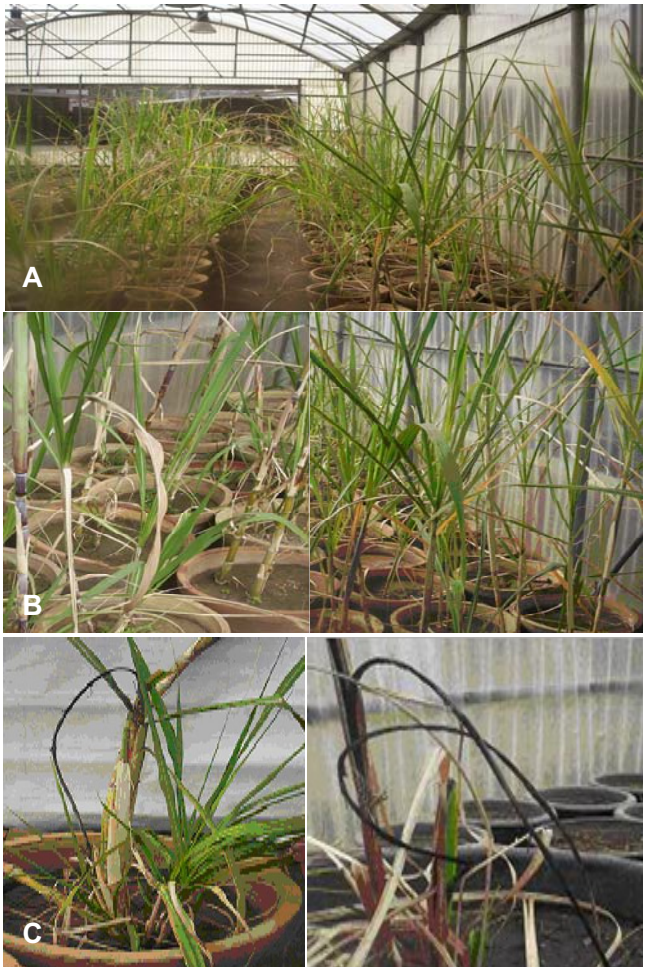

Fig. 1 Response of sugarcane cultivars to artificial inoculation with smut (*Ustilago scitamine*). (**A**) experiment overview, (**B**) non-infected plants (**C**) infected plants.

Table 3 Reaction of the 10 sugarcane cultivars to artificial inoculation with *Ustilago scitaminae*.

Cultivars	No. of infected plants	No. of healthy plants	Infection %	Degree	Reaction
GT54-9	2	22	9	3	R
PH8013	1	20	5	2	R
G99-165	2	17	11.7	4	S
G95-19	1	18	5.5	2	R
G95-21	1	22	4.5	2	R
G98-28	1	17	5.8	2	R
G98-24	1	18	5.5	2	R
G84-47	1	23	4.3	2	R
G85-37	1	19	5.2	2	R
NCo310	7	18	38.8	7	HS

Numerical rating model, response and degree according to Satya Vir and Beniwal (1978):

Reaction (Response)	Infection %	Degree
Highly resistant (HR)	0-3	1
Resistant (R)	4-6	2
	7-9	3
	10-12	4
Moderately susceptible (MS)	13-25	5
Highly susceptible (HS)	26-35	6
	36-50	7
	51-65	8
	66-100	9

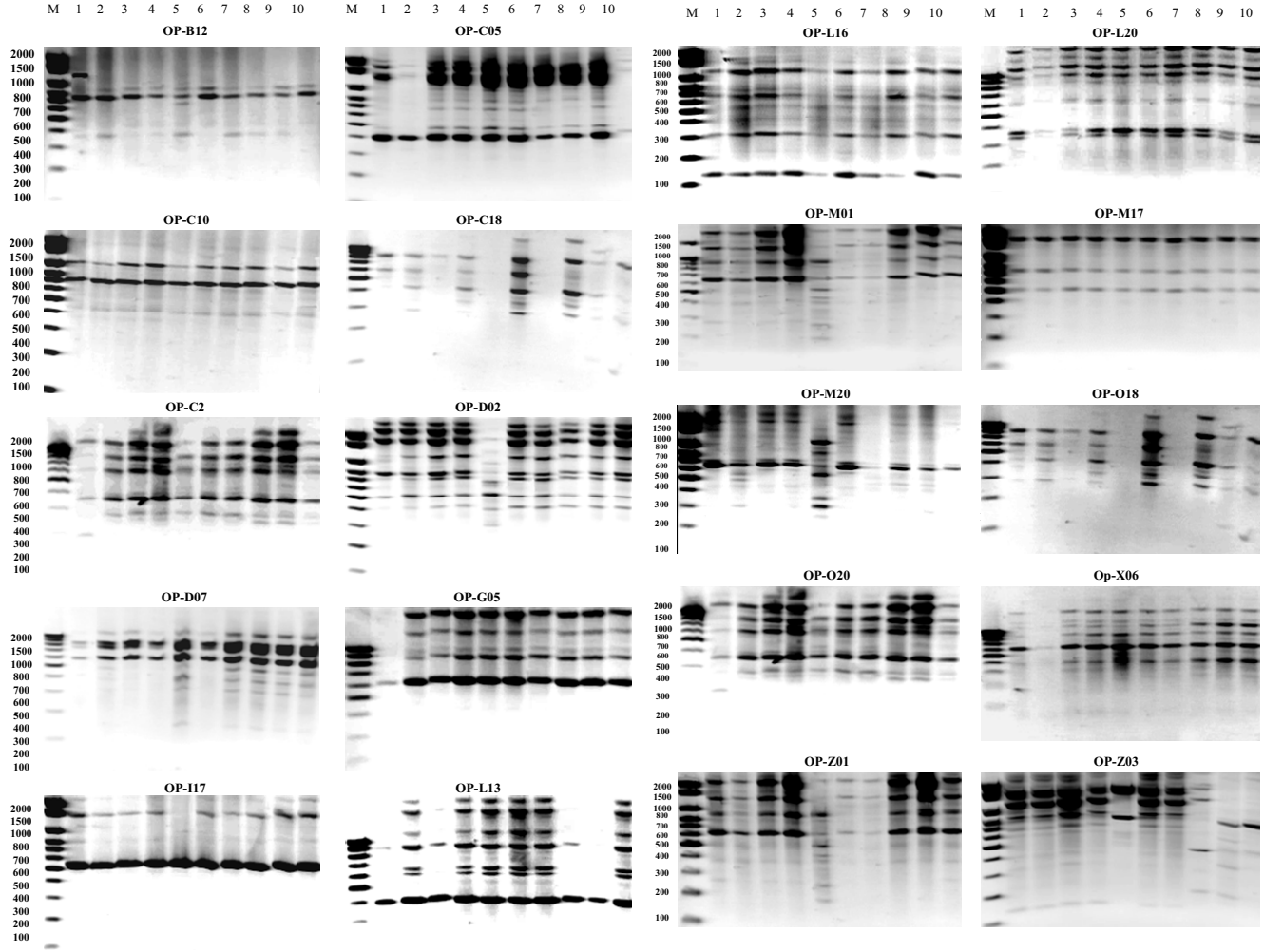

Fig. 2 RAPD profiles of the 10 sugarcane cultivars amplified with 20 random primers. M = DNA ladder, 1 = 'GT54-9', 2 = 'PH8013', 3 = 'G99-16'5, 4 = 'G95-19', 5 = 'G95-21', 6 = 'G98-28', 7 = 'G95-19', 8 = 'G84-47', 9 = 'G85-37', 10 = 'NCo310'.

determine the sugarcane reaction to the disease. Ten cultivars reacted from moderately resistant to resistant, while another 10 ranged from susceptible to highly susceptible.

RAPD-PCR analysis

A total of 233 amplified fragments were detected using the 20 random primers (**Figs. 2a, 2b; Table 4**), whereas 182 polymorphic fragments were detected and polymorphism levels differed from one primer to the other in the 10 cultivars with 78.1%.

Among the 20 RAPD primers, five exhibited eight smut resistance-fragments that were absent in the susceptible cv. 'NCo310' while they were existed in the other 9 resistant cultivars as shown in **Table 5**. For instance, primer OP-M20 showed three fragments with (430, 300 and 170 bp) and primer OP-C05 (1470, 1360 and 400 bp) while primer OP-M01 displayed two fragments with (2400 and 800 bp) and the other two primers (OP-C20 and OP-M17) displayed one fragment in each primer (430 and 400 bp, respectively). Among the eight resistant cultivars, some were not displayed the smut resistance-fragments while the other revealed these fragments with their disappearance in 'NCo310', for instance 'G98/28' (370 bp with primer OP-B12), 'GT 54/9' (390 bp with primer OP-O20) and 'PH8013' (1230 bp with primer OP-C05). In contrast, one fragment existed in 'NCo310' and was absent in the resistant cultivars in four primers, such as 420 bp in (OP-C05), 450 bp in (OP-C10), 700 bp in (OP-L20) and 2055 bp in (OP-Z03). Moreover, among the 10 cultivars some displayed cultivar-specific fragments that appeared uniquely and were absent in the other nine cultivars. For example, 'G95/21 revealed one unique fragment with 430 bp in primer OP-X06 and five unique fragments in OP-Z01. 'G95/19' showed a fragment with 230 bp in primer OP-O20.

ISSR-PCR analysis

DNA isolated from the 10 cultivars was tested against 9 ISSR primers (**Table 2**). The total number of fragments developed through the PCR reaction was 146. One primer showed a monomorphic pattern (HB12) while 8 primers gave 102 polymorphic fragments 69.9% (**Fig 3; Table 4**). Two primers exhibited 3 fragments, primer HB9 displayed a fragment with molecular size 600 bp that absent in 'NCo310' cultivar only, while appeared in all resistant cultivars. Primer B98 exhibited 1 fragment with molecular size 180 bp in 'NCo310' cultivar only, while it was absent in all other cultivars. At the same time primer HB13 exhibited 1 fragment at molecular size 1500 bp which appeared in 'NCo310' cultivar only, while it was absent in all resistant cultivars.

On the other hand, some primers showed ISSR fragments in most of the resistant cultivars as follows; primer HB-09 showed 3 fragments at molecular sizes of 510, 430 and 380 bp in 8, 7 and 6 resistant cultivars, respectively. Primer HB-10 gave 2 fragments at molecular sizes 480 and 400 bp in 7 and 6 resistant cultivars. Two fragments at molecular sizes of 700 and 180 bp with primer HB-11 appeared in 8 resistant cultivars, while one fragment at molecular size 630 bp was present in 6 resistant cultivars. The total specific fragments with their molecular sizes (bp) that appeared in smut resistant sugarcane cultivars only using the 20 RAPD and 9 ISSR primers are summarized in **Table 5**.

Table 4 RAPD, ISSR and SSR analyses of the 10 sugarcane using 20 RAPD, 9 ISSR and 4 SSR primers.

Markers	Primers	TAF	Pf	Sugarcane cultivars																			
				Commercial				Promising cultivars														Susceptible	
				GT54-9		PH8013		G99-165		G95-19		G95-21		G98-28		G98-24		G84-47		G85-37		NCo310	
				AF	*	AF	*	AF	*	AF	*	AF	*	AF	*	AF	*	AF	*	AF	*	AF	*
RAPD	OP-B12	6	5	3	3	3	3	4	2	4	2	5	1	3	3	4	2	4	2	4	2	3	0
	OP-C05	18	15	13	5	7	0	16	0	16	0	16	0	16	0	14	0	14	0	16	2	5	4
	OP-C10	8	5	3	0	4	0	5	0	5	0	3	0	5	1	5	0	5	0	4	0	6	1
	OP-Ml 7	5	1	5	0	5	0	5	0	5	0	5	0	5	0	5	0	5	0	5	0	4	1
	OP-L16	11	6	7	1	10	0	11	0	11	0	6	2	9	0	8	0	10	0	10	0	10	0
	OP-117	5	1	5	0	5	0	4	1	4	1	4	1	4	1	4	1	4	1	4	1	5	0
	OP-D02	16	14	10	0	9	0	10	0	9	0	9	4	8	0	8	0	8	0	8	0	8	0
	OP-M01	14	11	10	0	10	0	10	0	10	0	13	4	8	0	9	0	9	0	8	0	6	2
	OP-M20	19	16	12	0	12	0	12	0	13	0	15	4	11	0	7	2	12	0	12	0	4	2
	OP-Z01	16	11	9	0	9	0	10	0	11	0	13	6	7	0	7	0	9	0	10	0	10	0
	OP-C18	10	10	1	1	8	1	2	1	3	0	7	0	9	0	3	0	6	0	5	0	7	0
	OP-C20	14	11	5	4	9	0	10	0	12	1	8	0	7	0	9	0	10	0	11	1	7	1
	OP-Z03	22	22	12	0	11	0	12	0	10	0	6	0	12	0	12	0	7	1	5	1	3	1
	OP-D07	9	6	3	1	6	0	4	0	4	0	8	0	4	0	8	0	9	1	8	0	8	0
	OP-018	8	8	3	0	4	0	1	0	5	0	0	0	7	0	0	0	7	0	4	1	2	0
	OP-O20	14	12	4	2	8	0	9	0	11	1	7	0	6	0	7	0	9	0	9	0	6	0
	OP-G05	8	7	1	3	6	2	6	0	6	0	6	0	6	0	6	0	6	0	6	0	6	0
	OP-L13	8	7	2	0	7	0	4	0	7	0	7	0	7	0	7	0	2	0	2	0	7	0
	OP-L20	9	2	7	0	7	0	8	0	8	0	7	0	7	0	8	0	8	0	8	0	9	2
	OP-X06	13	12	5	2	1	0	7	0	7	0	10	0	6	0	6	0	8	0	10	0	10	0
	Total	233	182	120	23	141	6	150	6	161	5	155	22	147	5	137	5	152	5	149	8	123	13
ISSR	HB-9	25	23	11	0	11	0	11	0	11	0	13	0	14	1	15	0	15	0	12	0	12	1
	HB-10	21	17	11	1	15	1	11	2	14	0	17	0	15	0	13	0	18	0	16	1	12	0
	HB-11	20	18	16	1	14	0	14	0	16	0	14	0	17	0	9	1	16	0	14	0	14	0
	HB-12	7	7	7	0	7	0	7	0	7	0	7	0	7	0	7	0	7	0	7	0	7	0
	HB-13	25	25	10	1	13	4	0	0	13	1	10	0	9	2	8	0	6	0	5	0	7	1
	HB-14	10	4	8	0	8	0	8	0	9	0	9	0	9	0	9	0	9	0	10	1	10	0
	b44	10	7	4	1	4	0	3	0	5	0	8	0	9	0	8	0	7	0	6	0	6	0
	b98	14	0	3	0	3	0	4	1	1	0	0	0	1	0	6	0	1	1	4	1	6	1
	b99	14	1	1	0	1	0	5	1	4	0	9	0	9	0	7	1	8	1	9	0	7	0
	Total	146	102	71	4	76	5	63	4	80	1	87	0	90	3	81	2	87	2	83	3	81	3
SSR	SMC222CG	5	3	2	0	4	0	4	0	2	0	3	0	3	0	4	0	4	0	5	0	5	0
	SMC226CG	3	3	0	0	0	0	0	0	1	0	2	0	2	0	2	0	0	0	3	0	3	0
	SMC319CG	5	5	0	0	0	0	3	0	0	0	3	0	1	0	3	0	2	0	1	0	3	0
	SMC477CG	4	4	0	0	0	0	3	0	2	0	2	0	2	0	4	0	4	0	3	0	4	0
	Total	17	15	2	0	4	0	10	0	5	0	10	0	8	0	13	0	10	0	12	0	15	0

TAF = total amplified fragments; Pf = polymorphic fragments; AF = amplified fragments; * = specific fragment

Table 5 Total specific fragments with their molecular sizes (bp) for smut resistance using the 20 RAPD and 9 ISSR primers according to their presence in most resistant cultivars.

	9 cultivars	8 cultivars	7 cultivars	6 cultivars
RAPD-specific fragments	OP-C05$_{1470}$	OP-B12$_{370}$	OP-M01$_{270}$	OP-M20$_{1000}$
	OP-C05$_{1360}$	OP-C05$_{1230}$	OP-M20$_{480}$	OP-M20$_{580}$
	OP-C05$_{400}$	OP-M01$_{1060}$	OP-Z03$_{2210}$	OP-O18$_{410}$
	OP-C20$_{430}$	OP-M20$_{1500}$	OP-Z03$_{1840}$	OP-Z03$_{560}$
	OP-M01$_{2400}$	OP-M20$_{1240}$	OP-Z03$_{1550}$	OP-Z03$_{160}$
	OP- M20$_{300}$	OP-O20$_{390}$	OP-Z03$_{690}$	
			OP-Z03$_{350}$	
ISSR-specific fragments	HB-09$_{600}$	HB-09$_{510}$	HB-09$_{510}$	HB-09$_{510}$
		HB-11$_{700}$	HB-10$_{480}$	HB-10$_{400}$
		HB-11$_{180}$		HB-11$_{630}$

SSR-PCR analysis

The SSR analysis of the 10 cultivars using the four specific SSR primers was presented in **Fig. 4**. The total number of fragments developed through the PCR reaction was 17 with 88.2% polymorphic fragments (**Table 4**). Primer SMC222 CG produced five polymorphic fragments with molecular sizes ranged from 280 to 180 bp. Two cultivars, 'G85-37' and 'NCo310' revealed the five fragments, while the other showed from 2 to 4. Primer SMC226CG exhibited 3 fragments with molecular sizes ranged from 170 to 140 bp. Fragment 1 of 170 bp appeared uniquely in 'G85-37' and 'NCo310', however 'GT54-9', 'PH8013', 'G99-165' and 'G84-47' showed no fragments and 'G95-19' showed one fragment with 140 bp. Moreover, primer SMC319CG showed five polymorphic fragments (100%) with molecular sizes ranged from 280 to 160 bp. Fragment 3 with 230 bp appeared in cultivars 'G98-28' and 'G84-47', while fragment 5 with 160 bp displayed in cultivars 'G99-165' and 'G98-24'. No fragments were displayed in the two commercial cultivars, 'GT54-9' and 'PH8013'. Primer SMC477 CG showed four polymorphic fragments with molecular sizes ranged from 300 to 190 bp. Three cultivars showed the 4 fragments, while no fragments were displayed in 'GT54-9' and 'PH8013'.

Genetic similarity and UPGMA cluster analysis based on RAPD, ISSR and SSR markers

Each of the RAPD, ISSR and SSR marker analysis was used to estimate the genetic similarity among 10 sugarcane cultivars as shown in **Table 6**. The highest genetic simi-

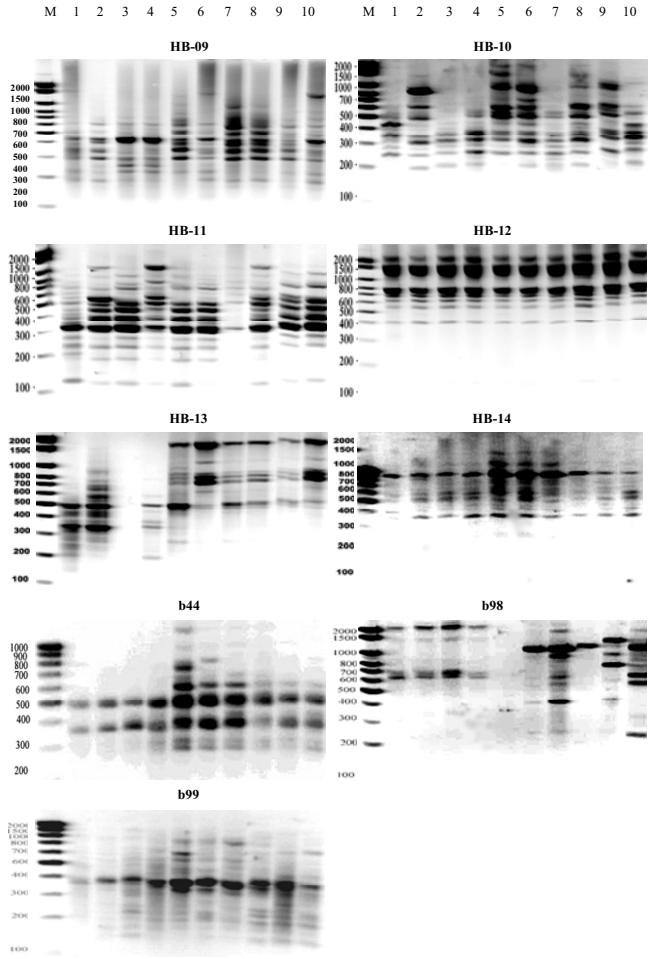

Fig. 3 ISSR profiles of the 10 sugarcane cultivars amplified with 9 primers. M = DNA ladder, 1 = 'GT54-9', 2 = 'PH8013', 3 = 'G99-16'5, 4 = 'G95-19', 5 = 'G95-21', 6 = 'G98-28', 7 = 'G95-19', 8 = 'G84-47', 9 = 'G85-37', 10 = 'NCo310'.

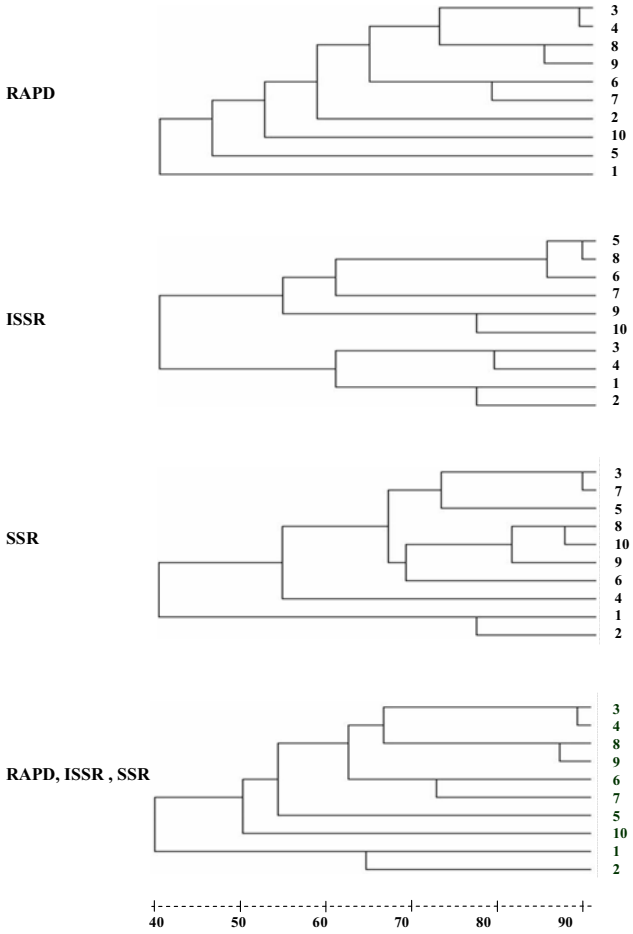

Fig. 5 Dendrograms for the genetic distances relationships among the 10 sugarcane cultivars based on similarity percentages of RAPD, ISSR and SSR and combined analyses. 1 = 'GT54-9', 2 = 'PH8013', 3 = 'G99-16'5, 4 = 'G95-19', 5 = 'G95-21', 6 = 'G98-28', 7 = 'G95-19', 8 = 'G84-47', 9 = 'G85-37', 10 = 'NCo310'.

larity index from RAPD data was 0.928 that observed between 'G99-16' and 'G95-19', while the lowest similarity index was 0.588 between 'GT54-9' and 'NCo310'. The highest genetic similarity from ISSR data was 87% that observed between 'G95-21' and 'G98-28', while the lowest similarity index 58.8% was between 'GT54-9' and 'G85-37'. The highest similarity index recorded from SSR data was 80% that was observed between 'G84-47' and 'NCo310' while the lowest similarity index was zero percentage between 'GT54-9' and 'G95-19' and 'PH8013' and 'G95-19'. Moreover, combining the analyses of the three markers; RAPD, ISSR and SSR showed that the highest

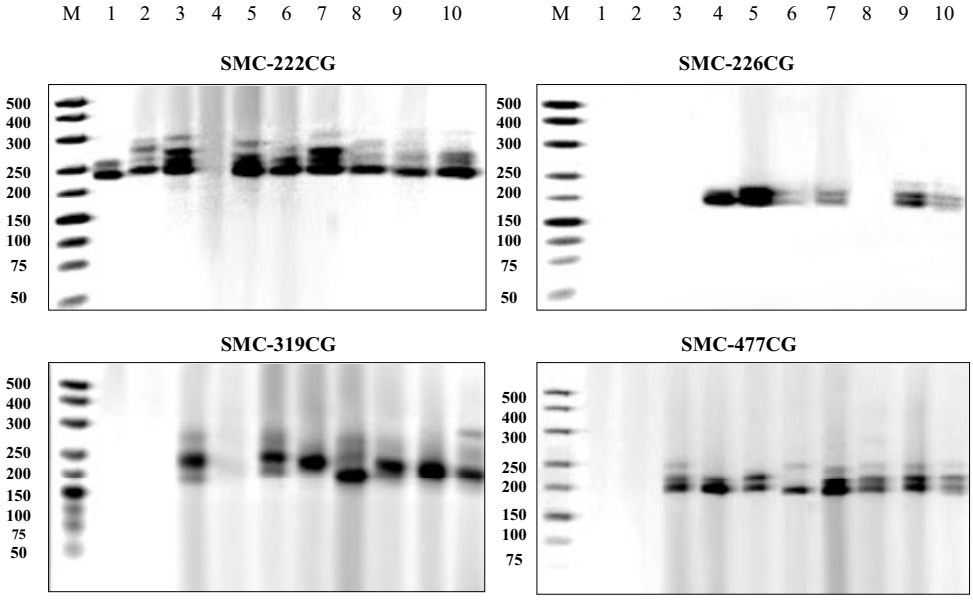

Fig. 4 SSR profiles of the 10 sugarcane cultivars amplified with four specific primers. M = DNA ladder, 1 = 'GT54-9', 2 = 'PH8013', 3 = 'G99-16'5, 4 = 'G95-19', 5 = 'G95-21', 6 = 'G98-28', 7 = 'G95-19', 8 = 'G84-47', 9 = 'G85-37', 10 = 'NCo310'.

similarity index was 0.888 between 'G99-16' and 'G95-19' while the lowest similarity index was 58.4% between 'GT54-9' and 'NCo310'. The dendrograms for the genetic relationships among the 10 sugarcane cultivars were performed as presented in **Fig. 5**.

RAPD markers were used for identification of molecular markers linked to hybrids or cultivars (Yang *et al.* 2001; Ranade *et al.* 2002; Abde-Tawab *et al.* 2003; Zhang *et al.* 2008). The characteristic features of the ISSR fragments suggest their potential applicability to the classification, phylogenetic analysis and construction of PCR-based genome maps in sugarcane (Li *et al.* 2002). ISSR could produce more polymorphic fragments compared with RAPD (Qi *et al.* 2003). ISSRs molecular markers were used to detect the genetic diversity among 30 clones of sugarcane (Sheji *et al.* 2006). ISSRs are ideal as markers for genetic mapping and population studies because of their abundance, and the high degree of polymorphism between individuals within a population of closely related genotypes. Those properties indicate their potential role as good supplements for RAPD based genome analysis (Korbin *et al.* 2002). Moreover, Kaye *et al.* (1991) initiated to assess the feasibility of using microsatellite or simple sequence repeat (SSR) markers as both a mapping tool, and a method for varietal identification and pedigree control in sugarcane. The search for microsatellite containing sequences includes the production and screening of enriched libraries. Several small insert libraries enriched for microsatellite containing sequences have been constructed from the cultivar R570. Several clones have been sequenced and specific primers have been designed from the sequence flanking the SSR. Successful primer pairs were used to amplify products from DNA of several sugarcane cultivars as well as several clones of *S. officinarum* and *S. spontaneum*. The resulting patterns range from simple, monomorphic patterns with few fragments to highly complex multi-fragment "ladder-like" patterns. Simple sequence repeats or microsatellite (SSR) markers are small segments of DNA that can be used as genetic markers to identify varieties of sugarcane and the unique markers of a variety are inherited by the off spring from these crosses. Using an additional 66 SSR markers, 277 of the original 286 progeny from the selfing of the sugarcane variety LCP 85-384 were verified as true selfs (Grisham *et al.* 2008). The progeny were multiplied and screened in greenhouse and field tests for susceptibility to ratoon stunting disease (RSD), mosaic, smut and rust. Only two out of 277 were susceptible to smut by inoculated test in the greenhouse; however, approximately 5% of the progeny were susceptible to natural inoculation in the field. Susceptibility to RSD ranged from resistance to highly susceptible. The 25 most susceptible and the 25 most resistant were identified for future genetic analysis. More than 250 sugarcane microsatellite markers are being applied in fingerprinting sugarcane lines, testing hybrid identity in breeding and in populating sugarcane genome maps by Henry and Cordeiro (2002). However, the authors reported that SSR analysis is complicated by the nature of the sugarcane genome but this has not prevented successful application of this technology. To ensure the identity of Louisiana sugarcane parental clones, Pan *et al.* (2007) genotyped 116 clones with 21 microsatellite (SSR) markers. A total of 144 distinctive SSR alleles were scored and the presence or absence of these SSR alleles in a sugarcane clone was recorded into a DNA sequence of presence and absence to represent its SSR genotype. SSR markers were developed from 2005 sugarcane consensus sequences derived from the

Table 6 Genetic similarity percentages of the 10 sugarcane cultivars based on 20 RAPD, 9 ISSR and 4 SSR primers.

Markers		**Cultivars**								
		GT54-9	**PH8013**	**G99-165**	**G95-19**	**G95-21**	**G98-28**	**G98-24**	**G84-47**	**G85-37**
RAPD	PH8013	78								
	G99-165	79	83							
	G95-19	76	82	93						
	G95-21	63	68	73	76					
	G98-28	74	79	84	85	78				
	G98-24	72	77	82	83	80	88			
	G84-47	69	79	85	85	73	83	82		
	G85-37	68	75	86	85	74	79	79	91	
	NCo310	59	74	75	75	68	76	77	79	81
ISSR	PH8013	82								
	G99-165	82	75							
	G95-19	82	78	84						
	G95-21	66	74	69	78					
	G98-28	68	69	71	78	87				
	G98-24	61	59	62	71	75	76			
	G84-47	68	72	73	80	88	85	78		
	G85-37	59	63	65	72	75	74	72	83	
	NCo310	59	62	69	73	71	71	71	75	83
SSR	PH8013	67								
	G99-165	20	36							
	G95-19	0	0	33						
	G95-21	15	29	67	40					
	G98-28	33	29	57	25	47				
	G98-24	22	40	82	36	60	46			
	G84-47	33	29	57	50	47	60	62		
	G85-37	29	25	53	67	67	55	43	73	
	NCo310	33	29	71	50	59	60	62	80	73
RAPD + ISSR + SSR	PH8013	79								
	G99-165	77	79							
	G95-19	76	80	89						
	G95-21	63	69	72	75					
	G98-28	71	75	79	82	80				
	G98-24	76	70	76	78	78	83			
	G84-47	68	76	81	83	77	84	80		
	G85-37	64	71	78	80	74	83	75	88	
	NCo310	58	69	73	74	69	74	74	78	82

SUCEST database (http://sucest.lad.ic.unicamp.br/en/) and the details of primer pair sequences and foreseen product size with SSR motifs for the selected markers in sugarcane were described by Oliveira *et al.* (2009).

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