Molecular genetic markers associated with salt tolerance in grain sorghum

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ABSTRACT

The present study was carried out to evaluate two inbred lines of grain sorghum for their environmental stress tolerance (salinity) at the field. These two inbred lines were chosen as salt tolerant (ATX631) and salt sensitive (ICSR89038) for hybridization to obtain the F_1 generation and then selfed to obtain the F_2 generation. These contrasting genotypes and their F_1 and F_2 generations were utilized to detect DNA-based molecular markers associated with salt tolerance via RAPD and ISSR techniques, following the bulked segregant analysis. This analysis revealed some genetic markers associated with salt tolerance in grain sorghum that can be utilized during breeding programs via marker-assisted selection.

***Key words**: Genetic markers, RAPD, ISSR, Sorghum, salt tolerance.*

INTRODUCTION

Grain sorghum is an important staple food throughout semi-arid Asian and African regions (Ahmed *et al*., 2000). A large number of different landraces, well-adapted to low-input conditions as well as to biotic and abiotic stress factors, are still cultivated. The frequent occurrences of food shortage in sorghum growing areas and the extension of sorghum cultivation to marginal lands require extensive breeding programs to introduce new varieties fitting small-scale farms needs (Haussmann *et al*., 2000). Compared to maize, sorghum breeding has been neglected in recent decades and the availability of high yielding maize varieties has led to the displacement of sorghum. However, maize is less adapted to drought conditions and thus had lower yield stability (Wenzel *et al*., 2001). Instead, sorghum may bear advantageous genes that are especially useful in conferring resistance to biotic as well as abiotic stresses.

Molecular markers developed by analysis of random amplified polymorphic DNA (RAPD) and inter simple sequence repeats (ISSR) have recently shown excellent potential to assist selection of quantitative trait loci (QTLs) associated with economically important traits. In addition, marker-assisted breeding can offer an efficient and rapid means to identify and incorporate adapted germplasm into Egyptian cultivars. Miche-lmore *et al.* (1991) developed the bulked segregant analysis of F_2 plants as a simpler alternative to isogenic line analysis, where the highest and lowest extreme groups of the F_2 population are bulked for the development of molecular markers associated with a given characteristic. Abdel-Tawab *et al.* (2004) performed bulked segregant analysis in sweet sorghum using SSR on the two contrasting parents (in their total biomass), their F_1 and F_2

bulks using 19 pairs of specific primers to detect the co-segregation of pre-mapped SSR markers with total biomass (TBM).

The main objectives of this paper are:

1. Hybridize the most salt-tolerant *Sorghum bicolor* and the most salt-sensitive inbred lines, based on previous screening, to obtain the F_1 plants followed by selfing to obtain F_2 plants.
2. Identify molecular markers associated with salt tolerance via RAPD and ISSR.

MATERIALS AND METHODS

Field experiments

Two inbred lines of grain sorghum (*Sorghum bicolor* L. (Moench), i.e., ATX631 (salt- tolerant) and ICSR89038 (salt-sensitive), chosen based on previous screening experiment (Ahmed and EL-Menshawi, 2006), were obtained as pure lines from Sorghum Res. Section, Field Crop Res. Institute, ARC, Giza, Egypt. These two inbred lines were grown in the field and crossed to obtained the F1 grains. Some of the F1 grains were sown in the field and selfed to obtained the F_2 grains. The two parental lines and their respective F_1 hybrid were evaluated in the field under two sites (normal and saline areas) at Nubaria Station (ARC, Egypt). F_2 grains were sown under saline area during 2005-2006 growing season Experiments were conducted in a randomized complete block design with three replicate to study the effect of soil salinity on growth and yield of different genotype. Each replicate consisted of 29 ridges for saline area and 9 ridges for normal area. Three ridges were planted for each of P_1, P_2 and F_1 and 20 ridges for F_2. Each ridge was four-meter long and 70-cm width. Planting was done in hills spaced at 20 cm apart and hills were thinned at two plants/hill. Days to 50% heading, plant height (cm), leaf area (cm^2) and grain yield per plant (g) were recorded on a sample of 10 guarded plants in the middle row for each of the P1, P2 and F1 and all guarded plants in all rows of F_2 for each replicate. The F_2 generation was represented by 1030 individual plants. The common agricultural practices of growing grain sorghum were applied properly as recommended in the district. Three soil samples were obtained at different depths from the soil surface. The mechanical and chemical analyses of the soil and water properties are shown in Table (1). The analysis of variance and multiple comparison were performed according to the method described by Snedecor and Cochran (1968).

Molecular genetic studies

Genomic DNA extraction

Genomic DNAs were isolated on a small scale from 200 mg of one-week-old etiolated seedlings of both cultivars along with their F_1 and F_2 generations. Leaves were ground to a powder using liquid nitrogen in microfuge tubes and DNAs were isolated using plant genomic DNA Mini Prep Kit (V-gene Biotechnology, China, cat. no. 110420-25) according to the manufacturer manual.

PCR conditions and electrophoresis

Ten out of 38 primers for RAPD and 10 out of 11 primers for ISSR were used in the study. Names and sequences of the selected primers are as the following:

RAPD Primers	
Name	Sequence
C02	GTGAGGCGTC
C04	CCGCATCTAC
C05	GATGACCGCC
C16	CACACTCCAG
C20	ACTTCGCCAC
O12	CAGTGCTGTG
O16	TCGGCGGTTC
O18	CTCGCTTCC
Z12	TCAACGGAC
O09	TCCCACGCAA

ISSR Primers	
Name	Sequence
814	(CT)8TG
844A	(CT)8AC
844B	(CT)8GC
17898A	(CA)6AC
17898B	(CA)6GT
17899A	(CA)6AG
17899b	(CA)6GG
HB15	(GTG)3GC
HB11	(GT)6CC
HB12	(CCA)3GC

Table (1): Mechanical and chemical analyses of the soil at the experimental sites.

Properties and components	Type of soil	
	Normal area	Saline area
Sand %	69.50	69.50
Silt %	18.00	18.50
Clay %	12.30	12.60
Texture	Sandy loam	Sandy loam
Organic matter %	00.36	00.21
CaCO3 %	23.60	23.05
Soil:		
EC(Mmhos/cm)	01.56	06.33
pH	08.31	07.87
Water:		
EC(Mmhos/cm)	00.80	00.80
pH	07.10	07.10
Soluble cations(-equiv./L):		
Ca^{++}	02.16	19.30
Mg^{++}	01.40	11.35
Na^{+}	07.78	35.03
K^{+}	00.81	04.20
Soluble anions(-equiv./L):		
$HCO3^{-}$	02.90	08.30
Cl^{-}	06.00	28.70
$SO4^{-2}$	03.00	33.00

PCR for both analyses was performed in 25 µl volume containing 2.5 mM $MgCl_2$, 0.2 mM dNTPs, 20µM primer, 50 ng genomic DNA and 1 unit Taq DNA polymerase (Bioron, Germany). All reactions were performed in a Perkin Elmer 2400 thermal cycler. RAPD Program was performed as 1 cycle of 94^0C for 4 min and 40 cycles of 94^0C for 1 min, 35^0C for 1 min, and 72^0C for 2 min. ISSR program was performed as 1 cycle of 94^0C for 4 min and 35 cycles of 94^0C per 30 sec, 44^0C for 45 sec,72^0C for 1.5 min. Then, a final extension step of 72^0C for 10 min was done for both analyses.

To visualize the PCR products, 15 µl of each reaction was loaded on 1.2% agarose gel. The gel was run at 90V for 1 h and visualized with UV Transilluminator and photographed using UVP gel documentation system (GelWorks 1D advanced software, UVP).

Data analysis

Data of polymorphic and monomorphic bands for both analyses was scored using the UVP gel documentation system. Amplicon sizes were estimated using 100-bp and 1-kb DNA standards (Bioron, Germany).

RESULTS AND DISCUSSION

Evaluation of salt-stress tolerance

The results for days to 50% heading, plant height, leaf area, and grain yield per plant for the two sorghum genotypes along with their cross under normal and saline conditions are shown in Table (2). The results reflected the significant differences between parental lines for all agronomic characters under normal and saline conditions indicating the variability existed between the two parental lines. Mourad *et al*. (1999), EL-Menshawi *et al.* (2003; 2005) and Ahmed and EL-Menshawi (2006), in their salt stress experiments on grain sorghum, indicated the high genetic potentiality of some genotypes based on yield components.

It was obvious that the parental line ATX631 was less early heading (with the means of 71.3 and 74.43 days under normal and salinity treatment, respectively), taller (with the means of 111 and 96 cm, respectively), has larger leaves (with the means of 417 and 370 cm^2 for both treatments, respectively), higher yielding ability (with the means of 84.5 and 61 g, respectively) and consequently more salinity tolerant than the other parental line ICSR89038. F_1 plants had higher performance as for plant height, leaf area and grain yield/plant.

Table (2): Mean performances of two grain sorghum parental lines and their cross for yield and some agronomic traits under normal and saline conditions.

Genotypes	condition	Days to 50% heading	Plant height (cm)	Leaf area (cm^2)	Grain yield /plant (g)
ATX631	**normal**	71.30	111.00	417.00	84.50
	salinity	74.43	096.00	370.00	61.00
ICSR89038	**normal**	74.50	105.00	381.00	68.10
	salinity	81.55	074.00	317.00	30.40
F1	**normal**	70.43	146.00	444.00	96.30
	salinity	76.18	130.00	390.00	78.30
Mean	**normal**	72.09	120.67	414.00	82.97
	salinity	77.39	100.00	359.11	56.58
LSD (0.05)	**normal**	01.30	002.620	002.62	02.06
	salinity	01.00	002.620	012.16	01.64

After the two selected inbred lines, i.e. ATX63 (tolerant, T) and ICSR89038 (sensitive, S) were crossed to obtain F_1 grains, some of these F_1 grains were sown in the field and selfed to obtain F_2 grains. Salt experiment was conducted to investigate the response of the F2 segregating population to salinity stress.F_2 plants were classified in a descending order, based on their tolerance to salt stress, into groups of 10 F_2 individuals in which the most four tolerant and the most four sensitive groups (Table 3) were selected for subsequent molecular analysis. The four most salt-tolerant groups had means of 74.3 days, 83 cm, 392.8 cm^2 and 64.8 g for different traits, respectively, while the four most salt-sensitive groups had means of 78 days, 52.5 cm, 303 cm^2 and 20.8 g for the above mentioned characters, respectively. It was obvious from the data across the two seasons, to evaluate the two parents and their F_1 plants in the first season and the F_2 segregating population in the second that other environmental factors have negatively affected the performance of F_2 individuals. Accordingly, it was recommended to study the two extreme groups only for their

performance under salinity stress for further molecular analysis to detect molecular genetic markers related to salt stress following the bulked segregate analysis first mentioned by Michelmore *et al.* (1991), was adopted in this respect.

Table (3): Mean performances of the most tolerant and the most sensitive F_2 groups with respect to yield and some yield-related traits under soil salinity stress.

Group	Days to 50% heading	**Plant** Height (cm)	Leaf area (cm^2)	Grain yield per plant (g)
The most tolerant groups				
1	77.0	95	414.0	84.0
2	75.0	89	401.0	77.0
3	73.0	77	386.0	58.0
4	72.0	71	370.0	40.0
Mean	74.3	83	392.8	64.8
The most sensitive groups				
1	81	43.0	256	13.0
2	79	49.0	282	19.0
3	78	55.0	317	23.0
4	74	63.0	357	28.0
Mean	78	52.5	303	20.8

Molecular genetic analysis

The bulked segregant analysis (BSA) was adopted in this investigation (Michelmore *et al.*, 1991) to detect markers for salt tolerance in grain sorghum. BSA identifies markers linked to a molecular trait of interest in the segregating F_2 population generated from the hybrid between the two contrasting genotypes (tolerant and sensitive in this study). Two DNA bulks from the most two contrasting F_2 groups were used along with their parents and F_1 plants to develop RAPD and ISSR markers associated with stress tolerance.

RAPD markers associated with salt stress tolerance

The DNA bulks of F_2 of the two extreme groups, for their performance under salinity condition, F_1 and their parents (ATX631, T and ICSR89038, S) were tested against thirty-eight 10-mer random primers. Data were considered for only ten primers (Figure 1) the lowest number of bands was developed by primer O12 (4 bands), while the highest was developed by primer C04 (13 bands). These 10 primers developed a total of 78 bands, in which 49 of them were polymorphic (63 % polymorphism). A great deal of band polymorphism was arbitrary; however, 14 bands were found to be useful markers related to salt stress (4 positive and 10 negative) as shown in Table (4). Primer C20 seemed to be the only one to generate no stress-related markers. A positive marker (coupling) is a band generated in the tolerant parent, F_1 plants and tolerant F_2 bulk, while a negative marker (repulsion) is a band generate in the sensitive parent, F_1 plants and sensitive F_2 bulk. The results of the present investigation are in accordance with those of Abdel-Tawab *et al.* (1997; 1998), who detected one RAPD marker for salt tolerance and three for drought tolerance in maize, respectively, while they developed two positive and two negative molecular markers for salt tolerance in maize (Abdel-Tawab *et al.*, 2001) using bulked sergeants analysis. Many other successful

attempts to detect RAPD markers for salt or drought tolerance were reported (Breto *et al.*, 1994; Rahman *et al.*, 1998). RAPD offers the simplest and fastest method for detecting a great number of genomic markers in a short period of time.

Table (4): Number of markers for grain sorghum based on RAPD and ISSR analysis.

	Positive	No.	Negative	No.
RAPD	C02-715, C05-1585, O16-210, Z12-2000	4	C04-2015, C04-1770 C05-300, C05-240 C16-270, C16-200 O12-1100, O16-465 O09-1700, O18-855	10
ISSR	17898A-210, 17899A-430 844A-2170, 844B-2170 HB11-820, HB11-290	6	17898A-620, 17899B-2415 17899B-1675, 814-2105 814-1355 , 844B-810 844B-730 , 844B-480 HB12-805	9

ISSR markers associated with salt stress tolerance

ISSR, as a relatively new class of molecular markers, is based on inter tandem repeats of short DNA sequences. These inter repeats are highly polymorphic in their sizes even among closely related genotypes, due to the lack of evolutionary functional constraints in these non-functioning regions. The DNA bulks of F_2 under salt treatment, F_1 and their parents (ATX631, T and ICSR89038, S) were tested against 11 ISSR primers. Data were considered for only 10 primers (Figure 2). The lowest number of bands was developed by primers 844a and 844b (6 bands), while the highest was developed by primer 17899a (12 bands). These 10 primers developed a total of 77 bands in which 37 of them were polymorphic (48 % polymorphism). A high percentage of band polymorphism was arbitrary, while 15 were found to be useful markers related to salt stress (6 positive and 9 negative) as shown in Table (4). Primers HB15 and HB13 resulted in the recovery of no molecular markers related to salt stress. Our results are in harmony with the findings of Yang *et al*. (1996), who used the inter-simple sequence repeats (ISSR) and microsatellites in soybean cultivars discrimination and reported that microsatellites represent an excellent technique to study the genetic polymorphism. It is evident from the aforementioned results that the high number of molecular markers related to salt tolerance reflects the complexity in this trait besides being affected by the environment. However, the use of marker-assisted selection could enhance the identification of sorghum genotypes tolerant to environmental stress. This approach would enable the molecular plant breeder to detect the promising lines with more confidence in their merits as line selection based on genetic rather than phenotypic basis, with the elimination of the environmental factors as much as possible. Moreover, this process is fast, reliable and cost-effective, reducing the required time for sorghum breeding programs in Egypt.

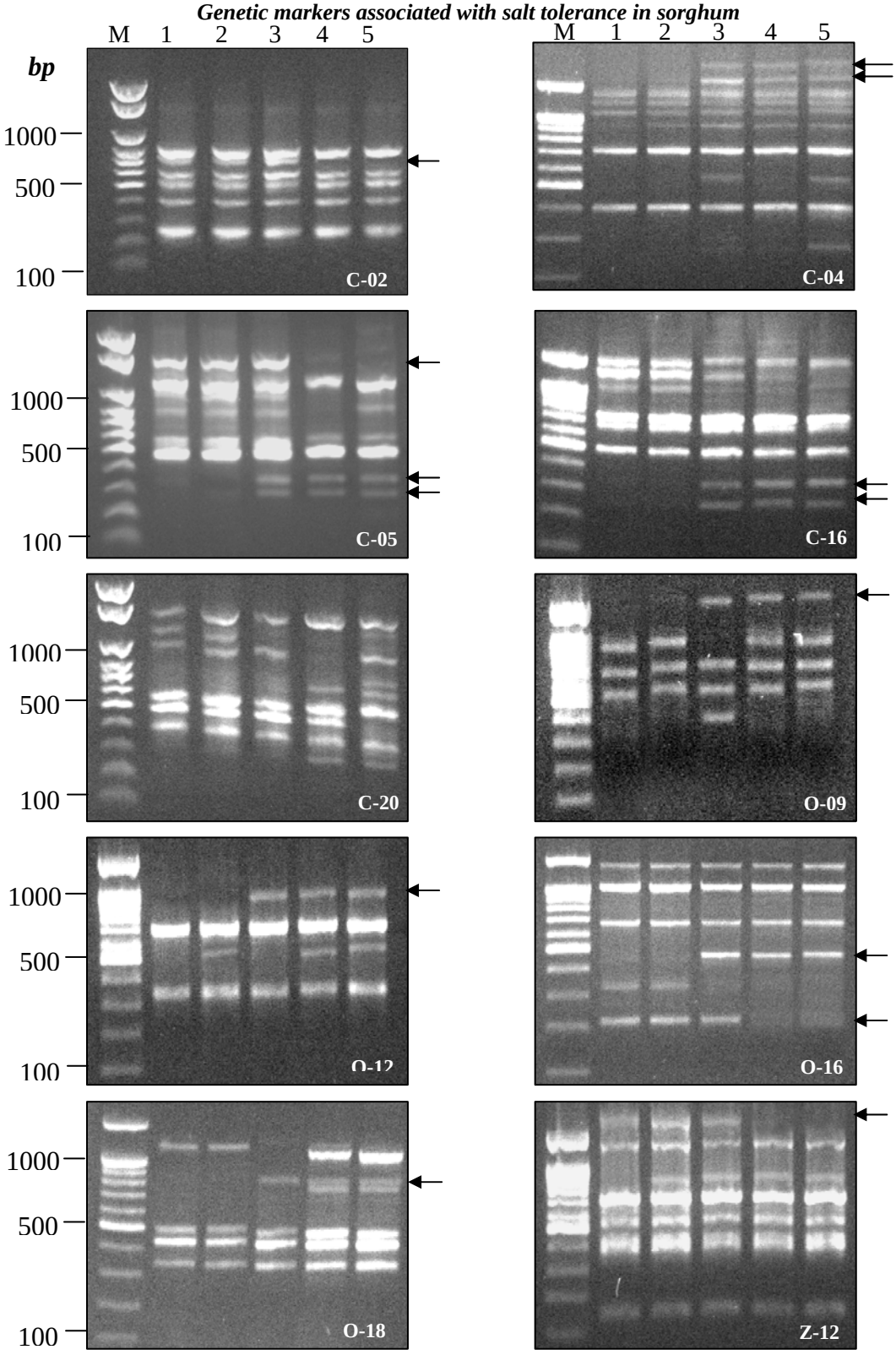

Fig. (1): RAPD profiles for the tolerant parent (1), tolerant F_2 bulk (2), F_1 (3), sensitive parent (4) and sensitive F_2 bulk (5) using ten random 10-mer primers.

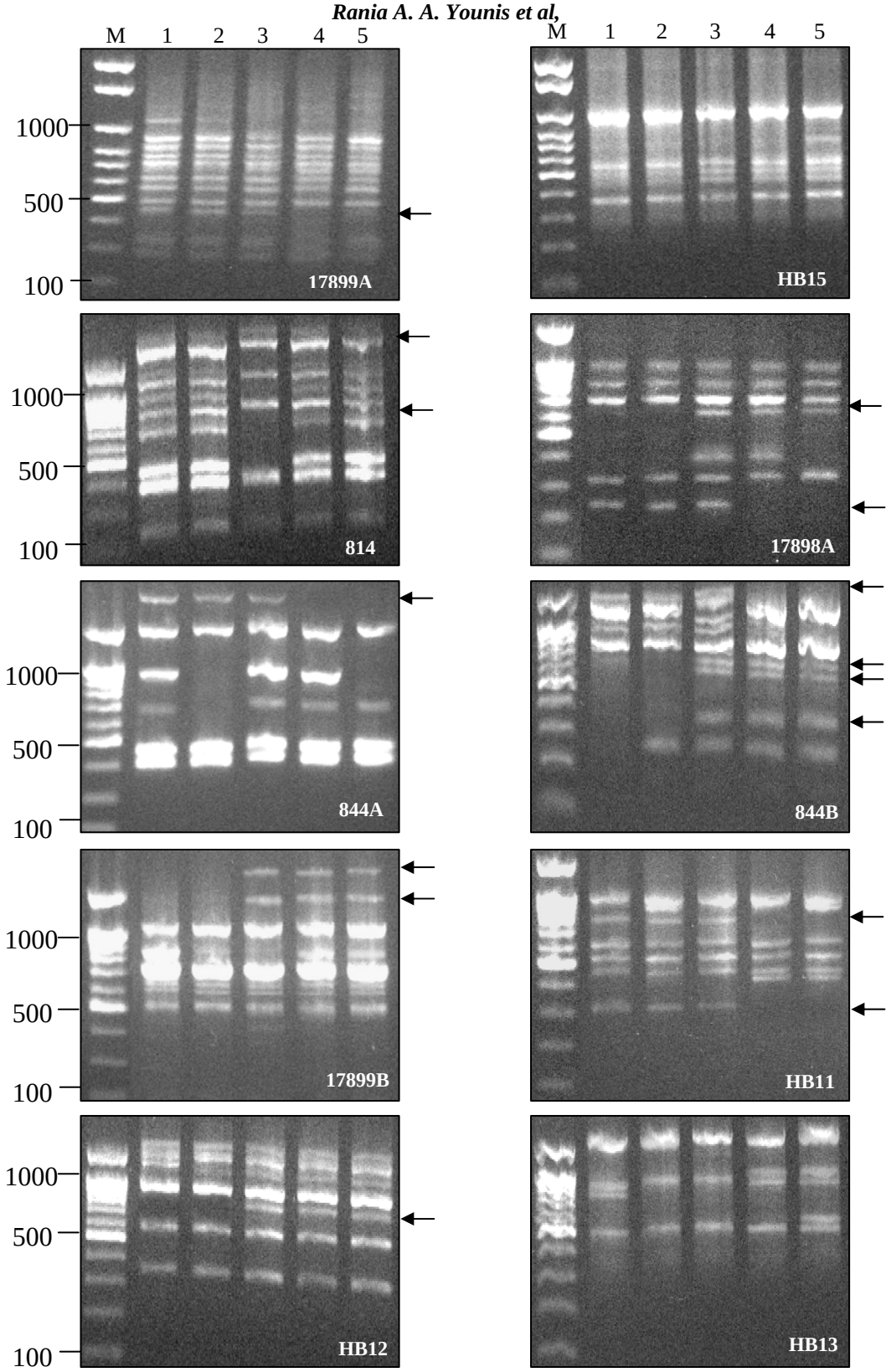

Fig. (2): ISSR profiles for the tolerant parent (1), tolerant F_2 bulk (2), F_1 (3), sensitive parent (4) and sensitive F_1 bulk (5) using 10 primers.

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الملخص العربي

الأدلة الوراثية الجزيئية المرتبطة بتحمل الملوحة فى الذرة الرفيعة

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تهدف الدراسة الحالية إلى تقييم مدى تحمل الضغوط البيئة المعاكسة (الضغط الملحى) لصنفين من أصناف الذرة الرفيعة هما (ATX631) كصنف متحمل للملوحة و (ICSR89038) كصنف حساس وكذلك الهجين الناتج منهما على مستوى الجيل الأول و الثاني وذلك للحصول على الواسمات الجزيئية المساعدة للانتخاب(MAS) Marker Assisted Selection عن طريق تقدير تلك الواسمات الجزيئية بتقنيتين: الأولى حساب التباين العشوائى للـ DNA المجسم (RAPD) والثانية تقدير التباين بين التتابعات البسيطة المكررة (ISSR) فى جينومات تلك الأصناف والجيل الأول والثانى الناتج من تهجينهما. وقد أظهرت تلك الدراسة عدداً من الواسمات الجزيئية المرتبطة بتحمل الملوحة.