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Inter-Simple Sequence Repeat (ISSR) Markers to Study Genetic Diversity Among Cotton Cultivars in Associated with Salt Tolerance

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Abstract

Developing salt-tolerant crops is very important as a significant proportion of cultivated land is salt-affected. Screening and selection of salt tolerant genotypes of cotton using DNA molecular markers not only introduce tolerant cultivars useful for hybridization and breeding programs but also detect DNA regions involved in mechanism of salinity tolerance. To study this, 28 cotton cultivars, including 8 Iranian cotton varieties were grown in pots under greenhouse condition and three salt treatments were imposed with salt solutions (0, 70 and 140 mM NaCl). Eight agronomic traits including root length, root fresh weight, root dry weight, chlorophyll and fluorescence index, K^+ and Na^+ contents in shoot (above ground biomass), and K^+/Na^+ ratio were measured. Cluster analysis of cultivars based on measured agronomic traits, showed 'Cindose' and 'Ciacra' as the most tolerant cultivars, and 'B-557' and '43347' as the most sensitive cultivars of salt damage. A total of 65 polymorphic DNA fragments were generated at 14 inter-simple sequence repeat (ISSR) loci. Plants of 28 cultivars of cotton grouped into three clusters based on ISSR markers. Regression analysis of markers in relation with traits data showed that 23, 33 and 30 markers associated with the measured traits in three salt treatments respectively. These markers might help breeders in any marker assisted selection program in order to improving cotton cultivars against salt stress.

Keywords: cotton, genetic variation, salt tolerant, ISSR marker

Introduction

Cotton is one of the most economically important crops which is cultivated for about 85 years in Iran, mostly on irrigated lands (according to report of Iran Cotton Union). *Gossypium herbaceum* and *G. hirsutum* are mostly cultivated species in Iran.

One of the major abiotic stresses responsible for low yield in arid and semi-arid regions of the world is soil salinity which according to the FAO (2008), Land and Plant Nutrition Management Service affects about 20 percent of irrigated agriculture. About 90 percent of Iran cultivation area falls in arid climate where no crops can be grown without irrigation. Half of the irrigated agriculture is influenced by soil salinity which causes more than 50 percent yield reduction in arid regions of Iran. More than 7.6 milliard cubic meters of salt water is flowing annually in 12 rivers of Iran (Qureshi *et al.*, 2007).

Cotton is relatively a salt tolerant crop. Most of the cotton cultivars and germplasms could withstand salinity levels of 7 to 8 ds/m (deci Simens per meter) without significant reduction in growth and yield. Early seedling stage is more sensitive to saline condition than later stages of growth. However there is variation exists for salt tolerance. *G. herbaceum* and *G. barbadense* were found to be more tolerant than *G. arboreum* and *G. hirsutum* (CICR Technical Bulletin No: 2). Asiatic cotton is more tolerant than upland cotton to salinity. Salt tolerance is a quantitative trait which is affected by the environmental factors. However, selection based on genetic rather than phenotypic characteristics (marker assisted selection; MAS) is a fast, reliable and cost effective approach which can enhance the identification of tolerant cotton genotypes. Inter-simple sequence repeat (ISSR) fingerprinting is a PCR based method was developed such that no prior sequence knowledge was required. 16-25 bp long microsatellites such as $(GACA)_4$ are used as primers to amplify the inter-simple sequence repeats of different sizes (Pradeep *et al.*, 2002). This technique has proven to be a simple, quick and inexpensive method which can generate high percentages of polymorphic loci. In cotton knowledge of molecular markers is very limited due to the several factors reviewed by Preetha and Raveendren (2008). Lack of a high density molecular map and a polymorphism detection methods

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are most important factors. ISSR technique reported as an easy and informative genetic marker system for revealing both inter and intraspecific variations in cotton (Liu and Wendel, 2001), yielding a multi locus marker system useful for fingerprinting, diversity analysis, and genome mapping. In this investigation genetic diversity of 28 cotton cultivars is studied under salt stress conditions, compared with genetic distance estimated from ISSR markers and explained reliable molecular markers of salt tolerance.

Materials and methods

Plant cultivars and treatments

Seeds of 28 cultivated varieties of cotton (*G. hirsutum*) (Tab. 1) were obtained from Moghan agro-industry institute, Ardabil, Iran. Individual plants were grown in three replication in a factorial experiment based on Randomized complete block design. Pots (37×27×24 cm) placed in a temperature-controlled greenhouse (32°C/ humidity 60%). All pots watered with distilled water first and in five and ten days later three salt treatments of 0, 70, and 140 mM NaCl were applied to plants according to Munns *et al*. (1995).

Morphological and physiological traits

Three weeks after first salt treatment, at the 6-leaf stage, chlorophyll and fluorescence indexes were measured with Chlorophyll Meter SPAD-502 and Chlorophyll Fluorometer OS-30P respectively. After these measurements aboveground and underground parts were harvested separately. Above-ground part oven- dried at 70°C for 48 hours. One gram of dried parts was grinded and heated in an oven at 550°C. Ten ml Hcl was added to dry-grinded material to extract sodium and potassium ions and was adjusted to a final volume of 100 ml with double distilled water. Na^+ and K^+ were determined with the help of flame photometer (Darwish *et al*., 2007). Freshly harvested roots were washed three times with distilled water to wash off attached minerals and dried on filter paper. Root length and root fresh weight were measured then roots oven- dried at 70°C for 48 hours to measure root dry weight. Data analysis was done by SPSS19. For cluster analysis of cultivars, Ward's method based on Euclidean distances was conducted.

DNA extraction and amplification

Leaf tissues of plants at four-leaf stage were ground to a fine powder in liquid nitrogen and used for DNA extraction by using CTAB method with minor modifications (Doyle and Doyle, 1990). 34 ISSR primers were supplied from Bioneer Company (South Korea). The PCR reaction volume was 20 ul containing 30 ng genomic DNA, PCR buffer (2 µl), dNTP (0.2 µl), MgCl2 (0.8 µl), each primer (1.6 µl), and 1U of *Taq* DNA polymerase. The temperature cycles were programmed as 94°C for 5 min followed by 35 cycles of 94 for 1 min (45-55)°C for 1 min, 72°C for 2 min, and finally 5 min at 72°C for final extension. Amplified products were separated by electrophoresis on 2 percent agarose gels and visualized under UV light. ISSR fragments were scored as present (1) or absent (0).

Marker's data analysis

Cluster analysis, drawing the cluster produced by UPGMA clustering and principle component analysis was performed using NTSYS 2 Software. GenALEx6.3 was used to calculate Shannon Diversity Index, Marker Index (MI) and Polymorphism Information Content (PIC) and to perform Mantel's test (Liedloff, 1999). PopGene 1.32 software was applied to calculate Nei's gene diversity index. Other statistical analysis to investigate possible relations of molecular markers and measured traits was done by SPSS19.

Results and discussion

Cluster analysis of 28 cotton commercial studding cultivars based on agronomic traits performed based on WARD method. Three distinct clusters at 0 salt treatment

Tab. 1. Name and origin of the genotypes used

Cultivar Number	Cultivar Name	Origin	Cultivar Number	Cultivar Name	Origin
1	'Avangrd'	Bulgaria	15	'Cindose'	Greece
2	'Opal'	America	16	'Shirpan 539'	Bulgaria
3	'Oltan'	Iran Trade	17	'Shirpan 603'	Bulgaria
4	'B557'	Pakistan	18	'Mehr'	Iran Trade
5	'Bakhtegan'	Iran Trade	19	'Mutazhenez'	Mutant
6	'Bolghar 539'	Bulgaria	20	'Nazil'i	Turkey
7	'Bolghar 996'	Bulgaria	21	'Varamin'	Iran Trade
8	'Beliisovas'	Turkey	22	'Varamin 349'	Iran Trade
9	'Tabladika'	Spain	23	'No-200'	Greece
10	'Tashkand'	Uzbekistan	24	'No-228'	Greece
11	'Chegurava 15:18'	Turkey	25	'010'	Uzbekistan
12	'Khordad'	Iran Trade	26	'4.S.4'	Greece
13	'Sahel'	Iran Trade	27	'4325'	Greece
14	'Ciacra'	Iran Trade	28	'43347'	Greece

were indicated (Fig. 1-A). There were significant differences between the means of three clusters (α=1%). The mean of first and third cluster showed the lowest and the highest deviations from the total mean respectively (Fig. 2- A). Four clusters identified in 140 mM salt stress treatment (Fig. 1-B) which among them group 1 and 4 showed the lowest and the highest deviations from the total mean respectively (Fig. 1-B and 2-B). Negative deviation of Na^+ content and positive deviation of other agronomic studied traits from the total mean was represented a high-yield, salt-tolerant cultivar. 'Cindose' and 'Ciacra' at control salt treatment were in the group of low-yield while at salt shock treatment they showed the highest yield. This means these two cultivars are the most salt- tolerant genotypes. 'B557' and '43347' are the most salt-sensitive genotypes in the other way around.

ISSR data

14 ISSR primers from 34 studding ones produced 85 visible bands. Of these, 65 bands were polymorphic and

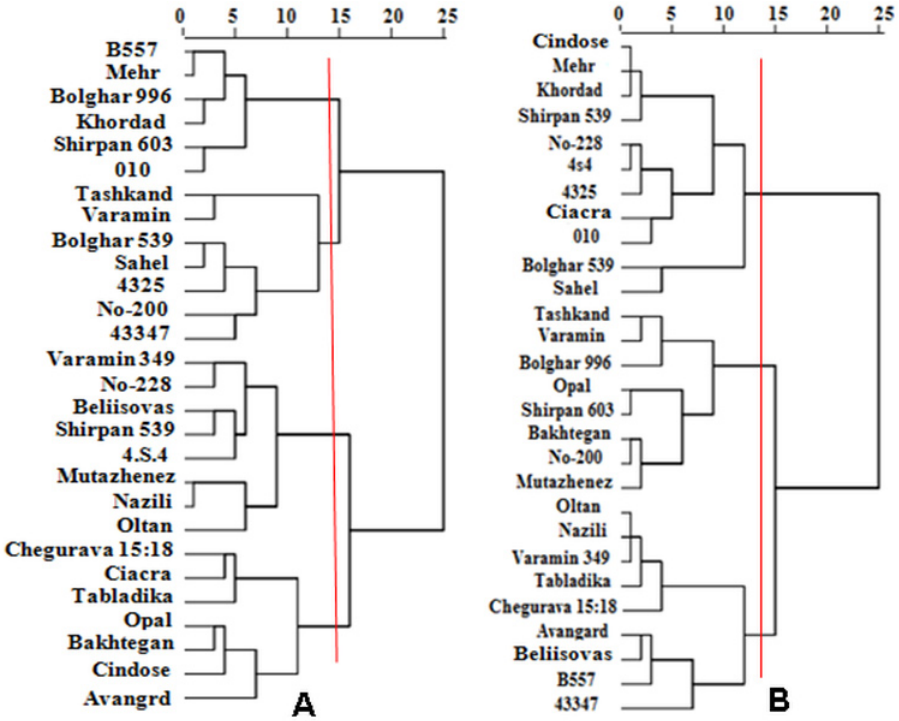

Fig. 1. Cluster analysis dendrogram of 28 cotton commercial cultivars based on agronomic traits (A. Control B. 140 Mm NaCl)

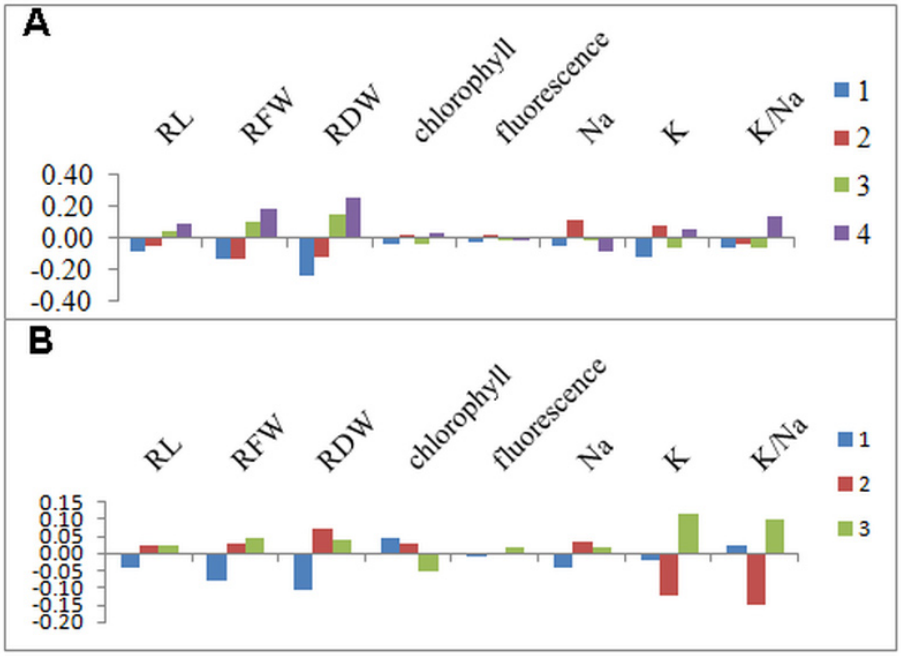

Fig. 2. The average deviation of the mean cluster: A. Control B. 140 mM

20 bands were monomorphic. Number of polymorphic bands per primer ranged from 3 (ISSR-2 and ISSR-28) to 8 (ISSR-1, ISSR -5, ISSR -8, ISSR- 14 and ISSR 19). Fig. 3, shows ISSR-19 banding pattern. ISSR-30 and ISSR-9 revealed the highest MI (2.028) and PIC indexes (0.378) respectively (Tab. 2). The marker index is used to evaluate the utility of marker system and is the product of the total number of loci per primer pair and the arithmetic mean heterozygosity (Nei, 1973).The PIC value provides the value of a marker for detecting polymorphism introduced by Botstein *et al*. (1980). When frequency of two alleles in a population is equal then PIC value gets the heights value of 0.5 (Mateescu *et al*., 2005).

Estimation of genetic diversity

Nei's gene diversity index (Nei, 1973) is commonly estimated parameter as a measure of gene diversity. The average Nei's gene diversity index in ISSR data set was 0.299 and ISSR-8 showed the highest value (0.398) (Tab. 2). In Similar study by Sharaf *et al*. (2009) this index was 0.228, 0.284 and 0.272 respectively for RAPD, ISSR and AFLP markers used.

Cluster analysis based on molecular data was generated by UPGMA using Jaccard's similarity coefficients (Fig. 4). The correlation coefficient was statistically significant (r= 0.84, α=1%). The cluster analysis of ISSR markers separated the cotton genotypes into three distinct clusters (Fig. 4). First cluster included 18 cultivars: 'Avangard', 'Sahel', 'B557', 'Bakhtegan', 'Khordad', 'Opal', 'Bolghar 996', 'Varamin', 'No-200', 'Varamin 349', 'Cindose', '4325', 'Shirpan 539', 'Mehr', 'Tabladika', 'Tashkand', 4.S.4' and '43347'. Two cultivars 010 and Beliisovas clusterd together and the third cluster included 8 remained cultivars.

Molecular data and agronomic traits

The success of any selection scheme relies on the availability and identification of agronomical beneficial alleles for the target traits. Traditionally, the genetic variability exploited by modern breeding to improve quantitative traits has been derived from highly selected, elite materials with a genetic basis much narrower than that of the wild relatives from which crops were originally domesticated (Tanksley and Nelson, 1996). However, beneficial QTL alleles have also been identified among wild relatives of crops (Tanksley and Nelson, 1996); therefore, the same should also hold true for salt tolerance.

Stepwise regression analysis between molecular data (0, 1) as fixed variable and studied agronomic traits data

Tab. 2. Polymorphic and Nei's (1973) gene diversity

Primers	Primer sequences	Number of amplified bands	Number of polymorphic bands	Polymorphic / amplified bands (%)	PIC	MI	Nei's gene diversity
ISSR- 1	5′ AGAC AGACGC 3′	8	8	100	0.189	1.512	0.189
ISSR- 2	5′ GACAGACAGACA GACA 3′	3	3	100	0.225	0.675	0.223
ISSR- 3	5′ AGAGAGAGAGAGAGAGC 3′	5	4	80	0.101	0.404	0.100
ISSR- 5	5′ AACAACAACGC 3′	8	8	100	0.115	0.920	0.151
ISSR- 8	5′ GACGACGACGACG 3′	8	4	50	0.378	1.512	0.398
ISSR- 9	5′ TCTCTCTCTCTCTCTCC 3′	6	4	66	0.376	1.504	0.390
ISSR- 14	5′ CACACACACACAGT 3′	8	8	100	0.375	3	0.393
ISSR- 15	5′ ACGACGACGACGAAC 3′	6	3	50	0.349	1.047	0.387
ISSR- 16	5′ CACACACACACAAG 3′	5	4	80	0.193	0.772	0.238
ISSR- 19	5′ AGAGAGAGAGAGAGAGT 3′	8	6	75	0.338	2.028	0.358
ISSR- 28	5′ GAGGAGGAGGC 3′	3	1	33	0.337	0.337	0.375
ISSR- 30	5′ GAGAGAGAGAGAGAGAC 3′	6	5	83	0.343	1.715	0.338
ISSR- 31	5′ CACCACCACGC 3′	6	5	83	0.336	1.680	0.334
ISSR- 32	5′ AGAGAGAGAGAGAGAC 3′	5	2	40	0.296	0.592	0.305
Total		85	65		3.95	17.64	4.179
Mean		6	4.6	76	0.282	1.26	0.299

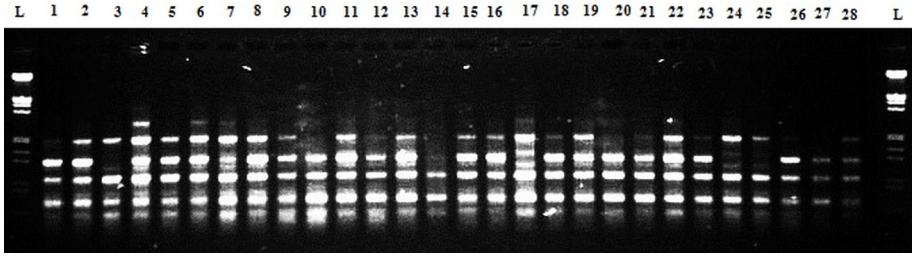

Fig. 3. Example of ISSR gel with ISSR-19 primer. The first twenty-eight cultivars represented in Tab. 1 are displayed from left to right. Ladders were run in the outside two lines

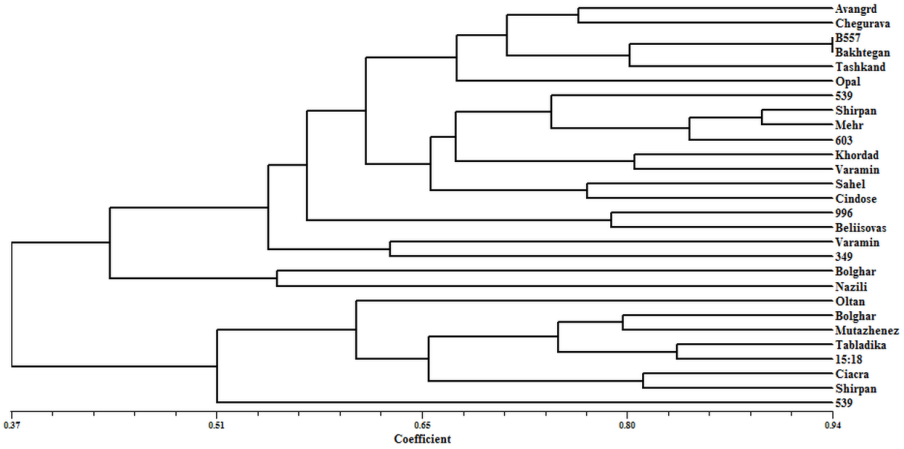

Fig. 4. Phenogram showing genetic diversity among 28 cotton cultivars using ISSR data

as variable function was performed to identify salinity associated markers with a high value of R^2. In total 23 and 30 ISSR markers associated with measured agronomic traits in 2 salt treatments detected (Tab. 3 and 4).

At control salt treatment (0 mM NaCl), there was only one marker related to K^+ content whereas 8 markers were associated with Na^+ and K^+/Na^+. Positive markers related to Na^+ content and K^+/Na^+ could explain 86% of variation (the highest) compare to K^+ and chlorophyll content related markers which was 28% (the lowest). ISSR14M4 was the most effective marker associated with studied agronomic traits at control salt treatment (Tab. 3).

Under severe salt stress (140 mM) two markers were associated with K^+/Na^+ and 8 markers were associated with root fresh weight. Markers in association with root fresh weight explained 91% of variation whereas amount of explained variance by K^+/Na^+ associated was 40%. At salt shock ISSR1M1 marker was the most effective marker associated with studied agronomic traits (Tab. 4).

Natural diversity provides a rich source of genetic recombination and mutations which can be analyzed for

Tab. 3. Regression coefficients and adjusted R square in the multiple regression between the agronomic triats and location ISSR gene in control

	RL	RFW	RDW	chlorophyll	fluorescence	Na^+	K^+	K^+/Na^+
Intercept	120.06	0.164	0.03	50.25	0.81	0.81	37.97	0.53
IS1M1	0.67					-0.41		
IS1M2				-0.64				
IS1M4							0.53	
IS1M7			0.31					
IS1M8						-0.32		0.80
IS2M1								-0.45
IS2M2								-0.30
IS3M2						-0.50		
IS5M3						-0.41		
IS8M2		0.37				-0.31		
IS9M1								-0.35
IS14M4		-0.62	-0.53			-0.50		
IS14M5					0.86			
IS14M7	-0.50							
IS15M1	0.26			-0.32				
IS16M1								-0.32
IS19M3								0.60
IS19M4								-0.49
IS30M1			0.31			0.86		
IS30M4	-0.31					0.34		
IS31M1								-0.27
IS31M3					0.53			
R2	0.73	0.41	0.50	0.28	0.62	0.86	0.28	0.86

Tab. 4. Regression coefficients and adjusted R square in the multiple regression between the agronomic triats and location ISSR gene in 140 mM NaCl stress

	RL	RFW	RDW	chlorophyll	fluorescence	Na^+	K^+	K^+/ Na^+
Intercept	87.48	0.05	0.05	42.93	0.79	46.13	32.45	1.12
IS1M1	0.44	0.43	0.64					
IS1M2		0.39						
IS1M3							0.40	
IS1M4			-0.72					
IS1M7				-0.39				
IS1M8		0.30				-0.56		
IS2M1			-0.27	0.46				
IS3M4	-0.33							
IS5M7		-0.51						
IS8M2						-0.48		
IS8M4		0.45						
IS9M1	0.29							
IS9M4						0.26		
IS14M1					0.37		0.34	
IS14M3	0.38							
IS14M4		-0.46						
IS14M5		-0.22						
IS14M6						-0.49		
IS14M7		0.20						
IS15M1			0.38			0.33		
IS15M3				0.46				
IS16M1					-0.44			
IS16M2					-0.47	-0.78		
IS19M6								-0.49
IS28M1			-0.38					
IS30M3							-0.56	
IS30M4				-0.41				
IS30M5						-0.27		
IS31M2					0.28			0.50
IS32M1				0.59				
R2	0.73	0.78	0.91	0.68	0.65	0.81	0.41	0.40

salt tolerance (Galpaz and Reymond, 2010; Katori *et al*., 2010). DNA molecular markers are an important tool which can be incorporated in this kind of analysis. There is not enough marker data in cotton to screen cotton genotypes tolerant for salt stress.

Conclusion

The results showed that ISSR molecular marker could be served as useful method in breeding for salt tolerance in cotton but it could be used in future studies with more primers.

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