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Assessment of genetic diversity among wheat selected genotypes and local varieties for salt tolerance by using RAPD and ISSR analysis

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Abstract

Three genotypes of wheat developede for saltetoleranceethroughe plant breeding program and two local cultivars were screened for genetic variation under salinity conditions through RAPD and ISSR markers. Eight selected primers (OP1-06, OPE-16, OPN-07, OPO-17, OPD-20, OPL-05, OPI-01 and OPJ-13) were used in randomeamplifiede polymorphic eDNA (RAPD-PCR reaction) and three selected primers (UBC 809, UBC 810 and UBC 811) were used in ISSR markers. According to the results of the amplification and ISSR markers, the genetic distance and dendogram illustratedegeneticefingerprint and relationshipsebetweeneselected genotypes and local cultivars were determinant. Results revealed that there are genetics differences between the selected genotypes and the local cultivars in some specific segment at different size (bp) with all primers which used in this study, except the result of the primer (OPE-16) showed that there are no bands appeared in all selected genotypes and local cultivars. Other results of RAPD markers showed that there are differences among the selected genotypes in their banding patterns only with primers (OP1-06, OPN-07 and OPO-17) at different size. The results of ISSR markers showed differences that there are also differences between the selected genotypes and local cultivars in specific segment with the three primers which used, the selected genotypes were similar in banding patterns with UBC 809 and UBC 811primers. Genetically, the results showed that all the selected genotypes and local cultivars differed in their genetic distance, variations among the selected genotypes in their genetic distance. In conclusion the selected genotypes (salt tolerant) genetically differed from the local cultivars (salt sensitive).

Keywords: wheat genotypes, RAPD and ISSR analysis, PCR.

تقدير التباين الوراثي بين التراكيب الوراثية المنتخبة والاصناف المحلية لصفة تحمل الملوحة باستخدام تحاليل ISSR و RAPD

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الخلاصة

اجريت دراسة لتقييم صفة تحمل الملوحة في ثلاث تراكيب وراثية مستنبطة لصفة تحمل الملوحة من خلال برامج التربية والتحسين تحت ظروف الشد الملحي وبالمقارنة مع صنفين محليين بأستخدام تقنية RAPD و

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ISSR.استخدمت ثمانية بادئات منتخبة هي(OP1-06) ، OPE-16 ، OPN-07 ، OPO-17 ، OPD-20 ، OPL-05 ، OPI-01و (OPJ-13 في تقنية التغاير الوراثي العشوائي RAPD-PCR واستخدمت ثلاثة بادئات منتخبة اخرى(UBC 809 ، UBC 810و UBC 811) في تقنية ISSR-PCR.استنادا الى نتائج تقنية RAPD و ISSR تم تقدير البعد الوراثي بين التراكيب الوراثية والاصناف المحلية المستخدمة، اوضحت النتائج بان هناك اختلافات وراثية بين التراكيب الوراثية المنتخبة والاصناف المحلية المستخدمة في بعض الحزم مختلفة الاحجام مع جميع البادئات المستخدمة في هذه الدراسة وما عدا البادئ (OPE-16) لم يظهر اي حزمة في جميع التراكيب الوراثية المنتخبة والاصناف المحلية المستخدمة. ايضا اظهرت نتائج تقنية RAPD-PCR بان هناك اختلافات بين التراكيب المنتخبة في نمط الحزم التي ظهرت في بعض البادئات(OPI-06 ، OPN-07و OPO-17 مختلفة الاحجام. اشارت نتائج مؤشر ISSRأبأن هناك ايضا اختلافات بين التراكيب الوراثية المتحملة للملوحة والاصناف المحلية في نمط الحزم التي ظهرت في البادئات الثلاثة المستخدمة. بينما كانت التراكيب الوراثية المنتخبة متشابه في نمط الحزم التي ظهرت مع بادئ UPC 809 و .UPC 81 وبشكل عام اظهرت النتائج بان جميع التراكيب الوراثية المنتخبة والاصناف المحلية اختلفت في البعد الوراثي وايضا هناك اختلافات بين التراكيب الوراثية المنتخبة في بعدها الوراثي. وبالتالي واستناداً الى هذه النتائج فان التراكيب الوراثية المنتخبة المتحملة للملوحة اختلفت وراثياً عن الاصناف المحلية الحساسة للملوحة.

1. Introduction

Wheat (Titicumaestivum L.) is one of the most important and widelye cultivated crops in the world, used for the preparation of bread and other bakede production. Soil salinity is one of the major abiotic stresses affecting crops productivity, and the degree of effect depends on the levels of salinity. Therefore, increasing the yield of crop plants under salinity condition is essential for feeding the word [1]. One of the important projects for increasing crops productivity under stress conditions is toe breed crops more tolerant to this stress However, success in breeding for tolerance has been limited because the tolerancee to stress is controlled by manye gene, and their heritability is low, and then the response to selection is difficulte[2,3]. Al-mishhadani (3) reported that there is a strong possibility to improve the salte tolerance in some wheat genotypes through plant breeding programs; also they reported that theree are some genotypes of wheat selected with high salt tolerance as compared with the unselected genotypes and local cultivars through plante breeding programs. Also Al- mishhdani et al. and Al-mishhdani [4,5], these selected genotype of wheat were derived from
F2 seedling after exposure to 30 ds/m drain age water for six cycles of screening and selection at germination and early seedling stages.

Application of DNA (RAPD) and (ISSR) have become excellent tools for plant breeders to improve salt tolerance character in plant [6]. In contrast to RAPD amplification , the ISSR markers are more feasible and reproducible. Reddy *et al.* [7], mentioned that ISSR is a simple, cost-efficient , rebuts, multilocus markers method which is extremely useful in determining genetic variability . RAPD and ISSR markers used for various applications and proved to be the most polymorphic markers in wheat [8]. Applying markers and recognition of polymorphic nucleotide sequences dispersed throughout the genome have providedgood possibility for determining diversity , intra –species genetic distance and relationships [9]. ISSR as a relatively new class of molecular markers , is based on inter tandem repeats of short DNA sequences and these are highlypolymorphic in their sizes even among closely related genotypes [10]. The development of molecular (DNA) marker provides new dimension , accuracy and perfection in the screening of germplasm [11]. According to the random amplified polymorphic DNA (RAPD- PCR reaction) result , the genetic distance and variation between selected genotype (Dijila) of wheat and local cultivar in their salt tolerance were determent under saline and non-saline conditions [5]. However , the determination of genetic diversity between the genotype and cultivars is very important factor for plant breeding programs to improve the tolerance, production, and quality in plant [12].

So, the amis of this study were to investigate genetic diversity and identifying molecular marker of three selected genotypes (1H, 2H, and 3H) and two local cultivars for salt tolerance by using RAPD and ISSR techniques and genetic distance analysis .

2. Materials and methods

2.1 Plant material and germination test

Three genotypes (1H, 2H, and 3H) and tow iraqi cultivars (Latefyi and Iraq) of *Triticum aestivum* L. were used in this study. The selected genotypes were selected through plant breeding programs. All works were done in Biotechnology Recerch Center/ Al-Nahrain University, Baghdad-Iraq.

2.2 Genomic DNA Isolation

The DNA was extracted from the seeds of five wheat plant by small-scale method using commercial kit (Bionner-Korea). The purity of DNA was measured dependent on optical density by using spectrophotometer, the DNA was detecting using agarose gel electrophoresis with ethidium bromide and visualized under UV light [13] .

2.3 RAPD assay

In this study six of RAPD pimers were used, the primers was synthesd by (Bioneer-Korea) in lyophilized form and dissolved in sterile distilled water to get final conc. of (10pmol/ml) [14]. The primers and their sequences are listed in Table-1.

Table ١-The primers and their sequences used in this study.

No.	Primers	Sequences(´5 -´3)
1.	OPI – 06	AAGGCGGCAG
2.	OPE-16	GGTGACTGTT
3.	OPN-07	GAGCCCGAG
4.	OPQ-17	GAAGCCCTTG
5.	OPD-20	ACCCGGTCAC
6.	OPL-05	ACGCAGGCAC
7.	OPI-01	AAGGCGGCAG
8.	OPJ-13	CCACACTACC

Amplification of genomic DNA was performed with the master amplification reaction showed in Table-2.

Table 2- the master amplification Reaction

Materials	Final concentration	Volume for 1 tube
PCR pre mix	1x	5µI
Deionised D.W	——	11µI
Primer(10pmol/ µl)	10pmol /µl	2µI
DNA template	100ng	2µI

RAPD – PCR premix (final reaction volume = 20 µI). No. of cycles = 40 cycles between initial denaturation and final extension, the following Table-3 shows the RAPD program Followed by a hold at 4°C [14]. Each PCR amplification reaction was repeated twice to ensure reproducibility the products analyzed by electrophoresis in 1.5% agars gels with 0.5µl stained ethidium bromide at 7vt/cm for 3hours [13].

Table 3- the RAPD program.

Steps	Temperature (°C)	Time (min.)
Initial denaturation	94	5
Denaturation	94	1
Annealing	36	1
Extension	72	2
Final extension	72	10

2.4 ISSR assay

Six of primers were used in ISSR assay which provided by (Bioneer – Korea) in lyophilized form and dissolved in sterile distilled water to get final concentration (10pmol/ml) [14]. Table-4 showed the primers and their sequences.

Table 4- The The primers and their sequences used in this study.

No.	Primers Name	Sequence(´5-´3)
1.	UBC 809	AGA GAG AGA GAG AGAGG
2.	UBC 810	GAG AGA GAG AGA GAGAT
3.	UBC 811	GAG AGA GAG AGA GAGAC

The master amplification reaction was performed in a volume of 25µl Table-5.

Table 5-The master amplification reaction.

Materials	Final concentration	Volume for 1 tube
PCR pre mix	1x	5µI
Deionised D.W	——	17µI
Primer(10pmol/ µl)	10pmol /µl	2µI
DNA template	100ng	2µI

In the following Table-6 shows the ISSR program,No. of cycles= 40 cycles between initial denaturation and final extension.Each PCR amplification reaction was repeated twice to ensure reproducibility the products analyzed by electrophoresis in 1.5% agars gels with stained ethidium bromide 0.5µl at 5vt/cm for 2hour [13].

Table 6- the ISSR program.

Steps	Temperature(°C)	Time (min.)
Initial denaturation	94	5
Denaturation	94	1
Annealing	50	1
Extension	72	1
Final extension	72	10

2.5 Estimation of genetic distance

Data generated from the detection of polymorphic fragment were analyzed. The amplification profile of all the used isolates for any given were compared with each other, the presence scored as (1) and the absence of the same band of the samee size in other isolate scored as (0). Only clear and reproducible amplified fragments were considered for genetic relationship analysis. Estimates of genetic distance (G.D) were calculated between the selected genotype and local cultivar according to [15] based on following formula: G.D=1-{2Nab/ (Na + Nb)}. Where Na= the total number of fragments detected in individual (a); Nb= the total numbere of fragments shown by individual (b) and Nab= the number of fragments shared by individuals (a) and (b). Cluster analysis was performed to construct genetic relationship tree diagrams among studied. Among *Triticum aestivum* L. cultivars using an Unweighted Pair Group Method with Arithmetic Average (UPGMA). All computations were carried out using the Numerical Taxonomy and Multivariate Analysis System (NTSYSpc), Version 1.7 package [16]. The percentage of polymorphic bands was defined as ratio of the number of polymorphic bands amplified by a single primer to that of the total number of bands produced by the same primer .

3. Results and Discussion

3.1 genetic variations

In the RAPD analysis, eight primers were used to amplify all the genotypes and cultivars. Each primer varied greatly in their ability to identify the variability between the genotypes and cultivars. In Figure-1 the results showed amplification of three genotypes and two cultivars using four primers

OPI-06, OPE-16, OPN-07 and OPO-17) The results revealed that there are differences between the selected genotypes and local cultivars in their banding patterns with OP1-06, OPN-07 and OPO-17 primers. This used to identify genetic variation between the selected salt tolerance genotypes and local cultivars (sensitive), while there are no bands appeared in these genotypes and cultivars with OPE-16 primer Figure-1. Also the results showed there are variation between the three primers in their band in patterns which appeared in these genotypes and cultures. With OP1-06 the selected genotypes differed with local cultivars in two primers at different size, and also there are variations between the selected genotypes in their banding size. However, 1H and 2H genotypes are similar in their banding size, but they differed with 3H genotypes at two bands one with 1000 bp and the other with 500 bp. With OPN-07 primers the sensitive cultivars are similar in their banding patterns, but they differed with the selected genotypes in two bands Figure-1. While with the OPO-17 primers there are no bands appeared in the Iraq cultivar, but the other local cultivar differed with the selected genotypes in two banding patterns. Also the results showed there are differences between the selected genotypes in their banding patterns. Three wheat genotypes were used in this study (1H, 2H and 3H) as compared with the local cultivars (Latefya and Iraq) which sensitive for salinity. These genotypes were selected through plant breeding programs for salt tolerance after six cycles of exposure and selection under high salinity levels 30 ds/m drainage water. These genotypes were derived from F2 generation, and there was significant improvement in salt tolerance achieved in selected genotypes through these cycles of exposure and selection. This improvement in salt tolerance in these genotypes may due to that this character is inheritable [17] these genotypes were selected from F2-F7 generations generally contain high genetic variation in salt tolerance, and also the exposure of seeds and seedling plants was carried out under high salinity level (30 ds/m drainage water) which allow only to high salt tolerant genes segregated in very few plants still survived after the period of exposure . These genes will be responsible for salt tolerance mechanisms after exhibited high expression when grown in saline conditions. Munns [18] reported that salt tolerant in plant is more correlated with the mechanisms of salt tolerance which controlled by segregated salt tolerant genes through cycles of exposure and selection. These genes may not found in the local cultivars which were sensitive to salinity.

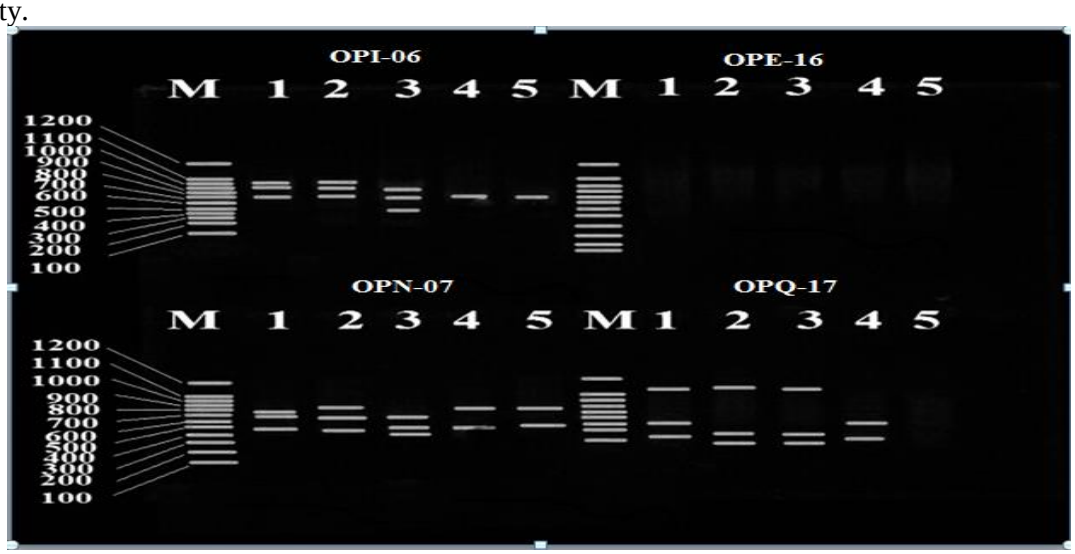

Figure 1- gel electrophoresis of RAPD-PCR primers (OPI-06, OPE-16, OPN-07 and OPQ-17) for wheat varities by 1% agarose gel (1X TBE, 75 v/cm, 1.15 hr) and visualized under UV light after staining with ethidium bromide 0.5µl. Lanes:M= DNA ladder; 1= 1H; 2= 2H; 3; 3H: 4= Latefyi; 5=Iraq

The results in the Figure-2 similar to the banding patterns with other four primers (OPD-20, OPL-05, OPI-01 and OPJ-13) in the selected wheat genotypes (salt tolerant) and local cultivars (salt sensitive). These results showed that the local cultivar (Latefyi and Iraq) were similar in their banding patterns with all primers except the Latefyi cultivar differed with Iraq cultivar in one band (750 bp) with OPD-20 primer, but the cultivars (salt sensitive) differed with all selected genotypes in one band with all primers except the primer OPD-20 they differed in 2-3 bands Figure-2. Also the results of

RAPD analysis showed that all the selected genotypes (salt tolerant) were proximately similar in their banding patterns with three primers (OPL-05, OPI-01 and OPJ-13) while they differed in their banding patterns with OPD-20 with OPD-20 primers.

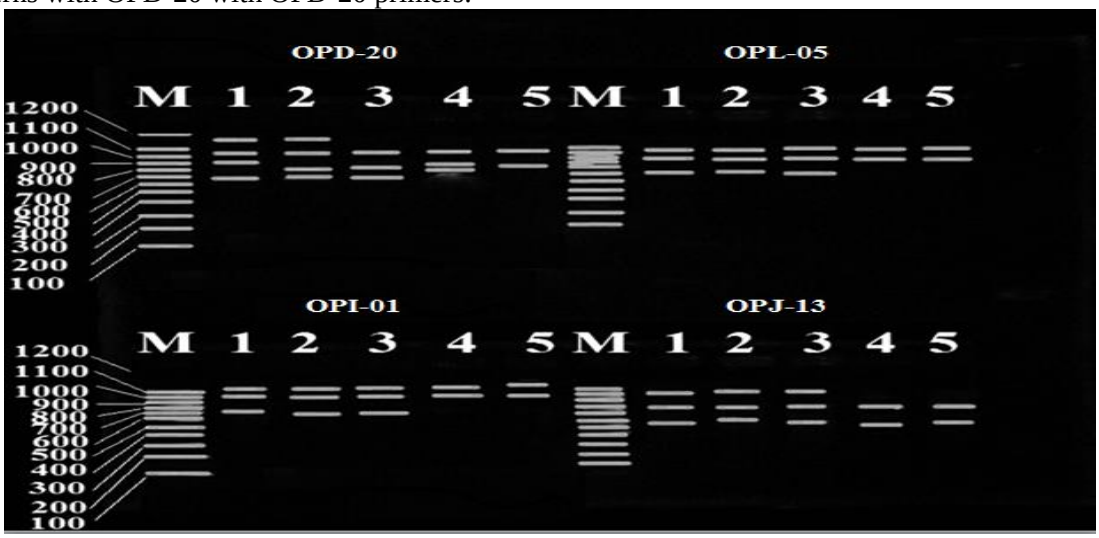

Figure 2- gel electrophoresis of RAPD-PCR primers (OPD-20, OPL-05, OPI-01 and OPJ-13) for wheat varities by 1% agarose gel (1X TBE, 75 v/cm, 1.15 hr) and visualized under UV light after staining with ethidium bromide 0.5µl. Lanes:M= DNA ladder; 1= 1H; 2= 2H; 3; 3H: 4= Latefyi; 5=Iraq.

3.2 ISSR-PCR Finger Printing:

The results in Figure-3 revealed the ISSR markers evaluated in this research provided sufficient polymorphism and reproducible fingerprinting profile of three selected wheat genotypes for salt tolerance and two local cultivars. Three ISSR primers detected specific band patterns, with UBC 809 and UBC 811 primers the selected genotypes differed with local cultivars only in one band (600 bp) and the others bands were similar in all selected genotypes and cultivars, but the 1H genotypes was differed with others selected genotypes and local cultivars in two bands one with UBC 811 primer (size 900 bp) and other with UBC 810 bp primer (molecular size 700 bp). However, the results of ISSR showed that all the selected genotypes and cultivars had the same two bands (molecular size 800 bp and 400 bp) with UBC 809 and one band (molecular size 800 bp) with UBC 811 primer, while with UBC 810 primer there is no similarity between the selected genotypes and local cultivars therefore all the primers gave specific bands patterns in selected genotypes (salt tolerant), while these absent in local cultivars.

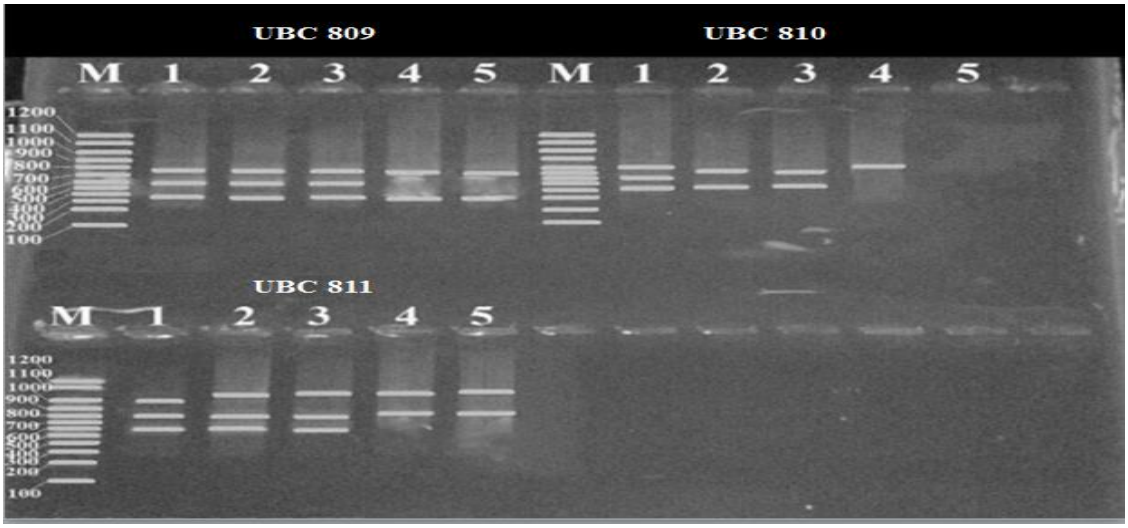

Figure 3- gel electrophoresis of ISSR-PCR primers (UBC 809, UBC 810 and UBC 811) for wheat varities by 1% agarose gel (1X TBE, 75 v/cm, 1.15 hr) and visualized under UV light after staining with ethidium bromide 0.5µl. Lanes:M= DNA ladder; 1= 1H; 2= 2H; 3; 3H: 4= Latefyi; 5=Iraq

The results of RAPD marker amplified to identify the genetic variation between the salt tolerant genotypes and sensitive cultivars by using 8 primers Figure-(1, 2) showed that three are differences in specific bands with all primers which used expect one (OPE-16) did not appear any band with the all selected genotypes and local cultivars. At each primer, the selected genotypes were differed in two or three bands with the local cultivars under high salinity condition. These bands showed high variability between the selected genotypes and local cultivars, this variability may be concern with the differences between them in their salt tolerance, because in the previous studies these selected genotypes considered as high salt tolerant while the local cultivars were sensitive to salinity [3,4]. On the other hands the results of amplification showed that there are differences between the selected cultivar (Dijila) and local cultivar in their banding patterns' with the two primers (OP1-01 & GB8) only under high salinity level, while there are no differences between them in their banding patterns under non-saline condition. This results reflecting that there are genetic differences between Dijila cultivar and local cultivar in their salt tolerance only under saline condition (16 ds/m), especially with GB8 primer [5]. Also the results of banding patterns indicated that there are variations among the selected genotypes in their salt tolerance.

3.3 Genetic distance

The dendrogram Figure-4 constructed on the basis of similarity and distance matrix showed that the genotypes and cultivars of wheat studied could be divided into two groups, 1H, 2H, 3H selected genotypes were clustered in first group and Latefyi and Iraq cultivars clustered in second group. Also the dendrogram of ISSR analysis Figure-5 showed that the selected genotypes and cultivars were divided into two groups. 1H, 2H, 3H and Latefyi were clustered in first group and Iraq cultivar was clustered in second group.

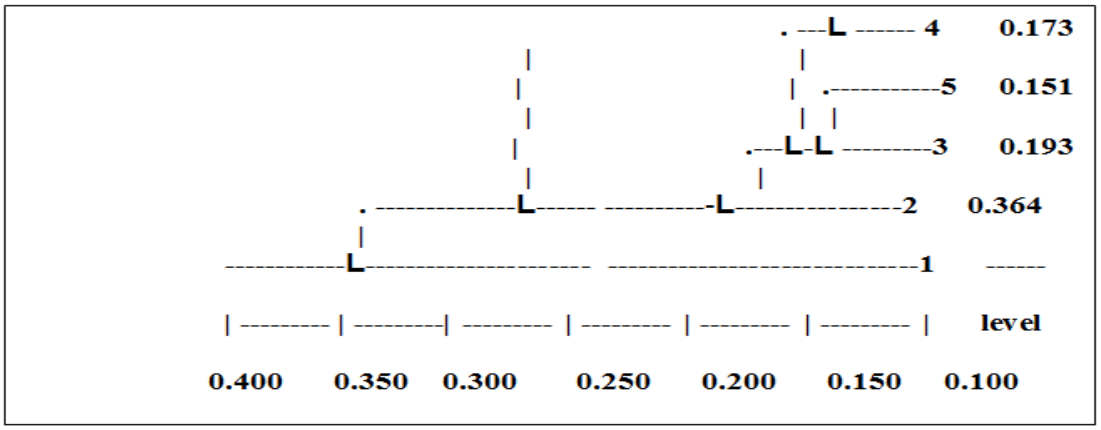

Figure 4-Dendrogram illustrated genetic fingerprint and relationships between varieties developed from RAPD data using unweight pair group method of arithmetic means (UPGMA).

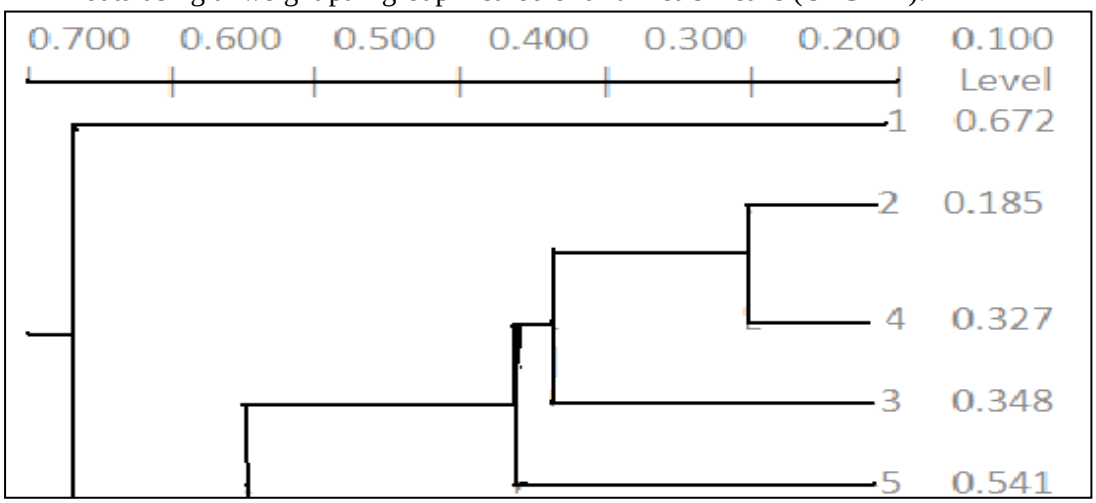

Figure 5- Dendrogram illustrated genetic fingerprint and relationships between varieties developed from ISSR data using unweight pair group method of arithmetic means (UPGMA).

The results in Figure-4 reflected that there are genetic distances between the selected genotypes and local cultivars under high salinity level and the genetic distance value was 0.980, this indicated that there are large genetic variations between them under high salinity conditions. Similarly, VanBecelaere *et al.* [19], reported that there are genetic distance estimated among the genotypes and used as indicator for selecting the parents, which be used in breeding program to create high genetic variation in F2 materials. The results of ISSR Figure-3 revealed that the selected genotypes (1H, 2H and 3H) differed genetically from the local cultivars in their salt tolerance when grown under high salinity level with the three primers which used. The genotype 1H differed genetically with 2H and 3H only with UBC 810 primer.

Genetic distance values were calculated to estimate the genetic divergence and relatedness among wheat genotypes and cultivars based on the RAPD Table-7 cluster analysis and on ISSR data Table- 8. The result in Table-7 showed that the genetic distance values ranged from 0.98521 to 0.33367, the highest value was between 3H selected genotype and latefyi local cultivar, while the lowest value was between 3H selected genotypes and Iraq local cultivar. However, the highest genetic distance was between these selected genotypes and local cultivars, but also there are genetic distances among the selected genotypes. The results in Table-4 which based on ISSR data revealed that the highest genetic distance was 0.99826 between the selected genotypes (1H and 2H), and the lowest value was 0.21556 between selected genotype (3H) and local cultivar (Iraq). Also the values of genetic distance indicated that there are distances between the local cultivars and also among the selected genotypes Table-8.

Table 7- Genetic distance matrix showing the relationship among the selected wheat genotype and local cultivars based on RAPD data.

	1	2	3	4	5
1	0.00000				
2	0.97773	0.00000			
3	0.62219	0.45770	0.00000		
4	0.48562	0.87972	0.98521	0.00000	
5	0.69881	0.67972	0.33367	0.58919	0.00000

Table 8- Genetic distance matrix showing the relationship among the selected wheat genotype and local cultivars based on ISSR data.

	1	2	3	4	5
1	0.00000				
2	0.99826	0.00000			
3	0.81082	0.68246	0.00000		
4	0.86553	0.4055	0.7	0.00000	
5	0.78038	0.74189	0.21556	0.55689	0.00000

The value of the genetic distance between the three selected genotypes and local cultivars was 0.990; this indicated that there is big genetic variation in their salttolerance under high salinity levels. The same RAPD technique hs been used by Manifeston, *et al.* [20] , to identifiy the improved genotypes and to estimate the genetic similarity and distance between wheat germplasm. The same determination of genetic variation in wheat genotypes and cultivares was reported by Sajida BiBi *et al.* [21] by using RAPD marker technique.

The conclusion of this study are, the selected genotypes (1H, 2H and 3H) were improved for salt tolerance through plant breeding programs, also there is large genetic variation and distance between these selected genotypes and local caltivars under salinity condition only.

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