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Using ISSR markers to build a phylogenetic of Barley Genotypes

Zeina S. M. Al_Hadeithi*
Biotechnology Research Centre, Al_Nahrain University, Baghdad, Iraq.

Abstract

This study is attempts to build a phylogenetic between nine Iraqi barley genotypes based on ISSR-PCR analysis by determine the level of genetic similarity among them. Nine issr primers used in this study produced 41 bands across nine studied varieties. Of these bands, 28 bands were polymorphic and the remaining monomorphic bands were 13. The average polymorphic rate was 70.5% ranged between 25%-100% , and average of polymorphic bands /primer was 4.5.The size of the amplified bands ranged 140-1600 bp. It was generated a 5 unique bands in this study, these bands can be used as a DNA profiling of all studied genotypes. The results were showed Genetic distances ranged between (0.0854-0.9897) among barley varieties. Based on phylogenic tree, a dendrogram were constructed among studied barley varieties, cluster analysis grouped the nine varieties into two main clusters depending on their ancestors and their morphological traits. The use of cultivars from various clusters and sub clusters offer the possibility of obtaining an appropriate genetic variability in hybrid population.

Keywords: Barley, Phylogenetic, ISSR, Molecular markers

استخدام مؤشرات التفاعل الضمني البسيط لبناء الشجرة الوراثية لأصناف الشعير

زينه سيف الدين محمد الحديثي*
مركز بحوث التقنيات الاحيائية، جامعة النهرين، بغداد، العراق .

الخلاصة

هدفت الدراسه الحاليه الى محاولة بناء شجرة السلالات الوراثية اعتماداً على تسعة مورثات لنبات الشعير العراقي، حيث استعملت المؤشرات الوراثية للتتابع الضمني لتحديد مستوى التشابه الوراثي للأصناف المدروسة. استعملت تسع بادئات ولوحظ ظهور 41 حزمه للأصناف المدروسه، كانت 28 حزمة متباينه في الحجم الجزيئي و 13 حزمة متماثله. وتراوحت نسب التباينات الوراثية بين 25%−100% وبمتوسط 70.5 % وكان معدل الحزم المتباينه لكل بادئ 4.5. تراوح الحجم الجزيئي للحزم المضخمه بين 140−1600 زوج قاعدي، وقد لوحظ ظهور خمس حزم فريده في هذه الدراسه يمكن استخدامها كمؤشر وراثي لدراسة اصناف الشعير المختلفه. اظهرت النتائج قيم البعد الوراثي اذ تراوحت بين (0.0854− 0.9897) لأصناف الشعير المدروسه. واعتمادا على شجرة تطور الطرز الجينية فقد تم بناء التحليل العنقودي لأصناف الشعير والذي جمع الأصناف التسع في مجموعتين رئيستين اعتمادا على اسلافهم وسماتهم الشكليه. استخداما لأصناف من مجاميع متنوعه ومجموعات فرعية توفر إمكانية الحصول على التباين الوراثي المناسب في المجتمع الهجين لنبات الشعير.

Introduction

Barley (*Hordeum vulgare L.*) was one of the most important crop species in the World, This crop is mainly used as animal feed, but in recent years, its use as a human food in non-traditional areas due to

*Email: zalhadeithi@yahoo.com

its high p-glucan content which acts as an inhibitor of cholesterol synthesis, and due to processing advantages, i.e. the absence of grain husks, for the barely food industry [1,2].

Barley had been subject to considerable genetic studies, it was a diploid (2n-2x-14), largely self fertilizing species with a large genome [3].

Assessment of the genetic diversity in a crop species is fundamental to its improvement. Genetic Diversity among and within plant species is in danger of being reduced. In wild species genetic diversity may be lost because of severe reduction in population size, whereas in domesticated crops genetic diversity may be lost because of the narrow genetic base in many breeding programs [4].
Estimates of genetic diversity can be based on different types of data. morphological quantitative traits have frequently been used for studying genetic diversity in barley [5,6].

Several biochemical techniques have been used to complement morphological examination of barley, [7]. Characterization with these kinds of markers was not very efficient for barley varieties due to the low levels of allelic variation. Molecular markers had been proved to be valuable tools in the characterization and evaluation of genetic diversity within and between species and populations. The advent of the polymerase chain reaction (PCR) favored the development of different molecular techniques such as inter-simple sequence repeat (ISSR) [8].

This molecular marker had been used in barley for detecting genetic diversity, genotype identification, and genetic mapping [9,10-11]. ISSR markers, which involve PCR amplifications of DNA using a primer to amplification of regions between adjacent, inversely oriented microsatellites, using a single simple sequence repeat (SSR) motifs (di-, tri-,tetra-, or pentanucleotides) containing primers anchored at the 3' or 5' end by two to four arbitrary, often degenerate nucleotides [12]. On the other hand, ISSR markers overcome the shortcomings of the low reproducibility of RAPD(random amplification polymorphic DNA), the high cost of AFLP(amplification fragment length polymorphism) and the complexity of SSR(simple sequences repeat) [13] and represents a fast and a cost-efficient technique [14].

In this study the genetic dissimilarity was evaluated using ISSR marker, in order to establish a base line to assist future conservation and breeding programs of this species, also aimed to report the usefulness of ISSR for the assessment of genetic diversity.

Materials and methods

Barley genotypes: Nine barley (*H. vulgare* L.) genotypes were used in this study. Among these varieties introduced from different regions and currently grow in central and north regions of barley cultivation areas of Iraq table -1.

DNA Isolation: Total genomic DNA extracted from fresh leaves plant by CTAB method for six barley varieties IPA265, IPA99, Tuwaitha, Hirta, Rayhan, Shuaa while DNA extracted from seeds by promega kit for other three barley varieties Bawadi, Samir, AL_khair to produce rapid extraction and high quality extracted DNA. Purity and concentration of DNA was measured by spectrophotometer [15].

Genomic DNA integrity was detected by running on 0.8% agarose gel electrophoresis followed by staining with ethidium bromide and visualized under UV light [16]. DNA samples were diluted to a working concentration of 100 ng/µl in order to be use in the ISSR-PCR experiments.

Table 1- Iraqi Barley varieties used in this study from*(SBSTC) and**(UST).

No.	Varieties Names	Pedigree	Breeding Institute	Rows No.
1	IPA 265	Brigs x 9cr.279 (OAP-2AP-4AP-03355_79/ICARDA)	*(SBSTC)	6 row
2	IPA 99	OAP-4AP-7L,sel /ICARDA	(SBSTC)	6 row
3	Tuwaitha	Radiation local black x Arevat-IRAQ	(SBSTC)	2 row
4	Hitra	Radiation local black x Arevat-IRAQ	(SBSTC)	6 row
5	Rayhan	Radiation Arevat seed-IRAQ	(SBSTC)	6 row
6	Shuaa	Radiation Arevat seed-IRAQ	(SBSTC)	6 row
7	Bawadi	Radiation hybrid (F3)local black x Arevat-IRAQ	(SBSTC)	6 row
8	Samir	Radiation local black x Arevat-IRAQ	**(UST)	6 row
9	AL_khair	Radiation local black x Arevat-IRAQ	(UST)	6 row

*(SBSTC) State Board of Seeds Testing and Certification. Ministry of Agriculture. Iraq. 2015
**(UST) Unit of seed technology. Ministry of Science and technology. Iraq. 2015

Primers: A set of 9 ISSR primers were screened using nine DNA samples from each cultivar and on the basis of that preliminary data, tested primers selected based on the studies used the same primers [17] table -2. These ISSR Primers were synthesis by (Bioneer – Korea) in lyophilized form and dissolved in sterile deionizer distilled water to get final concentrations of (10pmol/ml) Recommended by provider.

Table 2- The primers were tested in this study

No.	Primer Name	Primer Sequences	Annealing Temperature (C˚)
1	ISSR 5	5 ' AGAGAGAGAGAGAGAGAGAG 3'	50 C˚
2	ISSR 6	5 ' AGAGAGAGAGAGAGAGAGAGC 3'	50 C˚
3	ISSR 7	5 ' AGAGAGAGAGAGAGAGAGAGT 3'	50 C˚
4	ISSR 8	5 ' CTCTCTCTCTCTCTCTCTCTA 3'	50 C˚
5	ISSR 9	5 ' CTCTCTCTCTCTCTCTCTCTG 3'	50 C˚
6	ISSR 10	5 ' CTCTCTCTCTCTCTCTCTCTT 3'	50 C˚
7	ISSR 809	5 ' AGAGAGAGAGAGAGAGAGG 3'	40 C˚
8	ISSR 810	5 ' GAGAGAGAGAGAGAGAT 3'	40 C˚
9	ISSR 811	5 ' GAGAGAGAGAGAGAGAC 3'	40 C˚

ISSR analyses: final concentration reactions were performed in a volume of 25µl containing 5µl of PCR Master Mix (Bioneer-Korea), with concentration (1X) containing, *Taq* DNA polymerase 1U, dNTP (dATP, dCTP, dGTP, dTTP) 250µM, Tris-Hcl (pH9.0) 10mM, KCl 30mM, $MgCl_2$ 30mM, Stabilizer and tracking dye 1.5mM, 10pmol of the primer, and 100 ng of template DNA. Amplification was carried out using a Thermocycler (Applied Biosystems),reactions were submitted to the following PCR program: preliminary DNA denaturation step at 94°C for 5 min, followed by 45 cycles at 94°C for 45s., 40,50°C for 1 min according to used primer and 72°C for 45s. A final extension for 7 min at 72°C was Included. The ISSR products were separated by 1.5% agarose gels electrophoresis 5 Volt/cm for 2 hour in 1xTBE (10mM Tris-Borate, 1 mM EDTA) buffer, The size of ISSR-PCR products estimated by comparing with the DNA marker ladder (100-1500) bp provided by (Introne biotechnology-USA).Photo documentation was performed under UV light (365 nm) using a photo imaging system **(**Consort - Belgium).

Data analysis: Molecular weight was estimated by comparing the PCR products with the known size of DNA fragments ladder (bands from 100 to 1500bp).

Only data generated from the detection of polymorphic fragments were analyzed. The amplification profile of all the used varieties for any given primer were compared with each other, the presence of band scored as "1" and the absence of the same band of the same size in other varieties scored as "0". Only clear and reproducible amplified fragments were considered for genetic relationship analysis. genetic distance (G.D) were estimated between all pairs of the varieties according to Nei and Li [18]. based on the data matrix based on following formula:

$G.D = 1-\{2Nab/(Na + Nb)\}$

Where Na = the total number of fragments detected in individual 'a'; Nb = the total number 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 barley varieties using an Unweighted Pair-Group Method with Arithmetic Average (UPGMA) [19]. All computations were carried out using the Numerical Taxonomy and Multivariate Analysis System (NTSYS-pc), Version 1.80 package [20].

Results and discussion:

ISSR -- PCR analysis: ISSR markers are highly polymorphic and are useful in studies on genetic diversity, phylogeny, gene tagging, genome mapping and evolutionary biology [21].

In present study, the genetic diversity and relationships among nine barley varieties grown in Iraq were evaluated by ISSR technique, among the 9 primers used in this study, total of (41) bands appeared across the studied varieties. Figure -1-(A, B, C, D, E, F, G, H and I) , all details reported in Table -3. Among these (13) were monomorhpic bands while (28) were polymorphic bands. Total polymorphic band ranging between (1-10) band, the primer ISSR811 registered highest number 10 band while primer ISSR7 registered lowest number 1 band with polymorphic percentage (70.5%), primer ISSR8 showed highest percent of polymorphism (100%) compare with primer ISSR7 showed lowest percent of polymorphism (25%). Average number of polymorphic band per primer was (4.5) as

a mean polymorphism per/primer. The percentage of polymorphism expressed by ISSR primers from this study compared to the results of other researchers such as [22] who conducted molecular studies on barley using the same technique registered an average rate of polymorphism being (89.13%) with average number of polymorphic bands per primer was (9.2) while the other [23] showed number of band per primer with an average of (17.7.)

The size of amplification fragments ranged from 1600 bp for pimer ISSR 811 to 140 bp for primer ISSR8, the size of these fragments estimated by compared with the size of standard marker ladder fragments used in this study. some unique bands that generated which means the presence of a fragment in specific variety and the absence of the same fragment with the same size in all other varieties, Totally unique amplification fragments (5) produced by primers, ISSR811 (850,550) bp, ISSR809 (350) bp, ISSR10 (200) bp and ISSR8 (900) bp.

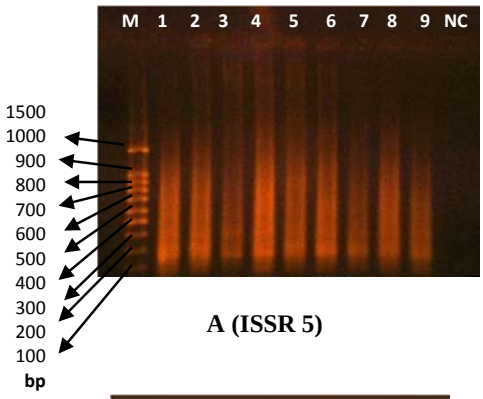
Genetic relationships between barley genotypes revealed by genetic similarity at ISSR levels were in agreement in agricultural production and breeding [24]. According to the ISSR data, genetic similarity among the nine *H. vulgare* varieties ranged from 0.9897to 0.0854 (table -4), the highest similarity 0.9897 (98.9%) was obtained between IPA 265 and Tuwaitha and lowest level of similarity 0.0854 (0.8%) was obtained in IPA 99 and Hitra, table 4. it was shown two main clusters in figure -2, that first cluster included 1IPA 265 variety formed a separate cluster appeared to be smoothly spike and 6 rows variety, This variety give heights value of genetic distance with Tuwaitha variety 98,9% because IPA265 variety descend from Brigs x 9cr.279 (OAP- 2AP-4AP-03355_79/ICARDA)While Tuwaitha variety descend from Radiation local black x Arevat-IRAQ appeared to be roughly spike and 2 rows variety (The number of rows is used as a phenotypic diagnosis of barley spike). The second cluster divided in to two sub clusters first sub cluster included (2 IPA 99, 4Hitra), (3Tuwaitha), and (5Rayhan). Second sub cluster included (6Shuaa), (7Bawadi, 8Samir), and (9 Al_khair). IPA 99 give lowest value of genetic distance with Hitra variety 0.8% because IPA 99 shared with Hitra by rows number 6 but differentiated by roughly spike and descend from OAP-4AP-7L,sel /ICARDA while Hitra appeared to be smoothly spike and descend from Radiation local black x Arevat-IRAQ**.** Rayhan shared with IPA 99 by rows number 6and roughly spike **.** Shuaa, Bawadi, Samir and Al_khair varieties shared by rows number 6**.** Shuaa, Bawadi and Al_khair smoothly spike while Samir appeared to be roughly spike. Bawadi variety and Samir variety descend from same pedigree was Radiation local black x Arevat-Iraq, Figure-2.

Depended on these results it can be concluded that the bulk analyses of ISSR markers were useful for study the genetic relationships between barley cultivars, providing the ISSR markers a powerful tool for the generation of potential fingerprinting diagnostic markers for cultivars.

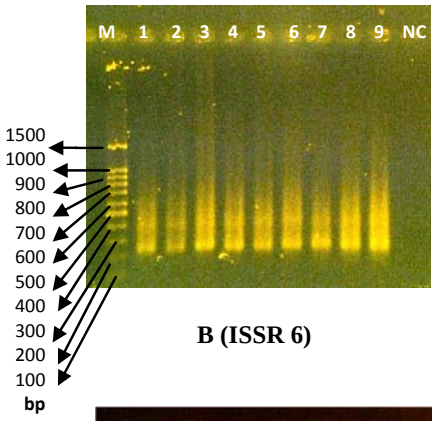
ISSR analysis through the 9 primers, attested the existence of a high genetic variability among cultivars involved in this study, which could be efficiently exploited in breeding programs of barley. The strongest correlation existed among oligonucleotides ISSR8, ISSR10, ISSR810 and ISSR811 were the best for differentiated between during detection polymorphic bands between barley varieties. It was found that some cultivars (Shuaa, IPA99, Tuwaitha, Hitra, and Al_khair) show unique bands and have molecular size range (900, 850, 550, 350 and 200 bp) and these bands could be used as a DNA markers to identify the barley genotypes.

Table3- Distinct characteristic of ISSR primers include in the study: primers name, total number of bands, number of main bands, number of polymorphic bands, number of monomorphic bands, number of unique band and percentage of polymorphism.

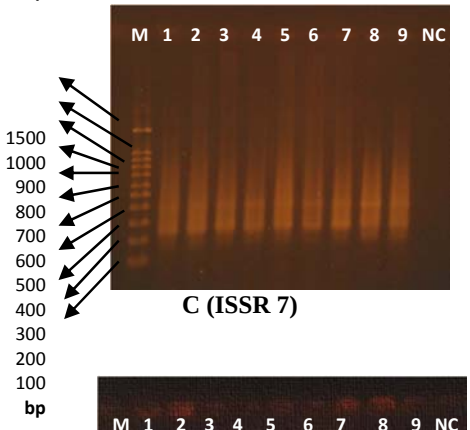
Primer No.	Total Bands	Main Bands	Polymorphic Bands	Monomorphic Bands	Unique Bands	Polymorphism %
1. ISSR 5	9	1	-------	1 (250 bp)	-------	-------
2. ISSR 6	18	2	-------	2 (400, 250 bp)	-------	-------
3. ISSR 7	29	4	1 (300 bp)	3 (400,250,190 bp)	-------	25%
4. ISSR 8	27	6	5 (600, 500, 300, 190,140 bp)	-------	1 (900 bp)	100%
5. ISSR 9	18	2	-------	2 (350, 250 bp)	-------	-------
6. ISSR 10	30	5	2 (490, 290 bp)	2 (550, 300 bp)	1 (200 bp)	60%
7. ISSR 809	14	4	2 (450, 300 bp)	1 (750 bp)	1 (350 bp)	75%
8. ISSR 810	25	5	4 (1500, 950, 750, 600 bp)	1 (650 bp)	-------	80%
9. ISSR 811	52	12	8 (1600 ,1400, 1100,675, 500, 380, 300,275 bp)	2 (1300,775 bp)	2 (850, 550 bp)	83%



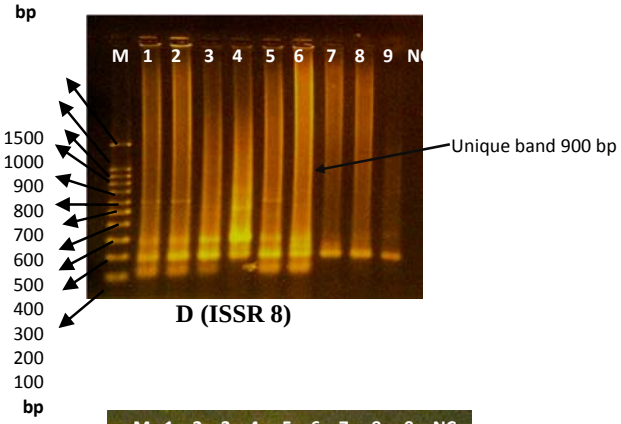
A (ISSR 5)



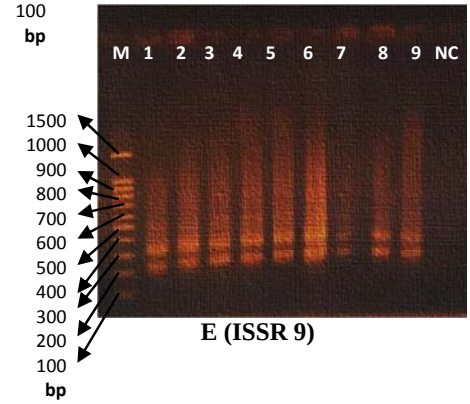
B (ISSR 6)



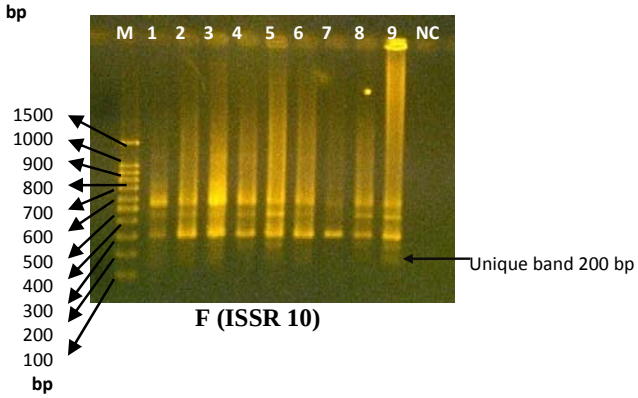
C (ISSR 7)



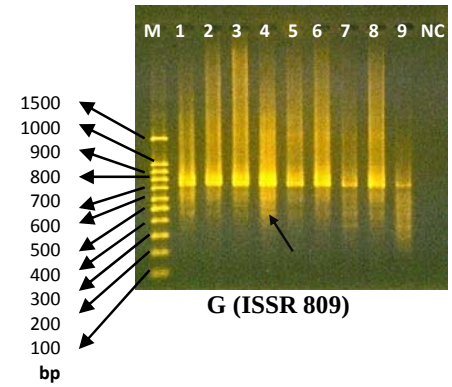
D (ISSR 8)



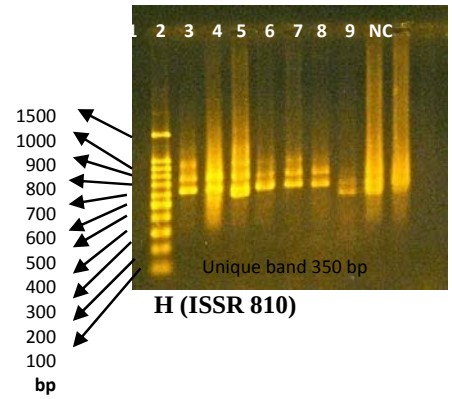
E (ISSR 9)



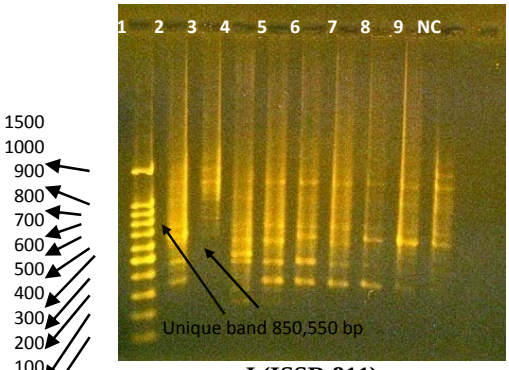
F (ISSR 10)



G (ISSR 809)



H (ISSR 810)



I (ISSR 811)

Figure 1- Agarose gel electrophoresis (A, B, C, D, E, F, G, H and I) of ISSR-PCR reaction for DNA of barley plant. Bands were fractionated by electrophoresis on a 1.5% agarose gel (2hr, 5V/cm, 1XTris-borate buffer) and visualized under U.V. light (365nm) after staining with ethidium bromide. 1.(IPA265),2.(IPA99), 3.(Tuwaitha), 4.(Hitra), 5.(Rayhan), 6.(Shuaa), 7.(Bawadi), 8.(Samir) and 9(Al_khair).

Table 4- Similarity coefficient matrix for pair wise comparsion of 9 barley genotypes 1.(IPA265),2.(IPA99), 3.(Tuwaitha), 4.(Hitra),5.(Rayhan), 6.(Shuaa), 7.(Bawadi), 8.(Samir) and 9.(Al_khair).

	1	2	3	4	5	6	7	8	9
1	0								
2	0.6063	0							
3	0.9897	0.2801	0						
4	0.8146	0.0854	0.3747	0					
5	0.5097	0.3197	0.3534	0.3823	0				
6	0.6861	0.5886	0.5672	0.7305	0.4207	0			
7	0.6779	0.4934	0.5050	0.6332	0.4126	0.2272	0		
8	0.7666	0.5773	0.5534	0.7277	0.4328	0.2349	0.2268	0	
9	0.6276	0.4611	0.5566	0.5951	0.4498	0.3406	0.2601	0.2986	0

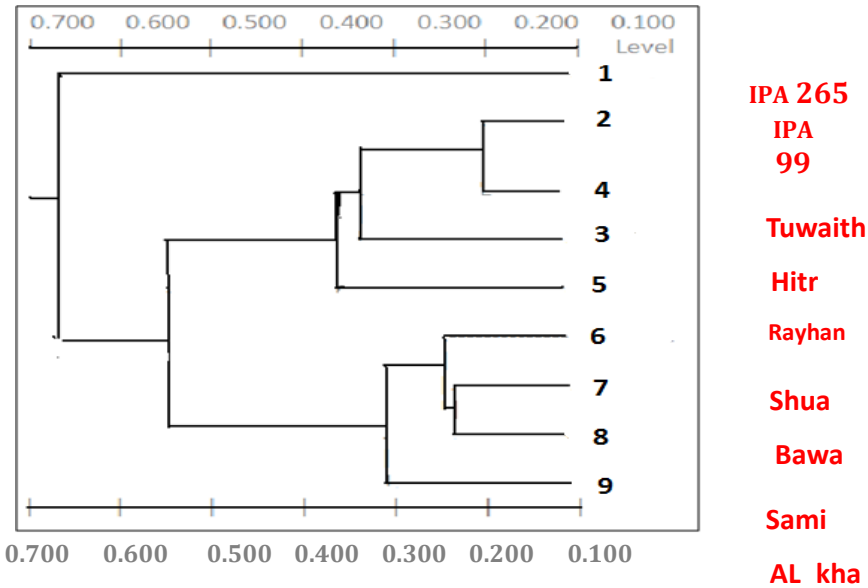


Figure 2- Dendrogram coefficient of 9 barley genotypes generated by UPGMA cluster analysis of the dissimilarity values.

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