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Molecular characterization of nine grapevine varieties cultivated in Salahaldin, Iraq by using RAPD-PCR marker.

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Abstract
Grapevine (Vitis vinifera L.) is one of important economic fruit crops found in Salahaldin province \ Iraq. To examine the Molecular characterization and genetic relationships among nine grape varieties by using random amplified polymorphic DNA (RAPD) marker. Fifteen RAPD primers produced polymorphic band (71, 56%), while two RAPD primers yielded monomorphic bands only. The size of the fragment ranged between 250-2700 bp, with an average of 5.47 band/primer. A total of 20 unique and absent bands used to identify seven cultivars. This study has found that genetic distance values ranged from 0.092 to 0.277 among studied grape cultivars. The cluster analysis showed there were two main groups (G_1 and $G_{2)}$. These results proposed RAPD marker as rapid, easy and power marker for Molecular characterization and genetic analysis among grapevine.
Keywords: Grapevine varieties, Vitis vinifera L., RAPD_PCR, Molecular characterization, genetic analysis.

Introduction
Cultivated grapevine (*Vitis vinifera L.*) is one of the oldest crops belongs to the family Vitaceae, with about 60 species distributed in Asia, North America and Europe under subtropical, Mediterranean and continental–temperate climatic conditions [1,2]. According to Sara, R. (2013), grapevine is a diploid plant that has genome size is approximately 475 Mb consisting of 19 chromosomes [3]. In Iraq, The estimated number of vine trees is about 6.5 million and the production is established to be 125 000 tons grape [4]. Grapevine is widely cultivated in middle and north of Iraq. In Salahaldin province, middle of Iraq, grapevines are mainly cultivated in the Balad town.

Previous research has established that grapevine misnaming is nearly up (5%) in the worldwide grapevine collections [5]. It is because in many regions farmers leading to synonyms and homonyms renamed cultivars. Morphological and biochemical markers for genotype characterization can be affected by environmental conditions and developmental stage of plant. The solution to the problem is only possible by DNA based molecular markers provide useful method for grapevine cultivars characterization. Various PCR-based DNA marker techniques, used in grape genome research, Such as Restriction Fragment Length Polymorphism (RFLP)[6], Amplified Fragment Length Polymorphism (AFLP) [7], Random Amplified Polymorphic DNA (RAPD _ PCR)[8], Inter Simple Sequence Repeats (ISSR) [9] [10], Sequence Related Amplified Polymorphism (SRAP) [11] Simple Sequence Repeats (SSR) [12] [13], and Single Nucleotide Polymorphism (SNP) [14].

RAPD technique have been extensively used molecular studies in animal, plant and bacteria, because it is easy and quicker to use, yet powerful, no prior knowledge of DNA and marker sequences is needs, it can produce abundant unique fingerprints of polymorphic fragments [15,16]. RAPD_PCR technique has been successfully used for various genetics purposes in grapevine cultivar, which include genetics Characterization [8] [17] [18], genetic diversity analysis [19] [20] and genetic fingerprint [21, 22].

The objective of present work are (i) Molecular characterization (ii) analyses the genetic diversity among nine local grape cultivars In Salahaldin province\Iraq.

Materials and Methods
Collection of grape samples:
A leaves sample (young and mature) collect from nine grapevine genotypes (Table 1) were taken from the farms in Balad, Iraq during the month May. The leaves samples were washed twice time in distilled water and stored at −286 °C (deep freeze) until used in DNA isolation.

DNA isolation and RAPD procedure:
Total genomic DNA was isolated from freezing leaves following the method described by Weigand et al.[23], based on the general principles of Saghai-Maroof, et al.[24], with some modifications. NanoDrop® ND 1000 Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) used to measured purity and concentration of DNA, this step is important because it determined if DNA suitable for PCR reaction. Samples of DNA were stored at –20 °C and diluted with distal water to 30 ng/µl to be use in the RAPD experiment.

Table 1. Common name of grapevine and region of collection.

number	Common Name	Town
1	Kishmishi	Balad / Salahaldin
2	Des–Alaniz	Balad / Salahaldin
3	Aswad	Balad / Salahaldin
4	Omeeri	Balad / Salahaldin
5	Buhrizi	Balad / Salahaldin
6	Zaitouni	Balad / Salahaldin
7	Halwani	Balad / Salahaldin
8	Kamali	Balad / Salahaldin
9	Shada Bedha	Balad / Salahaldin

In current study, from about one hundred primers available in laboratory of molecular biology (college

of science/ Tikrit university), randomly selected 17 RAPD primer (Operon Technologies Inc., Alameda, California, USA) (Table 2) were tested for PCR amplification. PCR reaction was performed in a final volume of twenty µL, containing (1) 1 µl of template DNA with 30 ng/µl concentration, (2) 1 µl of primer, (3) AccuPower® PCR PreMix tube (Bioneer_Korea), each tube contains a Top DNA polymerase in an easy to re-suspended, lyophilized premix of dNTPs, reaction buffer (Tris-HCl (pH 9.0), KCl, MgCl), a tracking dye, and a stabilizer. (4) 16 µl Nuclease-free water (Promega, USA). The amplification reactions were carried out using Applied Biosystems 2720 Thermal cycler (Singapore) and thermocycler was programmed as follows; initial step at 94 °C for 4 min. followed by 40 cycles of one min at 94 °C to denaturation double starand of DNA , one min at 36 °C to annealing primer with complementary site on DNA and two min at 72 °C to extension. An additional one final extension cycle of seven min at 72 °C. Electrophoresis of RAPD_PCR amplification products carried on 1.4% agarose gels (agarose dissolve in TBX 1X) and separated at 7 v / cm for 90 min; following this, the gel was stained with one liter of ethidium bromide stain solution (0.6 µg/ml) for 25-35 min. and viewed under a Gel Documentation System (ATTA-Japan).

Analysis of RAPD data:

After show images under gel documentation, image save in computer and Data were scored as 1 and 0 for present or absent bands among nine grapevines. After detect the number and the size of bands compared with DNA ladder (100 bp), 0 or 1 data matrix obtain from PCR product was used to calculate the genetic distance and similarity among grapevine cultivars using the (NTSYS) 1.8 program [25]**.** The dendrogram of nine grapevine cultivars was structured by using a distance matrix using the unweighed pair group method with arithmetic average (UPGMA) [26].

Results and discussion

As can be seen from the table 2, The RAPD profiles obtained from 17 RAPD universal primers; RAPD primers produced polymorphic fragment with range from100% in both OPA_06 and OPO_11 primers to 0% in OPB_10 and OPG_15. Total of 579 scorable bands were generated from those primers, which is an average of **5.47** loci per primer. The size of the RAPD amplified product bands occur in ranged from 250 bp in kishmisi cultivar with OPO-04 primer to 2700 bp in Omeeri, Buhrizi and Zaitouni with OPO_11 primer. Except primers OPB-10, and OPG-15, all primers generated polymorphic banding patterns. Each amplified fragment was considered as a recognized allele. The results, as shown in figure 1, arranged grapevine cultivars from smallest to largest according number of allele in each cultivar, therefore the large number of allele found in Zaitouni and low number of allele found in Kishmishi. In other hands the OPW-13 primer produce large number of allele while small number of allele produced by OPD-18 primer. Describe bands as polymorphic or monomorphic bands depend on present bands in some cultivar and was absent in other, it was considered as polymorphic, while if the band was present in all cultivar, it was considered as monomorphic [27,28].

Table 2. Primer's name, total number of bands, number of loci, unique bands and absent bands in grape genotypes studied

NO.	Primer name	Bands (NO.)	Loci (NO.)	Unique bands bp	Absent bands bp
1	OPA 06	31	6	**500 Kishmishi**	**1600 Kishmishi** **1200 Kishmishi**
2	OPA 13	34	6	-	-
3	OPB 10	27	3	-	-
4	OPB 15	48	7	**450 Kishmishi**	-
5	OPD 18	18	3	-	-
6	OPE 07	22	3	-	-
7	OPF 11	19	3	750 *Zaitouni*	
8	OPG 13	50	8	1300 Halwani 1200 Shada Bedha	2500 Des–Alaniz
9	OPG 15	36	4	-	-
10	OPH 08	25	5	600 Omeeri	-
11	OPL 20	26	3	-	950 Shada Bedha
12	OPO 04	32	9	**480 Kishmishi** **250 Kishmishi**	**650 Kishmishi**
13	OPO 11	20	5	950 Zaitouni	850 Aswad
14	OPP 01	50	7	400 Aswad	-
15	OPW 08	45	6	-	440 Omeeri
16	OPW 09	39	7	380 Buhrizi	
17	OPW 13	57	8	-	1100 Aswad 620 Aswad

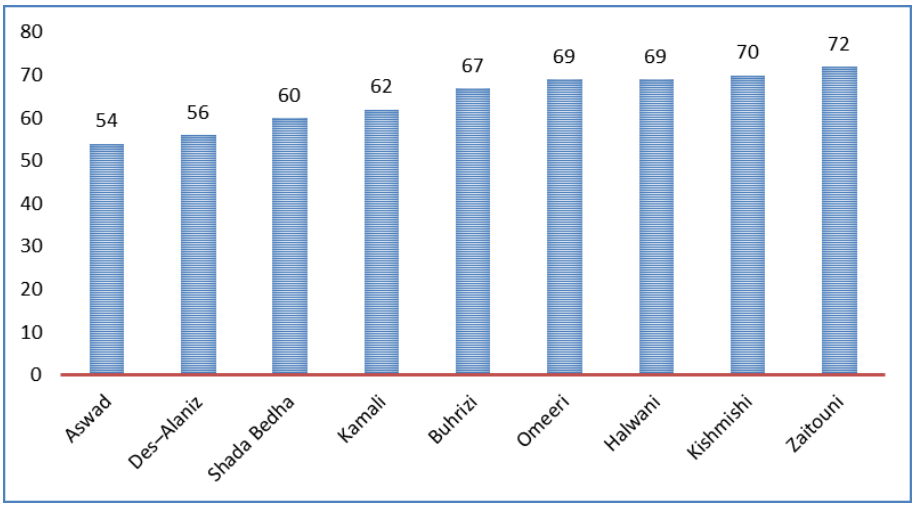

Figure.1. show number of allele for each grapevine cultivar in current study

In this study, the polymorphism level was higher than in previous molecular genetics studies on grapevine cultivars [15] [29][30]. On the other hand, the level of variation obtained in the present study were smaller than those reported by others studies [17][20]**.** Figure 1 and figure 2 shows the sample gel images of RAPD patterns.

Some primers gave unique or absent bands, which act as markers for a specific grape genotype as shown in (Table 2).therefore some cultivars could be distinguished for all other cultivars. Kishmishi cultivar showed seven specific markers, four of them unique fragments with primers OPA-06(one position), OPB-15(one position) and OPO-04(two position), respectively. This cultivar also showed 3 absent bands with primers OPA-06 (two position) and OPO-04 (one position). Aswad cultivar exhibited one unique bands with primer OPP-01, while primers OPP-01 and OPW-13 showed 1 and 2 absent bands, respectively. Shada Bedha cultivar exhibited one unique bands with primer OPG-13 and one absent bands with primer OPL-20.Omeeri cultivar recorded one unique band with primer OPH-08 and one absent band with primer OPW-08. Zaitouni cultivar Observed unique fragment with Primers OPF-11 and OPO-11. Buhrizi cultivar gave unique banding pattern with primer OPW-09. Des–Alaniz cultivars gave unique banding pattern with primer OPW-09. Maximum specific markers obtain from Kishmishi cultivar, this result was consistent with that obtain by previous RAPD marker [31]. The mainly important event lead to occur polymorphism among species; There are (1) mutation in primer_binding site, (2) an insertion or deletion (large or small) piece of DNA between inverted priming binding sites may alter the length of the amplified region, producing a length polymorphism of codominant alleles, rather than a presence or absence polymorphism and bands among species. [32, 33, 34].

The results of this study showed that the values of genetic distance among nine grapevine cultivars ranged from 0.092 to 0.277. The cultivar Kishmishi was highly divergent from Halwani with distances of **0.092**. The cultivar Buhrizi was very closely related to Omeeri with distances of **0.227** (table 3)**.** In the dendrogram eight genotypes were gathered into two distinct groups; groups 1 (G_1) and group 2 (G_2) with 3 and 5 genotypes, respectively, (Figure 3). G_1 contains the cultivar Des–Alaniz, Aswad and Omeeri, while G_2 contains Buhrizi, Halwani, Zaitouni, Kamali and Shada Bedha. Whereas Kishmishi genotype did not group into clusters, this means that biotic and abiotic environmental factors are not very powerful to make genetic variations in kashmishi, therefore, these factors may explain it is planted all over the world, despite Iran is original land of Kishmishi **[35].** Data from figure (1) can be compared with the data in figure (4), which shows relationship between number of bands for each cultivar and genetic distance. Results from several sources have identified the grapevine is genetically structured. Therefore, Genetic relatedness of grapevine cultivars has been take shape generally by human activity, natural select and geographical condition. [36, 37].

Although RAPD marker cover the entire genome revealing length polymorphism in coding or non-coding and repeated or single copy sequence, Variations among grape cultivars may be based on morphological traits, origin land, distribution patterns, and adaptive and agronomic characters are well documented.

In additional, the level of genetic distance and genetic similarity not alter depending in number of cultivars used in study but affect by sequence and number of primers that may be increase power of marker to revealing large region from genome. While presence or absent unique bands may be has less chance to found when increase number of cultivars in investigation.

In conclusion, RAPD data obtain from nine grape genotypes proved to be effective genetic markers for the identification of these genotypes.

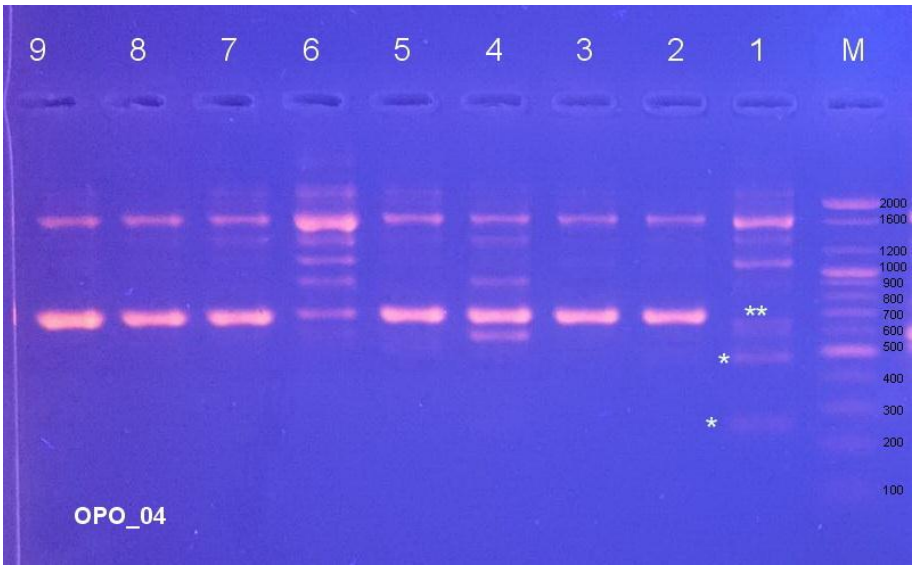

Fig (2): RAPD patterns of *grape cultivars* obtained with primer (OPO_04). M=100 bp DNA ladder,1:Kishmishi,2:DesAlaniz,3:Aswad,4:Omeeri,5:Buhrizi,6:Zaitouni,7:Halwani,8:Kamali,9:Shada Bedha, * = unique band and ** = absent band.

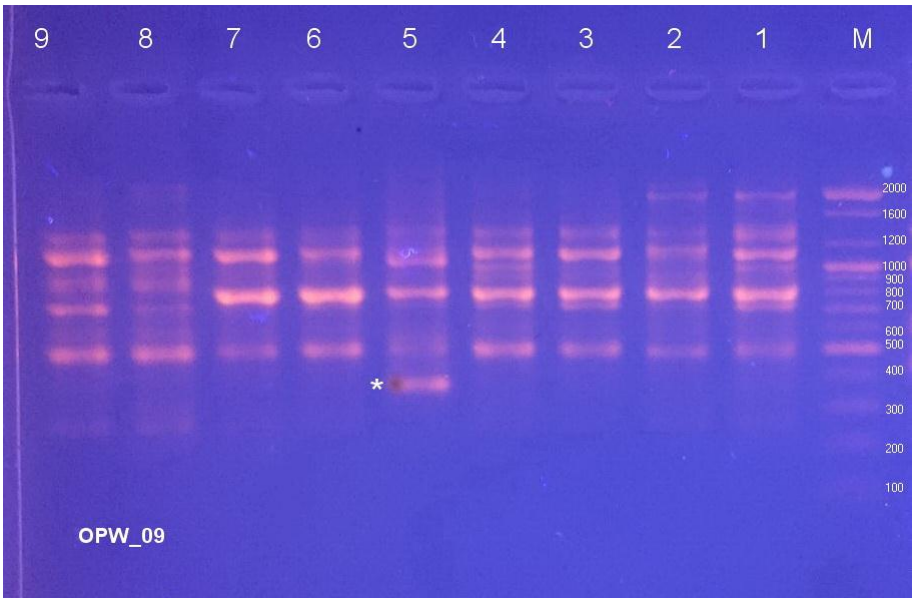

Fig (3): RAPD patterns of *grape cultivars* obtained with primer (OPW_09). M=100 bp DNA ladder,1:Kishmishi,2:DesAlaniz,3:Aswad,4:Omeeri,5:Buhrizi,6:Zaitouni,7:Halwani,8:Kamali,9:Shada Bedha and * = unique band

Table (3): The genetic distance values for nine grape cultivars.

	Kishmishi	**Des–Alaniz**	**Aswad**	**Omeeri**	***Buhrizi***	**Zaitouni.**	**Halwani**	**Kamali**	**Shada Bedha**
Kishmishi	0.000								
Des–Alaniz	0.205	0.000							
Aswad	**0.227**	0.115	0.000						
Omeeri	0.198	0.159	0.200	0.000					
Buhrizi	0.219	0.164	0.165	0.142	0.000				
Zaitouni	0.202	0.219	0.201	0.145	0.098	0.000			
Halwani	0.198	0.178	0.180	0.140	**0.092**	0.097	0.000		
Kamali	0.218	0.144	0.146	0.155	0.123	0.141	0.103	0.000	
Shada Bedha	0.201	0.168	0.150	0.213	0.124	0.142	0.157	0.122	0.000

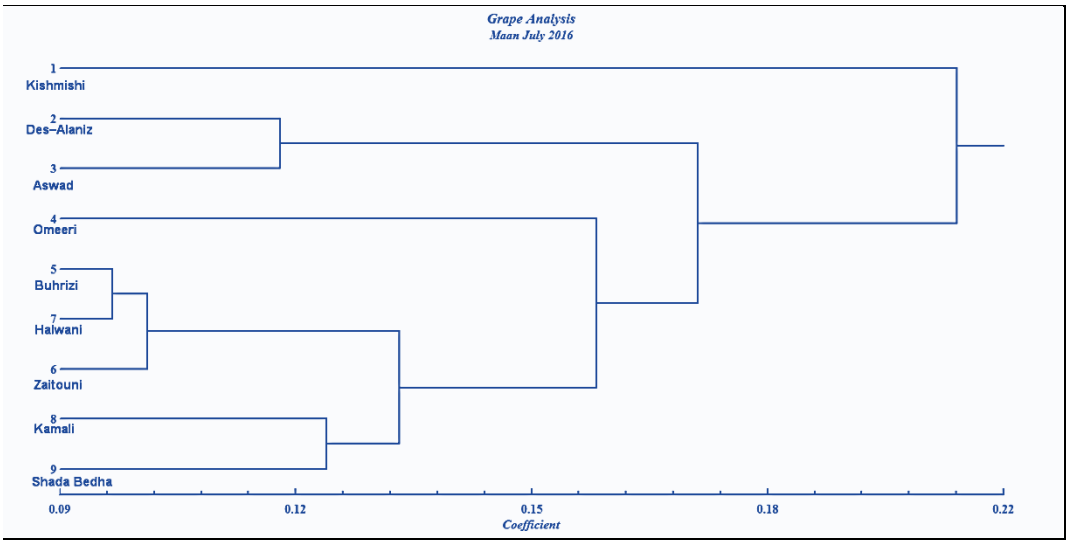

Fig (4): Cluster analysis of nine grapevine cultivars

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التوصيف الجزيئي لتسعة من أصناف العنب المزروعة في محافظة صلاح الدين، العراق باستخدام مؤشرات الـ RAPD-PCR

معن حسن الياسين

قسم علوم الحياة ، كلية العلوم ، جامعة تكريت ، تكريت ، العراق

الملخص

ان العنب *Vitis vinifera L* يعتبر من اهم الفواكه الاقتصادية في محافظة صلاح الدين/ العراق. لدراسة التوصيف الجزيئي والعلاقة الوراثية لتسعة أصناف من العنب استخدم مؤشر التضخيم العشوائي المتعدد الأشكال لسلسة ألدنا RAPD-PCR. 15 من البادئات المستخدمة أعطت حزم متباينة (71,56%) بينما اثنان من البادئات أعطت حزم متماثلة فقط. وان حجم القطع المتضاعفة تراوح بين 250_2700 زوج قاعدي وبمعدل 4.47 حزمة لكل بادئ. من حيث الحزم الفريدة والغائبة فقد تميزت سبعة أصناف بـ 20 موقع مميز. وفي هذه الدراسة تراوحت قيم الابعاد الوراثية بين 0.092 الى 0,277بين الأصناف الداخلة في الدراسة، بينما اظهر تحليل العلاقة الوراثية وجود مجموعتين رئيستين ($ج_1$ و$ج_2$). وبذلك يعتبر مؤشر RAPD-PCR مؤشر سهل وسريع وقوي للتوصيف الجزيئي والتحليل الوراثية بين أصناف العنب.