Molecular Characterization of some Genotypes of Pomegranate *(Punica granatum* L.) by RAPD Marker in Sulaimani region of Kurdistan -Iraq

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Abstract

Randomly amplified polymorphic DNA (RAPD) markers was used as a good tool to evaluate genetic diversity of pomegranate cultivars. Out of 12 random primers, only 10 primers amplified products. Primer products ranged from 6 to 10 bands. Total number of bands amplified was 82, out of which 71 were polymorphic and 11 monomorphic. The maximum, minimum and mean values of polymorphic bands were 9, 5 and 7.1, respectively. The PIC values were recorded range between 0.77 to 0.92. Dendrogram was constructed by using UPGMA method for the clustering for all the selections that the dissimilarity coefficients were ranged between 0.22 (G12 vs. G23) to 0.75 (G1 vs. G6), and 5 clusters such as (A, B, C, D and F) with a mean dissimilarity (0.54) were obtained for 24 pomegranate genotypes using 10 RADP markers. Molecular analysis showed high variation within accessions. Our data obtained in present study it can be concluded that RAPD studies can be useful in breeding programmers allowing the identification of different selections and assessing the genetic similarity among them, which would facilitate their use as identified genetic stock in future breeding programmers.

Keywords: *genetic relationship, genetic structure, random amplified polymorphic DNA*

1.Introduction

One of the most an important horticultural deciduous shrub, cultivated plant is pomegranate (*Punica granatum* L.) belongs to the Punicaceae family, which is used as ornamental and fruit tree with ecological and economic benefits. Chronological evidence indicates that pomegranate originates in Central Asia to northern India and has been cultivated since ancient times throughout the Mediterranean region of Asia, Africa and Europe (1). Pomegranate's domestication is reported to have begun around 3000-4000 BC in Iran and in India. In addition, more than 500 varieties of pomegranates are cultivated throughout the world (2) and (1). Genetically, Pomegranate's chromosome number is (2n = 2x = 16 and 18) with genome size approximately 328 Mb (3). Genetic diversity is an important tool for the breeder, by which all genotypes can be differentiated, also it is very useful for the improvement of the chances of selection better segregates for various characters (4). Therefore, several similar studies have been performed regarding genetically recognized cultivars and wild species of Pomegranate's, using several types of markers including RAPD, ISSR, SSR, SRAP and RFLP. Random amplified polymorphic DNA (RAPD) A single randomly chosen oligo nucleotide that Single RAPD primers are able to hybridize to several hundred sites within the target DNA; thus, the random amplified polymorphic DNA (RAPD) marker has been used to identify genetic diversity of many different plant species; the use of this reliable marker in pomegranate is limited (5). In Suleimani region of Iraqi Kurdistan, not much information is available about Pomegranate's genotypes, therefore the application of genetic diversity for this plant is special interested, the aim of the present study is to characterize 24 pomegranate cultivars using RAPD markers, which will help breeders in parental selection in a pomegranate cultivar-breeding program.

2. Materials and Methods

2.1. Locations and plant material

The plant material for present studies were collected from different geographical regions around of Sulaimani governorate. Twenty-four different cultivars of pomegranate as experimental materials were selected (table 1)

Table 1: sample and local names of pomegranates.

No	Sample name	Local name	No	Sample name	Local name
1	G1	mekhosh	13	G13	Twekl astury
2	G2	amareky,	14	G14	tweklastur mewkhosh
3	G3	salakhani trsh	15	G15	hanara sherna
4	G4	karaly twekl spy	16	G16	malesay 2
5	G5	Sazan	17	G17	hanary sur
6	G6	hanary trsh	18	G18	salakhni darajayak
7	G7	nawrsh	19	G19	kawa hanry sherin
8	G8	twekl spy	20	G20	malesay 1
9	G9	tweklastur	21	G21	malesay 3
10	G10	sury sherin	22	G22	hanary benawk trsh
11	G11	sury trsh	23	G23	sura hanry trsh
12	G12	hanart trsh drezh	24	G24	kawa hanry asly

2.2 *Genomic DNA Extraction*

Total genomic were extracted according to (6) with some modification that fresh leaves were ground by liquid nitrogen and then 1ml of Lysis buffer with 10 µl of RNase was added with incubated at 65ºC for 70min. After incubation, the sample was cooled and 200 µl of 5M potassium acetate (pH 6.5) was added and mixed very well then incubated at fridge for 10 min. The mixture was centrifuged at 12000-16000 rpm for 17 min and then 800 µl of chloroform was added in to supernatant, mixed gently by inverting for 1 min. The upper phase was taken and transferred to a new Eppendorf tube (2 mL), 1ml of buffer AW1 was added to the upper phase and mixed gently by inverting, and then 850 µl of mixture was loaded into a spin column and incubated at room temperature for 2min. Next spin column containing the mixture was centrifuged at 8000 rpm for 4 min, repeated this step, 500 µL of washing buffer AW2 was added to spin column and centrifuged at 8000 rpm, the spin column was centrifuged at speed (11000 rpm) for at least 5 min for drying, spin column was then inserted into a new 1.5 mL tube. 110 µL of Elution buffer (AE) was added to the spin column and incubated at room temperature for 5 min. The tube is centrifuged at 8000-9000 rpm for 5 min The Eluted DNA (at the bottom of tube) is collected and stored at -20° C. The quantity and quality of isolated genomic DNA was determine d by nanodrop spectrophotometer.

2.3 *RAPD Analysis*

10 primers were utilized in this work, (*Table 2)*. Primers were designed regarding many papers including (7) and (8) primers which were Germanys made. RAPD amplification was performed in a 25 µl reaction volume containing 50-100 ng genomic DNA, ×10 buffer (10 mM Tris-Hcl. pH 8.3, 50 mM KCl. 2 mM MgCl), 2.5 mM dNTPs, 10 pmol of single 10-base primer and 1U *Taq* DNA polymerase (Sigma, USA). The thermocycler was programed as follows; initial cycle of 5 min at 94°C followed by 38 cycles of 1 min at 94°C, an annealing temperature of 36°C for 1 min, extension step of 2 min at 72°C, and a final extension step of 10 min at 72°C. Polymerase chain reaction products were separated by gel electrophoresis on 1.5% agarose gels with ×1 Tris base, boric acid, ethylenediaminetetraacetic acid buffer, at 70 V/cm for 2 h. The gel was stained with 0.25 µg/ml ethidium bromide and stained gels were visualized by ultraviolet transilluminator and photographed. The molecular weights of bands were estimated using standard molecular markers. Amplification products were separated on 1.5% agarose gel in 1X TAE (Tris base, acetic acid and EDTA) buffer. Gels were run at a constant voltage of 100V for 60 minutes, then imaged using a UV trans-illuminator. The image was captured by a digital imaging system.

Table 2 Primer names, sequences and annealing temperatures of RAPD markers.

Prime number	Primer Name	Primer Sequences 3 ⟶ 5	Annealing Temperature (°C)
1	OPA-07	GAAACGGGTG	36
2	S093	CCACCGCCAG	36
3	S094	AGAGATGCCC	36
4	S087	GGTGCAGTCG	36
5	S088	GGTCCTCAGG	36
6	S089	CAGTTCGAGG	36
7	S090	TACCGACACC	36
8	S091	TCGGAGTGGC	36
9	S092	ACTCAGGAGC	36
10	S073	CCAGATGCAC	36

2.4 Statistical Analysis Data and Counting

The scorable bands were coded manually as either present (1) or absent (0). These data were used to create dendrogram using Jaccard method by using XLSTAT 2017 software (9). XLSTAT 2017 software was used to perform principal component analysis (PCA) to determine the association between different genotypes. Power Marker version 3.25 software was used to calculate PIC and gene diversity and major allele frequency (10). The software GenAlEx (version 6.5) was also used to evaluate molecular variance between and within populations (11). For the population structure, a model analysis was performed using the software STRUCTURE (version 2.3.4) to infer the genetic structure and clarify the number of sub-populations (12).

3.Results and Discussion

3.1Allelic Variation in Pomegranate Accessions Using RAPD Markers.

Molecular marker study was used to analysis of genetic diversity among the 24 selections of pomegranate cultivars using *12* random RAPD primers that among them 10 random primers were only able to amplify the genomic DNA successfully and their reproducibility and high polymorphism (*Table 3).* Number of bands for each primer ranged from 6 to 10 (Fig 1). Total number of bands got with all the primers was 82. Out of these 82 bands 71 were polymorphic and 11 monomorphic. Maximum numbers of bands (10) were obtained with primer (AR14, AR15) and minimum number of bands (6) was obtained with primer (AR13). The maximum, minimum and mean values of polymorphic bands were (9, 5 and 7.1) respectively. The PIC values were recorded range between 0.77 to 0.92. (Fig 2). Our results are nearly similar to those reported such as, (13) was documented those 25 primers was accepted to analysis genetic diversity among the 24 wild pomegranates that only 19 random primers were able to amplify the genomic DNA successfully. In addition, total number of bands obtained with all the primers was 142. Out of these 142 bands 116 were polymorphic and 26 monomorphic. Maximum numbers of bands (13) were obtained with primer OPA-14 and minimum number of bands (1) was obtained with primer-OPC-17. (14) investigate the genetic diversity among 13 cultivars of pomegranate cultivated in (RAPD) markers were used, that 12 out of 30 employed random primers showed good amplification and polymorphism on pomegranate samples with a total of 107 bands. The percentage of polymorphic DNA bands ranged from 66.6% (OPX-17) to 100% (OPO-07, OPS- 17, and OPE-18, OPP-02, OPK-08, OPL-17, and OPR-01) with an average of polymorphic rate of 94.39. In addition, (15) was documented that 25

polymorphic band among 28 DNA bands was observed and also the mean number of polymorphic bands per primer among eight pomegranate varieties was 5.6 and per cent polymorphism ranged from 75 to 100, with PIC value varied from 0.70 to 0.83. moreover, Fourteen RAPD primers were used to characterization of jordanian pomegranate, that among 157 fragments, 88 bands were polymorphic (56.0%). The Polymorphism information content (PIC) averaged over all loci ranged from 0.44 to 0.71 with an average of 0.56 (16). (17) reported that the 9 random primers generated a total of 63 RAPD bands, of which 31 were polymorphic that the highest polymorphism (80.0%) was observed for the OPBB-3 primer, while the lowest polymorphism (33.3%) was observed for the OPBB-4 and OPBC-8 primers. Therefore, our results confirmed the previous studies that concluding RAPD is effective technique to reveal genetic diversity among pomegranate accessions. PCR-RAPD markers were used to determine the diversity level among 24 Iranian pomegranate genotypes. That results produced 178 bands, 102 were polymorphic (18).

Table 3. Summary of PCR-RAPD amplified products including Markers name, number of amplified bands, number of polymorphic bands, number of monomorphic bands, major allele frequency, gene diversity and PIC value that obtained from 24 **pomegranate cultivars** ***using 10 random primers.***

Marker	**Number of Amplifed bands**	**Number of polymorphic bands**	**Number of monomorphic bands**	**Major Allele Frequency**	**Gene Diversity**	**PIC**
AR1	7	5	2	0.38	0.79	0.77
AR11	9	9	0	0.33	0.84	0.83
AR13	6	5	1	0.29	0.82	0.80
AR14	9	9	0	0.25	0.88	0.87
AR15	10	9	1	0.13	0.92	0.92
AR16	10	8	2	0.25	0.89	0.88
AR17	7	7	0	0.13	0.93	0.92
AR18	8	7	1	0.29	0.86	0.85
AR19	9	6	3	0.25	0.85	0.84
AR21	7	6	1	0.17	0.90	0.89
Total	82	71	11	-	-	-
Mean	8.2	7.1	1.1	0.25	0.87	0.86

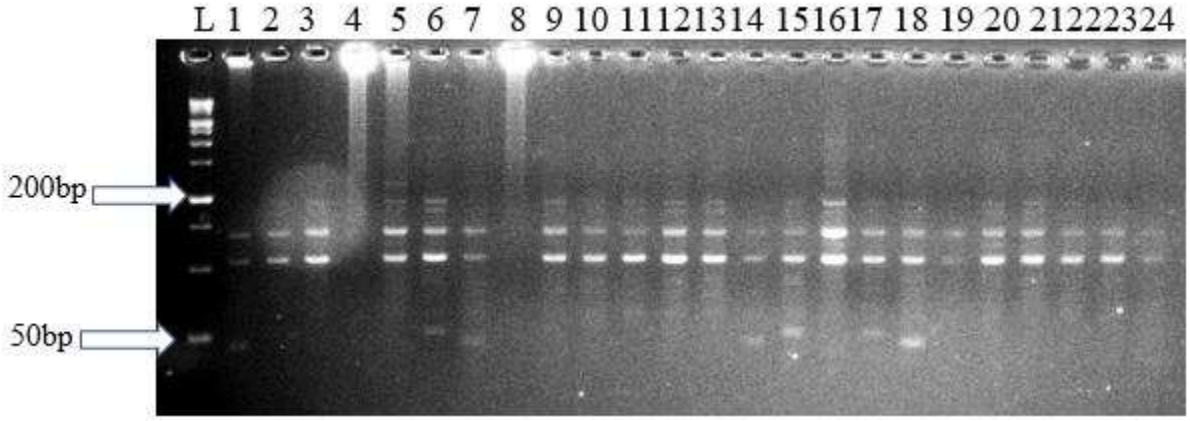

Figure 1: Amplification of PCR picture for 24 Pomegranate accessions using AR1 primer. L: DNA Ladder

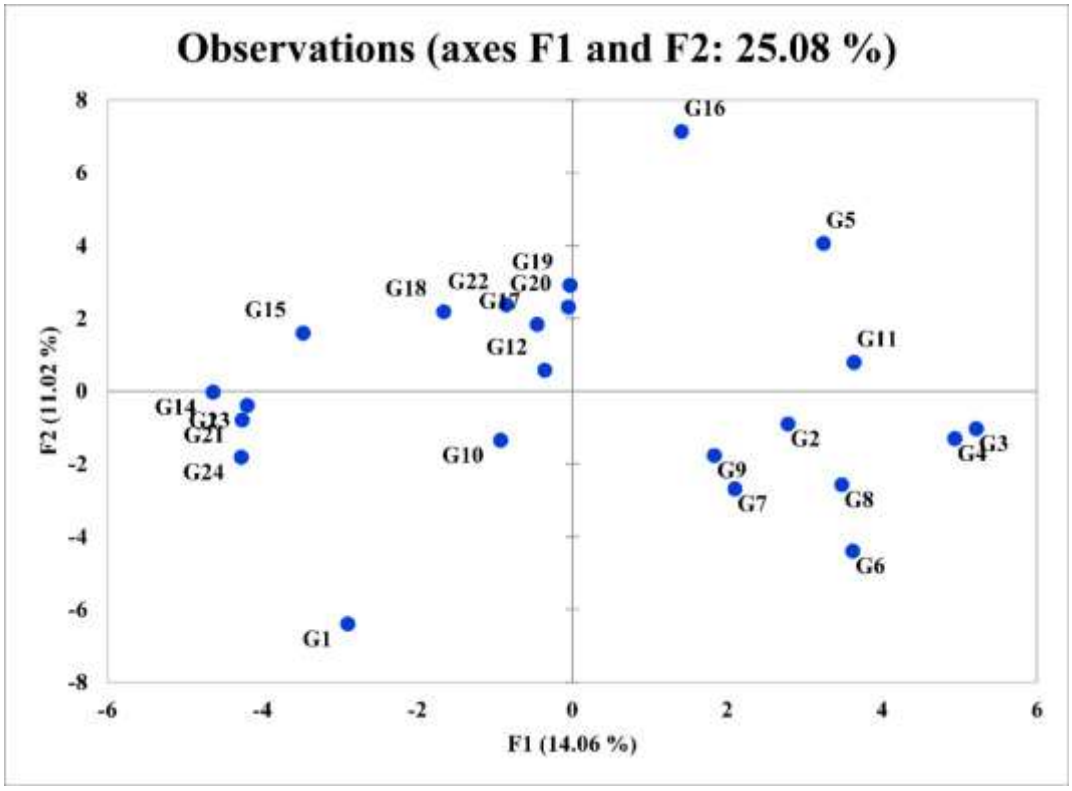

Figure 2. *PCA plot among 24 genotypes accessions of pomegranate based on different location*

3.2 Jaccard similarity and AMOVA analysis

Clustering analyses were performed for assessing the connection between pomegranate genotypes, based on the Jaccard similarity coefficients using the unweighted pair-group method (UPGMA). The dissimilarity coefficients were ranged between 0.22 (G12 vs. G23) to 0.75 (G1 vs. G6), wholly 24 pomegranate genotypes were

clustered into 6 groups (A, B, C, D ,E and F) with a mean dissimilarity (0.54) for 10 RADP markers (Fig. 3), cluster A includes only (G1) cluster B only (G6), cluster C (G15) cluster D (G24), cluster E(consent of all genotypes without cluster A, B, C, D and F) was observed and cluster F (G7, G10, G11, G14, and G21), Many researcher were recorded regarding pomegranate genetic diversity and their relationships including was documented that the dendrogram constructed using molecular data generated by five RAPD primers showed higher similarity in between Mrudula and Ganesh while lowest similarity found in S Bhagava and G-137, and also, the genetic similarities ranged from 0.32 to 0.72 and mean similarity co-efficient was 0.61 (16). In addition, serval research also confirmed that molecular characterization using RAPD markers proved to be a successful tool to determine genetic diversity among southern Jordan's pomegranate genotypes. UPGMA clustering of the genotypes showed three major groups. The highest genetic distance (0.481) was observed between Ma'an and Al Huseiniya populations and the lowest (0.429) was detected among Al Shoubak and Ma'an groups. About 39% of total genetic variation was detected among groups, whereas 41% of total variation was observed among populations within groups (17). In addition, (18) was demonstrated that the similarity matrix showed that the highest (0.920) and lowest (0.556) genetic similarities occurred between the APS13 and APS28 genotypes and the APS12 and APS42 genotypes, respectively. The dendrogram produced from the RAPD markers grouped the genotypes into 2 main clusters, which contained 2 and 17 genotypes, respectively, Cluster 1 contained the APS- 12 and APS-49 pomegranate genotypes. Cluster 2 was divided into 2 subclusters, with subcluster 1 containing just 1 genotype (APS-36), while subcluster 2 contained the remaining 16 genotypes. PCR-RAPD markers were used to determine the diversity level among 24 Iranian pomegranate genotypes. Cluster analysis of the genotypes was performed based on data from polymorphic RAPD bands, using Jaccard's similarity coefficient and UPGMA clustering method. The highest and lowest similarities detected between genotypes were 0.89 and 0.29, respectively. At a similarity of 60%, the genotypes were divided into four sub-clusters (18)

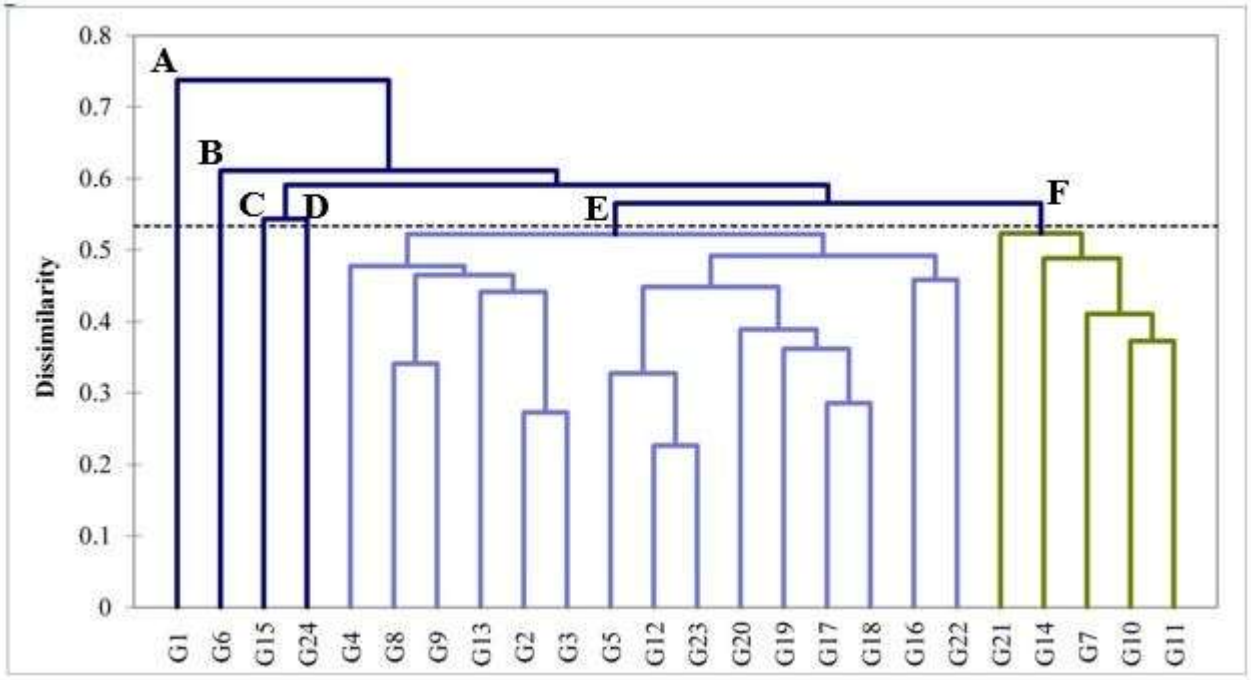

Figure 3. *Cluster tree created by UPGMA method based on 10 RAPD markers among 24 pomegranate genotypes*

Analysis of molecular variance (AMOVA) of the *24* pomegranate genotypes in RAPD analysis demonstrated 89% of the total variation within the populations), and 11% credited to differences between populations (Table 4).

Table 4. Analysis of molecular variance (AMOVA) of the five populations for 24 pomegranate genotypes.

Source	df	SS	MS	Est. Var.	%	**P-Value**
Among Population	4	109.242	27.310	2.100	11%	0.001
Within Population	19	333.550	17.555	17.555	89%	0.001
Total	23	442.792		19.655	100%	

3.3 Genetic Structure for all genotypes using RAPD markers.

Allele frequencies using STRUCTURE analysis was utilized to determine for *24* pomegranate genotypes (19) and results shows that Delta K genotypes were divided into six groups or sub-populations, and represented by color including group 1 (green line) and group 2 (red line) group 3 (Dark blue line) group 4 (yellow line) and group 5 (pink line) and group 6 (Light blue line) (Fig. 4A) In addition, all genotypes combination were observed more than one background only genotypes 3, 7, 16, 19, and 24 were shown one background that may be possibly had a complicated history linking intercrossing or practicably resulting from the gene flow between taxa, in addition, the high variability between genotypes may consequences from the changing climates found within the locations. Determine the true number of clusters (K) in a sample of individuals was observed that the peak was started at 3 and the real K value with the highest value of K= 5 for 10 RAPD markers (Fig. 4B).

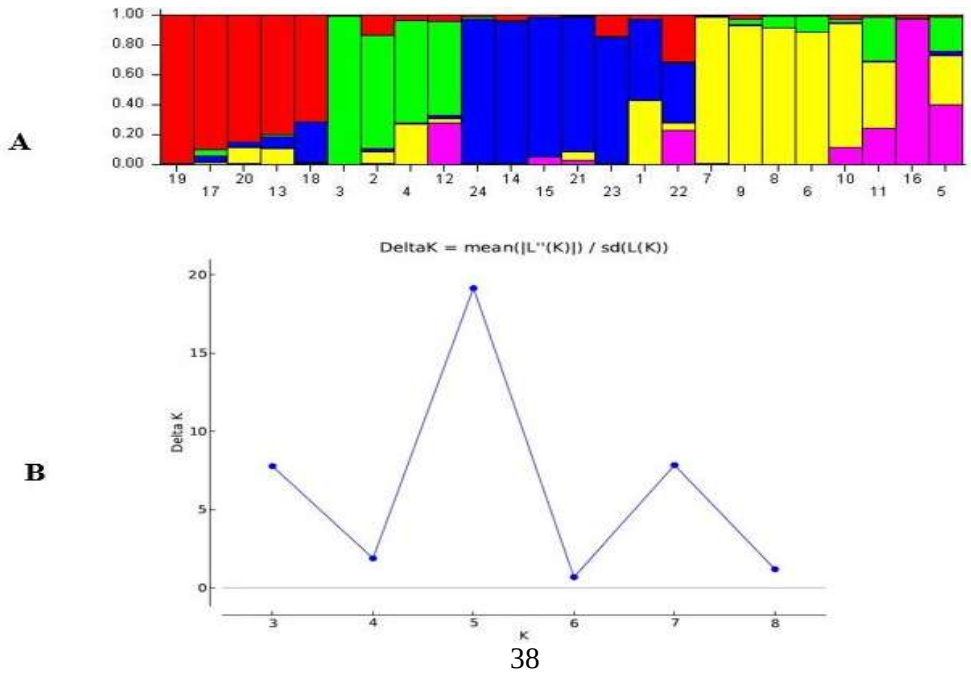

Figure 4. *twenty-four pomegranate genotypes clustered into different sub-populations by STRUCTURE software. RAPD. Accessions are coordinated as per estimated membership coefficients (q) in K= 5 clusters, Determining the optimal value of K by the (the (ΔK) procedure described by (18).*

4.Conclusions

The objective of present study was evaluated and assess the genetic diversity using RAPD marker to 24 pomegranate accessions grown around Sulaimani Iraq region. Because of development of a molecular method is suitable into pomegranate genetic polymorphisms surveying due to the relatively low number of cultivars studied and primers tested are seems in this area.

Genetically, the number of polymorphic and monomorphic bands, major allele frequency, gene diversity and the polymorphism information content (PIC), were demonstrated. The polymorphic bands of mean value were 7.1 and 1.1. for monomorphic bands, The PIC values were recorded range between 0.77 to 0.92. Jaccard similarity coefficients were achieved between 0.22 (G12 vs. G23) to 0.75 (G1 vs. G6) and clustered into 5 clusters (A, B, C, D and F) with a mean dissimilarity (0.54) for 10 RADP markers. Therefore, the consequence of the genetic diversity in Sulaimani pomegranate accessions is important for breeding as well as to the implementation of degree addressed to their usages and preservations, our recommendations for future investigates will be used quantitative traits loci (QTL) analysis and genome wide associated, and also for breeding programmers can be use more different locations and accessions with using various kinds of markers including SNIPs SRAPs, ALFPs, ISSR and SSRs.

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