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Comparative study of the morphological and molecular characteristics of 10 Iranian olive (*Olea europaea* L.) cultivars

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ABSTRACT

Iran itself has a well-documented history of olive (Olea europaea L.) growing dating back more than two thousand years. This country also contains a large variety of unknown olive cultivars. Morphological studies based on the method prepared by the EU RESGEN CT96/97 project, coordinated by the International Olive Oil Council (IOC) revealed that there are some homonymous in the main Iranian olive cultivars. Analysis of endocarp characteristics, which displays the most robust features, reveals a considerable degree of intra-cultivar variation within traditionally recognized Iranian cultivars. Based on the analysis carried out so far, the 10 traditional cultivars studied have been reclassified into 27 cultivars. Homonyms and mislabeling was clearly observed in 5 of 10 studied cultivars by morphological traits contain: Khormazeitoon, Rwoghani, Shengeh, Dakal and Golooleh. To evaluate the accuracy of the new morphological classification, the morphological classification examined the genetic diversity of these cultivars using 17 RAPD primers. The data analyzed using Dice similarity coefficient and the WPGMA clustering method. 4 of 17 primers (OPC 08, 15, 19, 20) produced high polymorphic bands that able to classify some new cultivars. As a conclusion, Molecular results proved the accuracy of the new morphological classification in some cultivars.

Keywords: RAPD, *Olea europaea*, Khormazeitoon, Rwoghani, Shengeh, Dakal, Golooleh, morphologic and molecular

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INTRODUCTION

Olive (*Olea europaea* L.) is a plant that adapted properly to the various climates of Iran from cold and mild elevations of Zagros and Alborz mountains to dried desert of Yazd. The genetic variation in most spices of plants shows erosive process during the time. However there is nothing like genetic erosive in olives and it is the key of severe adaptation power of olive to various environmental situations [2].

In addition to high adaptation power, binary usage (oil and canned) and many medical benefits will boost the under grown field of olive from 90000 hectares to 600000 hectares in 2014 that scheduled in Iran development plans (The report of development olive cultivation in Iran in 4th five-year plan, Olive office, agricultural ministry). The increase needs about 150,000,000 olive transplants product. This plan is performing while there are many obscurities of classification of olives type in Iran and it causes that one type of olive has several names (synonyms) and/or several types of olives classified only with one name (homonyms). This global problem has resulted in used more than 1200 types of well-known olives in the world were labeled with 3000 various names [6]. Forgetting information of gardens re-labeling of imported cultivars, doing inaccurate labeling during the reproduction and specially using nonstandard morphological attributes have caused these obscurities [5,7,10].

An important issue in identification of cultivars by morphological methods is the use of features receiving the least effects from the environmental factors. Today the use of molecular markers based on DNA are able to prepare a chance of direct comparing and determination of genetic independent from environmental effects. Being Independence of environmental effects and plants growth levels have caused the markers to be useful naturally for specific classification and source finding [4,8].

Although nowadays many do not trust on RAPD markers, there are some evidence that this marker can be useful. For instance one of the considerable researches that has been done by RAPD [1,11] on the classification of population of Iranian Taodar wheat in which the ability of RAPD in investigation of genetic variation has been proved. In that research after floristic investigation and determining protein finger printing of these populations, 100 chosen populations were classified in 7 florestic groups by RAPD while these 7 groups showed 6 groups by proteins finger printing in maximum. RAPD markers determined that 85-90% of these population belong to a big genotypic group that almost have no polymorphism. These populations are distributed in six cold regions of the west and North West of Iran which have some different environmental characteristic and they have created six proteins finger prints under these minor environmental conditions. But 10-15% of populations belonged to a little genotypic group that are scattered in ultra-cool microclimates in those sextuple cold regions. In fact RAPD managed distinguishing between the populations of cold regions and ultra-cool regions.

In this research we have studied the classification of 10 types of Iranian olives by considering the above mentioned Items at first step by using morphological properties then molecular RAPD markers.

MATERIALS AND METHODS

Plant material: In this study the trees which were classified in 10 common traditional groups (table1) were collected from different northern regions of Iran.

Morphological studies: morphological study of olives types were performed according to IOC (International Olive Council) and instruction presented by seed & plant certification and registration institute (2007-2008). Finally 32 morphological properties were recorded for 10 types of Iranian olive and were analyzed by SPSS software (table 2 and 3). Cluster analysis and PCA classification by using of all properties (quantitative and qualitative of fruit and core) didn't show a good separation among samples under investigation. In next step in order to determine which groups of properties (quantitative or qualitative) had important role in classification of these types in other word with which types of properties cluster analyses have complete accordance with morphological classification were done with various properties

In investigating all these dendrograms it was demonstrated that the cluster analyses by using qualitative properties of fruit and core are so power full in separating types from each other.

Molecular studies: after morphological investigation and classification of types, 54 trees were chosen among 10 studied types for molecular study.

DNA extraction: the samples of DNA were extracted by optimizing the Kang & Yang (2004) methods[9].

Polymerase Chain Reaction (PCR): After investigating the results of previous researches [14] among 3 sets of RAPD primer (A, B, C) group C of Operon Company which had shown better polymorphism were selected.

PCR temperature program was done by optimizing the temperature program of Samaei *et al*. article (2004) in Touchgne gradient thermal cycler. with 5 min for primary denature at a temperature of 94 ^{0}C, 36 cycle consist of 1min at 94^{0}C, 1 min at 36^{0}C, 2 min at 72^{0}C and 10 minutes of final extension at temperature of 72^{0}C add 25µl PCR reactive for final volume (primer 0.4 pm, Taq DNA polymerase 1.25 u, DNA 0.4 ng $Mgcl_2$ 1.5 mM, dNTPs 0.2 mM, PCR buffer 1x)

PCR products electrophoresis: electrophoresis was done with PCR products on both 2% agarose gel and 6% polyacrylamide gel because of the low number of band polymorphism in agarose gel.

The analyses were done by 2.02 version of NTsys software. In order to have best dendrogram and similarity matrix different calculating was done by methods of similarity matrix and clustering method. Two cases were important in choosing the best dendrogram: 1.The correlation coefficient 2. The most accordance of produced dendrogram with new morphological classification so the best method of calculating the similarity matrix by Dice method and dendrogram resulted from cluster analyses was selected by WPGMA.

RESULTS

Morphological results: Homonyms and mislabeling was clearly observed in 5 of 10 studied cultivars contain: Khormazeitoon, Rwoghani, Shengeh, Dakal and Golooleh. New classification of these cultivars shown in table 1 is confirmed by Spanish experts[3]. As it is illustrated in figure 1, for instance, in Khormazeitoon different morphological traits are obvious. Based on morphological study 10 traditional cultivars are split to 27 new cultivars (table 1).

Molecular results: Using 17 RAPD primers gave a total of 144 reproducible DNA fragments on 6% polyacrylamide gel. The data were analyzed using DICE similarity coefficient and the WPGMA clustering method. 4 of 17 primers (OPC 08, 15, 19, 20) produced high polymorphic bands that able to classify some new cultivars. 17-primer dendrogeram with Coffenetic Corelation = 0.93 and 4-primer dendrogeram with Coffenetic Corelation = 0.84 are presented in figure 2 and 3.

Table 1- traditional and new classification and labeling of Iranian olive cultivars based on standard morphological traits

Traditional classification	New classification and labeling based on standard morphological traits	Abbreviation	code of Replications (trees)
Zard	Zard	Z	322, 372, 1109,1157
Fishomi	Fishomi	F	255,293, 294, 297, 1051,1052
Khormazeitoon	KhormazeitoonI	KhoI	1128,1130, 1133
	KhormazeitoonII	KhoII	1148
	KhormazeitoonIII	KhoIII	299
	KhormazeitoonIV	KhoIV	402
Mari	Mari	M	341,368,397
Rwoghani	RwoghaniI , RwoghaniI´	RI, RI´	1047I´, 321I
	Rwoghani II G, Rwoghani II fadak	RII G, RII fadak	209IIG , 13(9) II fadak
	Rwoghani III	RIII	112,119
	Rwoghani IV	RIV	330,331
Shengeh	Shengeh I	SHI	128,1094
	Shengeh II	SHII	1082,1096
	Shengeh III	SHIII	452, 1115
	Shengeh IV	SHIV	1098
	Shengeh V	SHV	1083, 1086
	Shengeh VI	SHVI	1102
Dakal	Dakal I	DkI	283,285
	Dakal II	DkII	281G
Khara	Khara	Kha	352,356
Dezfool	Dezfool	Dz	287,291
Golooleh	Golooleh I	GI	303, 317
	Golooleh II	GII	387,391
	Golooleh III	GIII	374,502
	Golooleh IV	GIV	497
	Golooleh V	GV	1117,1160
	Golooleh VI	GVI	316

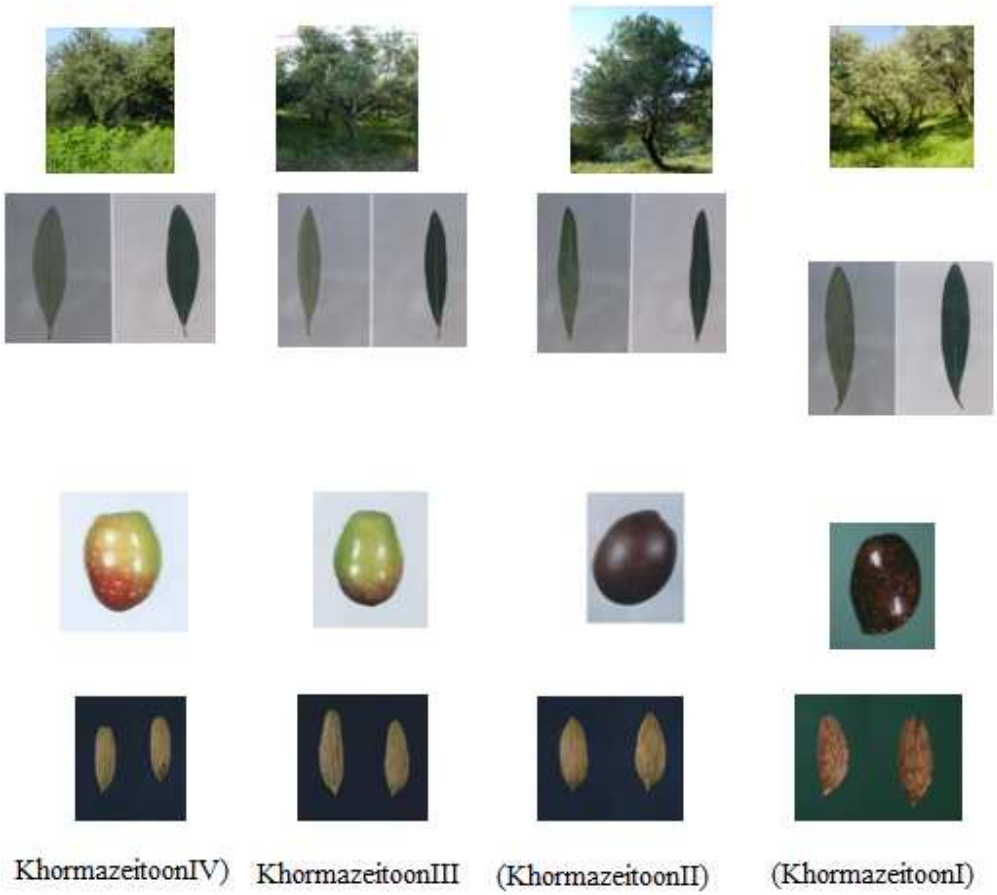

Fig. 1- four distinguished new cultivars of Khormazeitoon based on morphological traits

Table 2a- Morphological fruit profile in different new cultivar of Khormazeitoon Fruit

Acc. Code	F-shape	F-symA	F-pmtd	F-apex	F-base	F-nipple	Plt	Slt	Lst
KH-I	2	3(2)	3(2)	2	1	2	1(2)	2	3
KH-II	2(1)	3(2)	3	2	1	2	1(2)	2	3
KH-III	2	3	3	2	1	2	2	2	3
KH-IV	2	1	2	2	1	1	1	1	3

Table 2b- Morphological fruit profile in different new cultivar of Khormazeitoon Stone

Acc. Code	S-shape	S-symA	S-symB	S-pmtd	S-apex	S-base	S-urf	Groove	Dgroove	Top
KH-I	3	3	2	3	2	2	3	2(1)	2	2
KH-II	2(3)	2	1	3	2	2	3	2	2	2
KH-II	3	2	2	3	1	1	3	2	2	2
KH-V	3	2	1	2	2	1	2	2	2	2

Table 3- Abbreviation

Fruit	***Stone***
F-shape = *Fruit Shape*	***S-shape*** = *Stone Shape*
F-symA = *Fruit Symmetry A*	***S-symA*** = *Stone Symmetry A*
F-pmtd = *Fruit Position of maximum transverse diameter*	***S-symB*** = *Stone Symmetry B*
F-apex = *Fruit apex*	***S-pmtd*** = *Stone Position of maximum transverse diameter*
F-base = *Fruit Base*	***S-apex*** = *Stone Apex*
F-nipple = *Fruit Nipple*	***S-base*** = *Stone Base*
Plt = *Presence of lenticels*	***S-urf*** = *Stone Surface*
Slt = *Size of Lenticels*	***Groove*** = *Number of grooves*
Lst = *Location of start of colour change*	***Dgroove*** = *Distribution of the grooves*
	Top = *Apex Termination*

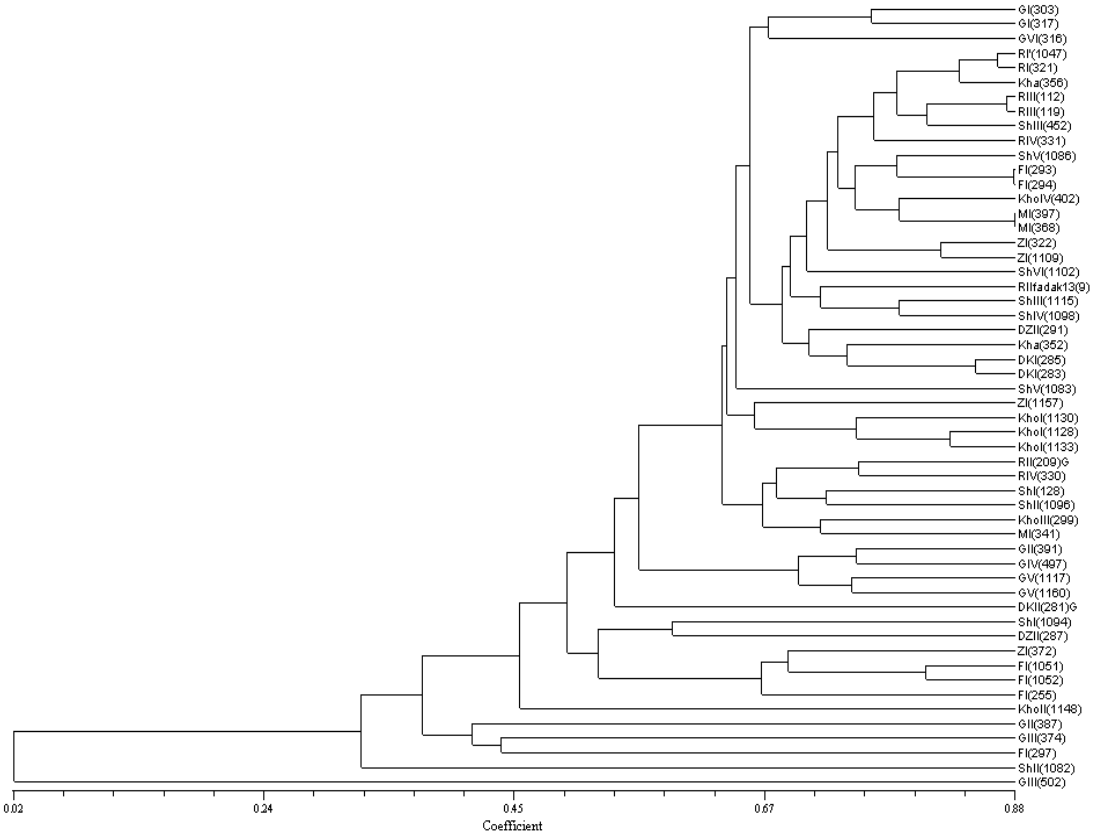

Fig.2- Dendrogeram of all cultivar based on 17 RAPD primers (Dice-WPGMA, R= 0.93)

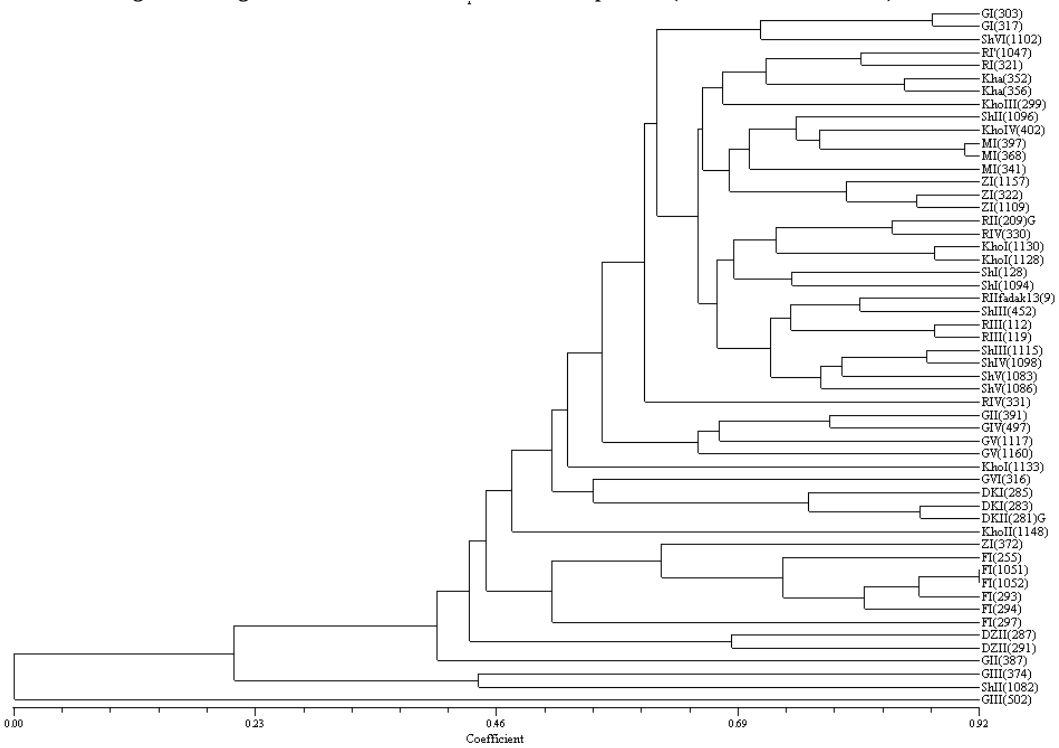

Fig.3- Dendrogeram of all cultivar based on 4 selected RAPD primers (Dice-WPGMA, R= 0.87)

DISCUSSION

According to new morphological classification we checked molecular-derived dendrograms. We expected replications of every new cultivar grouped together or were located nearer. In order to present clear discussion on the ability of 17-primer dendrogeram and 4-primer dendrogeram to classify new cultivars together, they are showed in table 4. There are two interesting case, first Fishomi 297 has at least similarity to other Fishimi replications (37% similarity in 17-primer dendrogeram and 49% similarity in 4-primer dendrogeram). Second case is Golooleh III 502 is really far from all cultivars of the research (99% dissimilarity in 17-primer dendrogeram and 100% dissimilarity in 4-primer dendrogeram).

On previous study of our research team which is placed in NIGEB, Iran [12] Ninety two accessions belonging to 10 main (old) olive cultivars were screened by 13 microsatellite markers revealing high genetic variability both within and between cultivars. The existence of homonyms, synonyms, or mislabeling as well as intra cultivar polymorphism was revealed by allele differences between accessions of the same denomination. The phonogram showed variability among as well as between some cultivars, but most accessions with the same generic names were grouped together.

The dendrogeram of SSR study (not shown here) is compared with our dendrogerams (figure 4) which only contain shared cultivars in both study in table 5.

Table 4- Comparing the ability of every dendrogeram on classification new cultivars together

Abbreviation of new naming	Dendrogram based on 17 RAPD primers	Dendrogram based on 4 selected RAPD primers
Z	3 of 4 replications are grouped together (min.73-max.88% similarity)	2 of 4 replications are grouped together (80%similarity)
F	Every two replications are grouped together (min.67-max.88% similarity)	All 6 replications are grouped together (min.75-max.- 92% similarity)
KhoI	All 3 replications are grouped together (min.75-max.- 83% similarity)	-
KhoII	Not enough replication	Not enough replication
KhoIII	Not enough replication	Not enough replication
KhoIV	Not enough replication	Not enough replication
M	2 of 3 replications are grouped together (88%similarity)	All 3 replications are grouped together (min.70-max.- 90% similarity)
RI, RI´	2 replications are grouped together (85%similarity)	2 replications are grouped together (75%similarity)
RII G, RII fadak	2 replications are separated	2 replications are separated
RIII	2 replications are grouped together (86%similarity)	2 replications are grouped together (89%similarity)
RIV	2 replications are separated	2 replications are separated
SHI	-	2 replications are grouped together (72%similarity)
SHII	-	2 replications are separated
SHIII	-	2 replications are separated
SHIV	Not enough replication	Not enough replication
SHV	-	2 replications are grouped together (73%similarity)
SHVI	Not enough replication	Not enough replication
DkI	2 replications are grouped together (84%similarity)	-
DkII	Although Not enough replication but II is located far from I	-
Kha	2 replications are separated	2 replications are grouped together (87%similarity)
Dz	2 replications are separated	2 replications are grouped together (68%similarity)

Parra-Lobato *et al.* (2012) studied 32 olive Spanish cultivars by using morphological traits and 5 RAPD markers. Results showed all the cultivars could be identified by a combination of three primers (OPF-6, OPA-8, and OPK-16); the resulting dendrogram, using the Unweighted pair group method with arithmetic mean clustering algorithm, depicted the pattern of relationships between the local Extremadura cultivars and the cultivars from geographically connected regions. This analysis showed a correlation between most of the minor local cultivars and the

geographical origin. They found that there was no apparent clustering according to morphological traits or fruit use of olive cultivars when these parameters were used as analysis criteria[13].

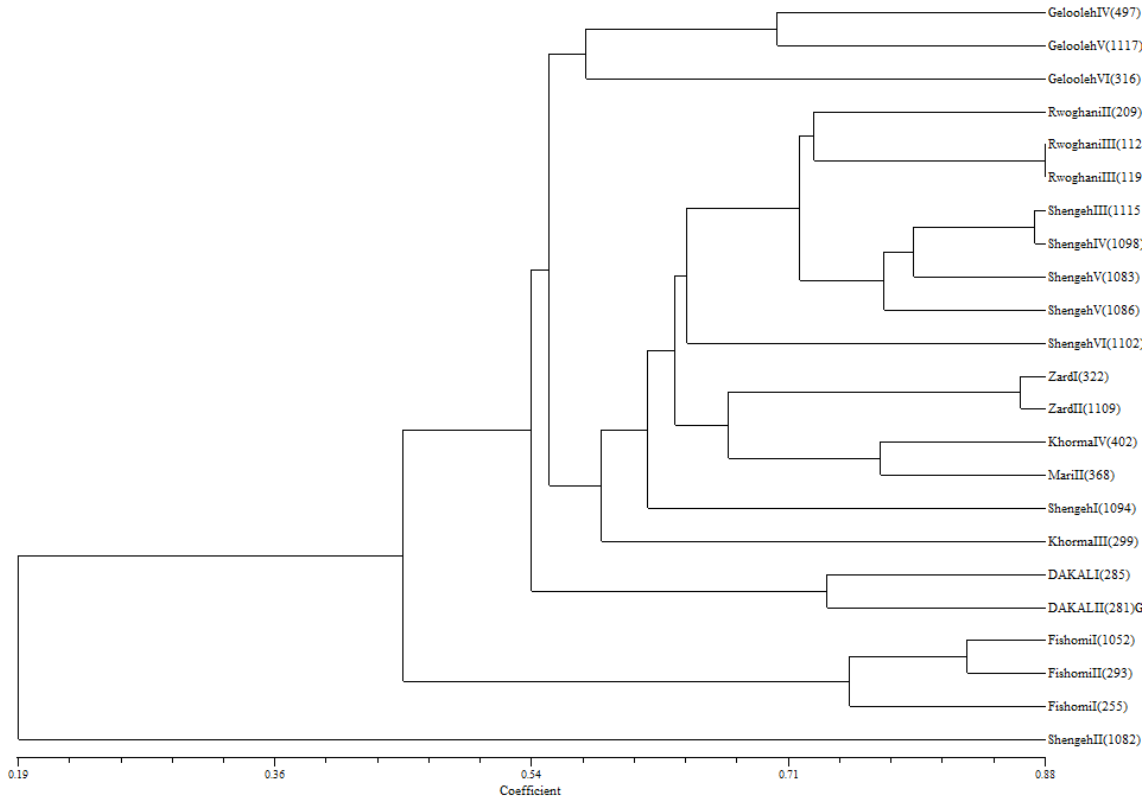

Fig.4- Dendrogeram of shared cultivars with SSR study [12] based on 4 selected RAPD primers (Dice-WPGMA, R= 0.84)

Table 5- Comparing the ability of RAPD or SSR dendrogarm on classification new cultivars together

Abbreviation of new labeling	Dendrogram based on 4 selected RAPD primers in current study	Dendrogram based on SSR primers of Noormohammadi *et al.*2007
Z	73% similarity	96% similarity
F	75% similarity	94% similarity
RII to RIII	72% similarity	48% similarity
RIII	2 replications are grouped together (88%similarity)	2 replications are grouped together (91%similarity)
SH II 1082 to SH VI 1102	20% similarity	100% similarity
SH II 1082 to SH IV 1098	20% similarity	80% similarity
SHV 1086 to SHV 1083	78% similarity	96% similarity
DkI to DkII	73% similarity	96% similarity
G	Distributed	Distributed

CONCLUSION

As it is provided our research in table 4, 5 and Parra-Lobato *et al.* (2012) research, it is important to achieve appropriate primers that have more linkage to selected morphological traits. In the other hand, apply a lot of primers can cause confusing because some primers don't have any relationship to selected morphological traits. Inappropriate primers only resulting in distribution whiten replications of a cultivar. 4 of 17 primers (OPC 08, 15, 19, 20) produced high polymorphic bands that able to classify some new cultivars. As a conclusion, Molecular results proved the accuracy of the new morphological classification in some cultivars.

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