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Molecular Characterization of Unknown Potentially Salt Tolerant Olive Genotypes Using RAPD Markers

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Abstract

Randomly amplified polymorphic DNA (RAPD) markers were used to study the genetic diversity and discriminate among 17 unknown genotypes (considered potentially salt tolerant) and 16 known olive cultivars. Fifteen decamer primers which produced 38 reproducible polymorphic bands in the genotypes were selected for analysis. The RAPD markers resulted in 93 distinct banding patterns. Based on either unique or combined patterns all genotypes could be identified. Genetic similarities between genotypes were estimated using Jaccard's similarity index indicating a high degree of diversity within the known and unknown genotypes. Using the unweighted pair-group method (UPGMA), most genotypes were clustered into two main groups according to their origin area of cultivation (native and foreign ones). The unknown genotypes mainly clustered with cv. Zard, one of the native Iranian olive cultivars. The presented results contribute to a comprehensive understanding of unknown olive genotypes, which potentially are tolerant to the high salinity of their cultivation area. These genotypes could be important for extension of cultivation purposes and breeding programs.

Keywords: Olive; Salt tolerance; RAPD marker

Introduction

Olive (*Olea europaea* L.) is a woody species found throughout the Mediterranean basin and is an important oil-producing species. Olive cultivation most likely originated from the eastern regions of the Mediterranean Sea as early as 3000 BC [4]. It spread westward, following the arches of the basin, to the southern parts of Europe and northern Africa. Because of its high quality oil, olive cultivation has gained importance in recent years in Iran. Recently high attention has been paid to increase the cultivation of the olive species in Iran [11]. Over the past years, some genotypes of olive have been examined for their quantitative traits as well as their ecological behavior such as: cold hardiness and salt tolerance in Iran [7,13]. One of the most important factors limiting the extension of olive growing area in Iran is the salinity of soil and water at some environmentally competent places. Some potentially salt tolerant olive genotypes at some lands of Iran with high

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salinity have been observed. However, there is a considerable uncertainty about the genetic status of these tolerant plants. Hence, identification of these genotypes, the aim of present study, is important for providing suitable material for propagation in order to extension of olive growing at potentially suitable areas which have a high level of soil salinity. Recently several molecular marker techniques have been applied to analyze olive germplasm variability and differentiate among cultivars [1,2,9]. The simplicity of laboratory assays and high efficiency of randomly amplified polymorphism DNA (RAPD) markers [17] make it an attractive method for fingerprinting of genotypes. This technique is already used for cultivar identification of other plants e.g. blueberry and persimmon [3,18]. In present study we used RAPD markers to identify some potentially salt tolerant but unknown genotypes parallel to known native and foreign cultivars.

Materials and Methods

Plant Materials

Plant materials are listed in Table 1 and included 17 unknown olive genotypes existing in an abandoned natural resource station at 40 km of Qom-Tehran highway with a saline irrigation water (8.5 ds/m) and 16 known native and foreign cultivars. All labeled known cultivars are maintained as representative genotypes at a germplasm collection at the private Fadak Company, Qom-Iran. The geographical origins of cultivars are given in Table 1. Tip shoots of olive with young leaves were collected and wrapped in moist cotton gunny for transportation. Samples were stored at −20°C for one week until use.

Experimental Methods

Genomic DNA was extracted from young leaves according to the modified CTAB method of Vroh Bi *et al.* (1996). Purity and quantity of the genomic DNA samples were determined spectrophotometrically (Perkin-Elmer Lambda, EZ. 201) and confirmed using agarose gel electrophoresis. Only UV absorption ratios A_{260}/A_{280} = 1.8 - 2 were accepted. In order to test about 80 decamer oligonucleotides (TIB MOLBIOL syntheseLabor GmbH), bulk DNA sample was established by pooling 10 µl (50 ng) per extracted DNA samples together. From the 80 tested primers, 18 resulted in sharp and good banding patterns and were used for RAPD reactions on separate DNA samples. At the end of RAPD analysis, 15 primers were found to give suitable polymorphisms among testing olive genotypes with reproducible banding patterns (Table 2). Polymerase chain reactions (PCR) were performed in a total volume of 25 µl containing 10 ng of template DNA, 0.4 µM of a single decamer primer, 200 µM of each dNTPs, 1.75 mM $MgCl_2$ and 1U of Taq DNA polymerase including 1X reaction buffer. A control PCR reaction containing all components except for template DNA was included to check for DNA contamination. Amplifications were performed in a thermal cycler (Bio-Rad) programmed as follow: 94°C for 4 min, followed by 35 cycles of 92°C/1 min, 37°C/1 min, 72°C/2 min and a final extension of 72°C for 10 min. Amplified products were separated by gel electrophoresis in 1.2% agarose and TBE buffer. PCR products were detected by ethidium bromide staining and photographed under UV light, with a Gel Doc camera system (UVP). Molecular size of the amplified fragments was estimated using 1kb DNA ladder (CinnaGen, Iran).

Data Analysis

Each gel was analyzed by scoring the presence (1) or absence (0) of polymorphic bands in individual lanes. In the case of some primers, PCR reactions were repeated three times. Only bands clearly polymorphic and reproducible were selected as markers. The NTSYS -pc software was used to estimate genetic similarities with the Jaccard's similarity coefficient. The generated matrix of similarities was analyzed by the unweighted pair-group method with arithmetic average (UPGMA), using the sequential hierarchical agglomerative and nested clustering (SHAN) module. The COPH module was applied to compute a cophenetic value matrix using the UPGMA matrix. The MXCOMP module was then used to compute the cophenetic correlation.

Results

The RAPD profiles of the 33 studied genotypes using 15 random primers (Table 2) produced a total of 93 bands. However, only 34 reproducible polymorphic bands, 33.5% of total bands, ranging from 750bp to 2500bp were selected as informative RAPD markers. The amplification products obtained by primer BA03 are illustrated in Figure 1 representing typical RAPD banding patterns. On average, each primer produced 2.2 informative markers. The primer BA03 showed the highest level of polymorphism (7 informative bands) and was the most discriminating primer (77.7%). A single primer was not adequate to discriminate among all genotypes, therefore multiple primers were used to identify majority of the genotypes. According to the

Table 1. Cultivars and genotypes of olive included in this study, their code and country of origin

Genotypes	Code No.	Country of origin	Cultivars	Code No.	Country of origin
Unknown 1	1	Iran	Valonia	18	Spain
Unknown 2	2	Iran	Arbequina	19	Spain
Unknown 3	3	Iran	Frontio	20	Italy
Unknown 4	4	Iran	Kronieki	21	Greece
Unknown 5	5	Iran	Bleidi	22	Syria
Unknown 6	6	Iran	Fishomi	23	Iran
Unknown 7	7	Iran	Concervolia	24	Greece
Unknown 8	8	Iran	Dezful	25	Iran
Unknown 9	9	Iran	Roghani	26	Iran
Unknown 10	10	Iran	Mari	27	Iran
Unknown 11	11	Iran	Leccino	28	Italy
Unknown 12	12	Iran	Lechio	29	Italy
Unknown 13	13	Iran	Bakozeitun	30	Azerbaijan
Unknown 14	14	Iran	Zard	31	Iran
Unknown 15	15	Iran	Sevillana	32	Spain
Unknown 16	16	Iran	Manzanilla	33	Spain
Unknown 17	17	Iran			

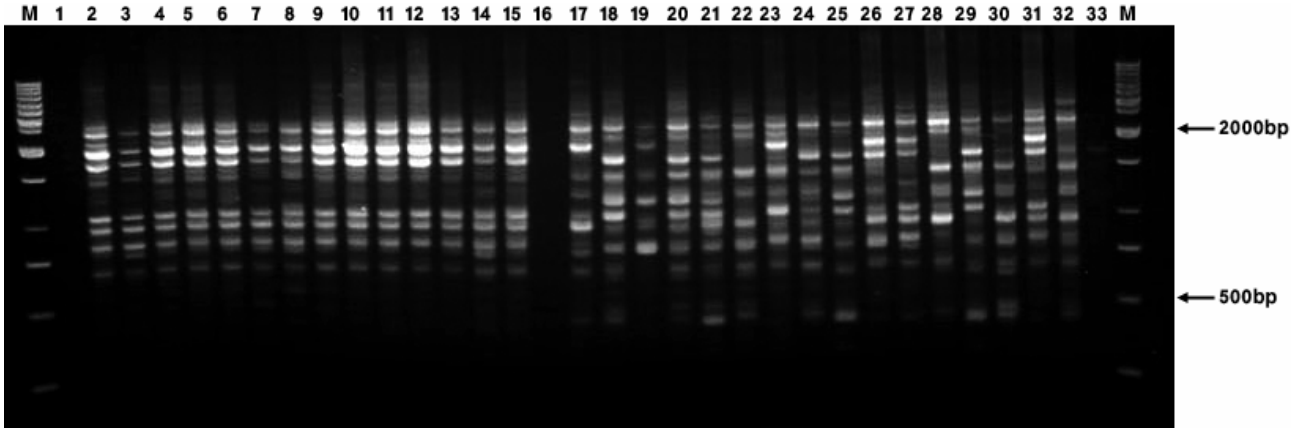

Figure 1. RAPD profiles of 33 olive genotypes amplified by 10 base primer BA03. Lanes: M, 1kb DNA ladder; 1-33 Olive genotypes listed in Table 1.

UPGMA dendrogram, based on the Jaccard's similarity coefficient, the cultivars were clustered into two main groups at 0.54 coefficients (Fig. 2). The co-phenetic correlation revealed a high degree of fit ($r = 0.94^{**}$) for the cluster analysis. Group A, included 23 genotypes, including all 17 unknown genotypes, and six (27%) native ones. Within this group and at a similarity coefficient of higher than 0.60 two subgroups (A1 and A2) were observed. Subgroup A1 was composed of 16 unknown genotypes clustered with the known native cultivar, 'Zard'. However, unknown genotype No.17 fitted within another subgroup besides cultivars 'Roghani' and 'Mari', two other native Iranian cultivars. Group B contained 10 cultivars, nearly all of foreign origin and cv. 'Dezful', a famous native Iranian cultivar, included in this group. Cultivars 'Manzanilla' and 'leccino' had less similarity with others and could be considered as a distinguished group. Principal coordinate ordination (PCO) of genotypes and cultivars is shown in Figure 3. The first and second dimensions explained 31.21% of the variation together. Addition of the third axis had no influence on grouping of the genotypes (data not shown). The maximum, minimum and average Jaccard's similarity coefficients among genotypes and cultivars were calculated from the pairwise marker data (Table 3). The value of the co-phenetic correlation was obtained nearly 0.94^{**} as a measure of the goodness of fit between dendrogram and the original similarity matrix.

Table 2. Selected primers and their sequence, number of polymorphic bands and percent of polymorphism on 33 olive genotypes

Primers	Sequence 5′→3′	No. of polymorphic fragments	Polymorphism (%)
BA03	GTGCGAGAAC	7	77.7
BA09	GGAACTCCAC	1	20
BA10	GGACGTTGAG	2	33.3
BA13	AGGGCGAATG	2	22.2
BA14	TCGGGAGTGG	2	50
BA20	GAGCGCTACC	1	14.28
BB05	GGGCCGAACA	4	28.57
BB07	GAAGGCTGGG	2	40
BB09	AGGCCGGTCA	3	60
BB10	ACTTGCCTGG	2	33.3
BC18	GTGAAGGAGG	2	18.8
BD09	CCACGGTCAG	3	50
BD17	GTTCGCTCCC	1	33.3
BD18	ACGCACACTC	4	66.6
BD20	AGGCGGCACA	2	66.6

Table 3. The maximum, minimum and average values of Jaccard's similarity coefficient among olive genotypes

Similarity coefficient	Olive genotypes		
	Native cvs.	Foreign cvs.	Unknown genotypes
Average	0.65	0.57	0.83
Maximum	0.85	0.80	0.97
Minimum	0.54	0.32	0.60

Discussion

Polymorphism and Identity

The frequency of polymorphism obtained was 38 markers/15 selected primers. This is higher than the ratio obtained in olive by Fabbri *et al.* (1995) of 47/40 and much lower than that reported by Wiesman *et al.* (1998) of 80/20. These 15 primers yielding 38 markers could reasonably discriminate all cultivars. Except for unknown genotypes, all of known genotypes were not closely related. A high discriminating ability of RAPD markers applied to olive species was also found by Wiesman *et al.* (1998). However, there is a difference between the rate of polymorphism yielded by primers in the Wiesman study (4 markers/primer) and present study (2.2 markers/primer). This could be explained by difference in the used primer sets, studied population and the criteria for selecting markers. The optimum number of primers needed to discriminate among genotypes depends on the level of genetic variability [9]. In present study because of preliminary experiments for selecting the 16 known different cultivars, only one primer, BA03 could discriminate nearly all of them. For other 17 unknown genotypes, which seemed to be two or at most three cultivars, only 2 or 3 primers among 15 used primers could classify them. Mekuria *et al.* (1999) found that six RAPD primers were adequate to distinguish 39 olive genotypes.

Grouping of Genotypes

The data obtained in the present study show a good fitness between the grouping of olive cultivars and their geographical origins for native and foreign ones. These results are in agreement with previous results for clustering cultivars according to their geographical origin based on isozyme markers and RAPD markers [12,14]. As indicated, only one native cultivar 'Dezful' was grouped with foreign cv. 'Lechio'. However, both of them were as a subgroup of native ones (group A). Based on this result, 'Dezful' and 'Lechio' at a similarity coefficient of 0.85 are close to each other. This finding can be interpreted as by renaming of 'Lechio' after interring at the main or first growing area as 'Dezful' by the native farmers or vise versa. It is noticeable that, Dezful is one of the major cities in sought of Iran. 'Lechio' and 'Dezful' cultivars have also a high degree of phenetic similarity. In Greece, over 170 cultivar names, mainly toponyms (place names) are in existence, although the number of true cultivars might be much smaller [9]. A 2-D PCO plot is useful to distinguish closely related individuals into groups as has been reported with RAPD data from plums [10] and olives [6]. The pattern shown in the biplot was comparable to the clustering of genotypes observed in the dendrogram. All unknown genotypes were clustered in native group (group A) separately from 16 known cultivars of foreign group (group B), with the exception of 'Lechio' which clustered in group A while as a foreign cultivar. The average similarity value across all unknown genotypes was 0.83. On the other hand, average similarity between unknown genotypes and 'Zard', a native olive cultivar, is 0.85; with minimum value of 0.63 corresponded to unknown genotype No. 17 and 'Zard'. These results are in agreement with results of dendrogram from the UPGMA cluster analysis. The average similarity value among foreign cultivars (0.57) was less than native ones (0.85) indicating the variation of original areas of foreign cultivars. However, minimum similarity coefficient (0.32) was observed between 'Manzanilla'

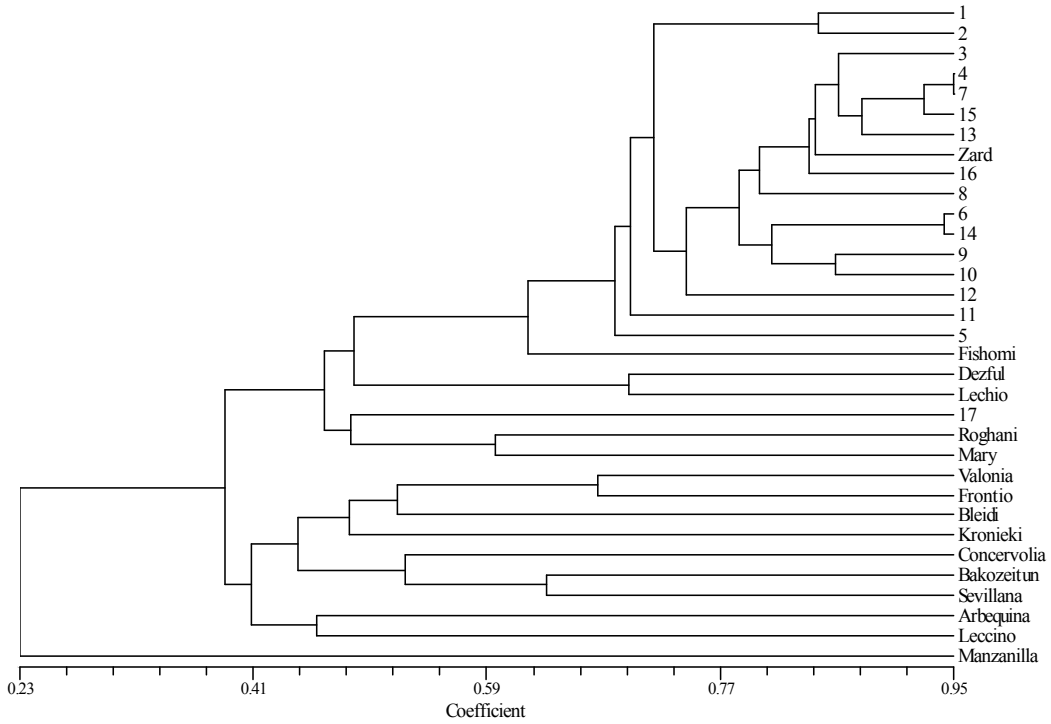

Figure 2. Dendrogram generated by UPGMA cluster analysis from the similarity matrix obtained by Jaccard's genetic distance for 33 olive genotypes and cultivars.

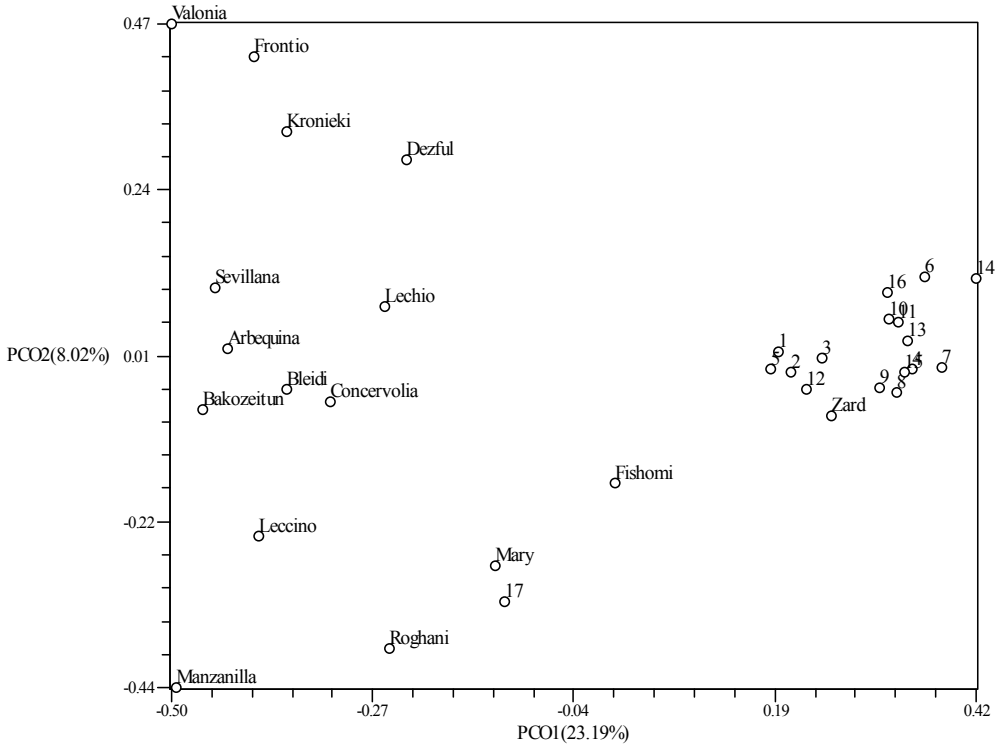

Figure 3. Principal coordinate ordination (PCO) of olive genotypes consisting of 17 unknown genotypes, 6 native (Iranian) and 10 foreign cultivars based on similarity coefficient from 38 RAPD bands.

and 'Valonia' originated from the same country (Spain). This can be explained regarding to this fact that the different geographical origin of plant materials within a country, influence the similarity value. As a result, by using RAPD markers, similarity of unknown genotypes to the known ones could be addressed. This result is in agreement with Burgher *et al.* (2002) who reported the use of RAPD markers to identify unknown genotypes of blueberry. Going towards comprehensive study and establishment of new modern orchards of olive in Iran, we have great problems about synonyms, homonyms and probably toponyms. In this experiment all unknown genotypes showed a high similarity with 'Zard' except genotype number 17 which had great similarity with 'Roghani' and 'Mary'. Cultivars 'Zard', 'Roghani' and 'Mary' are the most important Iranian olive cultivars. The close similarity of 16 from 17 unknown genotypes to the known cv. 'Zard' is confirming the more salt tolerance characteristic of 'Zard' cultivar. It is expectable that other cultivars, such as 'Roghani' were not enough salt tolerant and omitted within years from the population established at Qom highway place. This result is in agreement with Mosavi *et al.* (2004) who reported more salt tolerance of 'Zard' than 'Roghani' based on morphological and physiological studies. In conclusion, present study showed the high ability of RAPD markers for screening of olive genotypes. On the other hand, although the main goal of this study was the identification of unknown genotypes, the results confirmed the reports of Mosavi *et al.* (2004) and Soleimani *et al.* (2002) for more tolerance of 'Zard' to environmental stresses.

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