African Journal of Agricultural Research Vol. 5(15), pp. 2017-2022, 4 August, 2010
Available online at http://www.academicjournals.org/AJAR
ISSN 1991-637X ©2010 Academic Journals

Full Length Research Paper

Application of the RAPD technique and morphological characteristics in cultivated olives (*Olea europaea sativa*)

Meltem Sesli[1]*, E. Dilsat Yegenoglu[2] and Y. Gevrekci[3]

[1]College of Tobacco Expertise, Turkish Republic, Celal Bayar University, 45210, Akhisar, Manisa, Turkey.
[2]Akhisar Vocational College, Turkish Republic, Celal Bayar University, 45210, Akhisar, Manisa, Turkey.
[3]Faculty of Agriculture, Department of Animal Science, Turkish Republic, Ege University, 35100, Bornova, Izmir, Turkey.

Accepted 7 June, 2010

Five different morphological characteristics (width, length, width/length, stem length, stem thickness) were considered in 18 cultivated olives and discriminant analysis was applied. Using primers OP- I (1 - 20) and OP- Z (7, 8, 9, 10 and 11) in the same olives, evaluable bands were obtained from 12 primers out of 25. PCR analysis was repeated twice and those providing stable bands in the end of both analyses were evaluated. Genetic distances of cultivated olives were determined using neighbor-joining (Mega 4.1 program) method. SPSS 15.0 package program was used in the evaluation of morphological data of plants with the discriminant analysis; and in accordance with the discriminant results, while accurate estimations were made among the groups in olives, there had been misclassified estimations in some of them. The purpose of study is to determine the genetic distances of cultivated olives which economically important by applying Neighbor-joining method with the data obtained using RAPD analysis; and to classify the some morphological characteristics of same olives with the discriminant analysis.

Key words: Cultivated olives, RAPD, neighbor-joining method, discriminant analysis.

INTRODUCTION

Olea europaea sativa (cultivated olive) is cultivated in Turkey, having a rich potential for olive species, and used for oil production and consumption; and it is widely cultivated and has significant profits for the country's economy. In addition, Manzanilla, an important table olive type of Spain, is produced in the Aegean Region (MOARA, 1991). It is thus important to investigate the morphologic and genetic characteristics of this plant that is providing high profits. RAPD (Random amplified polymorphic DNA) is used being one of the dominant markers in determining the genetic relationship among wild and cultivated olives (Belaj et al., 2002; Lopes et al., 2009). In many studies, the data obtained in conclusion to RAPD analysis are determined by using Neighbor-joining method for identifying the genetic distances among the individuals (Joung et al., 2001; Mohammadi and Prasanna, 2003). After obtaining the RAPD profile, family trees are developed with phylogenetic analyses such as Neighbor-Joining by using different indexes for example Nei's genetic distance (Nei and Li, 1979). The Neighbor-Joining (NJ) method reconstructs phylogenetic trees from evolutionary distance data, under the principle of minimum evolution. This method provides both the topology and branch lengths of the phylogenetic tree. The Neighbor-Joining algorithm produces the correct unrooted tree (Saitou and Nei, 1987).

Canonic analysis is used to determine the optimum combination of variables (such as first function is most effective, second function is effective at second degree). In the analysis, the functions would be independent (orthogonal) that is; their contribution would not intersect with each other. In multi-discriminant functions, different functions are tested statistically and those found to be important are considered for further analysis. It is concluded that parameters with higher coefficients would have higher contribution (Kalaycı, 2005). Discriminant function analysis is a multivariate analysis of variance (MANOVA) method. In MANOVA, the independent

*Corresponding author. E-mail: meltem.sesli@bayar.edu.tr.

Table 1. Provinces where cultivated olives were supplied.

Type of olive	No. of sample	No. of leave	Place of supply	Province
Manzanilla	3	10	Olive production research institute	Izmir, Bornova, Turkiye
Gemlik	3	10	Olive production research institute	Izmir, Bornova, Turkiye
Domat	3	10	Olive production research institute	Izmir, Bornova, Turkiye
Memecik	3	10	Olive production research institute	Izmir, Bornova, Turkiye
Edremit	3	10	Sapling planters	Akhisar, Manisa, Turkiye
Uslu	3	10	Sapling planters	Akhisar, Manisa, Turkiye

variables are the groups and the dependent variables are the predictors. Main aim in the discriminant analysis is to determine whether the average of variable is different from the average of group and to search for the effect of that variable within the group (Kalaycı, 2005). Discriminant analysis uses continuous variable measurements on different groups of items to highlight aspects that distinguish the groups and to use these measurements to classify new items.

$$\lambda = \frac{|W|}{|T|} = \frac{|W|}{|W+B|}$$

If λ is found small it shows that there is a significantly important differences between groups. When the individuals are big in the groups in that way m=n-1-(p+k)/2 and using λ below test statistics is computed (Tatlıdil, 1996).

$$X^2 = -m\log(\Lambda) - X^2{}_{p(k-1);\alpha}$$

The model parameters are Wilk's Lambda, an index of the discriminating power ranging between 0 and 1 (lower means higher discriminating power), χ^2 is chi-square test statistics and F value indicates the statistical significance of a variable in the discrimination among the groups (Morrison, 1976). Coefficients of discriminant functions show the partial effect of each of variables on the discriminant function; and the structural coefficients show the simple correlation relation between each variable and discriminant function (Bektas and Hınıs, 2009). Discriminant analysis was also used in searching the relations between the RAPD markers and origin regions of varieties in the olives (Besnard et al., 2001).

MATERIALS AND METHODS

Plant material and DNA extraction

The cultivated olives were transferred to the glasshouse; young leaves were collected and stored in liquid nitrogen until DNA extraction. A total of 18 samples were extracted by Doyle and Doyle (1987) method (Table 1).

RAPD-PCR analysis

Twenty-five different decamer primers were used for RAPD analyses of *Olea europaea sativa*. A total of twenty-five primers from Kit OP-I (1-20) and OP- Z (7, 8, 9, 10, 11) (Operon Technologies, Alameda, CA, USA) were used for RAPD-PCR analysis. PCR was performed on an Eppendorf MasterCycler Thermal Cycler in a total volume of 25 µl. PCR mix including 25 ng template DNA, 2.42 µl. 10 X PCR reaction buffer (with $MgCl_2$, Sigma), 0.44 µl. dNTP (Sigma), 1 µM primer, and 0.13 µl Taq DNA polymerase (Sigma). The amplification reactions were carried out for 60 sec at 94ºC as an initial denaturation. The PCR program comprised 35 cycles with 20 sec at 94ºC; 20 sec at 35ºC; 30 s at 72ºC and a final extension performed at 72ºC for 5 min.

Amplification products were loaded onto 1.5% agarose gels (Sigma) in 0.5 x TBE buffer with 0.5 µg/ml ethidium bromide at a constant 100 V. For evaluating the base pair length of bands, a DNA ladder (Fermentas) was loaded on the first lane of each gel. After the separation of PCR products by agarose gel electrophoresis, gels were visualized with the Photo Print (Vilber Lourmat, France) imaging system and analyzed by BioOne D++ software (Vilber Lourmat, France).

Data analysis

The RAPD bands (markers) scored as 1 if present and 0 if absence. PCR analyses were duplicated. Only clear and reproducible bands were used for binary data matrix. The RAPD data was used to compute the genetic distances of cultivated olives according to Nei's distance index and MEGA ver. 4.1 (Molecular Evolutionary Genetics Analysis) (Tamura et al., 2007) was used to construct a Neighbor-Joining dendrogram (Saitou and Nei, 1987). Ten leaves were collected from each sample and discriminant analysis for the evaluation of the 5 different morphological features (Width, height, aspect, stem length, stem thickness) SPSS 15.0 for Windows package program was used (SPSS, 2006).

RESULTS AND DISCUSSION

Random amplification of polymorphic DNA (RAPD-PCR)

Twenty-five primers were used in the study and 12 of the primers of OP-I 1, 2, 3, 4, 7, 8, 9, 10, 11, 12 and OP-Z 9, 10 yielded scorable bands. A distance matrix was constructed using the Nei (1972) distance index (Table 2) and Neighbor-Joining analysis was done with Mega version 4.1 (Figure 1), dendrogram of cultivated olives based on RAPD polymorphism. According to this matrix, genetic distance values were found to be between 0.0364

Table 2. Distance matrix (Nei, 1972) of the cultivated olives.

	Gemlik 1	Gemlik 2	Gemlik 3	Manzanila 1	Manzanila 2	Manzanila 3	Domat 1	Domat 2	Domat 3	Memecik 1	Memecik 2	Memecik 3	Edremit 1	Edremit 2	Edremit 3	Uslu 1	Uslu 2	Uslu 3
Gemlik 1	*****																	
Gemlik 2	0.337	*****																
Gemlik 3	0.288	0.388	*****															
Manzanila 1	0.388	0.624	0.241	*****														
Manzanila 2	0.337	0.442	0.624	0.499	*****													
Manzanila 3	0.337	0.560	0.767	0.499	0.337	*****												
Domat 1	0.288	0.499	0.560	0.442	0.499	0.288	*****											
Domat 2	0.154	0.337	0.388	0.388	0.337	0.442	0.197	****										
Domat 3	0.154	0.337	0.388	0.388	0.337	0.442	0.197	0.000	*****									
Memecik 1	0.154	0.442	0.388	0.388	0.337	0.337	0.197	0.154	0.154	*****								
Memecik 2	0.113	0.388	0.337	0.337	0.288	0.388	0.241	0.113	0.113	0.113	*****							
Memecik 3	0.241	0.560	0.499	0.499	0.442	0.442	0.288	0.241	0.241	0.074	0.113	*****						
Edremit 1	0.113	0.388	0.337	0.337	0.288	0.388	0.241	0.113	0.113	0.036	0.074	0.113	*****					
Edremit 2	0.113	0.388	0.337	0.337	0.288	0.388	0.241	0.113	0.113	0.113	0.074	0.197	0.074	*****				
Edremit 3	0.241	0.560	0.499	0.499	0.442	0.560	0.388	0.241	0.241	0.154	0.197	0.154	0.113	0.113	****			
Uslu 1	0.113	0.388	0.337	0.337	0.288	0.388	0.241	0.113	0.113	0.113	0.074	0.197	0.074	0.074	0.197	****		
Uslu 2	0.113	0.388	0.337	0.337	0.288	0.388	0.241	0.113	0.113	0.113	0.074	0.197	0.074	0.074	0.197	0.074	****	
Uslu 3	0.241	0.560	0.499	0.499	0.442	0.560	0.288	0.241	0.241	0.241	0.197	0.337	0.197	0.197	0.241	0.113	0.113	****

and 0.6931. Hereunder, it was determined that the samples closest to each other based on their genetic distance values are Edremit 1 and Memecik1, and the samples most distant from each other are Manzanilla 3 and Gemlik 3. Sesli and Yegenoglu (2010) determined the genetic distances of wild olives by using one of the most widely used algorithms Neighbor-Joining for constructing dendrograms from a distance matrix

Morphological characteristics

Least square means ($\bar{x}$) of olive groups for 5 different morphological features are given in Table 3 and Table 4 gives discriminant test statistics as Wilks' Lambda, Chi square (χ^2) and probability (F) values. Wilk's Lambda statistics show the portion (rate) of total variance in separation scores that can not be defined by the differences among groups. All of the test functions were found significant ($p<0.05$). Table 5 shows the distribution of individuals per groups through discriminant analysis. All of the 18 samples in total, including 3 from each cultivar, were included in their respective groups. It has been determined as a result of discriminant analysis applied to the samples that 100% of such samples were included in their respective groups. It has been determined that the samples of cultivars used in the study are separated from each other with clear limits in terms of their morphologic values and that there was no overlapping. It has been observed that the discriminating performance of discriminant functions was high and that there was a high variation among the cultivars in terms of morphologic values. Güler et al. (1999), in their study in which they examined the relations in honey bee ecotypes in terms of morphologic characteristics, determined that such ecotypes were separated from each other with clear limits and that there was no overlapping. As a result, they pointed out that the discriminating performance of discriminant functions was high and that there was a high variation morphologically within the population.

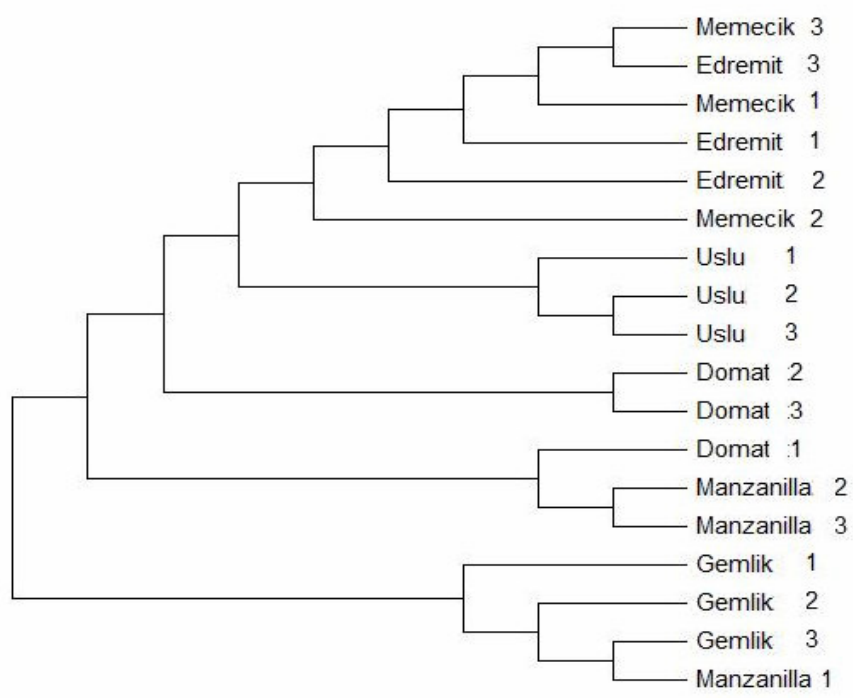

Figure 1. Dendrogram of cultivated olives based on RAPD polymorphism. Neighbor-Joining cluster analysis.

Table 3. Mean values and standard deviations of characteristics based on the groups.

	The mean values ($\bar{x}$) and standard deviations of characteristics				
Groups	**Width**	**Length**	**Width / Length**	**Stem length**	**Stem thickness**
Edremit	62.53 ± 0.011	12.18 ± 0.00425	5.29 ± 0.0017	4.47 ± 0.01	1.32 ± 0.0035
Domat	68.52 ± 0.011	11.83 ± 0.00425	5.62 ± 0.0017	3.97 ± 0.01	1.30 ± 0.0035
Gemlik	50.65 ± 0.011	11.83 ± 0.00425	4.28 ± 0.0017	3.38 ± 0.01	1.01 ± 0.0035
Manzanilla	57.66 ± 0.011	14.84 ± 0.00425	3.88 ± 0.0017	4.96 ± 0.01	1.28 ± 0.0035
Memecik	53.66 ± 0.011	10.83 ± 0.00425	4.95 ± 0.0017	3.89 ± 0.01	1.01 ± 0.0035
Uslu	59.35 ± 0.011	12.38 ± 0.00425	4.79 ± 0.0017	3.88 ± 0.01	1.01 ± 0.0035

Table 4. Test functions of discriminant analysis.

Test functions	**Wilks' Lambda**	χ^2	**F**
Between width-stem thickness	0.000	4551.38	0.000*
Between length-stem thickness	0.000	3001.88	0.000*
Between width/length rate-stem thickness	0.000	1640.22	0.000*
Between stem length-stem thickness	0.008	814.97	0.000*
Stem thickness	0.122	352.97	0.000*

*Significant at level P<0.05.

For the purpose of determining which characteristics caused the differences among groups, the Canonical Discriminant Analysis was applied. Canonical discriminant functions were calculated for the five characteristics considered in the study; and the Eigenvalues, percentage of variance, cumulative variance, and canonical correlation values of 5 canonical functions are summarized in Tables 6 and 7. It was determined that first of the functions analyzed had 74.6% of total variance; second had 24.3% of the same; third had 1%; fourth had 0.1%; and

Table 5. Results of discriminant analysis classification showing the number of specimens classified in each group.

Groups	Sample	Edremit	Domat	Gemlik	Manzanilla	Memecik	Uslu
Edremit	3	3	0	0	0	0	0
Domat	3	0	3	0	0	0	0
Gemlik	3	0	0	3	0	0	0
Manzanilla	3	0	0	0	3	0	0
Memecik	3	0	0	0	0	3	0
Uslu	3	0	0	0	0	0	3

Table 6. Canonical discriminant function coefficients.

	Functions				
	1	2	3	4	5
Width	16.874 *	-3.736	-20.651	-9.255	-8.317
Length	-6.380	60.113 *	83.419	46.853	38.492
Width length rate	-.825	43.152 *	254.001	127.560	103.581
Stem length	-.580	1.139	8.043	-5.224	-14.915
Stem thickness	-1.327	-13.586	9.833	-48.449 *	20.168
(Constant)	-904.564	-717.072	-1078.594	-568.573	-445.292 *

*Effective on functions.

Table 7. Eigenvalues statistics.

Function	Eigenvalue ([a])	Percentage of variance	Cumulative (%)	Canonical correlation
1	10411.366	74.6	74.6	1.000
2	3391.330	24.3	98.9	1.000
3	136.950	1.0	99.8	0.996
4	14.772	0.1	99.9	0.968
5	7.226	0.1	100.0	0.937

First 5 canonical discriminant functions were used in the analysis.

fifth had 0.1% of the same. As it is shown in Table 7, first two functions formed 98.9% of the total variation. It has been determined that width was effective of the first function; length and width/length was effective on the second function; stem length on the fourth function; and stem thickness on the fifth function. The canonic correlation was found as 1 in first function, 0.992 in second function, 0.341 in 3rd function, 0.275 in 4th function and 0.239 in 5 th function. In addition, as high as the Eigenvalue is found (higher than 0.40), so it shows that large part of the variance in dependent variable can be defined by the function (Kalaycı, 2005). In the classification performed through discriminant analysis, although the number of discriminant functions was taken as five in the beginning, it has been defined that the first two discriminant functions could demonstrate 98.9% of total variance. It has been determined that the width was a significant variable on the first function; whereas length, width/length on the second function. It is considered that such three characteristics can make discrimination in order to define the olive cultivars morphologically and to determine variation. It has been concluded that morphologic characteristics can be used in a sufficient way in order to define the differences among olive cultivars as a result of discriminant analysis. Width, length and width/length characteristics can be used in the discrimination of cultivars. In addition, since the morphologic characteristics are affected from environmental conditions, the studies to discriminate the olive types should be supported with DNA based marker analyses providing information on directly the genotype and not being affected from environmental conditions. Thus, the differences among olives may be examined in a more reliable way in terms of genetic and morphologic characters.

ACKNOWLEDGEMENTS

This study was supported by the Turkish Republic State

Planning Organization; the authors would like to thank the organization.

REFERENCES

Belaj A Stovic Z, Rallo L, Trujillo I (2002). Genetic diversity and relationships in olive (*Olea europaea* L.) germplasm collections as determined by randomly amplified polymorphic DNA. Theor. Appl. Genet., 105: 638-644.

Bektaş S, Hınıs MA (2009). Factors Affecting Seat Belt Use Predictive Model for Automobile Drivers. J. Sci. Technol. Inst. Erciyes Univ., 25(1-2): 208-222.

Besnard G, Baradat P, Bervillé A (2001). Genetic relationships in the olive (*Olea europaea* L.) reflect multilocal selection of cultivars. Theor. Appl. Genet., 102: 251-258.

Doyle JJ, Doyle JL (1987). A rapid DNA isolation procedure for small quantities of fresh leaf tissue. Phytochem. Bull., 19: 11-15.

Güler A, Kaftanoğlu O, Bek Y, Yeninar H (1999). Discrimination of some Anatolian Honeybee (*Apis mellifera* L.) Races and Ecotypes by Using Morphological Charachteristics. Trop. J. Vet. Ani. Sci., 23: 337-343.

Joung YH, Roh MS, Bentz SE (2001). International Symposium on Molecular Markers for Characterizing Genotypes and Identifying Cultivars in Horticulture. Characterization of *Acer griseum* and Its Putative Interspecific Hybrids. ISHS Acta Horticulturae, 1: 546.

Kalaycı Ş (2005). Discriminant Analysis. In: SPSS Applied Multivariate Statistical Techniques, Ankara.

Lopes PM, Gomes S, Brito JL, Lopes J, Pinto HG (2009). Assessment of clonal genetic variability in *Olea europaea* L. 'Cobrançosa' by molecular markers. Sci. Hort. 123 82-89.

MOARA (1991). Standard Olive Varieties Catalogue. Ministry of Agriculture and Rural Affairs Publishing, Ankara. p.13.

Mohammadı SA, Prasanna BM (2003). Review & Interpretation Analysis of Genetic Diversity in Crop Plants—Salient Statistical Tools and Considerations. Crop Sci., 43:1235-1248.

Morrison DF (1976). Discriminant Analysis. In: Multivariate statistical methods. Tokyo: McGraw-Hill. p. 415.

Nei M (1972). Nei's Original measures of genetic identity and genetic distance. Am. Nat., 106: 283-292.

Nei M, Li WH (1979). Mathematical models for studying genetic variation in terms of restriction endonucleases. Proc. Nat. Acad. Sci., 76: 5269-5273.

Saitou N, Nei M (1987). The neighbor-joining method: A new method for reconstructing phylogenetic trees. Mol. Biol. Evol., 4: 406-425.

Sesli M, Yeğenoğlu ED (2010). Determination of the genetic relationships between wild olives (*Olea europaea oleaster*) varieties grown in Aegean region. Genet. Mol. Res., 9(2): 884-890.

SPSS, 2006. SPSS For Windows Evaluation Version.

Tamura K, Dudley J, Nei M, Kumar S (2007) MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. Mol. Biol. Evol., 24: 1596-1599.

Tatlıdil H (1996). Discriminant Analysis. In: Applied Multivariate Statistical Analysis, Ankara, 1: 256.