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Genetic diversity and relationships in olive (*Olea europaea* L.) germplasm collections as determined by randomly amplified polymorphic DNA

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Abstract Genetic diversity studies using the RAPD technique were carried out in a set of 103 olive cultivars from the World Germplasm Bank of the Centro de Investigación y Formación Agraria (CIFA) "Alameda del Obispo" in Cordoba (Spain). A total of 126 polymorphisms (6.0 polymorphic markers per primer) out of 135 reproducible products (6.4 fragments per primer) were obtained from the 21 primers used. The number of bands per primer ranged from 4 to 11, whereas the number of polymorphic bands ranged from 3 to 10, corresponding to 83% of the amplification products. The dendrogram based on unweighted pair-group cluster analysis using Jaccard's index includes three major groups according to their origin: (1) cultivars from the Eastern and Central Mediterranean areas, (2) some Italian and Spanish cultivars, and (3) cultivars from the Western Mediterranean zone. The pattern of genetic variation among olive cultivars from three different Mediterranean zones (West, Centre and East) was analysed by means of the analysis of molecular variance (AMOVA). Although most of the genetic diversity was attributable to differences of cultivars within Mediterranean zones (96.86%) significant ϕ-values among zones ($\phi_{st} = 0.031$; $p < 0.001$) suggested the existence of phenotypic differentiation. Furthermore, the AMOVA analysis was used to partition the phenotypic variation of Spain, Italy (Western region), Greece and Turkey (Eastern region) into four categories: among regions, among countries (within regions), within countries, and among and within countries of each region. Most of the genetic diversity was attributable to differences among genotypes within a country. These results are consistent with the predominantly allogamous nature of *Olea europaea* L. species. This paper indicates the importance of the study of the amount and distribution of genetic diversity for a better exploration of olive genetic resources and the design of plant breeding programmes.

Keywords *Olea europaea* L. · Genetic diversity · RAPD · AMOVA · Germplasm bank

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Introduction

Olive (*Olea europaea* L.) is a very important oil-producing crop in the Mediterranean area. The origin of olive probably goes back to the Eastern region of the Mediterranean sea, over a thousand years ago, from where it spread around the basin (Zohary and Hopf 1994). Cultivar intercrossing and crosses between wild and cultivated forms, along with local selection of outstanding seedlings and subsequent olive cloning, could have led to a large number of varieties around their possible original areas of cultivation (Barranco 1997).

Knowledge of genetic diversity among existing olive cultivars is essential for the long-term success of breeding programs and maximises the exploitation of the germplasm resources. Traditionally, the variability of *O. europaea* L. has been evaluated by morphological methods (Barranco et al. 2000). Genetic diversity studies with enzymes (Loukas and Krimbas 1983; Ouazzani et al. 1993; Trujillo et al. 1995), RAPD markers (Fabbri et al. 1995; Mekuria et al. 1999; Besnard and Bervillé 2000; Belaj et al. 2001; Besnard et al. 2001; Sanz-Cortés et al. 2001), AFLP (Angiolillo et al. 1999) and chloroplast DNA markers (Amane et al. 1999, 2000; Lumaret et al. 2000) have been used to determine the genetic relationships among cultivated olive, wild forms and related species. Recently SSR markers are becoming the markers of choice for variability studies in olive (Rallo et al. 2000; Cipriani et al. 2002).

A previous knowledge of the structure of genetic diversity within the World Germplasm Bank of Cordoba

Table 1 Olive cultivars analysed including their register number (R.N.) and the countries of origin

Cultivar	R.N.	Origin	Cultivar	R.N.	Origin
Abu Satel Echlot	1040	Syria	Kalamon	105	Greece
Adramitini	102	Greece	Kalinjot	1171	Albania
Alfafara	605	Spain	Kan Çelebi	788	Turkey
Aloreña	829	Spain	Kiraz	679	Turkey
Amigdaloia	228	Greece	Konservolia	219	Greece
Amigdaloia Nana	696	Greece	Koroneiki	218	Greece
Arauco	833	Argentina	Lastovska	704	ExYugoslavia
Arbequina	231	Spain	Leccino	82	Italy
Ascolana Tenera	62	Italy	Leccio del Corno	83	Italy
Ayrouni	134	Lebanon	Lechín de Granada	54	Spain
Ayvalik	97	Turkey	Lechín de Sevilla	5	Spain
Barnea	711	Israel	Lucques	322	France
Beyaz Yaglik	683	Turkey	Manzanilla Cacereña	430	Spain
Blanqueta	11	Spain	Manzanilla de Sevilla	21	Spain
Bouteillan	63	France	Megaritiki	108	Greece
Buga	733	ExYugoslavia	Memeçik	93	Turkey
Cakir	96	Turkey	Menara	836	Morocco
Cañivano Blanco	52	Spain	Merhavia	102	Israel
Carolea	736	Italy	Meski	115	Tunisia
Carrasquenha	125	Portugal	Mixani	1079	Albania
Castellana	576	Spain	Moraiolo	78	Italy
Cellina	179	Italy	Morisca	955	Spain
Chalkidiki	220	Greece	Morrut	224	Spain
Changlot Real	15	Spain	Nabali	158	Palestine
Chemlali	744	Tunisia	Negrinha	123	Portugal
Chemlal de Kabylie	118	Algeria	Oblica	706	ExYugoslavia
Chetoui	113	Tunisia	Ouslati	114	Tunisia
Cobrançosa	124	Portugal	Picholine	70	France
Coratina	79	Italy	Picholine Marocaine	101	Morocco
Cordovil de Serpa	131	Portugal	Picual	9	Spain
Cornicabra	10	Spain	Picudo	3	Spain
Crnica	734	ExYugoslavia	Redondil	127	Portugal
Daebli	1044	Syria	Rosciola	88	Italy
Dam	1003	Syria	Salonenque	73	France
Domat	94	Turkey	Santa Caterina	72	Italy
Elmaçik	686	Turkey	Sevillenca	227	Spain
Empeltre	13	Spain	Sigoise	119	Algeria
Farga	12	Spain	Souri	858	Lebanon
Frantoio	80	Italy	Tanche	74	France
Galega	128	Portugal	Toffahi	721	Egypt
Gemlik	92	Turkey	U. Bardhëi Tiranës	1082	Albania
Gerboui	538	Tunisia	Uslu	95	Turkey
Gordal Sevillana	234	Spain	Valanolia	103	Greece
Grappolo	181	Italy	Vera	660	Spain
Hamed	722	Egypt	Verdale	76	France
Haouzia	835	Morocco	Verdial de Badajoz	988	Spain
Hojiblanca	2	Spain	Verdial de Huevar	6	Spain
Istarska Belica	735	ExYugoslavia	Verdial de Velez Málaga	883	Spain
Itrana	68	Italy	Villalonga	364	Spain
Izmir Sofralik	99	Turkey	Zaity	788	Syria
K. M. Berat	1080	Albania	Zalmati	117	Tunisia
Kaissy	975	Syria			

may be of great help to make decisions on management procedures, as well as on breeding strategies to use in current and future breeding programs.

The objectives of the present paper were to: (1) describe the RAPD diversity among olive cultivars of a world-wide germplasm collection, (2) determine the similarity of the cultivars within the collection, (3) study patterns of variation shown by olive cultivars from different Mediterranean zones and regions, and (4) use the information for the olive breeding program as well as to devise sampling strategies for future collection of olive germplasm.

Materials and methods

Plant material and DNA extraction

One hundred and three cultivars of olive from the World Germplasm Bank of the CIFA "Alameda del Obispo" in Cordoba, Spain, were studied (Table 1). The countries of origin of these cultivars were: Albania (4), Algeria (2), Argentina (1), Egypt (2), Ex-Yugoslavia (5), France (6), Greece (9), Israel (2), Italy (12), Lebanon (2), Morocco (3), Palestine (1), Portugal (6), Syria (5), Spain (26), Tunisia (6) and Turkey (11).

Genomic DNA was extracted from young leaf tissue collected in the spring of 1998, following the procedure of Belaj et al. (2001).

Polymerase chain reaction

RAPD amplifications were performed as described by Belaj et al. (2001). All the reactions were conducted three times, using DNA of various extractions and different lots of the Ampli*Taq* DNA polymerase. The amplification products were separated on polyacrylamide gels of 18 × 16 cm containing 10% acrylamide, 0.126% piperazine diacrylamide crosslinker in 0.375 M Tris–HCl, pH 8.8, using Tris glycine (0.025 M Tris, and 0.192 M glycine) and were visualised by silver staining as described by Bassam et al. (1991). Thirty primers from kits A, F, I, J, K, P, Q, X and Z (Operon Technology, Alameda, Calif., USA) were used in the study.

Data analysis

RAPD bands were scored as 1 (present) or 0 (absent) in a binary matrix for each primer. A conservative criterion for the selection of bands was used. Only reproducible and well-defined bands in each of the three replications were considered as potential polymorphic markers.

Jaccard's (1908) coefficient of similarity was calculated, and the cultivars were grouped by cluster analysis using the unweighted pair-group method (UPGMA). The computer program employed was NTSYS-pc version 2.02 (Rohlf 1998). The cophenetic correlation coefficient was calculated, and Mantel's test (Mantel 1967) was performed to check the goodness of fit of a cluster analysis for the matrix on which it was based.

The Analyses of Molecular Variance (AMOVA; Excoffier et al. 1992) were carried out on the RAPD data using the WINAMOVA 1.55 program (Excoffier 1992). A matrix of Jaccard distances between RAPD phenotypes was constructed, and the AMOVA variance components were used as estimates of molecular diversity at each hierarchical level. Three Mediterranean zones were established considering the countries of origin of the cultivars: (1) East Mediterranean (Egypt, Israel, Lebanon, Palestine, Syria and Turkey), (2) Central Mediterranean (Albania, Algeria, Ex-Yugoslavia, Greece, Italy and Tunisia) and (3) West Mediterranean (France, Morocco, Spain and Portugal). The variance among and within these Mediterranean zones was calculated by using a one-way AMOVA. The selection of four Mediterranean countries was made considering different criteria: their representativeness, geographical origin and relative economic importance in the industry of the olive fruits in the world. These were: Spain, Italy (here contained in the Western Mediterranean region), Greece and Turkey (here contained in the Eastern Mediterranean region). A two-way nested AMOVA was performed for the study of the partitioning of RAPD variation at each hierarchical level. Significance levels of the variance were obtained with tests including 1,000 permutations for each analysis.

The AMOVA procedure provides an estimate, ϕst, of population differentiation, which is equivalent to a *F*-st statistic when the degree of relatedness among the genetic variants is evaluated. To obtain a distance matrix, ϕst values between any two populations were interpreted as the inter-population distance average between any two populations (Huff 1997; Gustine and Huff 1999).

Homogeneity of intrapopulation molecular variances (homoscedasticity) was tested using the HOMOVA procedure (Barlett's test) also implemented in WINAMOVA (Stewart and Excoffier 1996). Barlett's statistics (Barlett 1937) null distributions were obtained after 1,000 permutations.

Results

Variability study with RAPD markers

Twenty one out of thirty decamer oligonucleotide primers were used for the RAPD fingerprinting of the 103 olive genotypes. A total of 126 polymorphisms (6.0 polymorphic markers per primer) out of 135 reproducible products (6.4 fragments per primer) were obtained, corresponding to 93% of the amplification products. Amplified DNA fragments ranged in length from 126 (OPI-12) to 964 (OPX-03) bp. The number of bands per primer ranged from 4 (OPA-01) to 11 (OPX-09), whereas the number of polymorphic bands per primer ranged from 3 (OPZ-07) to 10 (OPA-19; OPK-16; OPX-09).

Genetic relationships among olive cultivars

A relatively high range of similarity values among genotypes (data not shown) was observed, with most similarity measures falling between 0.38 and 0.70. A few pairs of cultivars ('Cakir-Valanolia'; 'Gordal Sevillana'-'Santa Caterina'; 'Cañivano Blanco'-'Picholine Marocaine'; 'Manzanilla de Sevilla'-'Redondil'; 'Manzanilla Cacereña'-'Negrinha'; 'Grappolo'-'Leccio del Corno'; and 'Cellina'-'Frantoio') clustered together at similarity values of 1. Apart from that, the greatest similarity (0.84) was observed between the cultivars 'Sigoise' and 'Menara'. The lowest similarity values were obtained between 'Redondil' and 'Grappolo' (0.18) and 'Santa Caterina' and 'Cellina' (0.20). The cophenetic correlation coefficient between the dendrogram and the original distance matrix was significant but relatively low ($r = 0.61$; $p < 0.01$).

Figure 1 displays the dendrogram constructed on Jaccard's distance. Thirteen clusters were defined. Most of these clusters could be classified into three main groups: group I (clusters 01 to 05), group II (cluster 06) and group III (clusters 07 to 09). The rest of the cultivars formed independent subgroups. Evidence of relationships for most of the cultivars according to their geographic origin was found.

Group I is constituted mainly by cultivars from the Eastern (Egypt, Israel, Lebanon, Syria and Turkey) and Central (Albania, Ex-Yugoslavia, Greece, Italy and Tunisia) Mediterranean zone. For instance, cluster 1 includes two Syrian cultivars ('Abu Satel Echlot' and 'Daebli'), three Turkish cultivars ('Ayvalik', 'Beyaz Yaglik' and 'Domat'), one Greek cultivar ('Adramitini') and one Albanian cultivar ('Kalinjot').

It seems interesting to mention that all French cultivars except 'Tanche' were grouped in different clusters of group I.

Group II was formed by just cluster 06. Three Italian cultivars ('Leccino', 'Moraiolo' and 'Rosciola') and three Spanish cultivars ('Alfafara', 'Hojiblanca' and 'Empeltre') clustered together.

Group III (cluster 07 to 09) was composed mainly by Western varieties (Morocco, Spain and Portugal) with some exceptions such as the cultivars 'Sigoise' (Algeria), 'Nabali' (Palestine), 'Ayrouni' (Lebanon), 'Buga' and 'Crnica' (Ex-Yugoslavia), and 'Coratina' (Italy).

The four subgroups that clustered independently (clusters 10 to 13) did not show a clear structure for the geographic origin with some exceptions.

Several pairs of cultivars represent some small groups relatively unrelated to the main clusters of the dendro-

Table 2 AMOVA and HOMOVA analysis for the partitioning of RAPD variation in olive varieties among and within Mediterranenan zones (Western/Central/Eastern)

Source of variation	*df*	Variance components	% Total variance	ϕ-Statistics	*p*-value	Bartlett's index	*p*-value
Among zones	2	0.009	3.14	0.031	<0.001	0.050	0.068
Within zones	99	0.265	96.86				

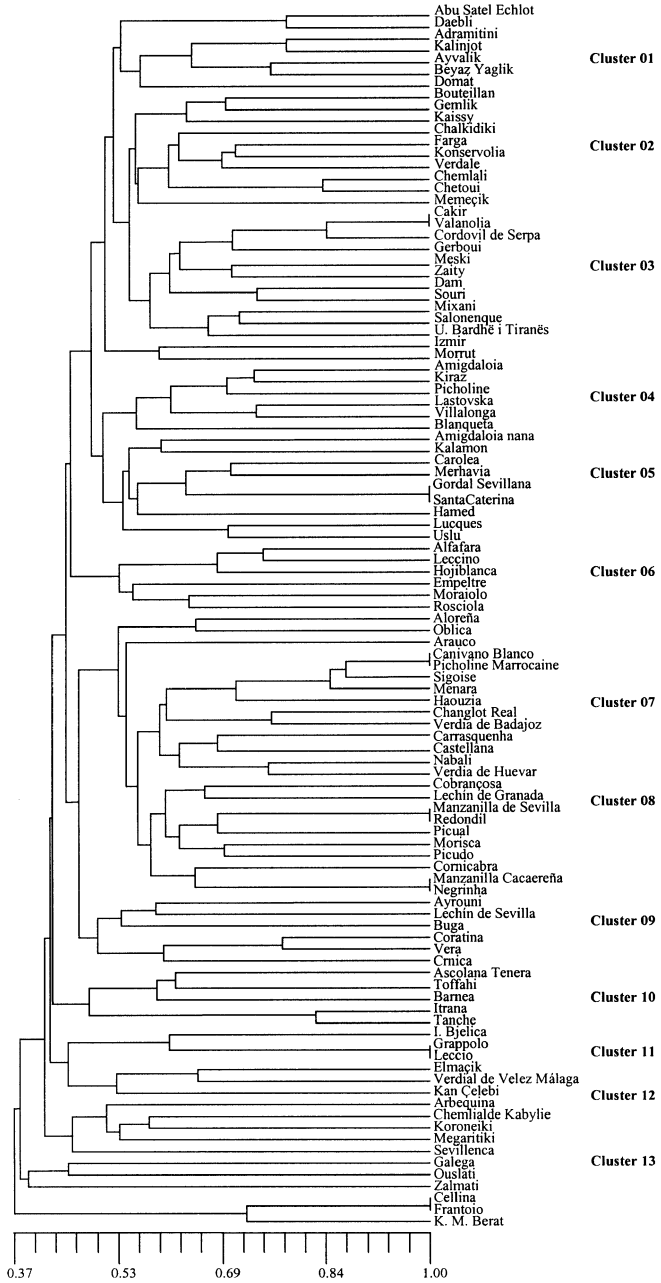

Fig. 1 A UPGMA dendrogram based on the Jaccard similarity index among 103 olive cultivars for 135 RAPD fragments

gram. This was the case for: 'Izmir Sofralik' and 'Morrut' which branched to the subgroup formed by the first three clusters of Group I, 'Aloreña' and 'Oblica' as well as the Argentinean cultivar 'Arauco', which clustered independently to the subgroup formed by clusters 8 and 9. And finally, the Italian cultivar 'Frantoio' clustered with the Albanian 'K. M. Berati'.

Table 3 Interregional distance matrix ϕ_{st} among three Mediterranean zones. Lower matrix diagonal: ϕ_{st} value = proportion of the total variance that is partitioned between two regions. Upper matrix diagonal: corresponding *p* values

Region	Western	Central	Eastern
Western		0.013	0.000
Central	0.018		0.000
Eastern	0.043	0.033	

AMOVA analysis

Hierarchical analysis of RAPD phenotypic diversity using AMOVA was performed to analyse the partition of RAPD variation in olive varieties among and within Mediterranean zones (Table 2). Although most of the genetic diversity was attributable to differences among cultivars within Mediterranean zones (96.86%), significant ϕ-valuesamong zones (ϕ_{st} = 0.031; $p < 0.001$) suggested the existence of phenotypic differentiation. Corresponding HOMOVA analysis revealed that the molecular variances were homogeneous among zones (B_p = 0.050, p = 0.068). ϕ_{st} values between each pair of Mediterranean zones were significant in all cases (Table 3).

The variance among regions (Eastern vs Western), among countries (within regions) and cultivars within countries were calculated from the corresponding RAPD matrix (53 cultivars) by means of hierarchical analysis of diversity using a two-way nested AMOVA analysis (Table 4).

The proportion of the total diversity found within countries was 93.33%, leaving only 6.77% of the diversity unequally distributed between regions (5.46%) and among countries/within regions (1.21%). However, differences between the Eastern and Western region were significant (ϕ_{st} = 0.055; $p < 0.001$).

For the within-Western region analysis, all the molecular variability could be attributed to the within-countries component (96.92%) since the among-countries component of variability was not significant (ϕ_{st} = 0.031; p = 0.063).

The same tendency, but more pronounced, was observed within the Eastern region where the proportion of diversity attributable to differences among countries had

Table 4 AMOVA and HOMOVA analysis for the partitioning of RAPD variation among regions (Western vs Eastern), among countries (within regions) and within countries as well as among and within countries of each of the two regions

Source of variation	*df*	Variance components	% Total variance	ϕ-Statistics	*p*-value	Bartlett's index	*p*-value
Among regions	1	0.016	5.46	0.055	<0.001	0.013	0.196
Among countries/within regions	2	0.004	1.21	0.013	0.176	0.017	0.611
Within countries	49	0.271	93.33	0.067	<0.001		
Among countries (Western)	1	0.009	3.08	0.031	0.063	0.009	0.436
Within countries	32	0.274	96.92				
Among countries (Eastern)	1	–0.004	–1.32	–0.013	0.622	0.003	0.622
Within countries	17	0.267	101.32				

Table 5 Distance matrix ϕ_{st} for four countries. Lower matrix diagonal: ϕ_{st} value = proportion of the total variance that is partitioned between two countries. Upper matrix diagonal: corresponding *p* values

Population	Spain	Italy	Greece	Turkey
Spain		0.031	0.000	0.000
Italy	0.031		0.000	0.000
Greece	0.065	0.072		0.825
Turkey	0.059	0.071	–0.013	

negative values (ϕ_{st} = –0.013; p = 0.622). ϕ_{st} values between each pair of the four countries were significant, except for the pair Turkey/Greece (Table 5).

Discussion

The high level of polymorphism observed in this study agrees with results of previous studies carried out in olive cultivars with RAPDs (Fabbri et al. 1995; Weismann et al. 1998; Belaj et al. 2001; Besnard et al. 2001; Sanz-Cortés et al. 2001). However, the polymorphism level yielded by RAPD markers in this study was higher than in other cases, possibly due to the better representativeness of olive cultivar diversity in the Mediterranean basin and a higher resolution provided by polyacrylamide gels.

The clustering of the cultivars originating from the same or nearby geographic areas suggests a common genetic base for these cultivars. This agrees with the hypothesis of autochthonal origin, as well as the limited diffusion of olive cultivars from their zones of cultivation (Barranco 1997; Belaj et al. 2000; Besnard et al. 2001; Sanz-Cortés 2001).

By contrast, lack of a clear clustering of cultivars according to their geographic origin was observed for 46 coincident cultivars analysed with SSR markers (Rallo 2001). These differences could be attributable to the low number of SSR markers employed.

Previous assumptions made by morphological and isozymes markers (Trujillo et al. 1995; Barranco et al. 2000) had defined as putatively synonyms the pairs of cultivars which clustered together at similarity values of 1 in our study. These results indicate the importance of using different descriptors as complementary tools for identification of the genetic material.

The three Moroccan cultivars ('Picholine Marocaine', 'Menara' and 'Houzia') clustered together, but were clearly discriminated by RAPD markers. The distinction of these cultivars by morphological descriptors is very difficult (Barranco and Trujillo, personal communication). The fact that Besnard et al. (1998), could not distinguish the couple of cultivars 'Sigoise' and 'Picholine Marocaine' is probably due to the low number of primers used (6). In our study, as well as in a study carried out with AFLP markers (Angiolillo et al. 1999), these cultivars proved to be different.

The high similarity and the clustering of almost all the French cultivars with the Eastern and Central Mediterranean cultivars is possibly due to their common origin. Besnard et al. (2001) did not find significant differences between RAPD profiles of the cultivars from Greece and France. Furthermore, Besnard and Bervillé (2000) found that the only mitotype (ME1) present in wild olive populations from the Near East, prevailed in the French cultivars. Loukas and Krimbas (1983) mention that olive cultivation was introduced into Southern France by Greek colonists.

The RAPD analysis detected a high genetic differentiation among cultivars within each Mediterranean zone (96.86%) and a low variability between zones (3.14). This higher variability within a zone agrees with the general observation that woody perennial outbreeding species maintain most of their variation within a population (Hamrick and Godt 1989; Lamboy et al. 1996; Bartish et al. 2000; Gauer and Cavalli-Molina 2000; Oraguzie et al. 2001). The low but significant level of differentiation among different zones (ϕ_{st} = 0.031; $p < 0.001$) agrees with the hypothetical multilocal selection of olive cultivars.

The significance of ϕ_{st} and HOMOVA values between each pair of Mediterranean zones in all of the cases suggests the presence of differentiation. This result can probably be attributed to the local selection of the cultivars for any kind of outstanding character and their posterior vegetative propagation.

The partitioning of phenotypic variance into four categories – among regions (Western vs Eastern), among countries (within each region), and within countries as well and among and within countries of each region by means of AMOVA analysis – showed the same tendency as in the case of the Mediterranean zones. Most of the

phenotypic variance was retained among cultivars within a country (93.33). Only 5.46% of the total diversity was attributed to the differences among regions. The significant ϕ-values among regions (ϕ_{st} = 0.055; $p < 0.001$) indicated the existence of phenotypic differences between Eastern and Western regions. The nonsignificant ϕ-values (ϕ_{st} = 0.031; p = 0.063) among countries of the Western region and its negative values (ϕ_{st} = –0.013; p = 0.622) among countries of the Eastern region, could probably be explained by a short geographic distance and the interchange of genetic material between countries of the same region.

The significance of ϕ_{st} and HOMOVA values between each pair of countries in all of the cases respectively indicate the presence of differentiation. The negative value of ϕ_{st} observed in the case of the Turkey/Greece pair suggests that some Turkish varieties are more similar to the Greek ones than to the varieties of their own country, and vice versa. The geographic proximity and the existence of commercial roads and organised migrations since ancient times could have influenced the exchange of genetic resources between these countries.

The results of AMOVA analysis indicate that collecting new cultivars from as many different countries of the Mediterranean basin as possible, can be a good strategy to represent the entire variability of the olive species. And it also suggests the design of correct sampling strategies from collectors of each country and the establishment of germplasm banks. Furthermore the AMOVA results show that breeders in each Mediterranean country may rely on the autochthonous olive genetic resources for the design of breeding programs, since there is sufficient genetic variability among cultivars of the same country.

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