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ORIGINAL PAPER

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# AFLP reveals structural details of genetic diversity within cultivated olive germplasm from the Eastern Mediterranean

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**Abstract** Amplified fragment length polymorphism (AFLP) analysis was used to assess genetic inter-relationships among olive varieties cultivated in the Eastern Mediterranean Basin. The genotypes sampled included most of the important cultivars from Turkey, Greece and the Middle East and selected genotypes from the Western Mediterranean area. A total of 119 polymorphic markers were generated from five selective primer-pair combinations. The combined data sets generated by just two primer-pairs were adequate to discriminate between all 65 genotypes, while each primer-pair could individually identify up to 64 genotypes. A factorial correspondence analysis (FCA) plot indicated that the cultivars clustered into two relatively modestly defined groups. The first broad group was dominated by cultivars from Turkey but also included genotypes originating from the Middle East (Syria and Lebanon) that collectively formed a tight subcluster. The second group comprised Greek cultivars and those originating from the Western Mediterranean. A significant genetic distance value between Greek and Turkish cultivars was provided by an analysis of molecular variance (AMOVA). There was also evidence of substructure here, with an apparent separation of most Spanish and Italian clones. These findings are in general accordance to previous suggestions of an East-West divergence of olive cultivars, although the dichotomy is less extensive than reported previously and complicated by regional variation within each group.

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## Introduction

The olive tree (*Olea europaea* L.) is among the earliest tree crops and was probably domesticated in the Middle East about 6,000 years ago (Zohary and Spiegel-Roy 1975; Zohary and Hopf 1994). Its subsequent spread as a cultivated crop to other parts of the Mediterranean Basin was rapid, largely because the olive tree has multiple uses (e.g. oil, fruits, wood and in practical medicine) and so became highly valued. It remains one of the most economically important trees in the region. Despite its economic and social importance, however, our knowledge of the history of olive domestication and cultivation and of the routes of dispersal is still limited. In addition, the extent and pattern of variation among the unknown number of cultivars is still undefined. Olive cultivars show a broad range of genetic variability for a large number of agronomic traits, including oil content, fruit size and degrees of adaptability to severe biotic or abiotic stresses (Hatzopoulos et al. 2002 and references therein). Thus, the ability to discriminate olive cultivars and estimate genetic variability are important factors for a better management of genetic resources and successful breeding programs.

The development of various molecular marker techniques and their application in genetic diversity studies have resulted in improved discrimination among or within several olive cultivars and facilitated the estimation of their genetic inter-relationships (Fabbri et al. 1995; Belaj et al. 2001; Sanz-Cortès et al. 2001; Cipriani et al. 2002; Banilas et al. 2003). Estimations of genetic diversity structure among cultivars and/or wild forms have also provided interesting insights into the origins of cultivated olives (Ouazzani et al. 1993; Besnard and Bervillé 2000; Lumaret et al. 2000; Besnard et al. 2001, 2002a, b; Bronzini de Caraffa et al. 2002). To date, most of the work in the field has been carried out by examining enzymes, random amplified polymorphic DNA (RAPD) markers and mitochondrial or chloroplast DNA markers, although Angiolillo et al. (1999) recently applied amplified fragment length polymorphism analysis (AFLP; Vos et al. 1995) to estimate relationships between a limited selection of olive cultivars, wild forms and related species. This latter technique has also been used to study the genetic diversity within and among a range of Spanish and Italian olive cultivars (Sanz-Cortés et al. 2003; Sensi et al. 2003).

Accumulated molecular or morphometric data suggest that there may be a disjunction between the Eastern and Western Mediterranean populations of both wild and cultivated olives (Besnard et al. 2002a, b; Terral et al. 2004; and references therein). However, sampling of the eastern populations has been modest to date, and so the pattern of olive diversity in the Eastern Mediterranean is as yet unclear. In the study reported here, we have sampled extensively from both sides of the East-West divide and used AFLP markers to investigate the patterns of cultivar biodiversity.

## Materials and methods

### Plant material

The olive germplasm investigated includes economically important accessions cultivated in the Eastern Mediterranean Basin. Most of these are native to their respective area, originating from Turkey (27), Greece (25) or the Middle East (one Syrian and two Lebanese), whereas some genotypes have been recently introduced from Western Mediterranean Basin (four Italian and six Spanish). The numerical code of the accessions, characterization on the basis of commercial use and likely country of origin (principal area of cultivation) are given in Table 1. The Turkish, Syrian and Lebanese cultivars were obtained from an olive collection established at the Kemalpasa Campus of the Institute of Olive Growing Research, Izmir, Turkey. All other accessions were derived from a collection maintained at the Institute of Subtropical Plants and Olive, National Agricultural Research Foundation (NAGREF), Chania, Greece.

**Table 1** List of olive accessions studied, their geographical origin and commercial use

| Origin | Code | Accession | Use[a] |
|---|---|---|---|
| Turkey | 1 | Ayvalik | O |
| | 2 | Buyuk Topak Ulak | T |
| | 3 | Cakir | O + T |
| | 4 | Cekiste | T |
| | 5 | Celebi | O + T |
| | 6 | Cilli | T |
| | 7 | Domat | T |
| | 8 | Edincik Su | T |
| | 9 | Egri Burun | T |
| | 10 | Erkence | O |
| | 11 | Gemlik | O + T |
| | 12 | Halhali | O + T |
| | 13 | Izmir Sofralik | T |
| | 14 | Kalembezi | O + T |
| | 15 | Kan Celebi | T |
| | 16 | Karamursel Su | T |
| | 17 | Kilis Yaglik | O |
| | 18 | Kiraz | O + T |
| | 19 | Memecik | O + T |
| | 20 | Memeli | O + T |
| | 21 | Nizip Yaglik | O + T |
| | 22 | Samanli | T |
| | 23 | Sari Hasebi | O + T |
| | 24 | Sari Ulak | T |
| | 25 | Tavsan Yuregi | O + T |
| | 26 | Uslu | T |
| | 27 | Yag Celebi | O + T |
| Greece | 28 | Adramytini | O + T |
| | 29 | Aggouromanakolia | O |
| | 30 | Amygdalolia | T |
| | 31 | Chalkidikis | T |
| | 32 | Dafnelia | O |
| | 33 | Gaidourelia | T |
| | 34 | Kalamon | T |
| | 35 | Karolia | O + T |
| | 36 | Karydolia | T |
| | 37 | Konservolia | T |
| | 38 | Koroneiki | O |
| | 39 | Koutsourelia | O |
| | 40 | Lianolia Kerkyras | O |
| | 41 | Mastoidis | O |
| | 42 | Mavrelia | O |
| | 43 | Megaritiki | O + T |
| | 44 | Myrtolia | O |
| | 45 | Picrolia | O |
| | 46 | Rahati | O |
| | 47 | Stroggylolia | O + T |
| | 48 | Thiaki | O |
| | 49 | Throumpolia | O |
| | 50 | Tragolia | O |
| | 51 | Valanolia | O |
| | 52 | Vasilikada | T |
| Spain | 53 | Gordal | T |
| | 54 | Manzanilla-Gr[b] | O + T |
| | 55 | Manzanilla-Tr[c] | O + T |
| | 56 | Manzanilla-Is[d] | O + T |
| | 57 | Picual | O |
| | 58 | Sevillano | T |
| Italy | 59 | Frantoio | O |
| | 60 | Leccino | O |
| | 61 | Oblonga | O |
| | 62 | San Francisco | T |
| Lebanon | 63 | Ayrouni | O + T |
| | 64 | Souri | O + T |
| Syria | 65 | Sourani | O + T |

[a]O, Oil; T, table; O + T, both uses
[b,c,d]Manzanilla accessions cultivated in Greece, Turkey or Israel, respectively

### DNA extraction and AFLP analysis

Genomic DNA was extracted from young olive leaves according to the method of Saghai-Maroof et al. (1984) as modified by Angiolillo et al. (1999). The AFLP technique was carried out as described by Vos et al. (1995) with the following modifications. The ligation mixture was diluted 1:5, and 2 µl of this dilution was used for PCR preamplification in a 10-µl volume with 50 ng of each preamplification primer. The preamplification products were also diluted 1:5, and 2 µl of this dilution was used as the template for selective amplification in 10-µl reactions with 25 ng each of two selective primers (for the primer sequences and the combinations used, see Table 2). PCR analyses were performed in a PTC-200 Peltier Thermal Cycler (MJ Research, Waltham, Mass.). Following the addition of an equal volume of formamide loading buffer and subsequent heat denaturation, 3 µl of each sample was loaded and run together with a molecular-weight marker (AFLP DNA ladder; Invitrogen, Carlsbad, Calif.) on a 6% (0.4-mm-thick) polyacrylamide gel (1× TBE buffer) in a sequencing gel electrophoresis apparatus (model S2; Whatman Biometra, Goettingen) at 60 W for 2 h, at 55°C. The primers were not radioactively labeled, and AFLP fragments were visualized using silver staining (Bassam et al. 1991). The reproducibility of the AFLP patterns was assessed for all genotypes by at conducting at least two replicate assays for each procedure.

### Data analysis

Each gel was analyzed by manually scoring the presence (1) or absence (0) of bands in individual lanes. Only polymorphic and reproducible bands were used to construct the original binary data matrix. Genetic similarities among accessions were evaluated by estimating the Dice (Dice 1945) or Simple Matching (Sokal and Michener (1958) coefficient, and the accessions were clustered by the unweighted pair-group method with arithmetic averages (UPGMA). The dendrograms generated on the basis of the above indexes were compared by computing the cophenetic correlation (Sneath and Sokal 1973). A cophenetic value matrix of the UPGMA clustering was also used to test for the goodness of fit of the clustering to the similarity matrix on which it was based by computing the product-moment correlation $r$ (Mantel 1967). In order to estimate the degree of independence between AFLP profiles generated from individual primer-pairs, we computed similarity matrices and subjected these to Mantel tests (Mantel 1967). Genetic interrelationships among accessions were ascertained and visualized to a better degree by analyzing the AFLP data by factorial correspondence analysis (FCA) (Benzécri 1982). The above analyses were conducted using different modules from the NTSYS-PC ver. 2.02 package (Rohlf 1998). In order to certify the significance of genetic divergence observed between the Greek and Turkish olive germplasm, we calculated both the molecular variance between and within these two groups of cultivars by one-way analysis of molecular variance (AMOVA, ver. 1.04; Excoffier et al. 1992) and the $\phi$ statistic ($\phi$-st), analog of the F statistic, as the genetic distance between the two groups, after 1,000 permutations.

## Results

### AFLP profiles

AFLP profiles of the 65 accessions studied using five primers-pairs produced a total of 287 fragments. Among these, 119 fragments (41.5%) ranging in size from 100 bp to 340 bp were polymorphic, clearly scorable and reproducible. Between 20 and 35 polymorphic markers were obtained per primer-pair, with an average of 23.8 (Table 2). No single primer-pair was capable of identifying all of the accessions, although individual primer-pairs typically produced high numbers of different banding patterns, ranging from 59 to 64. The combined patterns obtained by the primer-pairs E-ACT/M-CTG + E-AAC/M-CAA, E-ACT/M-CTG + E-AAC/M-CTG or E-AAC/M-CAA + E-AAC/M-CTG were adequate to discriminate all genotypes. A relatively high percentage (67%) of combined patterns generated by three primer-pairs could be used to identify all accessions, while every possible combination of patterns generated by four primer-pairs yielded 65 distinct patterns.

### Genetic similarities and phenetic inter-relationships

The UPGMA dendrograms based on similarity matrices generated with the Dice and Simple Matching indexes were highly correlated ($r = 0.85$, Mantel test), leading to analogous results. Therefore, only results obtained with

**Table 2** Amplified fragment length polymorphism selective primer-pairs used and the level of polymorphism obtained among the 65 olive accessions

| Primer-pair | Total scored bands | Polymorphic markers | Percentage of polymorphism | Electrophoretic patterns |
|---|---|---|---|---|
| E-ACT/M-CTG | 50 | 20 | 40.0 | 64 |
| E-ACT/M-CTT | 52 | 21 | 40.4 | 60 |
| E-AAC/M-CAA | 58 | 23 | 39.7 | 59 |
| E-AAC/M-CTG | 67 | 35 | 52.2 | 64 |
| E-ATG/M-CAA | 60 | 20 | 33.3 | 59 |

the Dice coefficient are presented here. Genetic similarity values between accessions ranged from 0.40 (Gordal–Rachati) to 0.98 (Sari–Ulak–Celebi and Ayrouni–Souri), with a relatively high proportion (53.1%) being higher than 0.70. A comparison of the similarity matrices based on data from individual primer-pairs revealed a very low correspondence ($r < 0.3$) between each pair, suggesting that different primer-pairs generated quite distinct banding profiles. In the UPGMA dendrogram based on the Dice coefficient, most accessions clustered into four main groups (Fig. 1). The cophenetic correlation between the dendrogram and the similarity matrix revealed a good degree of fit ($r = 0.75$). Group 1 included most of the Turkish cultivars (21) along with the accessions of the Middle East plus five Greek and three Italian cultivars. Seven clusters (1.1–1.7) could be identified within this group, which correlates with the grouping of accessions according to geographic origin: cluster 1.1 consisted of exclusively Greek cultivars; cluster 1.2, of Italian cultivars; clusters 1.3 to 1.7 included cultivars originating from Turkey or the Middle East, with the exceptions of the Greek cultivars Adramytini and Chalkidikis, which are closely related to Ayvalik and Cilli, respectively. Group 2 consisted mostly of Greek and Spanish accessions and was subdivided into three subclusters (2.1–2.3). Cluster 2.1 comprised Greek cultivars; cluster 2.2 included Manzanilla-Gr, Manzanilla-Is, Leccino and Kalamon; cluster 2.3, two Spanish accessions (Sevillano and Manzanilla-Tr). Group 3 consisted of Greek cultivars along with the Spanish accession Picual, and group 4 included four Turkish cultivars and the Greek accession Myrtolia. Among the accessions not included in the four main groups were Gordal, which clustered with Tavsan-Yuregi and

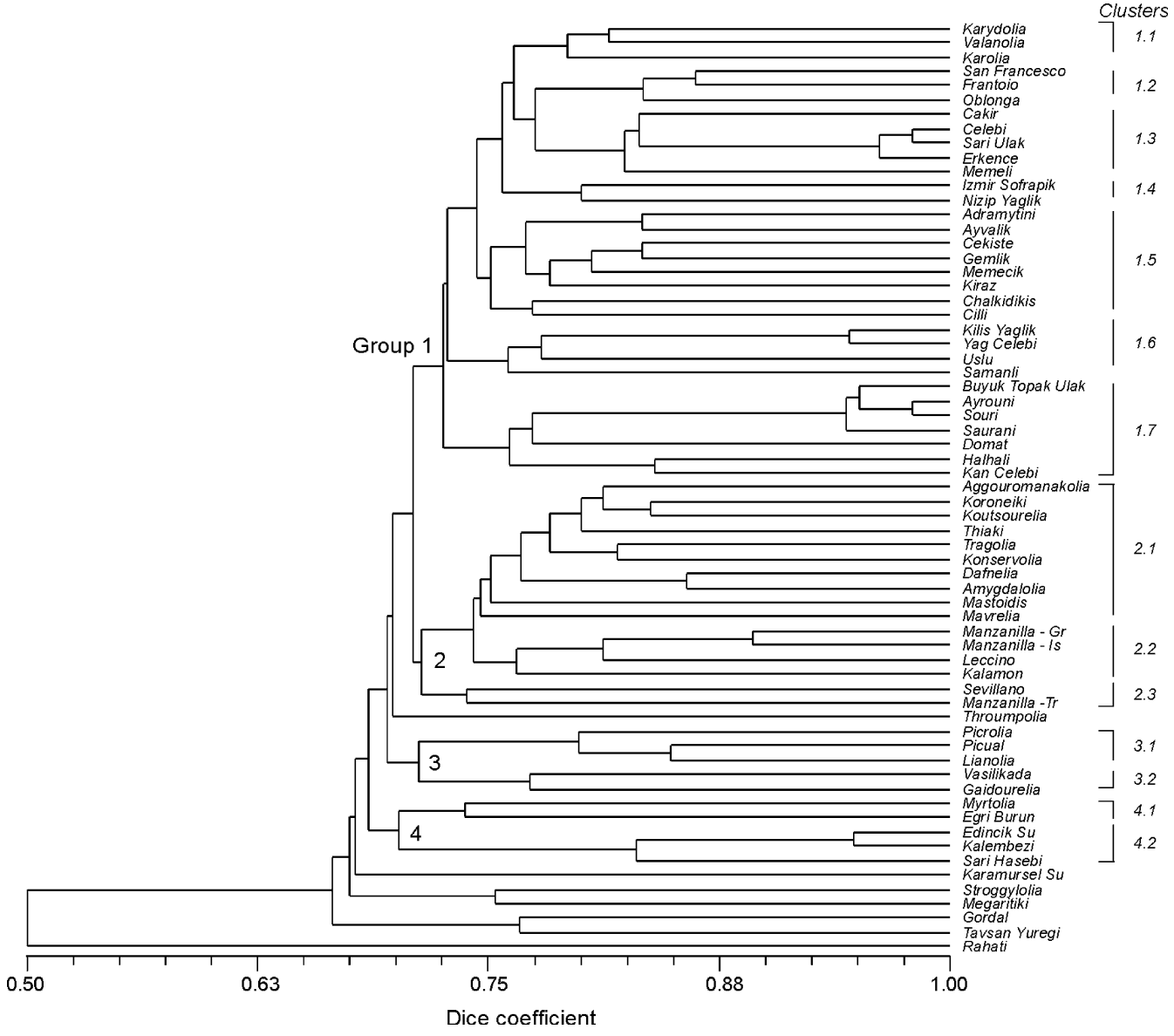


**Fig. 1** UPGMA cluster analysis of the 65 olive accessions using 119 AFLP markers

Strogylolia, which clustered with Megaritiki, whereas Rahati and Karamursel-Su remained rather unrelated to each other and to all other cultivars.

The FCA pattern (Fig. 2) was comparable to the clustering pattern of the accessions in the dendrogram, with most of the Turkish cultivars being located apart from the Greek ones. Analysis of the genetic diversity between and within the Greek and Turkish groups showed that a relatively high proportion of total genetic diversity was attributed within the two groups (89.84%; Table 3) with the variability within the Greek group (SS = 503.56) being higher than that within the Turkish group (SS = 482.07). However, AMOVA also revealed that the genetic distance between groups was significant ($\phi$-st = 0.102, $P < 0.001$). Five of the six Spanish accessions were located on the same side of the FCA plot, rather apart from both the Turkish group and most of the Greek cultivars. No particular inter-relationship was observed among the Italian cultivars, which were dispersed throughout the Greek group. Finally, cultivars from the Middle East were highly related to each other and located closer to the Turkish group.

In some cases the clustering of accessions according to fruit use was observed, particularly for the Greek cultivars. For example, nine of the 14 oil-producing Greek cultivars were included in cluster 2.1 of the dendrogram, and cluster 3.1 comprised three oil cultivars, two Greek and one Spanish (Fig. 1). On the other hand, the oil-producing Myrtolia was closely related to the table cultivar Egri Burun (cluster 4.1), and Valanolia clustered with Karydolia (cluster 1.1). Moreover, Turkish oil cultivars did not show any interrelatedness.

## Discussion

Molecular markers have been used to estimate genetic relationships among olive cultivars from different countries around the Mediterranean Basin. Of these, AFLP technology has been shown in earlier studies to be useful in discriminating between cultivars (Belaj et al. 2003a) and in addressing genetic diversity within the *Olea* complex (Angiollilo et al. 1999). In the present

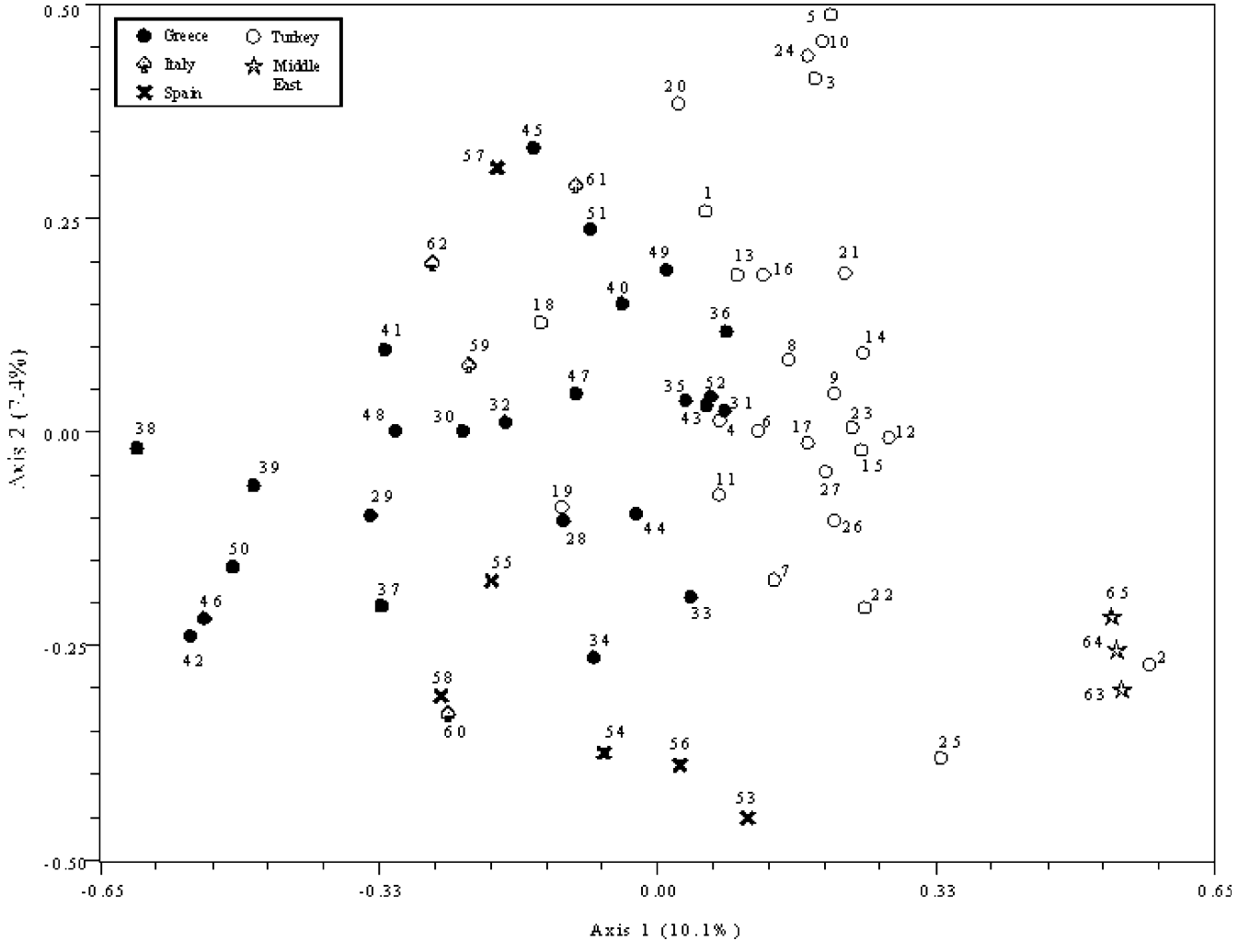


**Fig. 2** Factorial correspondence analysis of AFLP markers. The *numbers* refer to the codes of the olive accessions as shown in Table 1

**Table 3** The AMOVA analysis for the partitioning of AFLP variation between and within the Greek and Turkish groups of olive cultivars

| Source of variation | *df* | Variance components | Percentage of total variance | $\phi$-statistics | *P*-value |
|---|---|---|---|---|---|
| Between groups | 1 | 2.230 | 10.16 | 0.102 | $<0.001$ |
| Within groups | 50 | 19.713 | 89.84 | | |

study, we used AFLP markers to evaluate the structure of genetic diversity among common olive varieties cultivated in the Eastern Mediterranean. Although some of these cultivars had been evaluated previously using other molecular markers (see Belaj et al. 2002; Nikoloudakis et al. 2003), only three of the Greek and two of the Turkish varieties of the present study had been examined using AFLP technology (pioneering study of Angiolillo et al. 1999). In our investigation, five primer-pairs produced a relatively high number (119) of polymorphic bands among the 65 accessions, which indicates a high level of genetic diversity within the cultivated olive germplasm, even in a restricted geographic region such as the Eastern Mediterranean. The high level of genetic variability we detected among the Greek cultivars is consistent with results obtained by Nikoloudakis et al. (2003) using RAPD markers. Similarly, we found that the Turkish germplasm has a relatively broad genetic background and, most importantly for breeding programs, that it is distinct from the Greek germplasm.

AFLP proved to be a powerful method for revealing genetic diversity, as shown by the high discriminating capacity of the primer-pairs used. In a previous study, in which the first linkage map of olive was developed (la Rosa et al. 2003), a homogeneous distribution of AFLP markers was detected, which supports the use of AFLP as a means of detecting genetic diversity in olive.

We have shown that most Greek and Turkish cultivars can be separated into two distinct groups, as revealed by both UPGMA clustering and FCA analysis, and further justified by AMOVA. Such a clear-cut differentiation between the Greek and Turkish cultivated germplasm was surprising, as there has probably been an intense exchange of propagative material throughout the Mediterranean coastal region over a period of 5,000 years (Loukas and Krimbas 1983). In this region, where olive cultivation is as old as civilization, germplasm exchange would be expected to be profound. Belaj et al. (2002) examined olive cultivars originating from 17 Eastern and Western Mediterranean countries, including a limited number of cultivars of Greek or Turkish origin, and showed a genetic relationship between them. The distinction of overall Greek and Turkish cultivated germplasm that is observed can be attributed to the plethora of cultivars from the respective countries, since it is relative rather than absolute genetic similarities that are estimated by these molecular markers. Also in our study, two Turkish cultivars [Kiraz (no. 18) and Memecik (no. 19), Fig. 2] were more similar to the Greek ones than to cultivars of their own country. These results emphasize the need for representative sampling, particularly for genotypes from the same or nearby regions, in order that the genetic structure of the population be evaluated properly.

While the history of olive domestication and cultivation in Turkey is not yet clear, archaeological findings indicate that the first olive cultivars were introduced to Asia Minor by Ionian Greek colonists before 1,000 B.C.. Therefore, the clear-cut separation observed between the overall Turkish and Greek cultivated germplasm may be attributed to diverse anthropogenic processes throughout history, such as the changing of commercial routes and certain pressures on vegetation (Blondel and Aronson 1995). These data are in accordance with the suggestion that most olive cultivars have a local origin with limited diffusion outside their principal areas of cultivation (Besnard et al. 2001; Belaj et al. 2003b). Alternatively, due to the out-breeding nature of olive, the appearance of different variants could be the result of cross-pollination with local wild populations. Recent molecular data have shown that olive cultivars and wild plants of the same geographic origin have correlated genotypes (Besnard and Bervillé 2000; Contento et al. 2002). Similar AFLP profiles detected among cultivars from the Middle East and Turkey. However, because only three cultivars from the Middle East were included in the analysis, the above results are insufficient to support the claim that Turkish cultivars are more closely related to the Middle Eastern olive germplasm than to the Greek germplasm, even though such a hypothesis is supported by inter-related historical, geographical and environmental data.

Manzanilla, like some other important cultivars, has been introduced to other countries from its principal area of cultivation in the past. The AFLP data that we obtained were able to discriminate among the three Manzanilla accessions examined. However, although they showed a relatively close proximity, no inter-relationship was found. This raises questions about the accuracy of the 'Manzanilla' notation for all three genotypes, particularly for Manzanilla-Tr. On the basis of the FCA, the Spanish genotypes studied were located separate from the Turkish group and to some extent apart from the Greek cultivars, while three of the four Italian cultivars clustered together with the Greek cultivars in the dendrogram and were dispersed within the Greek group on the FCA plot. This suggests a common genetic base for the Italian cultivars and the Greek germplasm, thereby supporting the possibility of an early introduction into Italy of cultivars from the Eastern Mediterranean areas (Terral et al. 2004). Genetic relatedness between the Greek and Italian olive genotypes has been also documented previously using different types of molecular markers (Bronzini de Caraffa

et al. 2002; Belaj et al. 2002, 2003a). It has been suggested that the selection of olive cultivars occurred in different genetic pools in the East and West Mediterranean (Besnard et al. 2002a, b; Terral et al 2004). The results presented here do not refute this hypothesis but may indicate that a cline of variation may exist across the Mediterranean region rather than an abrupt separation.

In conclusion, the present analysis revealed the existence of a high level of genetic variability among the cultivars examined and that, overall, the Greek cultivated germplasm is genetically distinct from that of Turkey. Taking into account the geographic proximity of the two countries, this most likely reflects close regional and multiple selection of cultivars in the area. In agreement with this hypothesis, recent data suggest an autochthonous origin of Albanian cultivars (Belaj et al. 2003b). The examination of a greater number of cultivars and wild olives (oleasters) from the area may show if this is also the case in other countries and explain whether the autochthonous origin can be attributed to different local genetic background or to particular breeding pressures and the limited interchange of genetic material. The apparent unique nature of the Turkish olive germplasm revealed by our results supports the case for the implementation of more intense characterization and conservation strategies.

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