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Allozyme variation of oleaster populations (wild olive tree) (*Olea europaea* L.) in the Mediterranean Basin

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As a result of the early domestication and extensive cultivation of the olive tree throughout the Mediterranean Basin, the wild-looking forms of olive (oleasters) presently observed constitute a complex, potentially ranging from wild to feral forms. Allozyme variation was analysed at 10 loci in 31 large and 44 small oleaster populations distributed in various habitats of the Mediterranean Basin and in two populations of the wild subspecies *Olea europaea* subsp (ssp) *guanchica*, endemic to the Canary islands and closely related to oleasters. At eight polymorphic loci, 25 alleles were identified. Genetic evidence that nondomesticated oleasters still survive locally was provided by the occurrence of four and one alleles shared exclusively by the eight western and two eastern oleaster populations, respectively, which were

collected in forests potentially containing genuinely wild forms according to environmental, historical and demographic criteria. As reported previously from cytoplasmic and RAPDs analysis, substantial genetic differentiation was observed between the eastern oleaster populations genetically close to most olive clones cultivated in the Mediterranean Basin, and the western populations that are related to the wild Canarian populations. In addition, the occurrence of significantly lower heterozygosity in cultivated olive than in oleasters, whatever their origin, suggests that intensive selection involving inbreeding has taken place under cultivation to obtain particular characteristics in the olive cultivars. *Heredity* (2004) **92**, 343–351, advance online publication, 25 February 2004; doi:10.1038/sj.hdy.6800430

Keywords: allozyme variation; *Olea europaea* L. ssp *Sylvestris*; oleaster populations; olive domestication

Introduction

The olive tree (*Olea europaea* L.) is one of the most ancient domestic, cultivated plants characteristic of the Mediterranean Basin (Zohary and Spiegel-Roy, 1975). The crop (*O. europaea* subsp (ssp) *europaea* (= *O. europaea* var. *sativa* Lehr.)) has been of immense importance as the principal source of edible oil for the peoples of the Mediterranean region for many millennia. Palynological and anthracological (fossil charcoal) evidence supports the occurrence of wild olive forms (*O. europaea* sssp *sylvestris* (Miller) Hegi (= var. *oleaster* (Hoffmanns and Link) DC), in forests of the Mediterranean Basin during the Paleolithic (Lipshitz *et al.*, 1991). According to anthracological studies, during the Neolithic (10 000–7000 years ago), use of wild olive (oleasters) persisted in several parts of the Basin (Lipshitz *et al.*, 1991; Zohary and Hopf, 1993). Clear signs of olive domestication (olive oil presses) come from the Near East during the Early Bronze Age (second half of the fifth millennium BP) (Zohary and Spiegel-Roy, 1975; Lipshitz *et al.*, 1991). It is currently assumed that individual oleaster trees showing superior performance for size and/or oil content of fruit were selected empirically and propagated vegetatively as clones, using cuttings that were

planted directly or, more recently, grafted onto indigenous oleasters. From these propagated individuals, olive cultivars were developed and distributed by various successive human migrations throughout the Mediterranean Basin, especially from East to West although the selection of olive cultivars was multilocal and included originally western plant material, as shown by genetic studies based on allozyme polymorphism (Lumaret *et al.*, 1997), on RAPD profiles and on mitochondrial RFLPs (Besnard and Bervillé, 2000; Claros *et al.*, 2000; Besnard *et al.*, 2001a, b; Bronzini de Caraffa *et al.*, 2002a) as well as inter-simple sequence repeat (ISSR) markers (Vargas and Kadereit, 2001). Deforestation probably reduced native oleaster populations and the geographic dissemination of cultivars favoured multiple contact areas and hybridisation between cultivated olive and indigenous oleasters that are fully interfertile. This may have led to the progressive disappearance of the original genetic characteristics of the wild oleasters (Zohary and Spiegel-Roy, 1975).

Throughout the Mediterranean Basin, oleaster olives differ from the cultivated clones by the presence of spinescent juvenile shoots, smaller fruits characterised by less fleshy mesocarp and lower oil content, as well as by a long juvenile stage that may last for several decades in some individuals. As reported previously (Zohary and Spiegel-Roy, 1975), whatever their parental origin (wild or cultivated) any olive progeny grown from seed usually shows the morphological characters specific to oleasters. These therefore constitute a complex of

wild-looking olives potentially ranging from wild to feral forms (ie secondary sexual derivatives of the cultivated clones or products of hybridisation between cultivated trees and nearby oleasters) (Zohary and Spiegel-Roy 1975; Zohary and Hopf, 1993).

During the last five decades, controversy has developed as to whether the oleasters observed at present have originated exclusively by naturalisation from cultivated plants (Chevalier, 1948) or whether genuinely wild native forms still survive in some forests of the Mediterranean Basin. In this latter case, only the oleasters observed in secondary habitats (the edges of cultivated or abandoned orchards) would be feral (Zohary and Spiegel-Roy, 1975; Zohary and Hopf, 1993). In Australia, oleasters derived from olive cultivars introduced 200 years ago, and which are bird-dispersed plants, have proved to be successful invaders in numerous biotopes, including forests (Spennemann and Allen, 2000). Moreover, knowledge about the dissemination distance of olive pollen by wind (Griggs *et al*, 1975), from recent studies of seed dispersal of wild olive by birds in Spain (eg Alcantara *et al*, 2000), and from local reports about the extensive use of olive stones from olive groves for forestation in several Mediterranean countries suggest that genuinely wild olive populations may be restricted to very few isolated forest areas.

In several previous studies, the nuclear genetic variation of oleasters was analysed over the Mediterranean Basin using RAPD markers (Besnard *et al*, 2001a, b) and, in more restricted areas, using RAPDs (Bronzini de Caraffa *et al*, 2002a, b), AFLPs (Angiolillo *et al*, 1999) and allozyme polymorphism (Lumaret *et al*, 1997; Lumaret and Ouazzani, 2001). When wide areas were sampled, a clear nuclear genetic differentiation was observed between oleasters from the eastern and western parts of the Mediterranean Basin. In the present work, we analysed in detail the genetic variation of numerous oleaster populations distributed over the Mediterranean Basin. These populations were found to grow in various habitat conditions, such as the edge of olive groves, abandoned groves and noncultivated areas including forests. Moreover, several Mediterranean forests considered as good candidates for containing genuinely wild oleasters were selected according to several climatic, historical and demographic criteria. Allozymes at several independent loci analysed previously in numerous cultivated olive trees (Ouazzani *et al*, 1993; Ouazzani *et al*, 1995; Lumaret *et al*, 1997) were considered as appropriate codominant markers to characterise oleaster genetic variation. The objectives of the study were: (1) to analyse genetic variation in oleaster populations according to their geographical location and environmental conditions and (2), if possible, to identify allozymes specific to the oleaster populations selected as good candidates for containing genuinely wild material. Such specific allozymes were expected to be coded by low-frequency alleles and/or to be expressed by genes linked to loci coding for deleterious characters or for traits not suitable for olive domestication based exclusively on fruit characters. In both cases, allozymes would be expected to be absent in cultivated olives and in the feral oleaster forms derived totally or partially from them. A third objective was to compare allelic diversity and heterozygosity between oleasters and cultivated olive, two closely connected partners

of the same species, which reproduce sexually and vegetatively, respectively.

Materials and methods

Tree sampling

At least 20 trees (38.25 individuals on average) were sampled in 31 large oleaster populations distributed over the Mediterranean Basin (Figure 1 and Table 1). Two additional large populations (nos. 32 and 33) of the wild subspecies, *O. europaea* ssp *guanchica* P. Vargas, J. Hess, Muños Garm and Kadereit (*O. cerasiformis* (Webb and Berth) Kundel and Sunding), endemic to the Canary Islands, were sampled in two sites on Grand Canary Island (Figure 1). There is no evidence that this subspecies has been involved with olive domestication (Zohary, 1994), but it was found to be very closely related to Mediterranean oleasters (*O. europaea* ssp *sylvestris*) on the basis of chloroplast DNA variation and RAPD and ISSR markers (Hess *et al*, 2000; Lumaret *et al*, 2000; Médail *et al*, 2001).

Of the large populations, 10 were sampled in forest areas considered to be good candidates to contain genuinely wild oleasters according to three criteria: (i) past and present climatic conditions suitable for wild olive growth (humid and subhumid variants of the thermo-Mediterranean climate with an average minimum temperature of the coldest month and annual rainfall exceeding 5°C and 450 mm, respectively (Daget, 1977; Rivas-Martinez, 1981; Terral and Arnold-Simard, 1996); (ii) past and present isolation (usually more than 10 km) from areas of cultivated olive trees; and (iii) large oleaster populations (several thousands of trees) to reduce the influence of occasional pollen and seed flow from cultivated olive areas. In the eastern part of the Mediterranean Basin, where olive trees have been extensively cultivated for long periods, we used less restrictive criteria to identify candidate areas, more particularly for population size and isolation distance from cultivated olive areas. Candidate areas were not found in Egypt, Syria, Turkey, Crete or Greece, even though oleaster populations do occur in forested areas in those countries. Out of the 10 selected forests, two were located in the south of Spain (populations 22 and 23), two in central Morocco (populations 27 and 28), one in southern France (population 18), one in south Corsica (population 14), one in Sicily (population 10), one in Tunisia (population 30), one in central Cyprus (population 3) and one in Upper Galilee, Israel (population 1). Most populations were in evergreen oak forests located in uneven areas, and several were previously royal hunting grounds that have become natural reserves. Among the 21 other large sampled populations, 16 were located in habitats undisturbed by cultivation, including forests and degraded formations, and five (populations 7, 21, 25, 26 and 31) were in abandoned groves containing multicentenary oleaster trees that had been used to graft various olive cultivars.

In addition, 44 small populations were sampled. Their locations are indicated in Figure 1. Most of these populations consisted of a restricted number of oleasters that were sampled exhaustively (from six to 19 trees, 13.2 individuals on average). For two Israeli populations, a restricted number of trees was scored for the whole set of

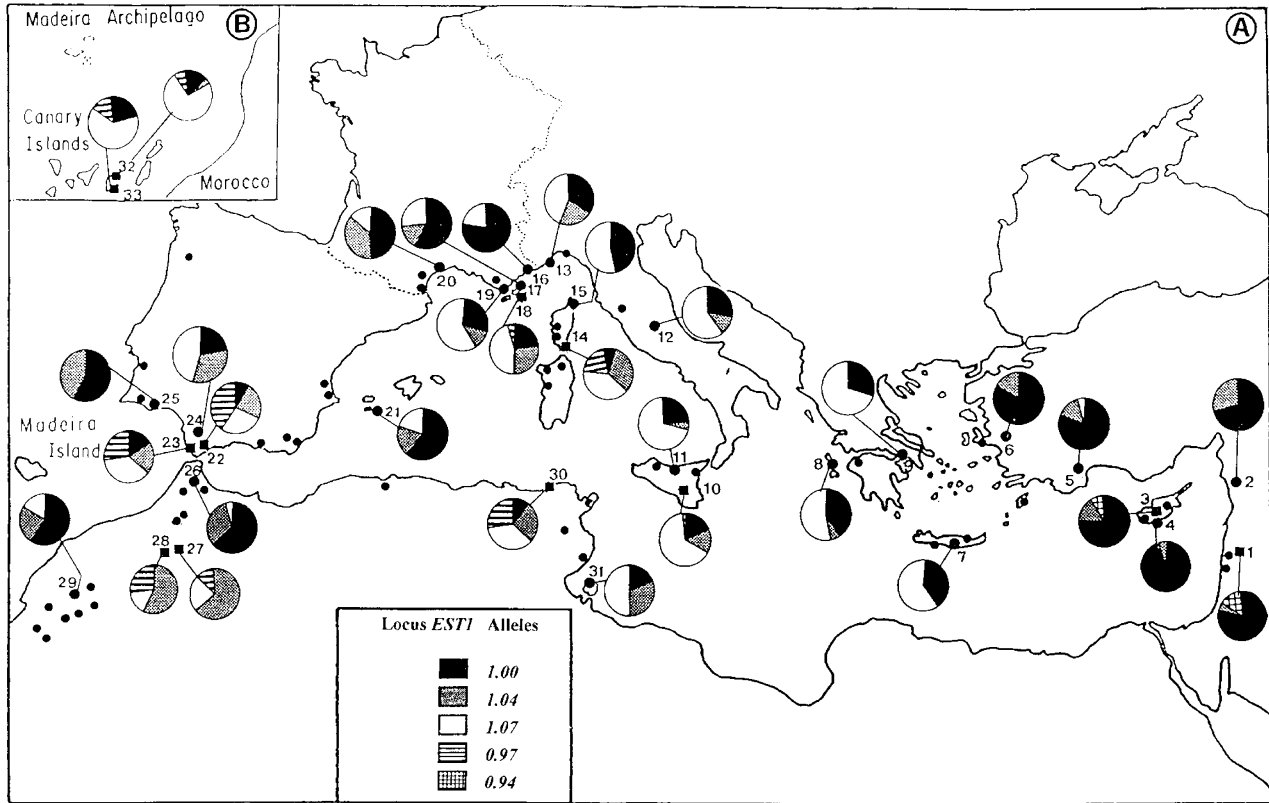


Figure 1 Geographical distribution of five alleles observed at the *Est-1* locus in the 31 large oleaster populations sampled over the Mediterranean Basin and in the two Canarian populations of *O. guanchica*. The location of 44 additional small populations is indicated by small black dots.

loci. Among the 44 small populations, 10 were located in forests, eight were groups of old trees used for grafting and 26 were small groups of oleasters growing at the edges of olive groves or within abandoned groves, that is, in environmental conditions where secondary feral forms of oleasters are expected to grow.

The results from analysis of oleasters populations were compared with data obtained previously at the same allozyme loci in the domesticated olive, including widely used cultivars, most of them from the olive germplasm world collection of Cordoba (Spain), and additional clones cultivated locally but not described as formal cultivars (Lumaret and Ouazzani, 2001). In all, 525 cultivated clones were analysed for the whole set of allozyme loci and most of the results were published previously (Ouazzani *et al*, 1993, 1994; Ouazzani *et al*, 1995; Trujillo *et al*, 1995).

Allozyme analysis

Proteins were extracted from leaves and used to perform horizontal starch-gel electrophoresis for six enzyme systems: phosphoglucose isomerases (EC 5.3.1.9. with two polymorphic duplicated loci, *Pgi-1-2*), alcohol dehydrogenases (EC 1.1.1.1., locus *Adh-1*), phosphoglucocomutases (EC 2.7.5.1., with two loci, *Pgm-1* and *Pgm-2*), leucine amino peptidases (EC 3.4.11.1., locus, *Lap-1*), esterases (EC 3.1.1.2., with two loci, *Est-1* and *Est-2*) and malate dehydrogenases (EC 1.1.1.37., with two loci, *Mdh-1* and *Mdh-2*). The protein extraction method, the composition of gels and electrode buffers, the techniques used to stain allozyme bands and the genetic determin-

ism of the systems analysed from controlled crosses were described previously (Ouazzani *et al*, 1993).

Data treatment

At each locus, genotypic and allelic frequencies were assessed from a survey of gel phenograms. Data from olive material cultivated in the eastern and the western part of the Mediterranean Basin and those from the 44 small populations were pooled, respectively. Allozyme data were used to calculate the percentage of monomorphic populations (*M*), the total number of alleles per locus (*At*), the mean (*A*), and the minimum and maximum number of alleles per locus and per population. The numbers of alleles and genetic diversity values were compared between populations or between groups of populations by Mann-Whitney *U*-tests. Allele genotypes were also used to calculate observed heterozygosity (*Ho*). Percentage comparisons were carried out for *M* and *Ho* values. Total genetic diversity (*Ht*), within-population genetic diversity (*Hs*) and the proportion of diversity resulting from gene differentiation among populations (*Gst*) and among groups of populations, were estimated by using GENETIX (4.2) software package (Belkhir *et al*, 2001).

Genotypic data were analysed using F-statistics (Wright, 1965). Departure of *Fis* values from zero was tested for each population at each locus (Li and Horvitz, 1953). All analyses mentioned above were performed using the GENEPOP (3.1) software package (Raymond and Rousset, 1995).

Table 1 Country, sample size, wild (W) and ferral (F) status hypothesised according to environmental, historical and demographic criteria, percentage of polymorphic loci (PL), mean number of alleles per locus (Al), genetic diversity within population (Hs) and Fis values in 31 large populations of oleasters and two populations of *guanchica* (nos. 32 and 33) analysed at eight allozyme loci

Pop.	Country/island	Sample size	Status	PL (%)	Al	Hs	Fis
1	Israel	38	W	100	2.29	0.346	+0.004
2	Syria	33	F	100	2.14	0.366	+0.009
3	Cyprus	20	W	086	2.29	0.379	-0.006
4	Cyprus	26	F	100	2.14	0.248	-0.010
5	Turkey	40	F	100	2.29	0.337	-0.005
6	Turkey	40	F	086	2.00	0.319	+0.004
7	Crete	32	F	100	2.14	0.420	+0.017
8	Greece	36	F	100	2.29	0.444	+0.005
9	Greece	34	F	100	2.14	0.414	+0.087
10	Sicily	42	W	100	2.86	0.483	+0.172
11	Sicily	52	F	100	2.57	0.468	-0.014
12	Italy	34	F	100	2.29	0.442	+0.072
13	Italy	32	F	100	2.29	0.461	-0.084
14	Corsica	39	W	100	2.86	0.480	+0.040
15	Corsica	44	F	100	2.43	0.423	+0.140
16	France	69	F	100	2.14	0.413	+0.122
17	France	37	F	100	2.43	0.450	+0.006
18	Port-Cros	40	W	100	2.57	0.429	+0.007
19	France	29	F	100	2.43	0.421	-0.046
20	France	38	F	100	2.29	0.432	+0.016
21	Ibiza	32	F	100	2.29	0.418	+0.190
22	Spain	45	W	100	3.14	0.499	+0.084
23	Spain	44	W	100	3.14	0.522	+0.140
24	Spain	40	F	100	2.43	0.459	+0.074
25	Spain	40	F	100	2.14	0.429	+0.042
26	Morocco	32	F	100	2.43	0.488	-0.063
27	Morocco	48	W	100	2.86	0.468	+0.144
28	Morocco	35	W	100	2.71	0.454	+0.180
29	Morocco	24	F	100	2.43	0.496	+0.001
30	Tunisia	38	W	100	2.86	0.450	+0.096
31	Tunisia	52	F	100	2.57	0.523	+0.112
32	Canary	26	W	100	2.86	0.392	-0.042
33	Canary	37	W	100	2.71	0.381	-0.021

Nei's (1978) genetic distances and, to maximise the effects of rare alleles, the χ^2 distances, weighted by allele frequencies averaged over all populations, were calculated between pairs of populations. The respective positions of the populations, estimated by the distances between them, were plotted in a multidimensional space and then projected onto a plane by nonmetric multidimensional scaling (or proximity analysis) (Escoufier, 1975). The same distances were also used as the basis for a hierarchical cluster analysis (UPGMA), with 'average distance' used as the clustering criterion. Clusters at specific levels of agglomeration (total range 0–100%) were mapped onto the diagram obtained from the multidimensional scaling, so that results from the two methods could be compared. These analyses were performed using the Biomeco computer package (Anon, 1989). Moreover, for each pair of populations, the relationship between genetic structure and geographical isolation was assessed by comparing geographical and genetic distances. Using GENETIX software (Belkhir *et al*, 2001), the correlation was then quantified (Z-value) using a Mantel test that compares the matrices of geographical and genetic distances. The measured Z was compared with that obtained with 1000 permutations by randomising one of the two matrices. Relationships between geographical distance and genetic structure were considered as significant when the true Z-value was higher than 99% of the Z-values obtained with 1000 permutations ($P < 1\%$).

Results

Allelic variation in oleaster populations

Among the 10 allozyme loci analysed, two (*Pgm-1* and *Mdh-1*) were monomorphic for the same allele in all populations studied. At the eight other loci, 25 distinct alleles were identified in oleasters, *Pgi-1-2^{1.60}*, *Pgi-1-2^{1.00}Pgi-1-2^{0.60}*, *Pgm-2^{1.14}*, *Pgm-2^{1.00}*, *Pgm-2^{0.90}*, *Adh-1^{1.12}*, *Adh-1^{1.00}*, *Adh-1^{0.92}*, *Est-1^{1.07}*, *Est-1^{1.04}*, *Est-1^{1.00}*, *Est-1^{0.97}*, *Est-1^{0.94}*, *Est-2^{1.03}*, *Est-2^{1.00}*, *Est-2^{0.94}*, *Est-2^{null}*, *Mdh-2^{0.79}*, *Mdh-2^{1.00}*, *Lap-1^{1.02}*, *Lap-1^{1.00}*, *Lap-1^{0.97}*, *Lap-1^{0.94}* and *Lap-1^{0.92}*. Of these alleles, 19 were also identified in the small oleaster populations and in the cultivars that have been shown to possess four additional rare alleles distributed very locally (Ouazzani *et al*, 1993, 1995). Allele distribution at the highly polymorphic *Est-1* locus in the 31 large oleaster populations and the two populations of *O. europaea* ssp *guanchica* is shown in Figure 1. At that locus, a clear genetic differentiation is observed between the populations from the eastern part of the Mediterranean Basin (Israel, Syria, Cyprus and Turkey), characterised by the predominance of allele *Est-1^{1.00}* and the other populations (including those from the Canary island) where several other alleles (particularly *Est-1^{1.07}*) were present at high frequency. In addition, two alleles (*Est-1^{0.97}* and *Est-1^{0.94}*) were observed exclusively in the oleasters of the 10 areas considered as good candidates for containing genuinely

wild olive. *Est-1^{0.97}* (ranging from 4 to 32%) was found in each of the eight candidate oleaster populations located in the western part of the Mediterranean Basin and in the two Canarian populations. In the eastern part of the Basin, *Est-1^{0.94}* was observed exclusively in the population 3 located in Cyprus (8%) and in a population from Upper Galilee (14%). At the other loci, three additional alleles were found to be specific to the selected areas, namely, *Lap-1^{0.94}* (from 7% to 22%) observed in the populations 22 and 23 from Andalusia and in the two Canarian populations, *Lap-1^{1.02}* identified in six populations (22 and 23 from Andalusia, 27 and 28 from Morocco and 32 and 33 from Canary islands) at a frequency ranging from 20 to 39% and *Pgm-2^{0.90}* (4–15%) found in the eight candidate areas located in the western part of the Mediterranean Basin.

The average genetic distance value among the 31 large populations scored at the eight polymorphic loci was 0.143 (range 0.011–0.321) for Nei's distance and 0.043 (range 0.012–0.087) for χ^2 distance. In Figure 2, the eight populations selected in the western part of the Mediterranean Basin as good candidates to contain genuinely wild oleasters and the two populations from Canary islands that share several specific allozymes (see above) are located on the left side of the plan, parted by axis 2, whereas the two populations selected in the eastern part of the Basin, the unselected populations (whatever their geographical origin) and the two groups of cultivars are located on the right side of the plan. Three sets of populations could be distinguished at the 75% level of agglomeration (a critical value in the agglomeration process) in the UPGMA analysis. A first set (on the left in Figure 2) consists of the four populations selected in the most western part of the Basin (South of Spain and Morocco) and the two Canarian populations. These six populations share several alleles (eg *Lap-1^{0.94}* and *Lap-1^{1.02}*) that were observed exclusively in the far western part of the Mediterranean Basin. The second

set clustered together most of the oleaster populations and consisted of three subsets identified at the 40% level of agglomeration. One subset clustered together four populations of selected areas in the more central part of Mediterranean Basin (South of France, Corsica, Sicily and Tunisia). In these populations, most of the alleles specific to the selected areas were observed at very low frequencies. The two other subsets consisted exclusively of unselected populations, one of two Moroccan populations (26 and 29) and the other of 15 populations from a range of geographical areas and environmental conditions in the western and central parts of the Mediterranean Basin. When the 15 populations were parted into 11 and four populations from undisturbed and disturbed areas, respectively, no significant difference could be observed between the allelic distributions of the two groups of populations. A third set gathered together the most eastern oleaster populations (1–6) and the groups of olive clones cultivated in the eastern and western parts of the Basin, respectively. As compared to the populations from the western and central part of the Mediterranean Basin, the eastern oleaster and cultivated material and most cultivated olive clones from western areas are characterised by higher *Pgm-2^{1.00}*, *Adh-1^{1.12}*, *Est-1^{1.00}* and *Lap-1^{0.97}* frequency, lower *Adh-1^{0.92}* frequency and the absence of *Est-1^{1.07}* and *Lap-1^{0.92}*. However, in a few local western cultivars (mainly from Sicily, Corsica and Andalusia), allelic distributions were close to those observed in the oleaster populations that were growing in the same areas. Within the third set, at 40% agglomeration level, two subsets were identified which parted the two populations selected for containing wild oleasters and characterised by a specific allozyme (see above) from the other populations.

For the 10 large populations selected to contain genuinely wild olive and for the eight populations of them growing in the western part of the Mediterranean Basin and considered separately, a positive relation

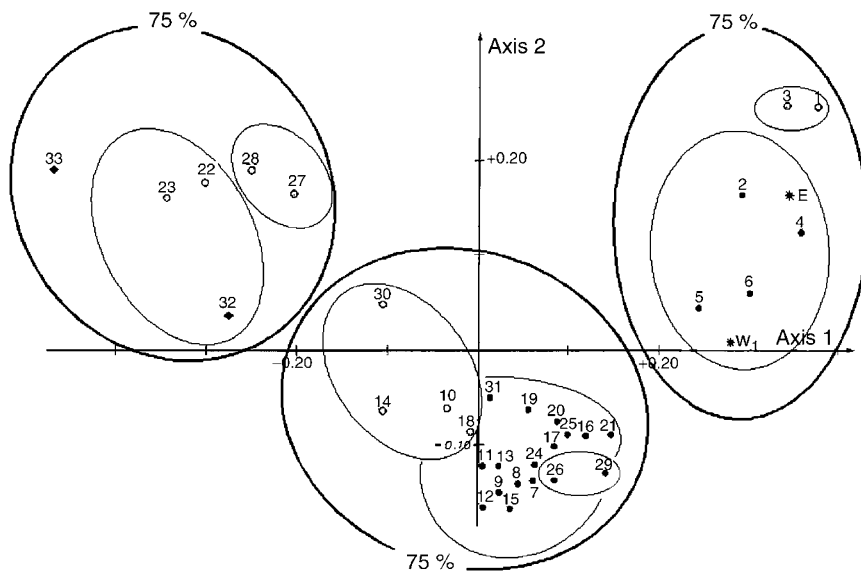


Figure 2 Position of 10 large oleaster populations selected as containing potentially genuinely wild olive (○), of 21 large unselected populations (●), of two Canarian populations of *guanchica* (◆) and of two groups of cultivated olive (○) according to polymorphism at eight polymorphic allozyme loci. Multidimensional scaling from χ^2 distances. Populations are clustered at the 40 and 75% levels of hierarchical clustering.

Table 2 Percentage of polymorphic populations (P), total number of alleles per locus (At), mean (A) (minimum–maximum) number of alleles per population, total genetic diversity (Ht), genetic diversity within populations (Hs), proportion of diversity between populations (Gst), observed heterozygosity (Ho) and Fis values at eight polymorphic allozyme loci in 31 large populations of oleasters

Locus	P-value (%)	At	A	Ht	Hs	Gst	Ho	Fis
<i>Pgi-1-2</i>	100.0	3	2.61 (2–3)	0.466	0.457	0.02	—	—
<i>Pgm-2</i>	100.0	3	2.22 (2–3)	0.499	0.459	0.08	0.372	–0.010
<i>Adh-2</i>	100.0	3	2.35 (2–3)	0.517	0.461	0.11	0.530	–0.030
<i>Est-1</i>	100.0	5	3.10 (2–4)	0.681	0.546	0.20	0.499	+0.123
<i>Est-2</i>	92.8	4	2.06 (1–3)	0.455	0.386	0.15	0.310	+0.191
<i>Mdh-2</i>	100.0	2	2.00 (2–2)	0.258	0.238	0.08	0.198	–0.070
<i>Lap-1</i>	100.0	5	2.90 (2–5)	0.559	0.485	0.13	0.522	+0.173
Mean 1	99.0	3.50	2.48	0.491	0.433	0.12	0.405	+0.063
Mean 2	97.5	3.37	2.77	0.506	0.449	0.11	0.428	+0.084
Mean 3	99.0	2.87	2.34	0.448	0.421	0.06	0.382	+0.032
Mean 4	86.2	2.62	2.33	0.356	0.339	0.05	0.346	–0.002
Mean 5	100.0	3.25	2.52	0.503	0.455	0.09	0.414	+0.077
Mean 6	100.0	2.75	2.75	0.421	0.385	0.08	0.430	–0.030
SP	95.4	2.75	—	0.402	—	—	0.392	—
EC	—	2.87	—	0.291	—	—	0.280	—
WC	—	2.87	—	0.323	—	—	0.324	—

Mean values calculated over the eight loci analysed in the 31 large populations (Mean 1), over the 10 oleaster populations selected for containing genuinely wild olive and the 21 unselected populations (Means 2 and 3, respectively), over the six and the 25 populations of eastern and western Mediterranean Basin (Means 4 and 5, respectively), over the two populations of *O. europaea* ssp *guanchica* (Mean 6), over the 44 small oleaster populations (SP) and over the olive clones cultivated in the eastern and western parts of the Basin (EC and WC, respectively).

between genetic and geographical distances was obtained ($P < 1\%$) from the results of the Mantel test ($Z = 11482$ and 3787 , Pearson's coefficient $r = 0.861$ and 0.672 , respectively). For the 21 large unselected populations, a significant positive relation ($P < 1\%$) was observed between genetic and geographical distances, but the correlation value was lower ($Z = 27252$, $r = 0.596$).

Table 2 shows the percentage of polymorphic populations, the mean and the range of alleles per population, several genetic diversity parameters, the observed heterozygosity and Fis values calculated at each of eight polymorphic loci analysed in the 31 large oleaster populations. Averages over these loci in these 31 populations (Mean 1), in the 10 populations from the selected forests (Mean 2), in the 21 unselected large populations (Mean 3), in the 25 populations located in the western and central parts of the Mediterranean Basin and in the six eastern populations (Means 4 and 5, respectively), and in the two populations from the Canary islands (Mean 6) are also indicated in Table 2. Maximum and minimum polymorphism and genetic diversity were observed at *Est-1* and *Mdh-2*, respectively. Of the average total diversity (0.491, range from 0.258 at *Mdh-2* to 0.681 at *Est-1*), 12% was attributable to differentiation among populations (range from 2% at *Pgi-1-2* to 20% at *Est-1*) (Table 2). Moreover, mean number of alleles per locus and per population and mean genetic diversity values were significantly higher ($P < 0.05$ and < 0.01 , respectively) in the 10 populations selected to contain wild oleasters than in the 21 unselected populations. The *Gst* value between the two groups of populations was 5%. For the same parameters, values were higher in the 25 large populations of the western and central distribution areas as compared to the six eastern populations ($P < 0.01$) and 10% genetic differentiation was observed on average between the two groups. Moreover, low polymorphism and low

genetic diversity values were observed in the 44 small populations and, more particularly, in the two groups of cultivated olive (Table 2).

In the 31 large populations, Fis values averaged over loci and/or populations were not significantly different from zero except at *Est-2* ($P < 0.05$) (Table 2). However, significant deviations from Hardy–Weinberg expectation were observed at some loci in several populations. Out of the 186 indices tested, 55 (four at *ADH-1*, 17 at *EST-1*, 22 at *EST-2* and 12 at *LAP-1*) positive deviations of Fis from 0 and 18 negative deviations (seven at *Adh-1*, nine at *Mdh-2* and two at *Pgm-2*) were observed at either the 5 or the 1% significance level. Fst values were 14% between the 31 large populations and 6% between the eight putative wild populations of the western distribution area. In the 31 large populations, mean observed heterozygosity was high (0.405, range from 0.198 at *Mdh-2* to 0.530 at *Adh-2*) (Table 2). A high heterozygosity value was also obtained in the two populations of *O. europaea* ssp *guanchica* from the Canary islands, whereas heterozygote frequencies were observed to be particularly low in the two groups of cultivated olive. Mean heterozygosity was significantly higher ($P < 0.01$) in the 10 populations from the selected forests than in the 21 other large populations.

Discussion

Genetic specificity of the populations identified to contain potentially genuinely wild form of oleasters

In the present study, substantial genetic variation was observed in oleasters. Average allele number per locus and heterozygosity values were particularly high. The highest genetic diversity values are observed in the 10 oleaster populations selected as the most likely to contain genuinely wild olive. In these populations, as compared to alleles occurring in both cultivated olive and

unselected oleaster material, five additional alleles (four and one in the western and eastern parts of the Mediterranean Basin, respectively) were identified, which do not come therefore from cultivated olive material. As these alleles are distributed over large areas in several isolated forests, they have a low probability of being of recent origin. Instead, these alleles probably constitute evidence of the survival of indigenous wild oleaster populations, especially in the western part of the Mediterranean Basin, as suggested by Zohary and Spiegel-Roy (1975). In addition, three of these alleles are shared by the Canarian populations of *O. europaea* ssp *guanchica* and by the most occidental oleaster populations (more particularly those from the selected forests of Andalusia and Morocco), suggesting that the populations growing in the most westerly part of the species distribution have a common origin. Except for four very rare alleles, oleaster populations possess all the alleles already identified in cultivars. This result is consistent with our interpretation that the domesticated olive represents a subsampling of the genetic variation in genuinely wild olive, which persists today in the Mediterranean Basin.

A very significant association between vigour (related to the duration of oleaster juvenile phase), and genotype, at the two specific loci *Est-1* and *Lap-1*, was observed previously in the progeny of a controlled cross between two cultivars and/or in oleaster populations grown for afforestation and in which individuals were the same age (Ouazzani *et al.*, 1993). In subsequent analysis, this association re-emerged systematically and exclusively at the same loci in various oleaster material, which suggests that genetic linkage cannot be ruled out. It should be noticed that four of the five alleles characteristic of genuinely wild olive were found at the two loci associated with tree vigour, suggesting that this genetic association may have prevented the establishment of several alleles at these loci within cultivated material.

Geographic allozyme variation in oleasters

In the present study, substantial genetic differentiation was observed between the oriental and occidental oleaster populations with lower total and average genetic diversity in the former group of populations. This result is consistent with previous reports based on the analysis of chloroplast DNA variation (Amane *et al.*, 1999; Besnard *et al.*, 2002), on the combination of mitochondrial DNA and RAPDs variation (Besnard and Bervillé, 2000; Besnard *et al.*, 2001a), and on RAPDs and rRNA genes polymorphism (Besnard *et al.*, 2001b). More particularly, very clear genetic differentiation based on the occurrence of distinct allozymes was observed between genuinely wild oleaster populations from the western and eastern parts of the Mediterranean Basin, respectively, suggesting that they may have distinct phylogeographical origins. In the present work, evidence is provided that the most western populations of the Mediterranean Basin are genetically close to the Canarian populations of ssp *guanchica*, which supports previous results obtained from plant material collected in the same areas and analysed using RAPD and ISSR markers (Hess *et al.*, 2000). A very high correlation was found between geographical and genetic distances, showing a gradual western–eastern variation within the occidental group of

populations (growing in the western and central parts of the Mediterranean Basin) and higher change in the eastern group of populations. However, the precise geographical boundary between the two population groups cannot be identified precisely because no candidate forest for containing genuinely wild olive was located in intermediate areas of the Mediterranean Basin and, more particularly, in Greece.

Within unselected oleaster populations, genetic differentiation based on both allele occurrence and frequency variation was also observed between the eastern populations (from Israel, Syria and Turkey) and the other populations (including those from Greece). The oriental populations are very close genetically to the two groups of olive clones cultivated in the eastern and in the western parts of the Basin, respectively, suggesting that most cultivars of the Mediterranean Basin originated from plants selected in the Middle East, as suggested previously from cpDNA, mtDNA and RAPDs analysis (Besnard and Bervillé 2000; Besnard *et al.*, 2001a, 2002). However, in the western group, a few cultivars (particularly from Sicily, Corsica and Andalusia) showed allozyme characteristics similar to those found in the western oleaster populations supporting the previous evidence of a multilocal selection of cultivars in olive (eg Besnard and Bervillé, 2000; Besnard *et al.*, 2001c; Bronzini de Caraffa *et al.*, 2002a,b; Contento *et al.*, 2002). In the present study, close genetic relationships were observed between the unselected oleaster populations of both the intermediate and western parts of the Basin, suggesting that most of these populations may derive, at least partly, from cultivated olive clones, many of them being close genetically and, as reported above, probably derived from selection in the same eastern area.

Genotype structure in oleasters and in cultivated olive

In addition to distinct allele distributions and frequencies, substantial genotype differences were observed between oleasters (whatever their origin) and cultivated olives with, at most enzyme loci, significantly higher heterozygosity in the former group than in the latter. Moreover, heterozygosity was particularly low in olive cultivated in the eastern Mediterranean Basin, as compared to the cultivated olives of the occidental part. This difference was due mainly to the occurrence of higher heterozygosity in the several areas where substantial olive material was selected and cultivated locally, for example, in southern Morocco (Ouazzani *et al.*, 1996). The trees originated very probably from hybridisation either between distinct cultivars or between cultivars and geographically close oleasters. In several recent studies using dominant nuclear markers (eg RAPDs, AFLPs and ISSRs), a substantial, but not complete, discrimination was observed between cultivars from various geographical origins and oleasters from populations distributed over the Mediterranean Basin, each represented by a single individual tree, although no genetic marker was identified by the authors to discriminate unambiguously between wild oleasters and the putative feral forms (Angiolillo *et al.*, 1999; Besnard *et al.*, 2001a; Vargas and Kadereit, 2001; Bronzini de Caraffa *et al.*, 2002a,b). Such results were observed even when the oleasters were collected in disturbed areas close to olive groves and were considered as being of feral origin (Angiolillo *et al.*,

1999), and also in feral oleasters growing in Australia, where genuinely wild olive (in *O. europaea*) is not expected to grow (Mekuria *et al*, 2002). From the results of the present work, we suggest that the nuclear genetic differentiation observed between oleasters (whatever their origin) and cultivated olive may be attributable mostly to heterozygosity level differences and, very occasionally, to distinct allelic composition between these two fully interfertile partners of the same species. In addition, the occurrence of lower heterozygosity in cultivated olive than in oleasters suggests that intensive selection involving inbreeding may have taken place under cultivation originally to obtain particular characteristics in the olive cultivars (very probably, larger sized fruits and/or higher oil content).

The substantial allele and genotype variation observed in the oleaster populations analysed in the present work suggests that a portion of that plant material may constitute genetic resources for further breeding of olive material adapted to the various climate conditions occurring in the species' distribution area. Moreover, our discovery of genuinely wild olive in a few areas of the Mediterranean Basin makes it necessary to develop conservation strategies to maintain environmental conditions for the survival of large populations of oleasters properly isolated from cultivated olive.

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