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Allozyme variation in cork oak (*Quercus suber* L.): the role of phylogeography and genetic introgression by other Mediterranean oak species and human activities

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Abstract Genetic variation in the cork oak (*Quercus suber* L.) was investigated using 11 loci from seven enzyme systems in 40 populations sampled over the entire distribution of this species in the western Mediterranean Basin. Mean heterozygosity values over the polymorphic loci (Ho = 0.283), the percentage of polymorphic populations (M = 0.76), and the total genetic diversity (Ht = 0.31) from which 11% was accounted for among-population variation, are among the highest recorded in oak species. In contrast to previous results in holm oak (*Q. ilex* L.), another evergreen species in the same area, cork oak possessed a smaller allele pool and a lower average number of alleles per locus and per population (A = 2.0). More particularly, very few low-frequency alleles were observed in cork oak except for eight populations in which allozyme polymorphism at locus *Pgi* 1, diagnostic between both species, indicates that these low-frequency alleles are introgressed from holm oak. On the basis of the genetic distance estimated from allozyme frequencies, 32 of the 40 cork oak populations studied were classified into two very distinct sets which also corresponded to distinct geographic areas. One set gathered together the 18 populations from the Iberian peninsula and two adjacent areas in France, i.e. the centre of origin of cork oak, according to paleobotanical data. This set was characterized by a larger allele pool, a higher within-population genetic diversity and a lower differentiation between populations than was observed in the other set, which comprised the populations from North Africa, Sicily, Sardinia, Corsica, continental Italy and the region of Provence (southeastern France). In these more southern and eastern disjunct areas, cork oak migration from Iberia may have occurred at different periods since the end of the Tertiary. The possible effect of human activity on cork oak genetic structure, i.e. the selection of good-quality cork, acorn over-use for animal food, and even human nutrition, is discussed.

Key words Allozyme polymorphism
Phylogeography · Human activity and population genetic structure · *Quercus suber*

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Introduction

In oaks, geographical patterns of allozyme variation have generally been observed whenever range-wide studies were conducted. These patterns have been attributed to geographical discontinuities (Michaud et al. 1995) and, more importantly, to evolutionary footprints, particularly re-colonizing routes (Zanetto and Kremer 1995). Such allozyme surveys were made mostly in red and white oak groups from North America (e.g. Manos and Fairbrothers 1987), in European white oaks (Kremer and Petit 1993; Zanetto and Kremer 1995), and in holm oak (*Quercus ilex* L.) (Michaud et al. 1995) a species of the evergreen oak group. In the Mediterranean area, this latter group is composed of four species, three of them, namely *Q. ilex* L., *Quercus suber* L.(cork oak) and *Quercus coccifera* L./*Quercus calliprinos* Webb.(holly-oak), are distributed over both Southern Eurasia and Northern Africa whereas the fourth (*Quercus alnifolia.Poech*) is endemic to Cyprus. In this paper, we report data concerning the geographical organization of allozyme variation over the entire range of *Q suber* L.

According to Camus (1938) and the Flora Europaea (Tutin et al. 1964–1980), cork oak and holm oak, which are the two main evergreen oak species in the western part of the Mediterranean Basin, belong to the subgenus

Cerris (Spach) Oersted and the subgenus *Sclerophyllodrys* O.Schwarz, respectively. These species show substantial overlap in their geographical distribution. However, as compared to holm oak which shows a very high ecological amplitude, cork oak is restricted to hot variants of the humid and sub-humid Mediterranean areas with at least a 450 mm mean annual rainfall and it avoids limestone substrates. Cork oak distribution (see Fig. 1) is therefore more occidental and reduced than that of holm oak which constitutes a continuum from Turkey to Portugal, including all the larger Mediterranean islands such as Crete and Sicily (Michaud et al. 1995). Moreover, cork oak geographic distribution shows several geographic discontinuities: (1) between Europe and Africa, (2) between the Mediterranean islands and these two continents, and (3) within Europe, between the Iberian peninsula and two adjacent French areas, namely Roussillon (French Catalogna) and Atlantic Landes, on one side, and Italy and the adjacent French area of Provence, on the other side. In their common distribution area, cork oak and holm oak often grow together and the local occurrence of trees morphologically intermediate between cork and holm oak has been reported (Natividade 1936; Camus 1938). By analyzing polymorphism at allozyme loci for which alleles are distinct in the two species when they grow in separate areas (diagnostic markers), evidence was obtained for the occurrence of hybrids between sympatric holm oak and cork oak in Spain (Elena-Rossello et al. 1992). Consequently, an analysis of allozyme variation within either species should refer to variation at the same loci in the other one.

Paleoecological data indicate that both species have been present in the South of Europe, and in the case of holm oak also in North Africa, since the end of the Tertiary period (Carvalho 1957). According to Sauvage (1961), *Q. suber* may have originated in the Iberian peninsula and have subsequently colonized North Africa from the Gilbraltar straits at the end of the Miocene. For the last glacial and postglacial periods, results from palynological data indicate the occurrence of cork oak in southern Spain since the Late Glacial period (17000–12000 years BP) and in North Africa since the early Postglacial (approximately 8500 years BP) (Reille et al. 1996).

With the exception of central Spain, where holm oak is considered as a fruit tree and has been selected for sweet acorn production to feed pigs (Ruperez 1957), holm oak forests can be regarded as rare cases of woodlands that have undergone very little or no silvicultural management. By contrast, the bark of cork oak, which protects the treed efficiently against fire, is used to produce the familiar cork and is mainly responsible for the important economic role of this partly domesticated species. Since ancient times, cork oak has been favored, and sometimes widely spread, by preferably using acorns from trees producing good quality cork (Montoya-Oliver in Elena-Rossello and Cabrera 1996). As cork oak is predominantly allogamous, with a life span of 500 years or more, and has a low replacement rate, it can be expected that, in some places at least, and mostly for selectively neutral characters, such empirical selection over millennia may have resulted in reduced genetic differentiation both among trees of the same population and between populations. However, at present, and more particularly in the southern part of its distribution, cork oak is facing a tremendous decline due to multiple factors such as the occurrence of very severe drought periods over several consecutive years (Montero-Gonzales 1987) and, more particularly in North Africa, to excessive pruning for fodder and bark over-exploitation (M'Hirit 1995). In these regions, overgrazing and, in some areas, the harvesting of sweet acorns for food also contribute substantially to prevent natural regeneration (M'Hirit 1995).

Due to the economic value of cork and because the maintenance of cork oak woodlands is crucial to avoid soil erosion, more particularly in poor and extremely acid soils of abrupt topography where it is one of the very few tree species that can survive, *Q. suber* populations represent valuable material for genetic studies and gene-conservation programs. Greater knowledge concerning the spatial organization of genetic variation within the species is necessary to permit decisions to be made about tree breeding and the conservation of genetic resources.

In the present study, we address five major questions: (1) What is the level of genetic diversity, within and among populations, over the entire distribution area of cork oak? (2) Is this diversity similar in the presumed primary distribution area of the species (the Iberian peninsula) and in the other parts of its present distribution area? (3) What is the effect of geographical discontinuities and of ancient spatial isolation on genetic variation? (4) To what extent does genetic introgression occur between holm oak and cork oak and what is the effect of such introgression on the genetic diversity of cork oak? (5) What is the effect of human activities, more particularly of the spreading over long distances of limited genotypes samples, and of the long-term limitation of natural regeneration, on the genetic structure of cork oak?.

Materials and methods

Forty cork oak populations were sampled from sites throughout the species' range (Fig. 1). In those populations; trees that were morphologically intermediate between cork oak and another oak species were not observed, except in population 23 from Coll Sacreu (Spain) where two intermediate individuals were found. These two trees were analyzed separately. The precise geographic co-ordinates of the 40 sites, as well as their elevation, parent rock, climate characteristics and the occurrence of other evergreen oak species within 30 km from the collecting site, are indicated in Table 1. Climate data were obtained from various sources (Walter and Lieth 1960–1967: Font Tullot 1983; Le Houérou 1989). A small leafy branch was collected

Table 1 Geographical, edaphic and climatic characteristics for the 40 sample populations of cork oak (*Q. suber* L.). The occurrence of other evergreen oak species, namely *Q. ilex* L. (I) and *Q. coccifera* L. (C), is indicated as follows: *rare; **abundant; ***predominant. **P** average annual rainfall (mm), **M** mean daily maximum temperature for the warmest month (°C), **m** mean daily minimum temperature for the coldest month (°C), **N** number of trees analysed for allozyme polymorphism

Site	Locality	Country	Co-ordinates	Altitude	Parent-rock	P	M	m	Oak species	*N*
1	Oulmès	Morocco	33°45 N 04°02 W	1050	Schist	670	27.0	2.1	I**	32
2	Maâmora	Morocco	34°20 N 06°40 W	110	Sand	627	34.8	8.1		25
3	Kemis Sahel	Morocco	35°11 N 06°03 W	80	Sand	683	30.8	6.0	I*	34
4	Chefchaouen	Morocco	35°07 N 05°14 W	300	Sandstone	1480	29.2	2.0	I*	30
5	Bab Azhar	Morocco	34°12 N 04°15 W	1100	Schist	970	26.5	1.1	I**	29
6	Aïn Draham	Tunisia	36°48 N 08°40 E	739	Gneiss	1561	30.8	3.8		30
7	Tabarka	Tunisia	36°55 N 08°45 E	200	Triasic limestone	1017	31.0	7.2	C*	36
8	Nefza	Tunisia	36°58 N 09°06 E	150	Triasic limestone	933	34.9	6.6		31
9	Caltagirone	Sicily	37°14 N 14°31 E	608	Volcanic	466	31.2	7.8	I*	24
10	Gibilmanna	Sicily	37°56 N 14°03 E	610	Volcanic	666	29.8	7.0	I*	24
11	Lama Coppa	Italy	40°40 N 16°37 E	280	Alluvial	617	33.4	5.7		24
12	Nettuno	Italy	41°28 N 12°40 E	25	Sand	800	31.2	3.7		26
13	Tuscania	Italy	42°45 N 11°53 E	120	Sand	780	28.4	2.8	I*	29
14	Calangianus	Sardinia	40°56 N 09°12 E	520	Granite	850	28.5	5.3	I***	36
15	San Giuliano	Corsica	42°16 N 09°28 E	180	Granite	480	29.0	3.8	I*	40
16	Grimaud-Bormes	France	06°23 E 43°16 E	350	Schist	1000	30.1	3.6	I*	36
17	Bormes	France	43°11 N 06°21 E	90	Schist	1000	29.9	3.7	I*	35
18	Mauguio	France	43°37 N 04°00 E	15	Sand	780	28.7	1.3	I***	36
19	St. Martin Londres	France	43°47 N 03°44 E	215	Sand	1202	28.1	0.9	I**	36
20	Banyuls	France	42°29 N 03°08 E	160	Schist	976	28.3	5.3	I***	36
21	Casteljaloux	France	44°19 N 00°06 E	80	Sand	780	27.5	1.3		32
22	Hossegor	France	43°39 N 01°25 W	25	Sand	1050	24.0	5.8		36
23	Coll Sacreu	Spain	41°34 N 02°42 E	450	Gneiss	598	27.6	6.1	I***	36
24	Cantallops	Spain	42°26 N 02°55 E	300	Granite	650	28.0	4.2		36
25	Escornalbou	Spain	41°07 N 01°02 E	500	Granite/Schist	630	28.1	6.6	I** – C*	36
26	Montseny	Spain	41°46 N 02°26 E	620	Schist	850	26.0	4.2	I***	36
27	Ponte Ulla	Spain	42°46 N 08°24 W	100	Schist	926	25.0	3.8		10
28	Merza	Spain	42°46 N 08°16 W	200	Granite/Schist	970	25.4	3.5		30
29	Toxa	Spain	42°45 N 08°16 W	320	Granite	1023	25.2	3.6		30
30	Valdelosa	Spain	40°58 N 05°41 W	790	Sand	470	24.7	– 0.6	I**	34
31	Navas de Estena	Spain	39°30 N 04°41 W	640	Precambrian	783	33.2	1.7	I*	30
32	Madrid	Spain	40°25 N 03°41 W	667	Granite	438	31.0	1.4	I**	30
33	Arcos	Spain	36°45 N 05°49 W	250	Sand	560	33.8	5.2	I**	21
34	El Berrocal	Spain	37°52 N 06°05 W	390	Granite	637	34.2	5.3	I*	24
35	Donãna	Spain	36°50 N 06°25 W	10	Sand	558	33.1	5.5		15
36	Ubrique	Spain	36°41 N 05°27 W	650	Gneiss	580	33.8	5.8		15
37	Pinet	Spain	38°59 N 00°20 W	480	Sandstone	450	30.2	5.5	I**	30
38	Espadàn	Spain	39°40 N 00°10 W	600	Triasic Sand	460	31.2	5.4	I*	30
39	Matinha de Queluz	Portugal	38°45 N 09°15 W	80	Granite	767	27.6	6.6	C*	30
40	Herdade da Parra	Portugal	37°11 N 08°26 W	380	Sedimentary	1076	30.5	7.5		30

from an average of 29.3 individual trees per population (a range of 10–40 trees) for analysis.

Proteins were extracted from leaves (from 1 to 12 months old) in a Tris-HCL buffer (pH 7.6) and were stored at − 80°C until analysis, as described in Yacine and Lumaret (1989). Horizontal starch-gel electrophoresis was performed for six enzyme systems revealing a minimum of 11 loci: phospho-glucose isomerases (EC 5.3.1.9 with two loci *Pgi1* and *Pgi2*), alcohol dehydrogenases (EC 1.1.1.1, *Adh1*), isocitrate dehydrogenases (EC 1.1.1.42, *Idh1*), peroxidases (EC 1.11.1.7, *Px1*, *Px4* and *Px5*), leucine aminopeptidases (EC 3.4.11.1, *Lap1*) and acid phosphatases (EC 3.1.3.2, *Acph1*). The composition of gels and electrode buffers and the methods used to stain allozyme bands were described in Lumaret (1981) for *Acph*, in Yacine and Lumaret (1989) for *Pgi*, *Adh* and *Idh*, in Michaud et al. (1992) for *Px*, and in Ouazzani et al., (1993) for *Lap*. For tetrazolium oxidases (EC 1.15.1.1, loci *To1* and *To2*), vertical zoned polyacrylamide gels were prepared following Gasques and Compoint (1976) and were stained according to Selander et al. (1971). The genetic interpretation of the banding patterns observed in the present study was based on previous reports by Wuehlisch and Nobrega (1995) and by Elena-Rossello and Cabrera (1996) who studied the same enzyme systems. Moreover, details of the zymograms observed in holm oak and their genetic determinism from controlled crosses was reported previously (Yacine and Lumaret 1989; Michaud et al. 1995). By analyzing allelic disjunction in progeny material from cork oak, holm oak, and from their hybrids (Elena-Rossello et al. 1992, and unpublished data) it was ascertained that both species had numerous common alleles (same electrophoretic mobilities) at most enzyme loci. However, at the *Pgi1* locus, as no allele was common to both species when grown separately, this locus was considered to be diagnostic for detecting

interspecific hybrids and/or genetic introgression between the species (Elena-Rossello et al. 1992). In addition, at *Lap1* and *Acph1* loci, a few additional alleles were also specific to one or the other species (Elena-Rossello et al. 1992; Toumi 1995). In the present study, to allow an allozyme comparison between cork oak and holm oak, an index value of 1.00 at each locus was given to the most frequent allele observed in holm oak, and the other alleles found in each of the two oak species were numbered according to their relative mobility. According to this nomenclature, alleles identified as $Pgi1^{\cdot 90}$, $Pgi1^{\cdot 70}$ and $Pgi1^{\cdot 50}$ in the present work correspond to alleles $PgiB^{1.00}$, $PgiB^{\cdot 75}$ and $PgiB^{\cdot 50}$ respectively in Wuehlisch and Nobrega (1995), and alleles identified as $Adh1^{1.00}$, $Adh1^{\cdot 76}$, $Lap1^{1.00}$, $Lap1^{\cdot 97}$, $Lap1^{\cdot 94}$, $Px1^{1.06}$, $Px1^{1.00}$, $Px1^{\cdot 93}$, $Px1^{\cdot 85}$, $Acph1^{1.00}$, $Acph1^{\cdot 76}$, $To1^{1.00}$ and $To1^{\cdot 92}$ in the present study correspond to alleles $Adh1^{\cdot 44}$, $Adh1^{\cdot 35}$, $Lap1^{\cdot 67}$, $Lap1^{\cdot 65}$, $Lap1^{\cdot 63}$, $Per2^{\cdot 49}$, $Per2^{\cdot 45}$, $Per2^{\cdot 41}$, $Per2^{\cdot 37}$, $Acp1^{1.00}$, $Acp1^{\cdot 76}$, $Sod2^{\cdot 87}$ and $Sod2^{\cdot 80}$ respectively in Elena-Rossello and Cabrera (1996).

At each of the 11 loci studied in cork oak, genotypic and allelic frequencies were assessed from a survey of gel phenograms. These data were used to calculate the percentage of polymorphic populations (M) at each locus and, at the 99% criterion (the frequency of the most common allele was not greater than 0.99), 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 were compared between populations, or between groups of populations, by Mann-Whitney U tests. Allele genotypes were also used to calculate the observed heterozygozity (Ho). Percentage comparisons were made 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), were estimated according to Nei (1973,1987).

Genotypic data were analyzed using *F*-statistics and the number of migrants exchanged per generation (Nm) was estimated from the Fst value (Wright 1965). The departure of Fis values from zero was tested for each population at each locus by the method proposed by Li and Horvitz (1953). All analyses mentioned above were performed using the GENEPOP (3.1) software package (Raymond and Rousset 1995). Because of the large size of the data set, allele frequencies and genetic parameters for individual accessions are not reported herein. The relevant tables may be obtained from the corresponding author.

The genetic distances of Nei (1978) and, to maximize the effects of rare alleles, the χ^2 distances weighted by allele frequencies averaged over all populations (Balakrishnan and Shangvi 1968) were calculated between pairs of populations. The respective positions of the populations, estimated by the distances between them, were plotted in a multi-dimensional space and then projected onto a plane by non-metric multi-dimentional scaling (or proximity analysis) (Escoufier 1975). The same distances were also used as the basis for an hierarchical cluster analysis (UPGMA), with the "average distance" used as the clustering criterion (Roux 1985). Clusters at specific levels of agglomeration (total range 0–100%) were mapped onto the diagram obtained from the multi-dimentional scaling, so that the results from the two methods could be compared. These analyses were performed using the Biomeco computer package (Anon 1989). Dendrograms obtained from each of the two genetic-distance estimations were computed using the NTSYS computer package (Rohlf 1987) and tested with a co-phenetic matrix correlation to determine the most significant one.

Genotype relationships within populations were also studied in a sub-sample of five cork-oak populations (nos. 6, 11, 21, 24 and 40) growing in pure stands and of five populations (nos. 10,18, 26, 33, 34) of cork oak and of holm oak where both species were growing together or were in very close proximity. Each individual tree was characterized by its multi-locus genotype over the seven polymorphic loci. Multi-locus genotypes were compared using correspondence analysis (C.A.) (Benzecri 1973) after being encoded according to the method of Mathieu et al. (1990). More particularly, at each locus, the values 0, 1 and 2 were used to indicate absence, presence of a specific allele in the heterozygous state, and presence in the homozygous state, respectively. The respective positions of the multi-locus genotypes estimated by the distances between them were plotted in multi-dimensional space and then projected onto a plane. The same data also constituted the basis for a Hierarchical Clustering Analysis, and clusters observed at the main levels of agglomeration were mapped onto the diagram obtained from the C.A. analysis, as described above. Data treatment was performed using the Biomeco package 3.7 (Anon 1989).

Results

Genetic variation in cork oak

Among the 11 allozyme loci analyzed, four (*Pgi2*, *Px4*, *Px5* and *To2*) were monomorphic for the same allele in all populations studied. In holm oak, these loci were also monomorphic for the same alleles as in cork oak (Michaud et al. 1995; Toumi 1995). At the seven other loci, 28 alleles were identified in cork oak. Allele frequencies for the eight alleles observed at the *Pgi1* locus in the 40 populations studied are plotted in Fig. 1. At this locus, the frequency of $Pgi1^{\cdot 90}$, which is predominant in the French Catalogna and Landes regions as well as in the Iberian peninsula, is lower in North Africa, in the Italian islands (Sardinia, Sicily) and continent as well as in Corsica and Provence, whereas allele $Pgi1^{\cdot 70}$ frequency increases in the southern and eastern parts of the species distribution. Of the 28 alleles identified in cork oak, five, namely $Pgi1^{\cdot 90}$, $Pgi1^{\cdot 70}$, $Pgi1^{\cdot 50}$, $Lap1^{\cdot 97}$ and $Acph1^{\cdot 92}$, can be considered to be specific to cork oak, since they were not found in any other oak species of the Mediterranean area investigated to-date (Toumi 1995). One allele ($Adh1^{\cdot 66}$), found exclusively in population 8 (from Tunisia), had already been observed in a few populations of *Q. coccifera/calliprinos* growing in the eastern part of the Mediterranean Basin (outside the cork oak area) and 22 were already observed in holm oak (Michaud et al. 1995; Toumi 1995). Among these, 12, namely $Adh1^{1.16}$, $Adh1^{1.00}$, $Adh1^{\cdot 76}$, $Idh1^{1.10}$, $Idh1^{1.00}$, $Px1^{1.00}$, $Px1^{\cdot 93}$, $Px1^{\cdot 85}$, $To1^{1.00}$, $To1^{\cdot 92}$, $Lap1^{\cdot 94}$ and $Acph1^{1.05}$, were found in many populations of both species even when these were growing in distinct areas. The other ten alleles, namely $Pgi1^{1.33}$, $Pgi1^{1.20}$, $Pgi1^{1.00}$, $Pgi1^{\cdot 80}$, $Pgi1^{\cdot 66}$, $Idh1^{\cdot 76}$, $Px1^{1.06}$, $Lap1^{1.00}$, $Lap1^{\cdot 96}$ and $Acph1^{1.00}$, which had been found previously at a substantial frequency in holm oak, were observed, albeit at very low frequency, exclusively in eight (nos. 1, 5, 18, 20, 23, 26, 33 and 34) of the 26 cork oak populations analyzed. These eight populations were all found growing in mixed stands with holm oak, or else located not very far from it. Moreover, the two trees which were morphologically intermediate between cork oak and holm oak combined alleles of both species, more particularly at the loci PGI1 and LAP1 for which alleles diagnostic of each species were identified.

Table 2 shows the percentage of polymorphic populations, the mean and the range of alleles per population, the observed heterozygosity, several

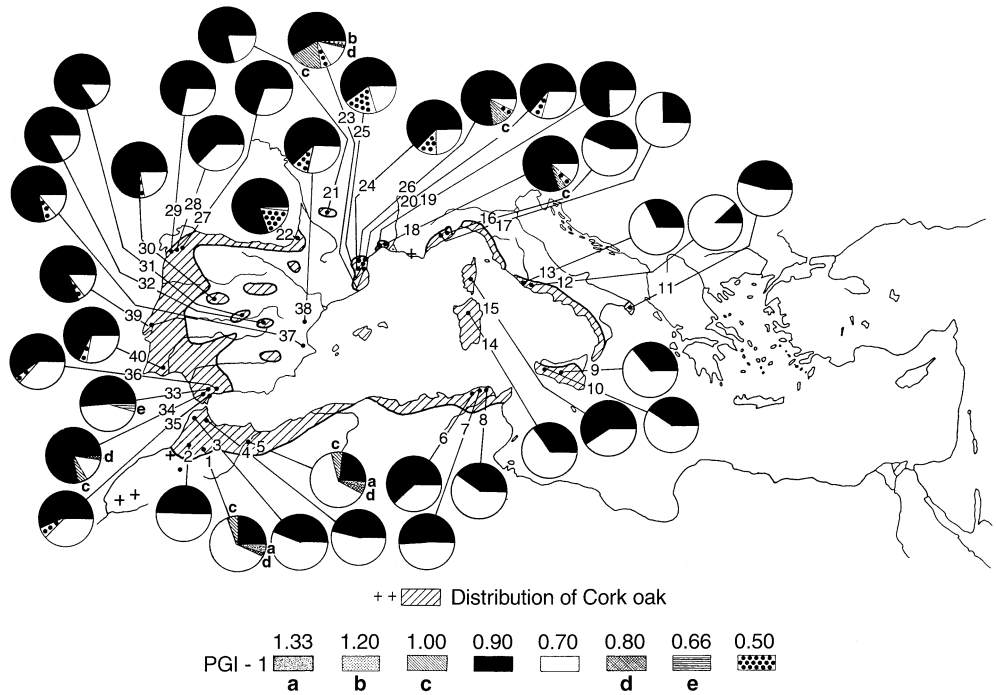

Fig. 1 Geographical distribution of allele frequencies at th *Pgi1* locus in 40 populations of cork oak. Overall species distribution areas are indicated by *hatched areas*

Table 2 Percentage of polymorphic populations (M), total number of alleles per locus (A_t), mean (A) (minimum-maximum) number of alleles per population, total genetic diversity (H_t), mean genetic diversity within populations (H_s), mean proportion of diversity among populations (G_{st}), mean number of migrants between populations per generation (N_m), observed heterozygosity (H_o), and F_{is} values at seven polymorphic allozyme loci in 40 populations of cork oak

Loci	M	A_t	A	H_t	H_s	G_{st} (%)	Nm	Ho	F_{is}
Pgi1	100	8	2.70 /(2–6)	0.52	0.44	16	1.27	0.424	0.02
Adh1	97	4	2.17 (1–3)	0.33	0.31	7	3.23	0.308	− 0.03
Idh1	95	3	2.02 (1–3)	0.47	0.43	9	2.66	0.479	− 0.10
Px1	87	4	2.37 (1–4)	0.27	0.24	11	2.10	0.248	0.02
To1	5	2	1.05 (1–2)	0.01	0.01	3	7.45	0.010	0.00
Lap1	97	4	2.12 (1–4)	0.46	0.43	7	3.37	0.394	0.07
Acph1	52	3	1.52 (1–2)	0.12	0.10	18	1.14	0.122	− 0.02
Mean 1[a]	76	4.0	2.00	0.31	0.28	11	2.09	0.283	− 0.01
Mean 2[b]	74	2.6	1.88	0.37	0.27	23	1.57	0.284	− 0.03
Mean 3[c]	48	2.9	1.63	0.20	0.18	7	–	0.180	− 0.01

[a] Mean 1: mean values over the seven polymorphic allozyme loci in the 40 studied populations
[b] Mean 2: mean values over the seven polymorphic loci analysed in the 14 cork-oak populations located far apart from other evergreen oak species
[c] Mean 3: mean values over the 11 loci (including the monomorphic *Pgi2, Px4, Px5* and *To2*) analysed in the 40 populations. – no data.

genetic diversity parameters, the mean number of migrants between populations per generation, and Fis values calculated at each of the seven polymorphic loci analyzed in the 40 cork oak populations. Averages over these seven loci either in the 40 populations (Mean 1) or in the 14 cork oak populations where the other evergreen oak species were absent (Mean 2), and over the total 11 loci studied in the 40 populations (Mean 3), were also calculated. At the seven polymorphic loci, maximum and minimum polymorphism and genetic diversity were observed at *Pgi1* and *To1*, respectively. Of the average total diversity (0.31, range from 0.01 at *To1* to 0.52 at *Pgi1*), 11% was attributable to differentiation among populations (range from 3% at *To1* to 18% at *Acph1*) (Table 2). The mean number of migrants among populations ($Nm = 2.09$) suggests that no differentiation among populations could be ascribed to the unique action of genetic drift (Wright 1965). Moreover, when the 14 cork oak populations isolated from other evergreen oak species were considered exclusively, a substantial decrease of the total allele number per locus (from 4.0 to 2.6, $P < 0.05$) and a slight

decrease of the average number of alleles per locus and per population were observed. Within-population genetic diversity also decreased. These results are due mainly to the absence in those 14 populations of ten alleles which were found frequently in holm oak. These ten alleles were identified in a few cork oak individuals in eight of the 26 localities where both species were observed to grow together or in close proximity. Conversely, an increase of the total diversity and of the level of differentiation among populations (from 11 to 23%) was also observed in the 14 cork oak populations. This change may be attributable to the smaller number of populations sampled in the same large distribution area.

Fis values averaged over loci and/or populations were not significantly different from zero. However, significant deviations from Hardy-Weinberg expectation were observed at some loci in several populations. Out of all the 257 indices tested, 17 positive deviation of Fis from 0, at either the 5% or the 1% significance level, were observed in populations 8, 9, 12, 15, 20, 23, 26, 30, 32, 33, 34 and 36, and 21 negative deviations, at either the 5% or the 1% significance level, were observed in populations 1, 3, 4, 6, 7, 10, 11, 17, 18, 19, 22, 28, 35 and 40.

The average genetic distance value among the 40 populations scored at the seven polymorphic loci was 0.038 (range from 0.001 to 0.164) for Nei's distance and 0.040 (range from 0.002 to 0.083) for the χ^2 distance.

Geographical variation in cork oak genetic structure

UPGMA coupled with χ^2 distance had the highest cophenetic correlation with the data. The diagram (Fig. 2) shows the respective positions of the 40 populations estimated by the χ^2 distance between them. Two large sets of populations could be distinguished at the critical 16% value of the agglomeration process in the UPGMA analysis. Set I, on the right side with regard to axis 2, consisted of 16 populations (Nos. 2–4, 6–17 and 28) from the geographic area including North Africa, Italy (including Sicily and Sardinia), Corsica, Provence (France) and from one population from Galicia (northwestern Spain). On the left side of axis 2, set II gathered together 18 populations from the second geographic area which includes the Iberian Peninsula and two adjacent French regions, namely Roussillon/Catalogne and Landes. The mean Nei's distance value between populations of the two sets was 0.033, and 32% of the Gst value calculated over the 40 populations was attributable to differentiation between the two population sets.

Several genetic differences were observed between the populations of these two large sets. The percentage of polymorphic populations was significantly lower in set I (70%) than in set II (78%), ($P < 0.05$), and a significantly lower ($P < 0.05$) average number of alleles per locus and per population was found in set I (A = 1.80) than in set II (A = 2.06). It should be noted that allele

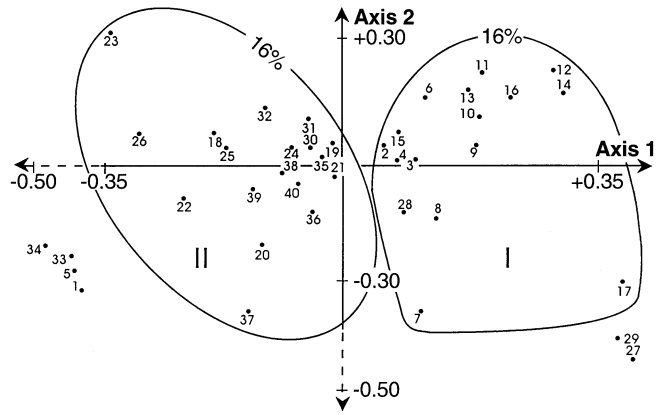

Fig. 2 Position of 40 cork oak populations according to polymorphism at the *Pgi1*, *Adh1*, *Idh1*, *Px1*, *To1*, *Lap1 and Acph1* loci. Multi-dimensional scaling is taken from χ^2 distances. Populations were clustered at the 16% level of hierarchical clustering

$Pgi1^{\cdot 50}$, specific to cork oak, was absent in set I whereas it was found in 14 of the 18 populations of set II. As mentioned above (see also Fig. 1), sets I and II showed distinct allele distributions at the *Pgi1* locus ($P < 0.01$). More particularly, the mean frequency of alleles $Pgi1^{\cdot 90}$ and $Pgi1^{0 \cdot 70}$ were 0.43 and 0.57, respectively, in set I and 0.72 and 0.20, respectively, in set II. The mean frequency of the four additional alleles was 0.08. The mean genetic diversity within populations was 0.26 and 0.28 in set I and set II, respectively, and the proportion of diversity attributable to the differentiation among populations was 9% in set I and 6% in set II. Consistently, mean Nei's distance values were 0.031 and 0.017 in set I and set II, respectively. Moreover, the mean observed frequency of heterozygotes was significantly lower in set I (25.0%) than in set II (28.1%) ($P < 0.01$), but the number of significant negative deviations of Fis values from zero (excess of heterozygotes) was significantly higher in populations of set I than in those of set II ($P < 0.05$).

Among the six additional populations which were not clustered at the 16% agglomeration level, two marginal populations from Galicia (Nos. 27 and 29), positioned on the lower right part of Fig. 2, were both characterized by a particularly low genetic diversity. Four populations, two from Andalousia (No 33 and 34) and two from Morocco (Nos. 1 and 5), were positioned on the lower left part of the diagram. In these latter four populations, cork oak occurred with holm oak in the same mixed stand, or else in close proximity, and a few individuals possessed several allozymes which are usually specific to holm oak.

Genotype distribution within populations

Similar patterns were obtained from all the populations studied and the diagram corresponding to

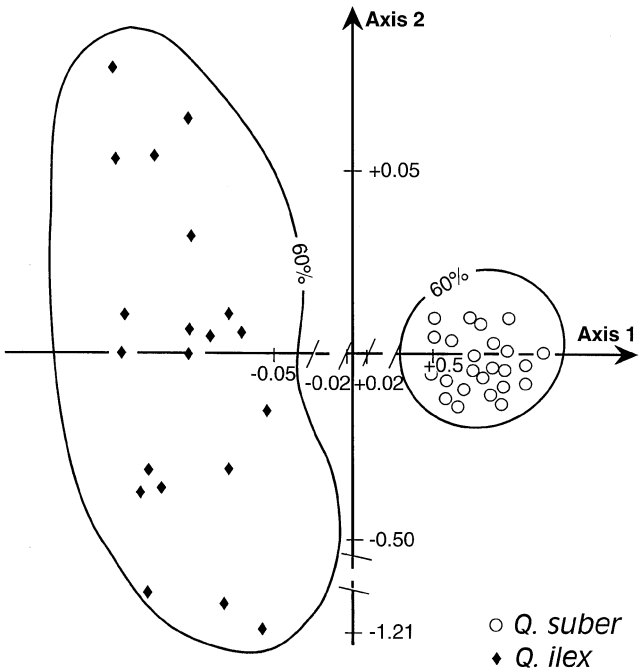

Fig. 3 Position of the multilocus genotypes of 24 cork oak trees and 20 holm oak trees from site 10 (Gibilmanna, Sicily), according to polymorphism at the *Pgi1*, *Adh1*, *Idh1*, *Px1*, *To1*, *Lap1 and Acph1* loci. Genotypes were clustered at the 60% level of hierarchical clustering

population 10 from Gibilmana (Sicily), where cork oak and holm oak populations were sympatric, is plotted in Fig. 3 as an example. There was very good discrimination between genotypes of each oak species, even when alleles specific to holm oak were present in a few cork oak individuals growing at the same site. Moreover, as shown in Fig. 3, the genotypes were always more similar in cork oak than in holm oak.

Discussion

Genetic diversity in cork oak

In cork oak, the average Fis value per locus, the total Fis value (-0.01), and the high percentage of observed heterozygosity, suggest a high outcrossing rate. This observation is also in agreement with results obtained previously in other oak species (Ducousso et al. 1993) and, more particularly, in holm oak for which 57 populations were scored for the same loci as in cork oak (Michaud et al. 1995). A relatively high genetic diversity, estimated by the several parameters mentioned above, was observed regularly in cork oak. As a point of comparison, the mean allele number per locus (At) averaged over 33 oak species was estimated to be 2.41 (Kremer and Petit 1993). Although the allele pool is smaller in cork oak (At = 4.00 and A = 2.00) than in holm oak (At = 5.70 and A = 2.31), the total genetic diversity and the within-population genetic diversity are higher in the former (Ht = 0.31 and Hs = 0.28) than in the latter (Ht = 0.26 and Hs = 0.23). This result is due to the occurrence of very few low-frequency alleles in the cork oak populations as compared to those of holm oak. In cork oak, 11% of the total genetic diversity measured over the polymorphic loci is attributable to differentiation among populations. This value is close to that estimated in holm oak (10%) (Michaud et al. 1995) but is higher than the mean value (7%) obtained over 25 species of oaks studied for enzyme polymorphism (Kremer and Petit 1993) and, more particularly, than the population differentiation (2.5%) estimated over eight polymorphic allozyme in *Quercus petraea* L., a European deciduous species (Zanetto and Kremer 1995).

Effect of genetic introgression from other evergreen Mediterranean oaks

Very rare cases of local hybridization between cork oak and some sympatric deciduous oak species, more particularly *Quercus cerris* and *Quercus faginea*, have been reported (Bellarosa et al. 1990; Gil-Sanchez et al. 1996). However, cork oak was reported to hybridize mainly with the other species of the evergreen oak-group and more particularly with holm oak (Camus 1938). The holly oak, which shows habitat preferences very distinct from those of cork oak and which is rarely observed to grow in the same sites (Gil-Sanchez et al. 1996), is not a good candidate for hybridization with cork oak. In the present study, in the 14 cork oak populations which were geographically isolated from holm oak and in 18 of the 26 cork oak populations growing in mixed stands or which were spatially close to that species, specific alleles not observed in any other evergreen oak species of the Mediterranean area were found exclusively at the diagnostic locus *Pgi1*, and alleles characteristic of holm oak or of other Mediterranean oak species were not observed at the other loci. This suggests that these 32 cork oak populations were not genetically introgressed by holm oak, or by any other species. Conversely, in the eight other cork oak populations in contact with holm oak, the occurrence of ten additional enzyme alleles (five at *Pgi1* and five distributed over four other polymorphic loci), all absent in the 32 other populations and all commonly observed in holm oak populations, constitutes evidence for the genetic introgression of holm oak into cork oak populations. In this study, as in previous allozyme surveys at the same loci (Toumi 1995), alleles diagnostic of cork oak and those diagnostic of holm oak were found in association in the very rare trees (from Catalogna, central Spain, Andalusia, Sardinia) which were morphologically intermediate between both oak species. However, no allele being diagnostic of cork oak

was found in the nearly 80 holm oak populations which were analyzed from the entire species distribution area, even when these populations were growing in mixture with cork oak (Michaud et al. 1995; Toumi 1995). All these results suggest the occurrence of unidirectional genetic introgression from holm oak into cork oak. This may be due to initial hybridization between both species and to subsequent backcrossing with cork oak. As stressed previously by Elena-Rossello et al. (1992), the flowering period occurs earlier in holm oak than in cork oak, usually with no overlap. However, high phenological plasticity was observed in both species (Corti 1959; Elena-Rosello et al. 1993) and occasional flowering overlap may occur, especially in sites where the reproductive period is disturbed or shortened by unusual environmental conditions such as very cold temperatures at high elevation.

Moreover, as trees in both species are protandrous (stamens ripen before the stigma matures), female flowers from the last flowering holm oak trees may be pollinated by the first flowering cork oak trees so that the occurrence of hybrids with holm oak as the mother tree are expected to be less rare than the reciprocal situation. Evidence that holm oak is usually (if not exclusively) the mother tree in hybridization events with cork oak was provided from an analysis of both mitochondial and chloroplast-DNA, which are cytoplasmic markers inherited maternally in oaks (Dumolin et al. 1995). For instance, chlorotypes characteristic of holm oak were systematically recovered in the trees identified as morphologically intermediate between both oak species, as well as in cork oak trees from local populations which were introgressed genetically by holm oak on the basis of enzyme polymorphism (Toumi 1995; Lumaret et al., unpublished). Moreover, in the present study, evidence that hybridization events most likely occurred in several distinct places was provided by the presence in the introgressed cork oak populations of alleles distributed locally and which were present exclusively in the holm oak populations of the same areas. For instance, in the Moroccan cork oak populations nos. 1 and 5, allele $Pgi1^{1.33}$ was identified in a few individuals. This allele was observed frequently in holm oak but was restricted to populations growing in the High and Middle Atlas (Michaud et al. 1995).

Several additional factors, such as the relative proportion of trees of each oak species present, may also play a role in the occurrence of inter-specific hybridization. Out of the eight cork oak populations analysed in the present study and for which evidence of genetic introgression by holm oak was found, four were located in French Catalogna/Roussillon and in Spanish Catalogna where the holm oak is predominant. Of the four others, two were situated in Andalusia (Spain) and two at high elevations in the Middle Atlas (Morocco). Both these areas are characterised by a high frequency of holm oak, either at present or else in the near past (Sauvage 1961).

Moreover, in regions where holm oak is grown in mixture with cork oak for acorn production (e.g. in central Spain), because most trees derived from hybridization between holm oak and cork oak either do not have any cork or are reported to produce a very low quality cork (Natividade 1936), they are generally eliminated as soon as they can be identified (namely, when they are from 10 to 15 years old, an age which marks the beginning of cork constitution in cork oak). In practice, the few morphologically intermediate trees which may show cork production, and/or quality, close to that of cork oak trees are very rarely identified and eliminated. The selective elimination of intermediate trees, intended to preserve cork quality, might also favor unidirectional genetic introgression from holm oak into cork oak. In several other regions (e.g. in northern Sardinia), holm oak trees growing in mixed stands with cork oak are regularly cut to avoid hybridization between the species (personal observation). The effect of such a practice may substantially reduce interspecific hybridisation in areas of intensive cork oak management but is probably less efficient in other areas, more particularly those situated at high elevation or those which, for any other reason, are not managed intensively. The results obtained from the present study of allozyme variation showed a localized genetic introgression of cork oak by holm oak with a genetic effect limited to a local increase of the allelic pool. The low ability of holm oak and cork oak to genetically introgress each other may support their classification as two distinct subgenera.

Phylogeographic structure of allozyme variation in cork oak

The most striking result of the present study was to identify genetically distinct groups of cork oak populations corresponding to two distinct geographical areas. The larger allelic pool, the higher within-population genetic diversity, and the lower differentiation among cork oak populations from the Iberian peninsula and the adjacent French regions, as compared to those of the rest of the distribution area, is not likely to be attributable to selective effects related to environmental factors since these did not differ significantly between the two parts of the geographical distribution. In the Iberian peninsula, which has been proposed to be the center of origin of cork oak (Sauvage 1961), the observation of several low-frequency allozyme alleles specific to cork oak and which were absent in the other parts of the species distribution area suggests the possible occurrence of a genetic bottleneck related to founder effects and genetic drift. This may be due to early cork oak migration from the Iberian Peninsula to North Africa through the Gilbraltar strait at the end of the Tertiary period or later, as suggested by Sauvage (1961). Moreover, the possibility cannot be ruled out

that, during the Quaternary, postglacial re-colonization from southern to northern regions may have occurred independently in the western and the eastern part of the present cork oak distribution from two distinct refugia located in southern Iberia and in North Africa, respectively. However, cork oak dispersal may also rely on the occasional transport of acorns by boat, since early Antiquity, from the Iberian peninsula to several other regions, and/or among those regions which, although being very disjunct geographically, showed genetically close cork oak populations. Conversely, in holm oak analyzed at the same enzyme loci and in the same areas as cork oak, geographic discontinuity was shown to be responsible for substantial genetic differentiation (Michaud et al. 1995). In addition, a frequency inversion concerning two alleles, $Pgi1^{\cdot 90}$ and $Pgi1^{\cdot 70}$, was observed between the cork oak populations from Iberia and the two adjacent French areas and those from the other parts of the species distribution. Consistent directional variation in allele frequencies was also recorded by Daubree and Kremer (1993) in populations of *Quercus rubra* L. following its introduction into Europe, compared with the native American populations. Michaud et al. (1995) found a similar trend in *Q. ilex* L. between populations from the Atlantic and the Mediterranean areas, respectively. In both studies, the authors suggested that the variation observed was most likely related to the different natural-selection pressures (on the enzyme loci or on loci tightly linked to them) occurring in the distinct regions or continents rather than to a founding effect, though this latter possibility was not completely ruled out.

The role of human activities on genetic variation in cork oak

Possible effects on overall genetic variation of the long-distance dissemination of cork oak by humans, and of the hypothesis of their consciously exercising at least partial control of its hybridization with holm oak, were already discussed above. As stressed by Elena-Rossello and Cabrera (1996), who analyzed allozyme variation in seven Spanish cork oak populations, the management and use of cork oak by humans should have modified its genetic structure in two main additional ways. One is due to the widespread practice of cork oak spreading on a local scale by sowing acorns from a very few trees which produce good quality cork (Sondergaard 1991; Montoya-Oliver in Elena-Rossello and Cabrera 1996). As the life span of cork oak may exceed 500 years, progeny from the same mother tree may be used many times to cover large areas. Based on allozyme analysis in the progeny of open-pollinated trees, cork oak mother trees were shown to be mainly outcrossed and, most often, by several (sometimes numerous) pollen trees (Wuehlisch and Nobrega 1995). Consequently, substantial genetic diversity is likely to be maintained even in a restricted number of tree progeny as shown previously in holm oak (Yacine and Lumaret 1989). However, in cork oak, directional selection over millenia may have reduced genetic differentiation both between populations and among genotypes within populations. As compared to the holm oak populations analyzed by Michaud et al. (1995) at the same allozyme loci and in the same geographic area as the cork oak populations, a lower genetic differentiation among cork oak populations was observed. For instance, in the area including the whole Iberian peninsula and the adjacent French regions, 6% and 9% of the total genetic diversity was attributable to differentiation among populations in cork oak and in holm oak respectively. This difference may be a consequence of the partial domestication of cork oak. Moreover, within populations, the effect of long-term selection was clearly reflected by the occurrence of a much closer relationships among allozyme genotypes in cork oak than in holm oak (Fig. 3). It will be recalled that holm oak has been submitted to low, or no, genetic selection over the main part of its geographical distribution.

As stressed by Gil-Sanchez et al. (1996), cork oak genetic structure may also have been affected by a considerable depletion of sexual reproduction due mainly to the over-use of acorns as animal feed and, in some areas, as food for people; consequently, many stands include exclusively very old trees, with no natural replacement in situ. In several long-lived tree species, a positive association has been observed between vigor and longevity on one hand, and heterozygosity on the other (Mitton and Grant 1984; Ouazzani et al. 1993). In cork oak, such an association may also occur. This was shown by the significantly higher number of cases of heterozygote excess in populations from the Southern and Eastern parts of the species distribution, which are characterized by extremely low or no regeneration, than in the other distribution areas.

In summary, this study has shown that life history traits (especially long life-span and out-crossing), phylogeographical events (more particularly intercontinental migration), genetic introgression, especially by holm oak, and human activity, have all played a major role in determining the genetic variation pattern observed in cork oak at present. These factors should be taken into account both in conservation programs and for the genetic improvement of this economically very important Mediterranean species.

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