

Effect of geographical discontinuity on genetic variation in *Quercus ilex* L. (holm oak). Evidence from enzyme polymorphism

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Allozymes at a maximum of eight loci were used to analyse the spatial patterns of genetic variation in 1942 holm oak trees (*Quercus ilex* L.) from 57 provenances distributed in the six main disjunct areas of the species distribution area. Polymorphism and genetic diversity were high except in the six marginal populations growing in various locations under unfavourable climatic conditions. Ten per cent of the total genetic diversity ($H_t = 0.262$) was accounted for by among-population variation. In this long-lived species, the effect of geographical discontinuities on gene flow restriction was shown by the occurrence of: (i) numerous rare alleles limited to a single disjunct region; and (ii) allele frequency variation among the disjunct regions for four alleles. This result was obtained by comparing spatial autocorrelograms from the 57 populations of the entire distribution area and from the 31 populations of the Mediterranean continental region which constitutes the largest continuous area in that distribution. Life history traits (e.g. long life span and high outcrossing rate) and past changes in climate and geographical continuity are assumed to be the main factors responsible for the present genetic variation patterns observed in the species.

Keywords: allozyme differentiation, long-term isolation, *Quercus ilex*.

Introduction

Many genetic studies using enzyme polymorphism in forest tree species have shown the occurrence of very high genetic diversity, especially within populations, whereas low differentiation has been observed among populations (Hamrick *et al.*, 1979). Spatial genetic homogeneity is usually attributed to the occurrence of high gene flow among trees in long-lived, wind-pollinated species which possess, therefore, very high dispersal ability (Levin & Kerster, 1974; Loveless & Hamrick, 1984; Govindaraju, 1988). However, population differentiation may also occur by mutation, differential selection or genetic drift, provided that seed and/or, more particularly, pollen dispersal are limited (Slatkin, 1987). In coniferous species, gene flow reduction has been shown to occur between populations isolated on islands and those growing on the continent (Fineschi, 1984), between populations distributed on two opposite sides of the same mountain (Chung, 1991) or in populations isolated in marginal areas (Furnier & Adams, 1986; Betancourt *et al.*,

1991; Zabinski, 1992). In broad-leaved tree species which have been shown to possess the same level of genetic diversity and geographical homogeneity as coniferous species (Hamrick *et al.*, 1992; Müller-Starck *et al.*, 1992), very little is known about the effects of long-term gene flow reduction from geographical discontinuity on genetic differentiation (e.g. Haase, 1992). The aim of the present study is to assess the effect of long-term spatial discontinuity on the genetic structure of the holm oak (*Quercus ilex* L.) by using allozyme markers.

The holm oak is a broad-leaved, evergreen, long-lived tree species able to resprout from roots and stumps. It is mostly wind-pollinated and outcrossing even though each tree bears both male and female inflorescences (Lumaret *et al.*, 1991). Acorn dispersal by small mammals and large birds is assumed to be very limited compared with pollen dispersal (Ducousso *et al.*, 1993). The holm oak is mostly distributed in the western part of the Mediterranean basin, in three distinct areas: (i) North Africa from Tunisia to Morocco; (ii) on several large islands, e.g. Crete, Sicily and Corsica; and (iii) in southern (continental) Europe, along a continuum from Turkey to Portugal.

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In addition, the species is regularly distributed along the Atlantic coast of France from the Bordeaux basin to the Loire river. Palaeoecological data indicate that the holm oak has occurred in the south of Europe and in North Africa since the end of the Tertiary period (Pons & Vernet, 1971; Palamarev, 1989). According to Pons & Vernet (1971), holm oak populations from the Atlantic area have a Quaternary history distinct from that of the Mediterranean populations. Since 4500 BP, at least in the wettest part of its Mediterranean distribution, the holm oak had been favoured by human activities such as pastoralism or clearing by burning (Pons & Thimon, 1987; Reille & Pons, 1992). However, in most of the distribution area, holm oak forests can be regarded as rare cases of woodlands that have undergone very low or no silvicultural management. By contrast, in central Spain, the holm oak is considered as a fruit tree and has been selected for sweet acorn production to feed pigs (Ruperez, 1957).

Two main morphological types of holm oak have been described. The 'rotundifolia' type is a small round-leaved morph occurring in inland parts of Spain and North Africa where it grows under Mediterranean climates ranging from semiarid (with markedly continental conditions) to perhumid. The 'ilex' type is a large, elongated leaf morph distributed from Turkey to the French Riviera, and along the Atlantic coast of France. This morph is restricted to humid or subhumid sites mainly in mild coastal areas (Barbero *et al.*, 1992). The two morphotypes have been considered either as two distinct species (Schwartz, 1964; Saenz de Rivas, 1972; Afzal-Rafii, 1988; Afzal-Rafii *et al.*, 1991a,b, 1992), two subspecies (Saenz de Rivas, 1967, 1970) or simply two varieties (Maire, 1961). In addition, trees showing morphological characters intermediate between those of the two morphs described above occur in the Mediterranean region of France and in the eastern and northern coastal areas of Spain.

Owing to the pollination system of the holm oak, its long fecundity period and its outcrossing reproductive system, substantial gene flow is expected to occur among populations. Conversely, discontinuities in its geographical distribution because of early colonization around the Mediterranean Basin (before the Gibraltar Detroit was formed, i.e. approximately five million years ago according to Bocquet *et al.*, 1978), may be responsible for genetic differentiation among the isolated areas. In the present study, we address three major questions. (i) Is the level of genetic diversity, within and among populations, similar over the whole distribution area? (ii) Do marginal populations which mostly grow in small discontinuous areas show reduced genetic variation, as has been observed in other species (Mayr, 1975; Hamrick *et al.* 1989; van

Treuren *et al.*, 1991)? (iii) What is the effect of large geographical discontinuities, and of ancient isolation, on the genetic structure of the holm oak?

Materials and methods

Origin of plant material and sampling procedure

Fifty-seven populations were sampled over the whole distribution area of the holm oak (Fig. 1). Populations were natural except those from nos 19–23 which were introduced into Brittany about one century ago (Dupont, 1990). Eleven populations were collected in marginal areas where the holm oak showed a patchy distribution. Six of those marginal populations, namely nos 9, 17, 37, 39, 44 and 47 (Table 1), were located in areas where the holm oak distribution was limited by climatic constraint such as frost or soil moisture (Quezel, 1976). The five other populations, nos 28, 29, 30, 31 and 54, were found growing under normal climatic conditions for the species, in areas where the patchy distribution was a result mainly of human activity (cultivation). Collecting sites were distributed over a great range of parent rocks and elevations (Table 1). Climatic data were obtained from various sources (Walter & Lieth, 1960–1967; Garnier, 1964, 1966; Lombardo, 1973; Font Tullot, 1983; Le Houérou, 1989). The populations were classified as 'ilex', 'rotundifolia' or 'intermediate type' according to Schwartz's morphological criteria (1964). A small, leafed branch was collected from an average of 34 individual trees per population (range 15–59 trees; Table 1).

Allozyme 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 & Lumaret (1989). Horizontal starch gel electrophoresis was performed for six enzyme systems: PGI, EC 5.3.1.9 (coding for phosphoglucose isomerase with two loci, *PGI-1* and *-2*), ADH, EC 1.1.1.1 (alcohol dehydrogenase, locus *ADH-1*), IDH, EC 1.1.1.42 (isocitrate dehydrogenase, locus *IDH-1*), PX, EC 1.11.1.7 (peroxidase, locus *PX-1*), LAP, EC 3.4.11.1 (leucine aminopeptidase, locus *LAP-1*) and AcPH, EC 3.1.3.2 (acid phosphatase, locus *AcPH-1*). The composition of gels and electrode buffers and the methods used to stain allozyme bands were described in Yacine & Lumaret (1989) for the PGI, ADH and IDH systems, in Michaud *et al.* (1992) for peroxidases, in Ouazzani *et al.* (1993) for leucine aminopeptidases and in Lumaret (1981) for acid phosphatases, respectively.

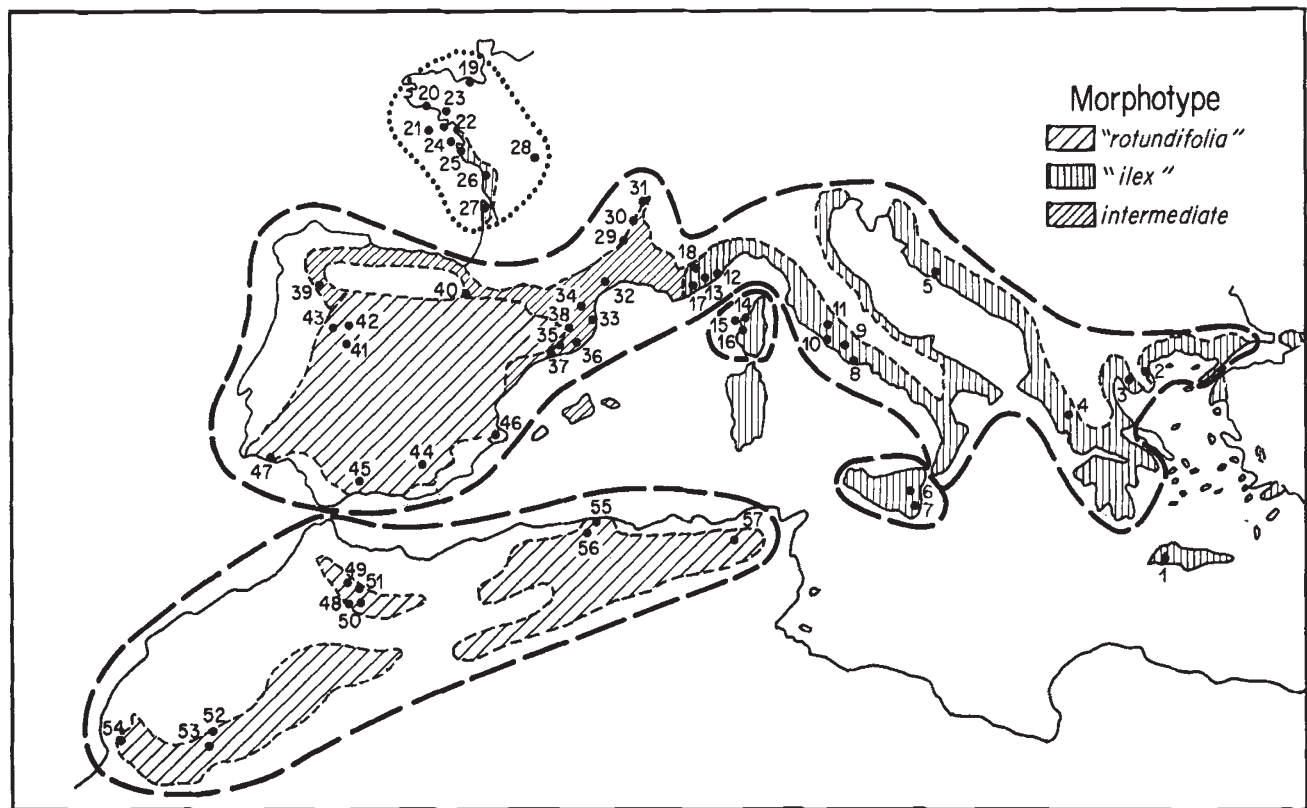


Fig. 1 Natural geographical distribution of holm oak (three morphotypes). Distribution areas corresponding to ancient (five million years) and recent (700 000 years) disjunctions are surrounded with dashes and dots, respectively. The 57 populations studied are also located and numbered on the map.

For tetrazolium oxidases, EC 1.15.1.1 (locus TO-1), vertical zonal polyacrylamide gels were prepared following Gasques & Compoint (1976) and were stained according to Selander *et al.* (1971). At each locus, the index value 1.00 was given to the most frequent allele whereas the other ones were numbered according to their relative mobility.

Inheritance of PGI, ADH and IDH isozymes was studied by Yacine & Lumaret (1989). To examine the genetic control of PX, LAP and AcPH isozymes, phenotype variation was analysed in the progeny of controlled crosses. At each locus, observed genotype frequencies in the progenies were compared with the frequencies expected under Mendelian segregation using a χ^2 -test. For TO, the study of genetic determination was limited by the lack of suitable parental genotypes.

The 57 populations were scored for polymorphism at six loci: *PGI-1*, *PGI-2*, *ADH-1*, *IDH-1*, *PX-1* and *TO-1*. Ten populations from the Atlantic area (nos 19–28 in Fig. 1) and 12 populations (nos 4, 6, 10, 11, 13, 29, 30, 31, 32, 37, 44 and 46 in Fig. 1) from the

European Mediterranean area were scored for two additional loci, *LAP-1* and *AcPH-1*.

Statistical analysis

Genotypic and allelic frequencies were assessed at each locus from a survey of gel phenograms. *G*-tests for heterogeneity of allele frequencies among populations were carried out according to Sokal & Rohlf (1981). The data were also used to calculate the total number of alleles per locus, percentage of polymorphic loci, real and effective number of alleles per population (A and A_e , respectively), observed heterozygosity (H_o), total genetic diversity (H_t), within-population genetic diversity (H_s), among-populations genetic diversity (D_{st}) and the proportion of diversity resulting from gene differentiation among populations (G_{st}) (Nei, 1973, 1987). Genotypic data were analysed using *F*-statistics (Wright, 1965). Departure of F_{is} values from zero was tested for each population at each locus by the method proposed by Li & Horvitz (1953).

Table 1 Geographical, edaphic and climatic characteristics and tree morphotypes for the 57 sample populations of holm oak (*Quercus ilex*)

Site	Locality	Country	Co-ordinates	Altitude (m)	Parent-rock	P (mm)	m (°C)	M (°C)	Bioclimate	Morph. type	Number of plants
1	Vlatos	Crete	35°28'N 23°38'E	320	Sericitic schist	557	7.7	30.3	SH-w	I	26
2	Olimbiada	Greece	40°35'N 23°47'E	8	Flinty	513	—	—	—	I	37
3	Vassilika	Greece	40°27'N 23°02'E	100	Flinty	602	4.8	30.3	SH-w	I	21
4	Monolithi	Greece	39°27'N 20°49'E	—	Limestone	1236	1.1	31.4	H-w	I	32
5	Marjan	Croatia	43°31'N 16°28'E	5	Limestone	914	4.6	27	H-w	I	41
6	Minardo	Sicily	37°50'N 14°55'E	1100	Lave	900	1	33	SH-w	I	30
7	Mandra	Sicily	37°07'N 15°32'E	450	Limestone	817	1	33	SH-w	I	30
8	Circeo	Italy	41°15'N 13°04'E	100	Limestone	1044	2.8	32	H-w	I	25
9	Monte Lepini	Italy	41°35'N 13°08'E	570	Limestone	1071	0.6	25	PH-c	I	29
10	Ostia	Italy	41°44'N 12°16'E	10	Sand	799	3.7	31.2	H-w	I	30
11	Cerveteri	Italy	42°02'N 12°08'E	150	Volcanic	760	3.5	33	H-w	I	30
12	Dolceacqua	Italy	43°51'N 07°37'E	150	Limestone	678	4.2	27.6	SH-w	I	23
13	Menton	France	43°07'N 07°30'E	200	Sandstone	819	4.4	27.8	SH-w	I	48
14	Fango	Corsica	42°22'N 08°45'E	370	Crystalline	686	3.9	25.3	SH-w	I	52
15	Galeria	Corsica	42°25'N 08°42'E	2	Sand	592	4.3	26	SH-w	I	38
16	Porto	Corsica	42°16'N 08°45'E	100	Crystalline	700	4.3	25.4	SH-w	I	40
17	Pas de la Faye	France	43°45'N 06°50'E	860	Limestone	1078	0.4	23.2	PH-c	I	50
18	Thoard	France	44°06'N 06°06'E	700	Crystalline	756	-3	28.8	SH-c	I	50
19	Saint-Servan	France	48°38'N 02°00'W	4	Granite	708	4.5	21.7	A	I	44
20	Combrit	France	47°53'N 04°09'W	8	Granite	1004	2.7	22.7	A	I	35
21	Belle île	France	47°20'N 03°10'W	50	Schist	616	4.7	21.4	A	I	19
22	Kervert	France	47°33'N 02°50'W	2	Sand	680	2.3	21.8	A	I	40
23	Sarzeau	France	47°32'N 02°46'W	6	Sand	680	2.3	21.8	A	I	15
24	Bois de la Chaise	France	47°01'N 02°15'W	2	Sand	718	4	22.8	A	I	40
25	Barre-de-Monts	France	46°52'N 02°07'W	9	Sand	718	4	22.8	A	I	20
26	La Rochelle	France	46°17'N 01°10'W	10	Limestone	687	2.1	25	A	I	26
27	Maubuisson	France	45°00'N 01°11'W	20	Sand	803	2.2	25.7	A	I	45
28	Le Porteau	France	46°35'N 00°20'W	90	Limestone	640	0.7	25	A	IR	39
29	Crussol	France	44°56'N 04°52'E	340	Limestone	904	0.2	28.8	SH-c	IR	36
30	Champagne	France	45°16'N 04°48'E	300	Gneiss	822	0.4	29.1	SH-c	IR	36
31	Sainte Colombe	France	45°31'N 04°52'E	200	Schist	786	-1	26.6	SH-c	IR	36
32	Montpellier	France	43°36'N 03°55'E	30	Limestone	857	0.6	28.7	SH-c	IR	50
33	Baillaurie	France	42°27'N 03°06'E	160	Schist	976	5.3	28.3	H-w	IR	59
34	Gorges de la Fou	France	42°27'N 02°35'E	350	Schist	860	2.1	28.9	H-w	IR	50
35	San Marti	Spain	41°32'N 01°02'E	550	Marl limestone	462	1.4	31.7	SA-w	IR	35
36	Coll Sacreu	Spain	41°34'N 02°42'E	450	Gneiss	598	6.1	27.6	SH-w	IR	39
37	Villafranca	Spain	41°20'N 01°42'E	300	Limestone	500	6.8	28.1	SA-w	IR	37
38	El Vilar	Spain	41°46'N 02°20'E	750	Schist	900	3.55	24.5	H-w	IR	40
39	Medeiros	Spain	41°56'N 07°25'W	600	Schist	893	0.4	23.2	PH-c	IR	17
40	Pancorbo	Spain	42°35'N 03°08'W	900	Limestone	860	1.3	25.4	H-w	IR	16

Table 1 Continued

Site	Locality	Country	Co-ordinates	Altitude (m)	Parent-rock	P (mm)	m (°C)	M (°C)	Bioclimate	Morph. type	Number of plants
41	Cristobal	Spain	40°28'N 05°55'W	740	Sand	460	0.6	30.5	SA-c	R	21
42	Finca	Spain	41°10'N 05°50'W	700	Alluvial	484	-0.6	29.7	SA-c	R	53
43	Carreros	Spain	40°55'N 06°00'W	770	Sand	421	-0.5	30.2	SA-c	R	22
44	Puerto del Mora	Spain	37°15'N 03°26'W	1350	Limestone	600	-2.5	30.3	SH-c	R	33
45	Cascajares	Spain	36°37'N 05°05'W	1300	Limestone	800	0.1	30.9	SH-c	R	30
46	Font Roja	Spain	38°40'N 0°32'W	1100	Limestone	726	-0.9	32.6	SH-c	R	43
47	Ria Formosa	Portugal	36°57'N 07°55'W	2	Sand	453	9.0	28.2	SA-w	R	24
48	Jamaa Bou Yalaa	Morocco	33°55'N 04°05'W	1350	Marl	500	-	-	-	R	35
49	Taineste	Morocco	34°35'N 04°05'W	1240	Sandstone	682	2	33.9	SH-w	R	31
50	Bab Larbaa	Morocco	34°01'N 04°03'W	1320	Volcanic	700	-	-	-	R	31
51	Bab Ou Idir	Morocco	34°03'N 04°07'W	1580	Gritty limestone	1200	-	-	-	R	36
52	Amizmiz	Morocco	31°13'N 08°14'W	1650	Schist	481	2.4	31.1	SA-w	R	35
53	Ounein	Morocco	31°02'N 08°01'W	2100	Limestone	544	1.8	29.8	SA-w	R	27
54	Imouzer	Morocco	30°41'N 09°29'W	1310	Limestone	532	2	32	SA-w	R	16
55	Tigzirt	Algeria	36°52'N 04°07'E	180	Schist	936	5.9	32.5	H-w	R	38
56	Meurdja	Algeria	36°30'N 03°09'E	900	Limestone	1129	3.8	36	H-w	R	40
57	Djebel Dir	Tunisia	36°16'N 08°42'E	500	Limestone	509	3.1	33.6	SA-w	R	21

— no data available; P: average annual rainfall; m: mean daily minimum temperature for the coldest month (January); M: mean daily maximum temperature for the warmest month (July/August). Mediterranean bioclimatic zones: PH hyperhumid, H humid, SH subhumid, SA semiarid; temperature variants: c indicates very cold to cold winters ($m < 1^\circ\text{C}$) and w indicates cool to very warm winters ($m > 1^\circ\text{C}$) following Emberger (1955) and Le Houérou (1989). A: Atlantic climate. Morphological types: I 'flex'; R 'rotundifolia'; IR intermediate between I and R.

The number of migrants exchanged per generation (Nm) was also estimated from the F_{st} value (Wright, 1951). All the analyses above were performed using the BIOSYS-1 computer program (Swofford & Selander, 1989).

The genetic distances of Nei (1978), Gregorius (1984) and a χ^2 distance were calculated between pairs of populations from their allelic distributions. The χ^2 distance, weighted by the average frequency of each allele in all the populations, has the property of maximizing the effects of rare alleles (Yacine & Lumaret, 1989). 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 the 'average distance' as the clustering criterion (Roux, 1985). Clusters at different levels of agglomeration (total ranging from 0 to 100 per cent) were mapped onto the diagram obtained from the multidimensional scaling so that the agreement between the results from the two methods could easily be compared. These analyses were performed using the BIOMECO computer package (Lebreton *et al.*, 1987). Dendrograms obtained from each of the 3 genetic distance estimations were also computed with the NTSYS computer package (Rohlf, 1987) and tested with cophenetic matrix correlation to establish the most significant one.

To assess the effect of geographical discontinuities on genetic variation, the populations were separated into five geographical groups (Mediterranean continental Europe, North Africa, Corsica, Sicily and the French Atlantic coast) which correspond to five disjunct distribution areas (Fig. 1). In North Africa, palaeobotanical studies have shown that the distinct holm oak distribution areas observed presently were connected in the very recent past (Reille, 1976; Lamb *et al.*, 1989). Therefore, these areas were considered as a whole in the present study. The single population from Crete was not considered in this study. Gene diversity between populations (G_{st}) was further subdivided into variation among groups (G_{gt}) and among populations within groups (G_{sg}). Moreover, spatial autocorrelation (SA) analysis (Sokal & Oden, 1978a,b) was carried out as a way of looking for correlated (similar or dissimilar) values of gene frequencies in populations that are a given geographical distance apart from each other. SA was carried out separately on the 57 populations located in the whole distribution area (set 1) and on the 31 populations from the Mediterranean continental Europe group (set 2) which constitutes the largest continuous distribution area for

the species. Latitudes and longitudes expressed in degrees were transformed into 'extended Lambert 2 co-ordinates' which enabled the computation of the distance in kilometres between the two members of any pair of populations (Petit *et al.*, 1993). For each set of data, a matrix of geographical distances between all pairs of populations was calculated. The distances were then grouped in discrete classes, with class intervals of 400 km. A Moran's (1950) autocorrelation index (I) which varies between -1 and $+1$ and a Standard Normal Deviate (SDN) were computed for each allele in each distance class. A significant spatial pattern is indicated at the 5 per cent probability level when $SND > 1.96$. A correlogram in which Moran's I values were plotted vs. distance classes was then built for each allele studied and correlogram significance was tested by the Bonferroni procedure (Oden, 1984).

Results

Survey of gel-banding patterns and enzyme genetic control

Peroxidases (PX). Of the four zones of activity observed on the gels, only the second fastest zone had interpretable banding patterns. Examples of observed phenotypes are given in Fig. 2a. Three classes of parental phenotypes were available; one was inferred to be homozygotes (single-banded phenotypes) and the others were inferred to be distinct heterozygotes (two-banded phenotypes). Crosses between an inferred homozygote and heterozygotes (crosses 2 and 3 in Table 2) produced two classes of progeny with single- or double-banded phenotypes in approximately equal proportions. Crosses between inferred identical homozygotes produced only the parental one-banded phenotype. These results suggest that the variability found in this zone of activity is controlled by a single polymorphic locus, *PX-1*. This locus codes for a functionally monomeric enzyme with seven codominant alleles, namely *PX-1*^{0.85}, *PX-1*^{0.93}, *PX-1*^{0.96}, *PX-1*^{1.00}, *PX-1*^{1.03}, *PX-1*^{1.06} and *PX-1*^{1.08}.

Leucine aminopeptidase (LAP). Two zones of activity were observed on the gels. Only the faster was regularly interpretable (Fig. 2b). The cross between an inferred homozygote (one-banded phenotype) and a heterozygote (double-banded phenotype) produced two classes of progeny with either single- or double-banded phenotypes in approximately equal proportions (cross 2 in Table 2). These results support the interpretation that LAP is coded by a single locus *LAP-1*, with three codominant alleles (*LAP-1*^{0.94}, *LAP-1*^{0.96} and *LAP-1*^{1.00}) which specify monomeric enzymes.

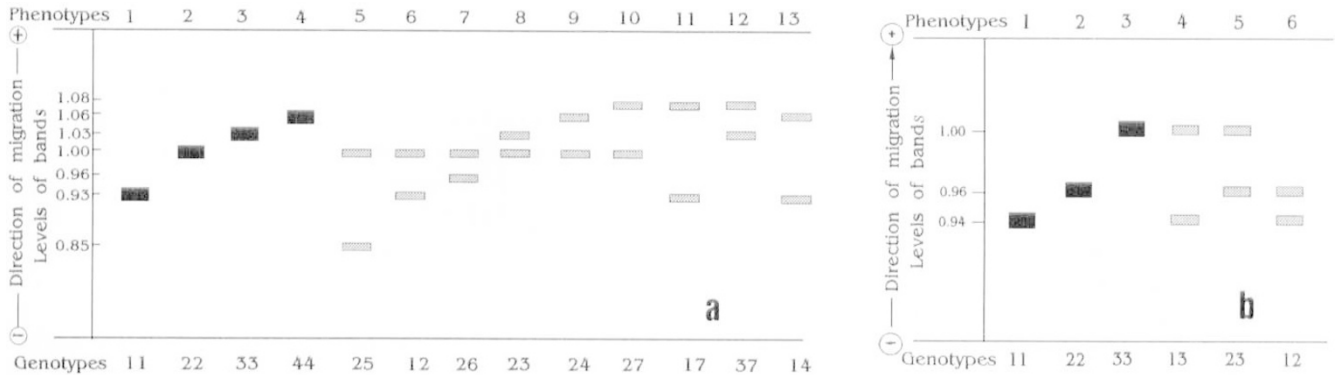


Fig. 2 Schematic pattern of (a) PX and (b) LAP zymograms. Band mobilities at *PX-I*, 1 = 0.93, 2 = 1.00, 3 = 1.03, 4 = 1.06, 5 = 0.85, 6 = 0.96 and 7 = 1.08; at *LAP-I*, 1 = 0.94, 2 = 0.96 and 3 = 1.00.

Table 2 Inheritance patterns at three loci in *Quercus ilex* L.

Locus	Cross no.	Presumed parental genotypes	Offspring genotypes† Observed (expected)	Total	χ^2
<i>PX-I</i>	1	1.00/1.00 × 1.00/1.00	1.00/1.00 6	6	
	2	1.00/1.00 × 1.00/1.06	1.00/1.00 1.00/1.06 13 (12.5) 12 (12.5)	25	NS
	3	1.00/1.00 × 1.06/0.93	1.00/1.06 1.00/0.93 7 (7.5) 8 (7.5)	15	NS
<i>LAP-I</i>	1	1.00/1.00 × 1.00/1.00	1.00/1.00 14	14	
	2	1.00/1.00 × 1.00/0.94	1.00/1.00 1.00/0.94 12 (9.5) 7 (9.5)	19	NS
<i>AcPH-I</i>	1	1.00/1.00 × 1.00/1.00	1.00/1.00 11	11	
	2	1.00/1.00 × 1.00/0.83	1.00/1.00 1.00/0.83 7 (5.5) 4 (5.5)	11	NS

†Genotypes are indicated with bold characters. Expected genotypic frequencies are in parentheses. NS = no significant difference between observed and expected frequencies.

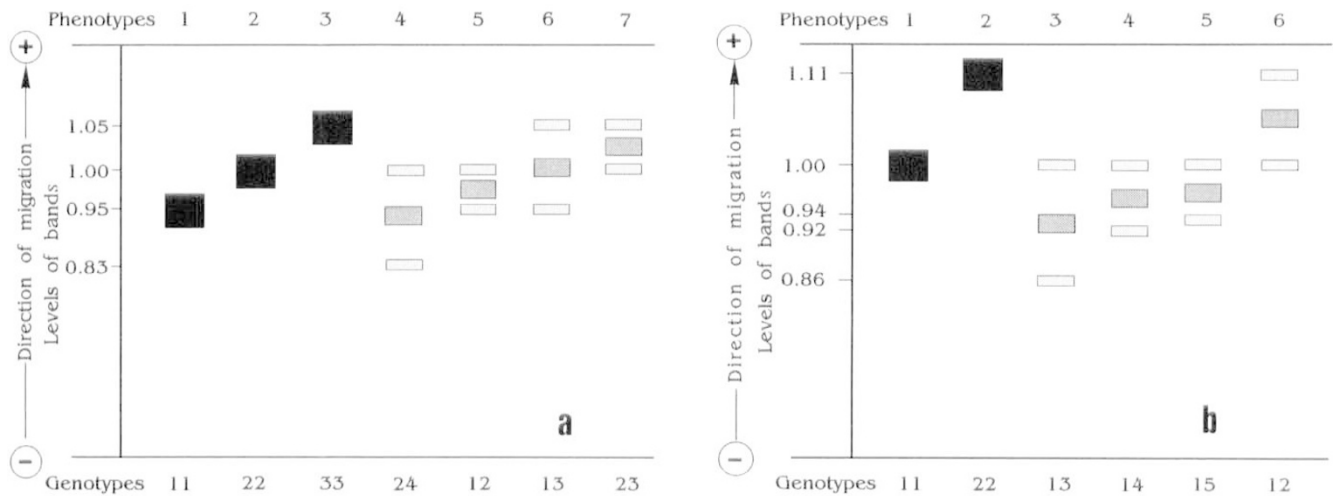


Fig. 3 Schematic pattern of (a) *AcPH* and (b) *TO* zymograms. Band mobilities at *AcPH-I*, 1 = 0.95, 2 = 1.00, 3 = 1.05 and 4 = 0.83; at *TO-I*, 1 = 1.00, 2 = 1.11, 3 = 0.86, 4 = 0.92 and 5 = 0.94.

Acid phosphatase (AcPH). Four zones of activity were observed on the gels, the fastest being the only one interpretable (Fig. 3a). The inferred homozygotes showed a single band of activity whereas the inferred heterozygotes possessed three bands with intense activity for the intermediate band. Genotypic segregation in cross 2 (Table 2) did not deviate significantly from that expected assuming a single-locus inheritance. These results support the interpretation that in that zone of activity AcPH is coded by a single locus (*AcPH-1*) with four codominant alleles (*AcPH-1*^{0.83}, *AcPH-1*^{0.95}, *AcPH-1*^{1.00} and *AcPH-1*^{1.05}) which specify dimeric enzymes.

Tetrazolium oxidase (TO). Three zones of activity were observed on the gels. Only the fastest zone was interpretable. Examples of observed single- and three-banded phenotypes are shown in Fig. 3b. A single class of parental genotype was observed and was inferred to be homozygote. Crosses between two of these inferred homozygotes produced a single class of progeny with the parental pattern. According to the banding patterns and to general agreement regarding enzyme substructure in higher plants (Gottlieb, 1977; Kephart, 1990), the tetrazolium oxidase system was considered to resolve one putative locus, *TO-1*, with five codominant alleles which code for dimeric enzymes. The alleles were *TO-1*^{0.86}, *TO-1*^{0.92}, *TO-1*^{0.94}, *TO-1*^{1.00} and *TO-1*^{1.11}.

Other enzyme systems. In addition, 11, four and six alleles were observed at the *PGI-1*, *IDH-1* and *ADH-1* loci, respectively, among the 57 populations. These alleles are: *PGI-1*^{0.66}, *PGI-1*^{0.74}, *PGI-1*^{0.80}, *PGI-1*^{0.90}, *PGI-1*^{0.92}, *PGI-1*^{0.95}, *PGI-1*^{1.00}, *PGI-1*^{1.09}, *PGI-1*^{1.20}, *PGI-1*^{1.33} and *PGI-1*^{1.40}; *IDH-1*^{0.76}, *IDH-1*^{1.00}, *IDH-1*^{1.10} and *IDH-1*^{1.30}; *ADH-1*^{0.55}, *ADH-1*^{0.76}, *ADH-1*^{1.00}, *ADH-1*^{1.16}, *ADH-1*^{1.25} and *ADH-1*^{1.36}.

Genetic variation in *Quercus ilex*

Among the eight loci analysed (with a total of 41 alleles), only *PGI-2* was monomorphic for the same allele in all the populations studied. Significant deviations from Hardy-Weinberg expectation were observed at some loci in a very few populations. Deviations of F_{is} from 0, at the 5 per cent significance level, were observed at *PGI-1* in populations 4, 7, 14, 38, 46, 56 and 57 (positive deviation) and in population 37 (negative deviation); at *ADH-1* in populations 8, 46 and 54 (positive deviation) and in population 15 (negative deviation). Deviations at the 1 per cent significance level were observed at *IDH-1* in population 46 (positive deviation) and in no. 49 (negative deviation), and at *PX-1* in populations 36 and 55 (positive deviation) and

in population 32 (negative deviation). Significant positive deviations were also observed (0.1 per cent level) at *TO-1* in population 57, at *LAP-1* in populations 32 and 38, and at *AcPH-1* in population 29. Although most of the F_{is} deviations from 0 indicate an excess of homozygotes, no particular factor responsible for heterozygote deficiency could be identified. The G -test for heterogeneity of allele frequencies was highly significant ($P < 0.01$ or lower values) for 19 alleles, namely *PGI-1*^{1.20}, *PGI-1*^{1.09}, *PGI-1*^{1.00}, *PGI-1*^{0.80}, *PGI-1*^{0.74}, *ADH-1*^{1.00}, *ADH-1*^{0.76}, *PX-1*^{1.00}, *PX-1*^{0.93}, *IDH-1*^{1.00}, *IDH-1*^{0.76}, *TO-1*^{1.00}, *LAP-1*^{1.00}, *LAP-1*^{0.96}, *LAP-1*^{0.94}, *AcPH-1*^{1.05}, *AcPH-1*^{1.00}, *AcPH-1*^{0.95} and *AcPH-1*^{0.83}, and was significant ($P < 0.05$) for two additional loci, *ADH-1*^{0.55} and *TO-1*^{0.92}. Heterogeneity could not be tested for the other alleles studied because these were observed at low frequency (less than 10 per cent) and in a very few populations. For the 57 populations scored at six loci, the mean proportion of polymorphic loci was 65 per cent, with values ranging from 33 per cent (in populations 35 and 47) to 83 per cent (in 16 populations). For the 22 populations analysed at eight loci, the mean proportion of polymorphic loci was 73 per cent with a range from 50 per cent (population 28) to 87 per cent (in five populations).

Table 3 shows the percentage of polymorphic loci, mean number of alleles per population, observed heterozygosity, several genetic diversity parameters, mean number of migrants between populations per generation and F_{is} values calculated either at each of the six loci analysed in 57 populations of holm oak, or at the two additional loci analysed in 22 populations. Averages over either the six or the eight loci studied were also calculated and indicate high genetic diversity, particularly at the *PGI-1* locus with its 11 alleles (see Fig. 4 for their geographical distribution). High genetic diversity was also observed at the *IDH-1*, *PX-1*, *LAP-1* and *AcPH-1* loci. Mean genetic diversity ($H_s = 0.126$) at the five polymorphic loci *PGI-1*, *ADH-1*, *IDH-1*, *PX-1* and *TO-1* was significantly lower ($P < 0.0001$) in the six marginal populations located in areas where climatic conditions constitute a limiting factor for the species than in the whole set of populations. Such low diversity is mainly because of the occurrence of a lower rate of polymorphism in those six marginal populations (mean allele number (A) = 1.87 over six loci) compared with the nonmarginal populations ($A = 2.50$). Conversely, the five other marginal populations growing under normal climatic conditions, but which were isolated by cultivation areas, showed a high mean genetic diversity value at the same loci ($H_s = 0.271$) that was not significantly different from the averaged value over the whole set of populations.

Of the average total diversity observed in the holm oak, 10 per cent was attributable to differentiation

Table 3 Percentage of monomorphic populations (M), total number of alleles per locus (A_t), mean (A) (minimum–maximum) number of alleles per population, mean effective number of alleles per population (A_e), observed heterozygosity (H_o), genetic diversity within populations (H_s), total genetic diversity (H_t), proportion (per cent) of diversity among populations (G_{st}), mean number of migrants between populations per generation (Nm) and F_{is} values at five polymorphic loci *PGI-1*, *ADH-1*, *IDH-1*, *PX-1* and *TO-1* in 57 populations and at loci *LAP-1* and *AcPH-1* in 22 populations of holm oak

Locus	$M(\%)$	A_t	A	A_e	H_o	H_s	H_t	$G_{st}(\%)$	Nm	F_{is}
<i>PGI-1</i>	0.0	11	4.28 (2–6)	1.69	0.340	0.381	0.408	6.5	3.60	0.091
<i>ADH-1</i>	36.5	6	1.89 (1–5)	1.11	0.072	0.084	0.095	11.9	1.85	0.093
<i>IDH-1</i>	11.3	4	2.10 (1–3)	1.45	0.275	0.275	0.332	17.1	1.21	–0.020
<i>PX-1</i>	0.0	7	2.99 (2–4)	1.58	0.328	0.335	0.369	9.3	2.45	–0.003
<i>TO-1</i>	55.6	5	1.39 (1–3)	1.04	0.035	0.034	0.037	6.0	3.91	0.010
<i>PGI-2</i>	100.0	1								
Mean	17.2	5.7	2.27	1.31	0.210	0.222	0.248	10.2	2.21	0.031
<i>LAP-1</i>	9.1	3	2.50 (1–3)	1.68	0.313	0.355	0.397	10.6	2.11	0.105
<i>AcPH-1</i>	4.5	4	2.68 (1–4)	1.44	0.311	0.287	0.308	6.9	3.37	0.058
Mean	14.6	5.2	2.31	1.34	0.224	0.235	0.262	10.0	2.25	0.045

Mean values were calculated over the six loci (including the monomorphic *PGI-2*) analysed in 57 populations, and over the eight loci analysed in 22 populations.

among populations (Table 3) ($G_{st} = 10.2$ per cent and 10.0 per cent according to the number of loci and populations analysed). The same conclusion is derived from the F_{st} average value (0.10) and the mean number of migrants among populations ($Nm = 2.2$), suggesting that no differentiation among populations could be ascribed to the unique action of genetic drift (Wright, 1951; Levin & Kerster, 1974). However, at the *IDH-1* locus, which showed high total genetic diversity, the G_{st} value was particularly high compared with values obtained for the other loci studied. Looking at the geographical distribution of allele frequencies at that locus (Fig. 5), evidence was found that most of the differentiation among populations is because of the occurrence of higher *IDH-1*^{0.76} frequencies in all the populations from the French Atlantic coast than in populations from the other distribution areas. In addition, total genetic diversity was 0.210 and 0.232 in groups of populations showing the ‘ilex’ and ‘rotundifolia’ morphotypes, respectively, and the G_{st} values within these groups (9.4 per cent and 9.3 per cent, respectively) were close to that found for the species as a whole.

The low rate of genetic differentiation among populations of holm oak was also obtained from genetic distance computation. Average genetic distance value among the 57 populations scored at six loci was 0.031 (range from 0.002–0.180) for Nei’s distance, 0.147 (range from 0.036–0.347) for Gregorius’s distance, and 0.047 (range 0.009–0.113) for the χ^2 distance. UPGMA coupled with χ^2 distance had the high-

est cophenetic correlation with the data. The diagram (Fig. 6) shows the respective positions of the 57 populations estimated by the χ^2 distance between them. Three sets of populations could be distinguished at the 47 per cent level of agglomeration (a critical value in the agglomeration process) in the UPGMA analysis. Eight of the 10 populations from the French Atlantic coast clustered closely together (set 1), as did six of the 10 populations from North Africa (set 3). In contrast, set 2 consisted mainly of populations from the Mediterranean part of the European continent and the three populations from Corsica. Among the 14 populations not clustered at the 47 per cent level, three are marginal populations (no. 9 from Italy, no. 39 from Galicia and no. 54 from Morocco) and two (nos 6 and 7) were collected in Sicily. These latter two populations differed from each other by the occurrence of several distinct alleles. In addition, most of the populations showing the ‘rotundifolia’ morphotype were located on the right side of the plan parted by axis 2 in Fig. 6, whereas most of the ‘ilex’ morphotyped populations were located on the other side. However, no consistent agreement was obtained between the genetic pattern based on allozyme variation and the morphological differentiation pattern as populations showing the ‘ilex’ and ‘rotundifolia’ morphotypes as well as those of the intermediate type clustered together in set 2.

Rare alleles may also contribute significantly to the whole genetic variation in the holm oak. Twelve of the 16 alleles observed in a limited number of populations were specific to a single disjunct region (Table 4).

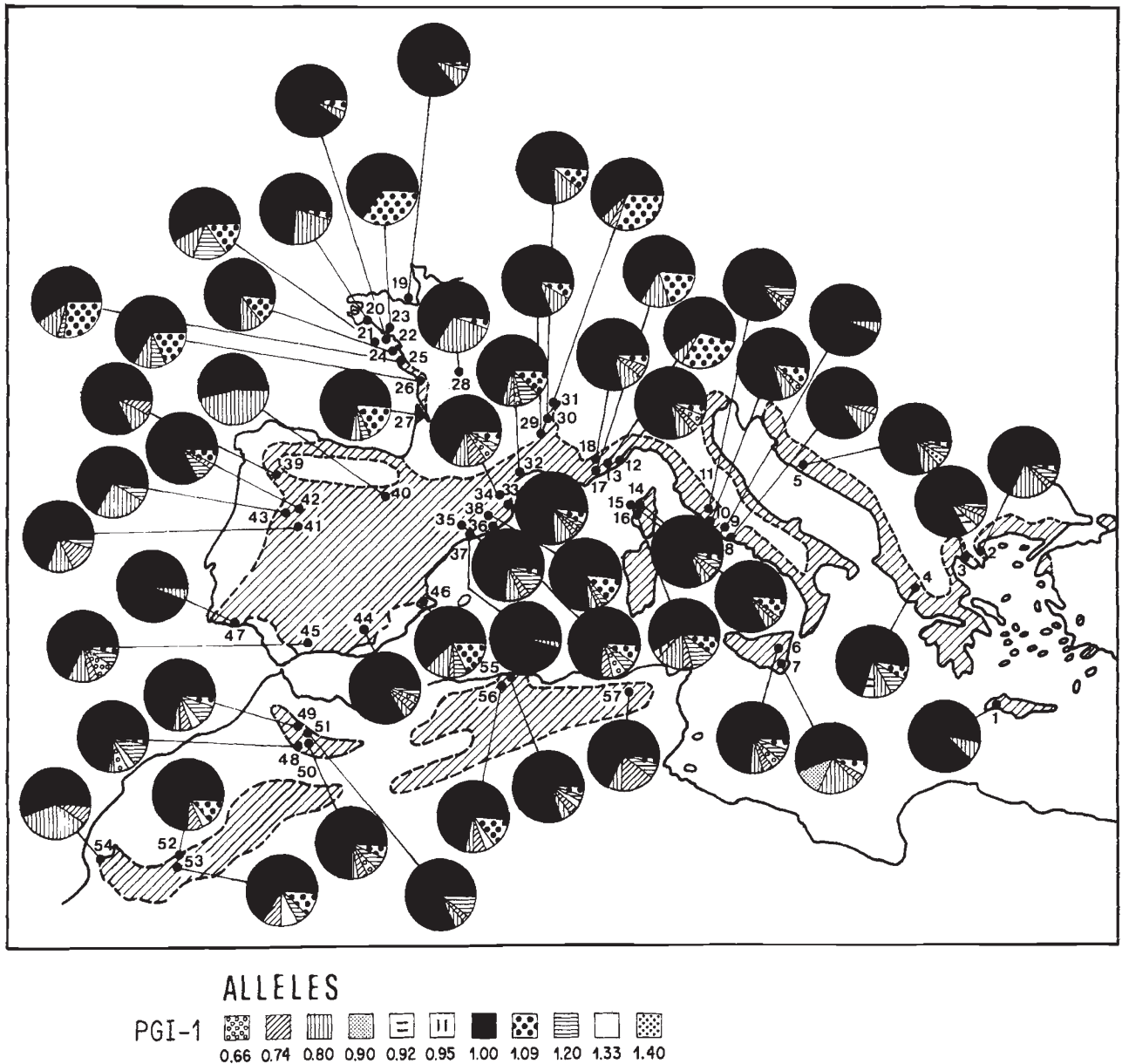


Fig. 4 Geographical distribution of allele frequencies at locus *PGI-1* in 57 populations of holm oak. On the map, overall species distribution areas are indicated by hatched areas.

The 16 alleles occurred at frequencies lower than 15 per cent. Of the eight alleles present in a single population, three were observed in islands (Sicily and Corsica) and one was found in a marginal population from Galicia. Five alleles present in several populations showed a local distribution: *PGI-1*^{1.33} in six of the ten studied populations from North Africa (Fig. 4), *PGI-1*^{0.92} in two of the three studied populations from continental Greece (Fig. 4), *ADH-1*^{0.55} in seven populations located in the eastern part of the holm oak distribution area, *ADH-1*^{1.36} in three populations from Sicily and central Western Italy and *PX-1*^{0.96} in two

populations from north-western Spain. The three remaining alleles were rare but not restricted to a particular area.

Geographical discontinuity and genetic differentiation among populations

When the 57 holm oak populations were separated into five groups corresponding to the disjunct regions, total genetic diversity averaged over the groups (H_{gt}) was equal to 0.404, 0.094, 0.364 and 0.036 at loci *PGI-1*, *ADH-1*, *PX-1* and *TO-1*, respectively, and remained therefore very close to the corresponding H_i

value obtained for the whole 57 populations (Table 3). In contrast, at the *IDH-1* locus, total genetic diversity decreased from 0.332 when calculated over the populations taken as a whole, to 0.287 when the data were averaged over the groups. The among-group diversity (G_{st}) values were very low at the four loci listed above (ranging from 0.3 per cent at *TO-1* to 1.5 per cent at *ADH-1*) whereas at *IDH-1*, differentiation among the five geographical groups corresponded to 13.5 per cent of the total diversity at that locus. When averaged over the five loci, only 35 per cent of the G_{st} value calculated over the whole populations (10.2) was attributable to differentiation among groups. The lowest genetic diversity (0.20; calculated over the five polymorphic loci and averaged over the populations in

each group) was observed in Mediterranean continental Europe whereas the two highest values (0.26 and 0.28) were obtained in the Atlantic and Sicilian groups, respectively.

Average genetic distances within and between the five disjunct regions were also calculated. With Nei's distance, which is more sensitive to substantial allele frequency variation than are the Gregorius and χ^2 distances, values within the disjunct regions ranged from 0.7 per cent for pairs of populations from Corsica to 2.1 per cent for the populations from the French Atlantic coast. Genetic distance between regions ranged from 1.3 per cent between populations from Sicily and those of Mediterranean continental Europe to 8.8 per cent between populations from North Africa

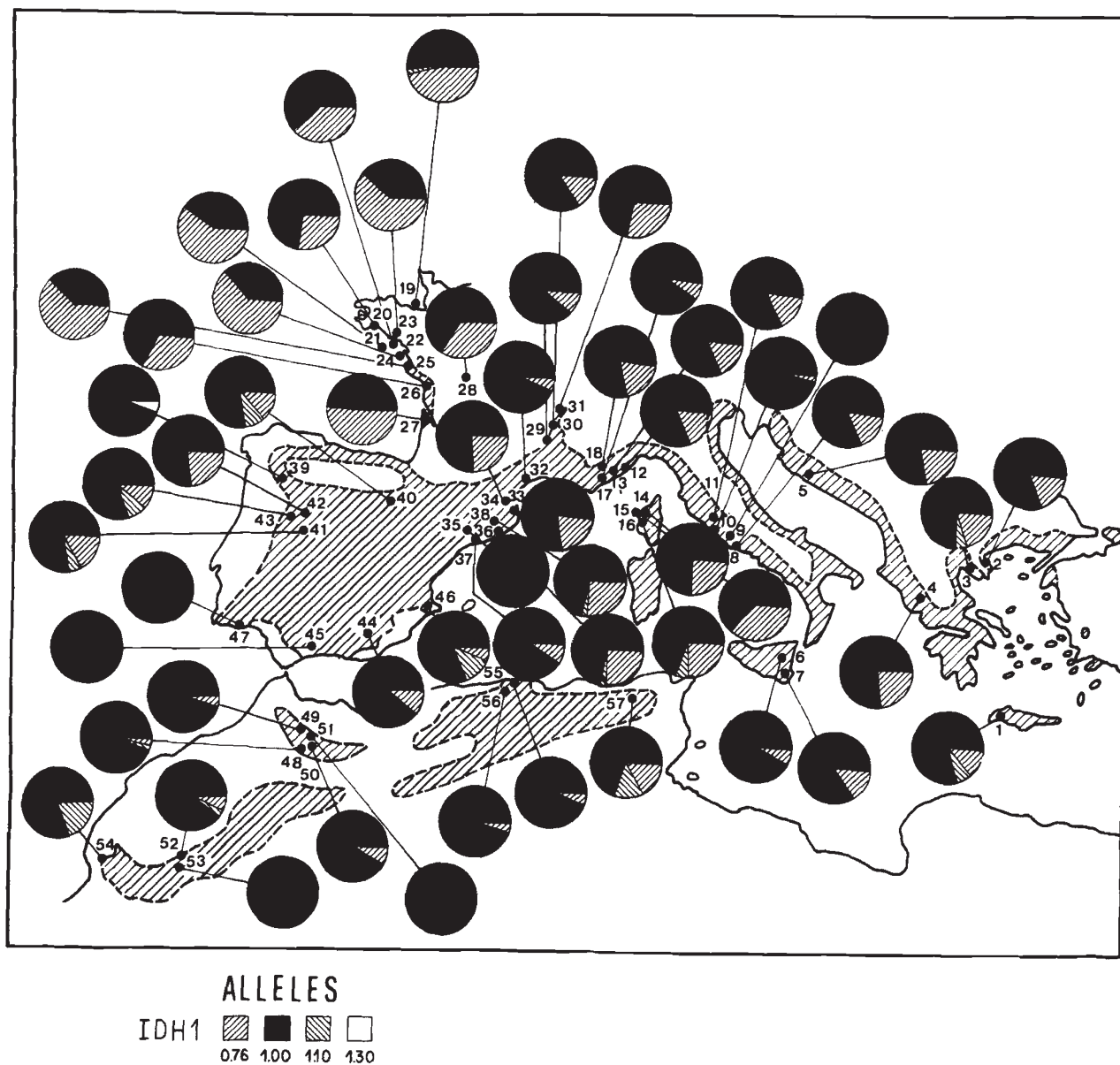


Fig. 5 Geographical distribution of allele frequencies at locus *IDH-1* in 57 populations of holm oak.

and those of the Atlantic coast. Using the χ^2 distance, which maximizes the effect of rare alleles, the lowest and the highest within-region values were observed for the Corsican and the Sicilian groups, respectively, whereas the lowest and highest between-group values

(1.8 and 8.2 per cent) were found between Corsica and Africa, and between Africa and Sicily, respectively.

In addition, spatial correlograms were obtained from autocorrelation analysis on the 21 alleles that showed significant heterogeneous spatial distribution

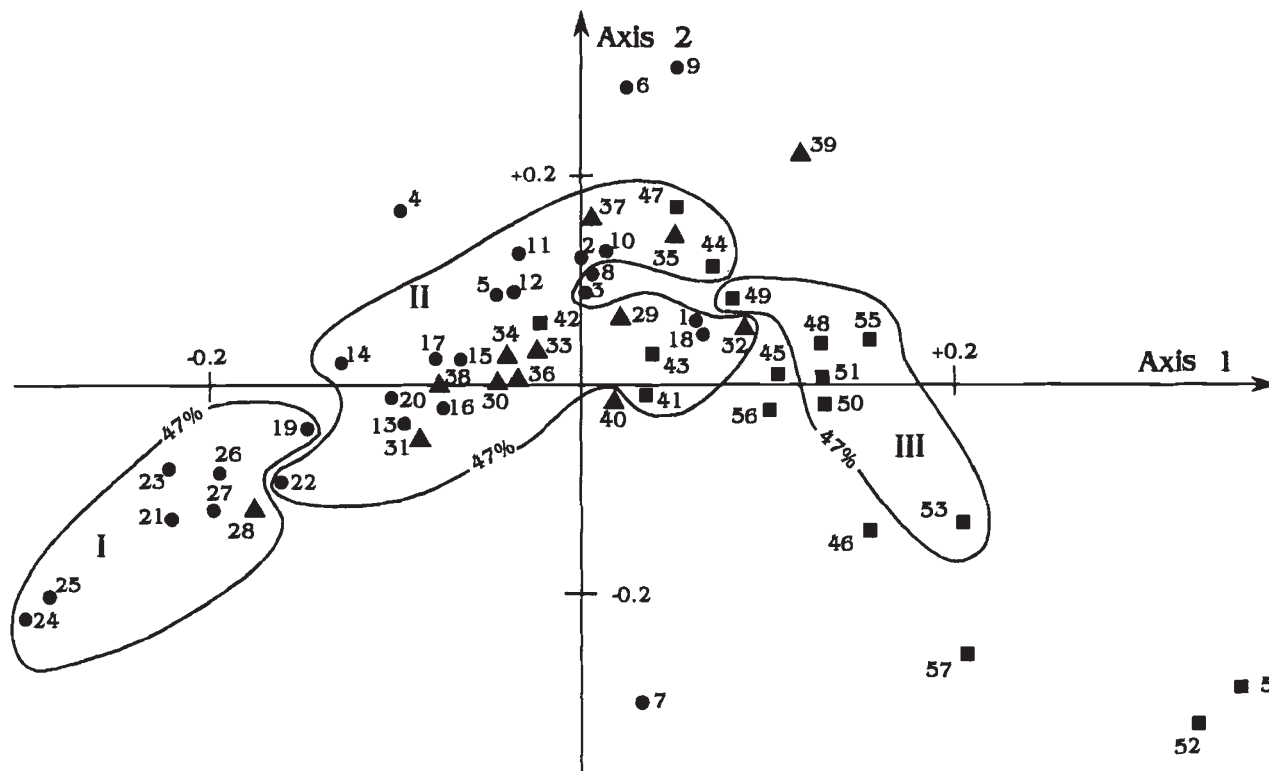


Fig. 6 Position of 57 populations of holm oak showing 'ilex' (●), 'rotundifolia' (■) and 'intermediate' (▲) morphotypes, according to polymorphism at loci *PGI-1*, *IDH-1*, *ADH-1*, *PX-1* and *TO-1*. Multidimensional scaling from χ^2 distances. Populations are clustered at the 47 per cent level of the hierarchical clustering.

Table 4 Identity and location of 16 rare alleles observed at five loci in 57 populations of holm oak

Locus	Alleles	Populations	Countries
<i>PGI-1</i>	0.90	7	Sicily
	0.92	3, 4	Greece
	0.95	16	Corsica
	1.33	48, 49, 52, 53, 55, 56	Morocco, Algeria
	1.40	5	Croatia
<i>ADH-1</i>	0.55	1, 2, 3, 4, 6, 7, 9	Crete, Greece, Italy, Sicily
	1.25	18, 38	France, Spain
	1.36	7, 8, 10	Sicily, Italy
<i>IDH-1</i>	1.30	39	Spain
<i>PX-1</i>	0.85	4	Greece
	0.96	40, 41	Spain
	1.03	7	Sicily
	1.08	56	Algeria
<i>TO-1</i>	0.86	6, 12, 32	Sicily, Italy, France
	0.94	48	Morocco
	1.11	33, 44, 52, 55, 57	France, Spain, Morocco, Algeria, Tunisia

(see above). Figure 7 plots the six significant correlograms obtained for $IDH-1^{1.00}$, $IDH-1^{0.76}$, $ADH-1^{0.76}$, $ADH-1^{0.55}$, $TO-1^{1.00}$ and $TO-1^{0.92}$ from the 57 populations located in the whole distribution area (set 1) and the six correlograms obtained for the same alleles from the 31 populations of the Mediterranean continental group (set 2). Six and five distance classes (400 km) were used for sets 1 and 2, respectively. Only two correlograms (for $TO-1^{1.00}$ and $TO-1^{0.92}$) were significant for the Mediterranean populations, suggesting that the variation observed in set 1 for the four other alleles ($IDH-1^{1.00}$, $IDH-1^{0.76}$, $ADH-1^{0.76}$ and $ADH-1^{0.55}$) may result from differentiation among the disjunct regions. As the significance level of each correlogram is 0.05, a single correlogram out of 20 is expected to be significant by chance. Consequently, the results of autocorrelation analysis obtained in the present study are

robust enough to be considered. Two different kinds of correlograms could be distinguished in set 1: (i) $ADH-1^{0.76}$, $ADH-1^{0.55}$ and, to lesser extent, $TO-1^{1.00}$ and $TO-1^{0.92}$ showed a typical clinal pattern with neighbouring populations tending to be characterized by similar frequencies. Similarity decreased with increasing geographical distance, up to the most distant populations which showed negatively correlated frequencies; (ii) a depression model seemed to apply for $IDH-1^{1.00}$ and $IDH-1^{0.76}$ (Sokal & Oden, 1978a), i.e. whereas the nearest populations (less than 800 km apart) had positively correlated frequencies, those in the third and fourth classes (between 800 and 1600 km) had negatively correlated frequencies. The autocorrelation values approached zero for the two largest geographical distances. The depression pattern may indicate a patchy distribution for the two alleles. As many distance values ranging between 800 and 1600 km involved one population located in the French Atlantic area and the other in another disjunct region, the depression pattern observed for alleles $IDH-1^{1.00}$ and $IDH-1^{0.76}$ may reflect the occurrence of frequency variation regarding these alleles between the Atlantic and the various other parts of the holm oak distribution area.

Discussion

Genetic diversity in the whole species distribution

Total and within-population genetic diversity. The average F_{is} value per locus (close to zero except at $LAP-1$) and the total F_{is} value (0.04) observed in the holm oak suggest a high outcrossing rate for that species. This observation is in agreement with results obtained previously in the holm oak from both direct experimental tests of self-pollination and crosses between half-sibs (Lumaret *et al.*, 1991), and indirect estimates of outcrossing rates using allozyme markers (Yacine & Lumaret, 1988).

A relatively high genetic diversity estimated by the parameters mentioned above was observed regularly in the holm oak. In this widespread species, the mean number of alleles per locus and the total gene diversity were similar to average values obtained in the other widespread species studied in the Fagaceae, e.g. in *Castanea sativa* L. (Villani *et al.*, 1991a,b), in *Fagus sylvatica* L. (Müller-Starck, 1991 and references therein) and in 33 oak species ($A = 2.41$; $H_s = 0.211$) (see Kremer & Petit, 1993 for a review). However, substantially lower genetic diversity was observed in *Nothofagus truncata* which has a restricted geographical and ecological distribution range (Haase, 1992), supporting the finding of Hamrick *et al.* (1992) that, in woody plant species, the level of genetic diversity

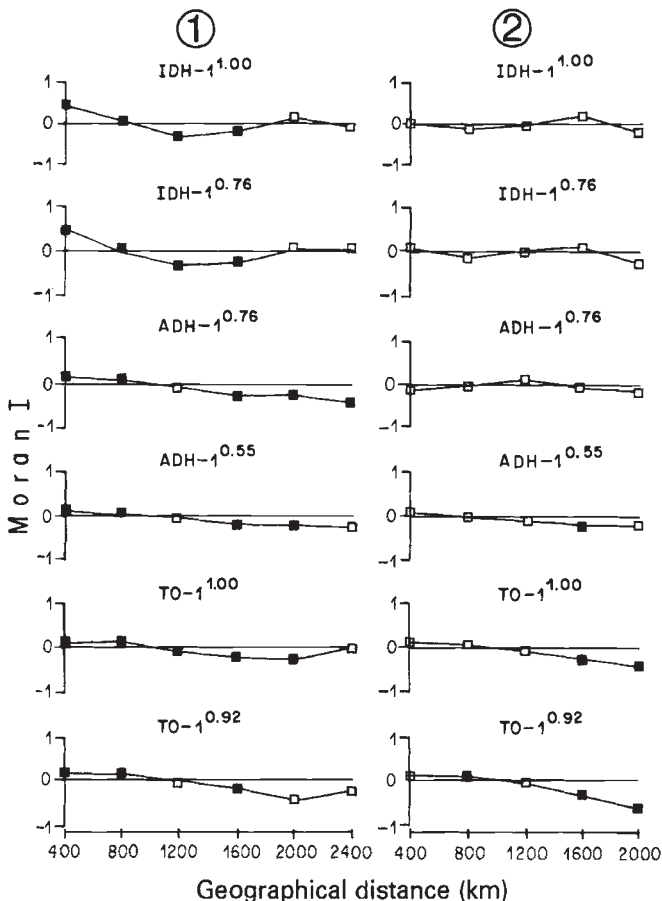


Fig. 7 Spatial autocorrelograms obtained in holm oak for six informative alleles in 57 populations from the whole distribution area (set 1) and in 31 populations from the Mediterranean continental area (set 2). The correlograms are significant except those obtained in set 2 for $IDH-1^{1.00}$, $IDH-1^{0.76}$, $ADH-1^{0.76}$ and $ADH-1^{0.55}$. Significant ($P \leq 0.05$) and non-significant Moran indices are indicated by black and white squares, respectively.

increases significantly with extension of geographical range. In addition, in the holm oak, studies of flowering phenology, breeding system and genetic diversity in progeny of several open-pollinated mother trees have shown that all the sexual reproductive characteristics of this species contribute to maintaining genetic diversity and can efficiently counteract genetic structuring within populations (Yacine & Lumaret, 1988; Michaud *et al.*, 1992).

Genetic differentiation among populations. In coniferous and in broad-leaved tree species, low genetic differentiation has been observed among populations compared with herbaceous species. Low differentiation in forest trees was attributed mainly to their long life span which favours extensive gene flow (Hamrick *et al.*, 1992). In the holm oak, ten per cent of the total diversity was attributable to differentiation among populations. That value is higher than the mean value (7 per cent) obtained over 25 species of oaks studied for enzyme polymorphism (Kremer & Petit, 1993) and than the genetic differentiation (5 per cent) observed among populations in *Fagus sylvatica* (Müller-Starck, 1991) and *Nothofagus truncata* (Haase, 1992). Moreover, in the holm oak, the location of distinct local alleles in three parts of the same continuous distribution area, i.e. in Greece, in both Italy and south-eastern France, and in both Spain and France, can be related to the refuge role played by the three north-south oriented Iberian, Italian and Balkan peninsulas, respectively, during the past glaciations (Bennett *et al.*, 1991; Tzedakis, 1993). In Italian populations of *Picea abies* such localized alleles were also found in relation to the past role of refugium played by central Italy (Giannini *et al.*, 1991). In the holm oak, clear genetic differentiation among populations growing in areas colonized from distinct putative refuge areas was also observed for chloroplast DNA variation (H. Michaud & R. Lumaret, unpublished data). Subsequent contacts between populations from distinct refuge areas may have occurred only in the northern distribution area which is restricted by climatic constraints to a narrow strip. Such a situation, which does not favour genetic introgression between the distinct groups of populations, may be responsible for the maintenance of genetic differentiation over long periods.

In the present work, the genetic distance of Nei averaged over all populations studied is either similar to or even higher than that obtained in other widespread outcrossing species in the Fagaceae, e.g. *Castanea sativa* in western Europe (Villani *et al.*, 1991a), suggesting that partial barriers to gene flow may occur more frequently in the holm oak. However, in the holm oak, allozyme and morphological differen-

tiation patterns were not consistent, indicating that morphological traits and enzyme polymorphisms probably have very distinct sensitivities to environmental selective pressures and therefore may have evolved independently. Discrepancy between variation in proteins and morphology is observed commonly in intraspecific studies of plants (see Linhart *et al.* 1989, for a review). However, in a few cases, e.g. in Turkish populations of *Castanea sativa* (Villani *et al.*, 1992), consistent variation patterns of differentiation among populations were observed for allozymic, morphometric and physiological traits, suggesting the occurrence of a certain degree of reproductive isolation within species.

Genetic diversity in marginal populations

In the holm oak, high genetic diversity was observed in all populations studied except in the six marginal ones growing under unfavourable climatic conditions. In these, only the predominant allele was observed at most of the loci, suggesting that higher selective pressure was occurring in such climatic conditions. Selection on adaptive characters may indeed reduce overall genotype diversity and contribute, therefore, to the loss of low frequency neutral alleles. Conversely, the five holm oak marginal populations growing in cultivated areas under normal climatic conditions for this species showed a high level of genetic diversity which, in such a long-lived species (Yacine & Lumaret, 1988), may reflect the previous genetic structure established before deforestation of those areas.

Genetic differentiation among the disjunct distribution areas

As shown in particular by the geographical distribution patterns and the spatial correlograms obtained for several alleles and by the comparison of genetic distances within and between the five disjunct regions of the distribution area, interpopulation genetic differentiation in the holm oak is partly the result of geographical discontinuity in its distribution range. Such discontinuity is responsible for the occurrence of both rare alleles specific for each disjunct area and regional variation for allele frequencies.

Seventy-five per cent of rare alleles were restricted to a single isolated region. For instance, the populations from Morocco and Algeria possess several alleles in common which are specific to North Africa. This result suggests the occurrence of efficient barriers to gene flow among the disjunct regions. Surprisingly, substantial genetic differentiation among populations closely located in the same region was also observed on a few occasions. For instance, the two populations from the

Etna region in Sicily differed by several rare alleles. Such a situation may be explained by the well-known characteristics of that volcano which releases frequent and successive flows of lava. The holm oak populations growing on Etna have to cope with frequent destruction and recolonization episodes and constitute a patchy distribution of relatively small, isolated subpopulations (Poli & Maugeri, 1974). These may be subjected to genetic drift.

In the holm oak, the occurrence of regional allele frequencies concerns mainly two alleles, *IDH-I*^{0.76} and *IDH-I*^{1.00}, the former being predominant in the Atlantic populations whereas the latter is predominant in all the other populations. Holm oak populations from the Atlantic part of France were considered by Pons & Vernet (1971) as a pre-Würmian relict. Predominance of *IDH-I*^{0.76} in the French Atlantic populations of holm oak, including those from central and northern Brittany which were introduced a few centuries ago at most, may result from recolonization of those areas from trees growing in a specific relictual region. This should be distinct from the regions from which the Mediterranean populations originate. Alternatively, because it concerns allele frequencies at a single locus, genetic differentiation in the French Atlantic populations may reflect environmental selection (on that locus or on loci tightly linked to it) in areas not subjected to a Mediterranean climate, in contrast to the other disjunct regions of the holm oak distribution. Consistent directional variation in allele frequencies was also recorded by Daubree & Kremer (1993) in populations of *Quercus rubra* L. following introduction into Europe, compared with the native American populations. The authors suggested that the variation observed was related to the different natural selection pressures occurring in the two continents.

According to Pons & Vernet (1971), isolation of the Atlantic populations occurred during the pre-Würmian period (i.e. about 700 000 years ago) and is therefore less ancient than the isolation of the several population groups by the Mediterranean sea which occurred approximately five million years ago (Bocquet *et al.*, 1978). However, genetic differentiation of the Atlantic populations is higher than that present among the Mediterranean groups which is limited to the occurrence of localized alleles which are always observed at low frequency.

The main conclusion to emerge from this study is that life history traits (especially long life span and outcrossing) and the evolutionary history of the holm oak related to changes in both climate and geographical continuity probably played a major role among the manifold factors responsible for the present genetic variation pattern observed in this species.

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