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Phylogeographical variation of chloroplast DNA in holm oak (*Quercus ilex* L.)

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Abstract

Variation in the lengths of restriction fragments (RFLPs) of the whole chloroplast DNA molecule was studied in 174 populations of *Quercus ilex* L. sampled over the entire distribution of this evergreen and mainly Mediterranean oak species. By using five endonucleases, 323 distinct fragments were obtained. From the 29 and 17 cpDNA changes identified as site and length mutations, respectively, 25 distinct chlorotypes were distinguished, mapped and treated cladistically with a parsimony analysis, using as an outgroup *Q. alnifolia* Poech, a closely related evergreen oak species endemic to Cyprus where *Q. ilex* does not grow. The predominant role of *Q. ilex* as maternal parent in hybridization with other species was reflected by the occurrence of a single very specific lineage of related chlorotypes, the most ancestral and recent ones being located in the southeastern and in the northwestern parts of the species' geographical distribution, respectively. The lineage was constituted of two clusters of chlorotypes observed in the 'ilex' morphotyped populations of the Balkan and Italian Peninsulas (including the contiguous French Riviera), respectively. A third cluster was divided into two subclusters identified in the 'rotundifolia' morphotyped populations of North Africa, and of Iberia and the adjacent French regions, respectively. Postglacial colonization probably started from three distinct southerly refugia located in each of the three European peninsulas, and a contact area between the Italian and the Iberian migration routes was identified in the Rhône valley (France). Chlorotypes identical or related to those of the Iberian cluster were identified in the populations from Catalonia and the French Languedoc region, which showed intermediate morphotypes, and in the French Atlantic populations which possessed the 'ilex' morphotype, suggesting the occurrence of adaptive morphological changes in the northern part of the species' distribution.

Keywords: cpDNA RFLP variation, evergreen Mediterranean oaks, morphological convergence, phylogeography, *Q. ilex* L.

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Introduction

Of the four evergreen oak species which grow in the Mediterranean area, *Quercus ilex* L. (holm oak) is the most widely distributed and shows an additional natural extension area along the Atlantic coasts of Portugal, Spain and France, up to the south of Brittany. In most of the distribution area, *Q. ilex* forests can be regarded as rare cases of woodlands that have undergone very low or no silvicultural management. By contrast, in the centre and, to a lesser extent, in the south of the Iberian Peninsula, *Q. ilex* is considered as a fruit tree and has been selected for sweet acorn production which is used to supplement feed for pigs (Ruperez 1957).

Q. ilex shows two main morphological types. The 'ilex' type is an elongated and large leaf morph distributed from Greece to the French Riviera, and along the Atlantic coast of France (Fig. 1). This morph is restricted to humid or subhumid sites mainly in mild coastal areas (Barbéro *et al*. 1992). The 'rotundifolia' morph is characterized by small

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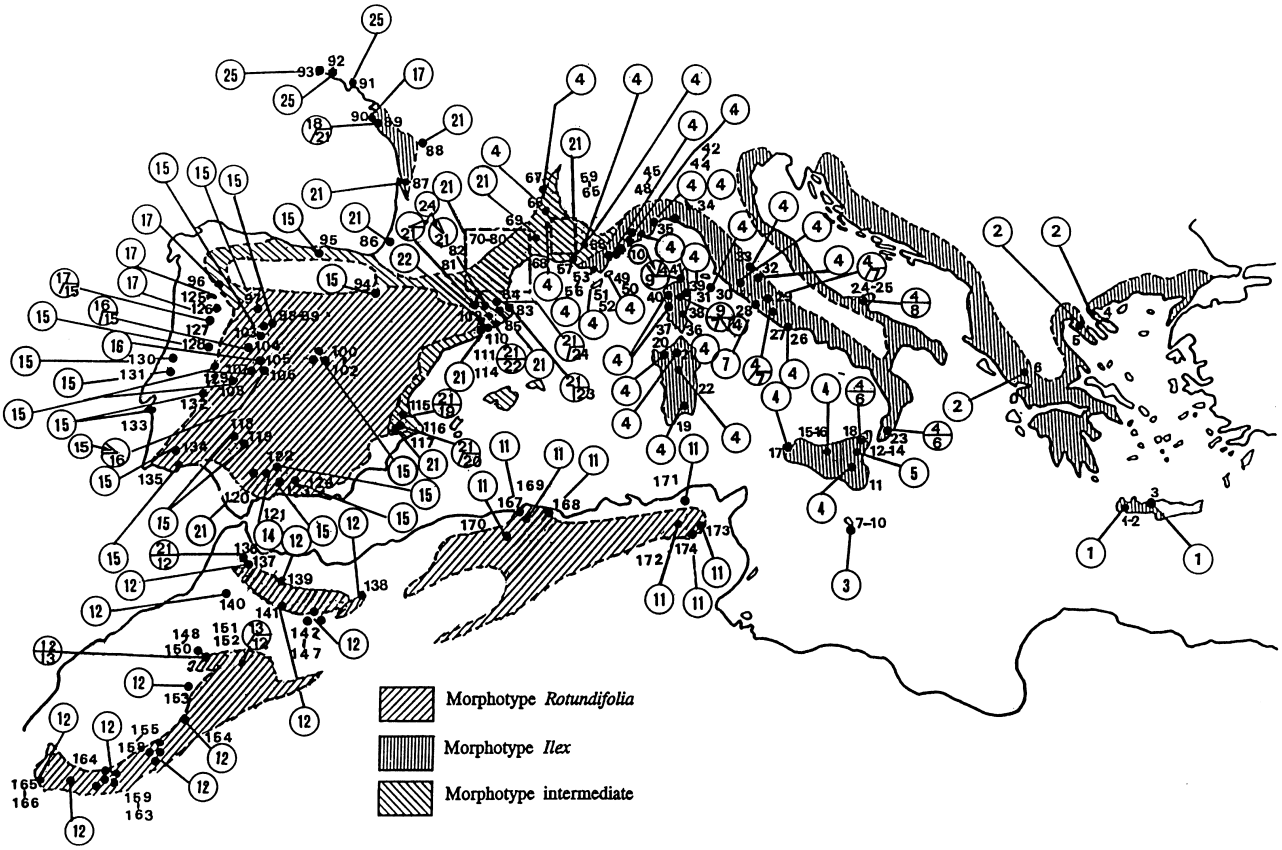

Fig. 1 Geographical frequency distribution of the 25 chlorotypes identified in the 174 *Q. ilex* populations scored for cpDNA variation. The main areas of the overall species distribution are indicated by tree distinct hatched areas according to population morphotype. The precise location of populations 59–65 and 70–80 (inside the squares) are indicated in Fig. 3.

and rounded thick leaves and occurs in inland parts of North Africa and Spain, where it grows under Mediterranean climates ranging from semiarid (with markedly continental conditions) to per-humid (Fig. 1). These morphotypes have been considered either as two distinct species (Tutin *et al*. 1993), two subspecies (Saenz De Rivas 1967), or simply two varieties (Maire 1961). In addition, trees showing morphological characters intermediate between the two morphs occur in the French regions of Languedoc and Roussillon, and in the eastern and northern coastal areas of Spain (Fig. 1). Evidence from allozyme polymorphism suggests the occurrence of a single species with two distinct subspecies. These differ mainly by adaptive characters related to the distinct climate conditions which occur in the distinct geographical areas of the Mediterranean Basin (Michaud *et al*. 1995). However, the possibility that the occurrence of the two morphs may involve phylogenetic differentiation due to postglacial migration from distinct ice-age refugia cannot be ruled out.

In a recent study based on the cpDNA variation of evergreen oaks in Morocco, cytoplasmic exchanges between *Q. ilex* and *Q. suber* (cork oak) were reported to occur in several areas (Belahbib *et al*. 2001). *Q. ilex* and *Q. suber* are not close genetically and belong to two distinct subsections (or subgenera), *Sclerophyllodrys* O. Schwartz and *S. cerris* (Spach), respectively (Schwarz 1937; Camus 1938). In morphological studies (Abel 1902; Natividade 1937) and in more recent analyses based on allozyme variation and cpDNA RFLPs, frequent hybridization and genetic introgression were also reported between *Q. ilex* and *Q. coccifera* (holly oak), which are closely related species and both belong to subsection *Sclerophyllodrys* O. Schwartz (Toumi & Lumaret 2001; unpublished data). From these studies, and because the cpDNA molecule is known to be maternally inherited in oaks (Dumolin *et al*. 1995), consistent results showed that, in the hybridization process with the other evergreen oak species, *Q. ilex* is very predominantly (but not exclusively) the maternal species and that hybridization is usually followed by unidirectional introgression, *Q. suber* and *Q. coccifera* being the pollen-bearing species. As a consequence, interspecific genetic exchanges are not expected to significantly affect the cytoplasmic variation of *Q. ilex*. In the present work, we analyse the phylogeographical variation of cpDNA in *Q. ilex*, over the whole distribution

of the species. The main objectives are: (i) to determine the probable route followed by *Q. ilex* during its spread over the Mediterranean Basin during the Tertiary; (ii) to identify ice-age refugia and the main postglacial re-colonization routes responsible for the present distribution area of holm oak; (iii) to analyse congruence between the phylogenetic structure based on cpDNA variation and the geographical distribution of the several morphs identified within the species.

Materials and methods

Plant material

The material used in the study was collected from 174 *Quercus ilex* populations distributed over the whole distribution area of the species (Fig. 1). Name, geographical coordinates and sample sizes of the localities may be obtained from the corresponding author. The populations were either pure (88 sites), or mixed with *Q. suber* in 60 sites (numbers 16, 20, 21, 22, 35, 36, 38, 39, 42, 44, 46–48, 52–55, 70, 72, 78–86, 97, 98, 103, 106–108, 111, 112, 118, 124, 127, 130–132, 134, 136, 137, 141–144, 146–152, 165, 166, 168, 174), with *Q. coccifera* (ssp. *coccifera*) in 17 sites (numbers 4, 6, 57–63, 65, 68, 69, 71, 74–77), *Q. coccifera* (ssp. *calliprinos*) in two Cretan sites (numbers 2 and 3), or with both oak species in seven sites (nos 49, 50, 56, 114, 115, 120, 133). A mean of 4.01 trees per population were analysed. This small sample size is consistent with the high level of interpopulation genetic differentiation (*Gst*) observed usually for cpDNA variation (Pons & Petit 1995). However, in the populations where cpDNA variation was observed, the sample size was increased to 11.10 trees per population, on average. In addition, by using several discriminate leaf characters described in *Flora Europaea* (Tutin *et al*. 1993) and by Llamas *et al*. (1995), 23 trees from 17 mixed populations (numbers 20, 49–52, 55, 78–82, 108, 111, 124, 137, 149) and 18 trees from five mixed populations (48, 49, 71, 72, 114) were identified as being morphologically intermediate between *Q. ilex* and either *Q. suber* or *Q. coccifera*, respectively. As shown by Natividade (1936) from experimental crosses, hybrid individuals between *Q. ilex* and *Q. suber* have no cork. Therefore, usual bark characters were unable to discriminate between *Q. ilex* and its hybrids with *Q. suber*. The hybrid origin of the trees identified morphologically as intermediate was confirmed by using several allozyme diagnostic markers identified previously (Elena-Rossello *et al*. 1992; Toumi & Lumaret 1998, 2001). Dry leaf vouchers from the populations where hybrids were detected morphologically are held in the herbarium of CEFE-CNRS in Montpellier. The individuals of hybrid origin were considered separately in the cpDNA analysis. As reported previously (Manos *et al*. 1999; Belahbib *et al*. 2001), the cpDNA haplotypes observed in *Q. ilex* and in *Q. suber*, respectively, belong to two very divergent lineages that can be identified easily. As a comparison, and using the same techniques as for *Q. ilex*, cpDNA was analysed in pure populations of several other evergreen Mediterranean species. Ten *Q. suber* individuals (two from Portugal, five from France, one from Italy and two from Morocco), five *Q. coccifera* (ssp. *coccifera*) individuals from French and Tunisian populations, five *Q. coccifera* (ssp. *calliprinos*) trees, four from Crete (one from a pure population, and three from two populations mixed with *Q. ilex*) and one from Cyprus (pure population), and three individuals of *Q. alnifolia* Poech (golden oak) were scored for cpDNA variation. *Q. alnifolia* is endemic to Cyprus island where *Q. ilex* was never observed and, on the basis of allozyme variation, this species was shown to be genetically close to *Q. ilex* and to *Q. coccifera* (Toumi & Lumaret 2001).

Isolation and restriction endonuclease analysis of cpDNA

Leaf-bearing branches collected on the trees were placed in the dark for 8 days to de-starch the leaves before they were ground in liquid nitrogen and freeze-dried. Chloroplasts were isolated from 4-g aliquots of freeze-dried powder, and cpDNA was extracted from chloroplasts as described by Mariac *et al*. (2000). Aliquots of 20 μg of chloroplast DNA were incubated for 5 h with four 6-cutter endonucleases (*Ava*I, *Bam*HI, *Dra*I, and *EcoRV*) and with a 4-cutter endonuclease (*Hha*I), according to the recommendations of the suppliers (Boehringer-Germany, Appligene-France). These restriction enzymes provided clear restriction patterns regularly, with a large number of fragments (usually over 45), except for *EcoRV* which generated fewer and larger-sized fragments, and which was used, more particularly, to estimate chloroplast DNA molecular size by adding together the size of the fragments. The digestion products were fractionated by electrophoresis on horizontal 0.85% agarose-slab gels. Additional 1.2% agarose gels were used, more particularly for *Hha*I which produced many small fragments. Raoul and 1-Kb Ladder DNA (Appligene) were used as size standards. Gels were stained with ethidium bromide and photographed under UV light. For each cpDNA restriction endonuclease pattern, DNA restriction fragment sizes were determined using BANDE software (Duggleby *et al*. 1981).

Identification of cpDNA mutations

The cpDNA restriction endonuclease patterns of individual trees were scored for fragment-length differences. The cpDNA changes were identified as either length or site mutations. The detection of specific changes, each revealed from an individual oak tree by several restriction enzymes, suggests that alterations in the length of the fragments may be due to DNA length mutations rather than site

mutations. By scoring those length mutations arbitrarily as the same mutation (same letter), we avoided counting the same deletion/addition several times and, thereby, overestimating the number of distinct mutations.

Relationships between chlorotypes

Considering the chlorotype identified in pure populations of *Q. alnifolia* as outgroup, the DNA changes observed in the several chlorotypes identified in *Q. ilex* were scored for presence/absence and were analysed cladistically by enumeration of the parsimonious trees using PAUP 4.0b (Swofford 1998). A strict consensus tree was obtained and 1000 bootstrap samples were used to place confidence limits on branching points in the tree.

Chlorotypes geographical distribution and genetic diversity analysis

The geographical distribution of the different chlorotypes identified from RFLPs was mapped. Nei's (1987) genetic diversity statistics adapted to small and unequal sample sizes ($h = n(1 - \Sigma pi^2)/(n - 1)$ where pi is frequency of the ith allele and n is population size) were calculated for each population (hs) and over all populations (ht), and the proportion of diversity resulting from genetic differentiation among populations (Gst) was estimated.

Results

Of all the *Quercus ilex* material analysed by digestion with five restriction enzymes, only one individual in population 49 showed extremely divergent patterns, identical to those observed in *Q. suber* from various geographical origins. Results from analysis of this isolated tree were not included in our data treatment. From the remainder of the holm oak material 54 different banding patterns were observed, giving a total of 323 different fragments. A single pattern was obtained for *EcoRV* whereas 21, 8, 10 and 14 distinct patterns were observed for *Hha*I, *Bam*HI, *Ava*I and *Dra*I, respectively. Overall, the restriction endonucleases *Hha*I, *Bam*HI, *Ava*I, *Dra*I and *EcoRV* generated an average of 55.0, 39.5, 40.0, 45.5 and 36.0 fragments, respectively. Chloroplast DNA molecular size was estimated by adding together the size of the fragments generated by each endonuclease, particularly those produced by *Bam*HI and *EcoRV* which provided fewer and larger-sized fragments. In holm oak, the cpDNA size was estimated to range between 142 and 143 kb.

To compare the restriction patterns identified in other regions, those observed for the several endonucleases in *Q. ilex* from Crete were used as a reference because they showed the highest similarity with the restriction patterns obtained from most the other Mediterranean evergreen oak species (*Q. suber*, *Q. coccifera* (ssp. *calliprinos*) and *Q. alnifolia*). By comparison with the restriction pattern observed in Cretan material for each of the four endonucleases which provided polymorphism, the mutations responsible for cpDNA variation in *Q. ilex* were identified and are listed in Table 1. Twenty-nine site mutations were found. In addition, 16 length mutations were identified using from two to four distinct endonucleases for each.

In *Q. ilex*, from all the mutations obtained with the five endonucleases, 25 distinct haplotypes (chlorotypes) were identified, all very divergent from those observed in *Q. suber*. The mutations which characterize each of the 25 chlorotypes are indicated in Fig. 2. In *Q. alnifolia* a single chlorotype was observed, which differed from the closest holm oak chlorotype, number 1, by six additional mutations (referred as 30, 31, 32, Q, R, S) (Fig. 2). Individuals of *Q. coccifera* ssp. *coccifera* possessed either chlorotype 4 or 21, both observed in *Q. ilex*. In *Q. coccifera* ssp. *calliprinos*, chlorotype 1 was identified in the two Cretan populations mixed with *Q. ilex*, and two additional chlorotypes, genetically close to chlorotype 1, were found in individuals from the pure populations of Crete and Cyprus, respectively.

The consensus tree obtained by the parsimony method from the analysis of presence/absence of the 51 cpDNA mutations, of which 26 were phylogenetically informative, with *Q. alnifolia* as an outgroup, can be observed in Fig. 2. Four most-parsimonious trees were obtained which required a minimum of 52 steps to account for the 51 mutations. The single reversion was observed for the site mutation 10. The consistency and retention indices were equal to 0.98 and 0.99, respectively. The consensus tree was obtained with confidence values ranging from 62 to 100% of the major branches (Fig. 2). Four clusters can be distinguished, with distinct geographical distributions (Fig. 1). Chlorotype 1 differs from the 24 other chlorotypes by nine site and two length mutations. In *Q. ilex*, this chlorotype is observed exclusively in the Cretan populations, either pure or mixed with *Q. coccifera* ssp. *calliprinos* (Fig. 1). Chloroplast 2 is also very distinct from most of the others and was observed exclusively in Greece (Fig. 1). A group of eight chlorotypes (from 3 to 8) is characterized by the phylogenetically informative mutations 2, 5, I and L. These chlorotypes were found exclusively in continental Italy, in the French region of Provence and in several nearby islands (Malta, Sicily, Sardinia and Corsica). Most of these chlorotypes are distributed locally. Chlorotype 3 was observed exclusively in Malta, chlorotypes 5 and 6 were specific of the Etna area and northeastern Sicily, respectively, chlorotypes 7 and 8 were discovered in Latium and central Corsica and in Calabria, respectively. Chlorotypes 9 and 10, which share mutation 6, were restricted to the Corte region (Corsica) and to a small locality of the Corsican Cape, respectively. Conversely, chorotype 4 is distributed widely in the entire continental and island area of Italy and in the

Table 1 Restriction fragment length changes (kb) compared to the fragment size observed in *Q. ilex* individuals from Crete, type of mutation (site, length) observed in *Q. ilex* material scored for RFLP cpDNA variation by using four restriction enzymes. Changes attributable to the same mutation are indexed with the same letter

Restriction enzyme	Mutation			
	No.	Site		Length
*Hha*I	1	2 × 6610 → 2 × (6400 + X[a])	A	5610 → 6120
	2	6400 → 6250 + X	B	4770 → 4810
	3	6400 → 6150 + X	C	4650 → 4850
	4	4600 + X → 4650	D	4650 → 4770
	5	4570 + 460 → 5030	N	4650 → 4790
	6	4200 + X → 4580	E	3820 → 3100
	7	3940 → 3820 + X	F	2110 → 1390
	8	3940 → 3880 + X	G	1650 → 1710
	9	3940 → 3840 + X	H	1510 → 1430
	10	3850 + X → 3870	I	1410 → 1370
	11	3850 → 2660 + 1190	J	1390 → 1360
	12	3500 → 3350 + X	K	1070 → 1030
	13	2110 + 530 → 2640		
*Bam*HI	14	5250 + 2350 → 7600	A	5500 → 6010
	15	5180 → 3230 + 1950	B	3280 → 3320
	16	5180 + 3260 → 8440	I	1670 → 1630
			N	1380 → 1520
*Ava*I	17	9180 → 8200 + 980	A	3510 → 4020
	18	5510 → 5210 + 300	B	1920 → 1960
	19	5440 + X → 5490	C	1780 → 1980
	20	2380 + 2130 → 4510	F	5440 → 4720
	21	2330 → 1580 + 750	H	1820 → 1740
			I	2100 → 2060
			L	3190 → 3260
			M	3120 → 3140
			O	4040 → 3990
			P	1740 → 1720
*Dra*I	22	7130 + 2150 → 9280	A	2150 → 2660
	23	7130 → 7540 + X	D	1690 → 1810
	24	6400 → 4940 + 1460	E	7420 → 6700
	25	3770 → 3600 + X	F	4840 → 4100
	26	3490 + 3030 → 6520	G	3390 → 3450
	27	2770 → 1730 + 1040	H	2950 → 2870
	28	1980 + X → 1220	I	1440 → 1400
	29	990 + 690 → 1680	J	1530 → 1500
			K	1660 → 1620
			L	3240 → 3310
			M	7550 → 7570
			O	960 → 910
			P	870 → 850

[a]X fragment not visualized because of either small size or superimposed bands.

nearby French region, Provence, and Corsica island. A fourth cluster, characterized by mutations 15, 21, 29 and H, can be subdivided into two subclusters. The first includes the three chlorotypes, 11, 12 and 13, which were observed exclusively in North Africa. More particularly, chlorotype 11 was found in Tunisia and Algeria, whereas chlorotypes 12 and 13 were observed in Morocco, the former being distributed in the whole country and the latter restricted to few trees growing in Central Plateau. The second subcluster gathers together the 12 chlorotypes which were observed in the Iberian Peninsula and in three southern and western regions of France, adjacent to this Peninsula. Six chlorotypes (from 14 to 19), characterized by mutation 10, were observed in the Iberian Peninsula, except in Catalonia. All the *Q. ilex* populations selected for sweet acorn production (see above) were shown to possess chlorotypes 15 or 16, according to the geographical area. The other six related chlorotypes (from 20 to 25), characterized by

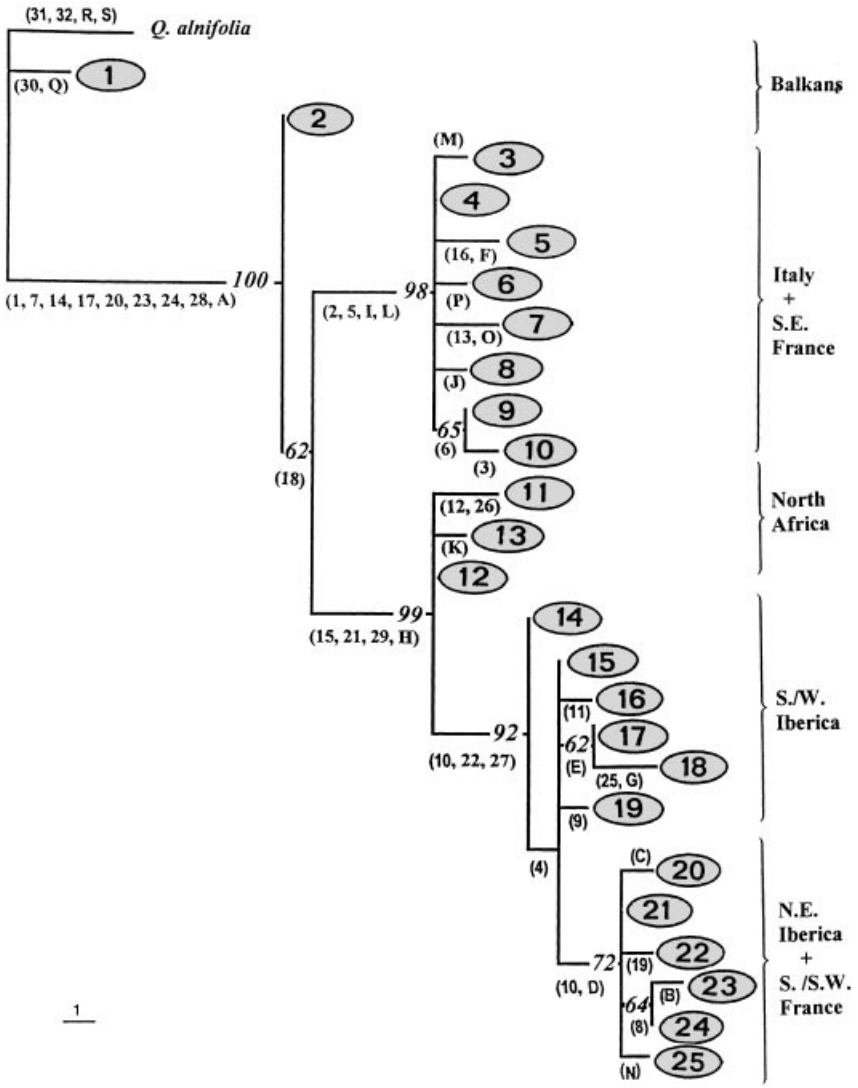

Fig. 2 Phylogram based on the consensus tree resulting from parsimony analysis of the 25 chlorotypes observed in the 174 *Q. ilex* populations scored for cpDNA variation, using *Q. alnifolia* as an outgroup. The mutations are identified (between parentheses) on the tree branches. For each major branch, the percentage of times that the defined group occurred in the 1000 bootstrap samples is indicated in italics.

mutation D, were found in a locality near Valencia (type 20), in Spanish Catalonia (types 21 and 22), in French Catalonia (21, 23, 24), in southwestern France (21) and along the southern coast of Brittany (chlorotype 25). With few exceptions, genetically close chlorotypes also showed geographical proximity. However, chlorotype 17, distributed widely in northern of Portugal, was observed in all the trees collected in the very distant French oceanic island of Noirmoutier (population 90 in Fig. 1) and chlorotype 18, which differs from chlorotype 17 by two specific mutations (25 and G), was observed exclusively in a locality of the continent, very close geographically to Noirmoutier (population 89). Chlorotype 21, widely distributed in Spanish Catalonia and in the south of France (except in Provence and in Corsica), was also observed in an Andalusian locality (population 120) and in a few trees of a population (136) located in northern Morocco. In addition, in the south of France, a contact area is observed between the populations of Languedoc (west of the Rhône river), which are morphologically intermediate between 'rotundifolia' and 'ilex' morphs and possess chlorotype 21, and the populations of Provence (eastern side of the river), corresponding to 'ilex' morph, and characterized by chlorotype 4. The two chlorotypes differ by 12 mutations (2, 4, 5, 15, 21, 22, 27, 29, D, H, I and L). As shown in Fig. 3, in the contact area, chlorotype 21 was able to colonize the eastern side of the Rhône Valley in the south, whereas chlorotype 4 spread, but less extensively, along the western side of the river in the north. The two chlorotypes were never observed in the same site.

A single chlorotype was observed in 150 of the 174 *Q. ilex* populations analysed. In each of the 24 polymorphic populations (nos 18, 23–25, 27, 29, 38, 41, 81–84, 89, 104, 106, 114, 116, 127, 136, 148–152) localized in various areas two chlorotypes were found, except in two Corsican populations (nos 38 and 41), where three distinct chlorotypes were

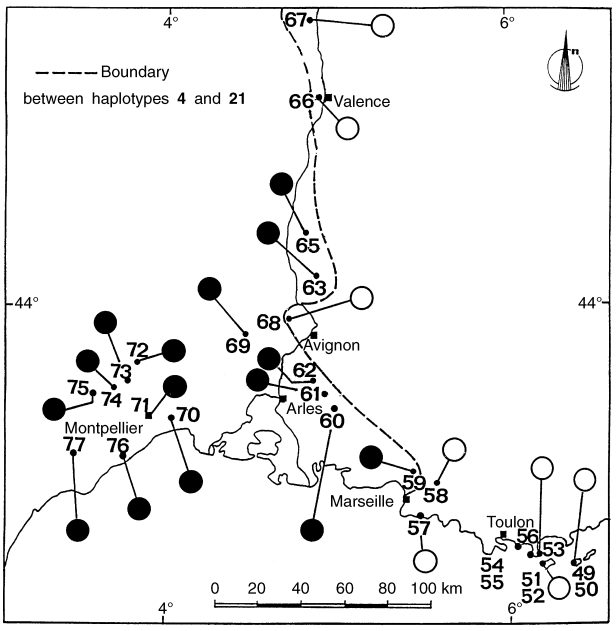

Fig. 3 Geographical distribution of *Q. ilex* chlorotypes 4 (○) and 21 (●) in the Rhône valley considered as being the contact area between the Italian and Iberian postglacial migration routes of the species.

identified (Fig. 1). Within each geographical region, the same chlorotypes were usually observed in pure and mixed populations, and the proportion of polymorphic populations was not significantly different between pure holm oak populations and those mixed with other evergreen oak species (χ^2 test). Average genetic diversity per population, total genetic diversity and the proportion attributable to differentiation between the populations were equal to 0.06, 0.82 and 92%, respectively.

Of the 23 individuals identified as being of hybrid origin between *Q. ilex* and *Q. suber* on the basis of morphology and allozyme diagnostic markers, only two, from populations 49 and 149, respectively, showed a chlorotype characteristic of the *Q. suber* cpDNA lineage. Four chlorotypes, 4, 12, 15 and 21, were observed. With no exception, the same chlorotype was identified in the hybrid and in the *Q. ilex* trees of the same site. In the *Q. ilex* populations mixed with *Q. coccifera* (ssp. *coccifera*), the same chlorotype (number 21) was observed in the 18 individuals morphologically intermediate between both species and in the *Q. ilex* trees including in the sites where a distinct chlorotype was observed in the *Q. coccifera*.

Discussion

CpDNA variation in Quercus ilex

In the present study, substantial cpDNA variation has been documented over the whole geographical distribution of *Quercus ilex*. The identification of nine specific mutations shared by all the chlorotypes (except chlorotype 1), which also show direct molecular relationships, supports the occurrence of a single specific cpDNA lineage. Moreover, since the chlorotypes observed in *Q. ilex* were identified predominantly in its hybrids with other evergreen oak species, the cpDNA variation observed in that species is probably not significantly affected by interspecific genetic exchanges. This result is in agreement with a previous report by Belahbib *et al*. (2001) who, in 97 *Q. ilex* and *Q. suber* Moroccan populations, observed two very distinct cpDNA lineages associated with each species. Of the 165 trees from 31 Moroccan *Q. ilex*/*Q. suber* mixed populations the authors identified as *Q. ilex*, and which were scored for cpDNA variation, only five trees (each from a distinct population) were shown to possess a chlorotype of the *Q. suber* lineage. Moreover, as species identification was based exclusively on bark characters (presence of cork) (Belahbib *et al*. 2001), the putative hybrids between the two evergreen oak species were not distinguished from *Q. ilex* individuals.

In the present work, chlorotype 1 is substantially divergent from the 24 other chlorotypes of the *Q. ilex* cpDNA lineage and its membership of that lineage may be questionable. Chlorotype 1 was observed exclusively in Crete, in pure *Q. ilex* populations and in mixed populations of *Q. ilex* and *Q. coccifera* (ssp. *calliprinos*). Chlorotype 1 is distinct but genetically close to the chlorotypes observed in *Q. coccifera* ssp. *calliprinos* (pure populations from Crete and Cyprus), in *Q. suber* and, to a lesser extent, in *Q. alnifolia*, and may therefore be considered as an ancestral *Q. ilex* chlorotype which has been maintained exclusively in Crete. Alternatively, as this chlorotype is shared by the *Q. coccifera* individuals which grow in mixed populations, the possibility that its occurrence in *Q. ilex* may be the result of cytoplasmic introgression from *Q. coccifera* into *Q. ilex* cannot be ruled out.

In *Q. ilex*, the proportion of cpDNA polymorphic populations is low, most chlorotype diversity being attributable to differentiation among populations. Similar results were obtained from cpDNA analysis in several European white oak species (Petit *et al*. 2002a).

Chlorotype phylogeographical variation

The chlorotype phylogeographical structure reported previously for the European deciduous oak species was characterized by the occurrence of unrelated variants located in the same refugia and by the concentration of most chlorotypes in the southern part of the distribution area (Petit *et al*. 2002b). By contrast, clear chlorotype phylogeographical patterns are observed in *Q. ilex* and variation is distributed regularly over the whole distribution area. Examination of both phylogenetic structure and present geographical distribution of chlorotypes

shows that in *Q. ilex*, the genetically closest chlorotypes (numbers 1 and 2) to those observed in related evergreen oak species (more particularly in *Q. alnifolia*) were found exclusively in the eastern part of the species distribution area. In addition, in the phylogram the several clades of chlorotypes which are differentiated successively from the root were identified in geographical areas distributed from east to west (i.e. Greece, Italy, North Africa and the Iberian Peninsula) suggesting that, in *Q. ilex*, migration and genetic differentiation occurred initially in that direction. Moreover, because each clade is defined by numerous mutations, this genetic differentiation may be very ancient and may characterize distinct geographical ice-age refugia (i.e. southern Balkan Peninsula, southern Italian Peninsula, North Africa and southern Iberian Peninsula) from which, after the last glaciations, *Q. ilex* began to migrate to more northern areas. Palynological and anthrachological data (based on coal fragments) have shown the occurrence of *Q. ilex* in all these areas since the end of the Tertiary period and its persistence in the regions corresponding to these four putative refugia throughout the ice-age period (Pons & Vernet 1971; Carrión *et al*. 2000). With the exception of North Africa, the same ice-age refugia were identified for European white oak species and for several other forest tree species (Ferris *et al*. 1993, 1998; Hewitt 1999; Taberlet *et al*. 1998; Brewer *et al*. 2002; Petit *et al*. 2001, 2002b). According to our data, the chlorotypes observed in the Iberian Peninsula are related to those identified in North Africa. This is particularly obvious for the very local chlorotype 14, occurring exclusively in the south of Andalusia, which may be considered as evolutionarily intermediate between the widely distributed Moroccan chlorotype 12 and the several other Iberian chlorotypes. In their study based on leaf morphology and flavonoid variation in *Q. ilex*, Lebreton *et al*. (2001) showed that the North African and the Iberian populations were close morphologically but that the former possessed more ancestral flavonoid molecules than the latter. In the present work, no African chlorotype was identified in Iberia and, in previous studies based on allozyme polymorphism, several widely distributed alleles were observed exclusively in North Africa (Michaud *et al*. 1995). This high cytoplasmic and nuclear genetic differentiation between the African and the European *Q. ilex* populations supports the occurrence of efficient barriers to gene flow between the two groups of populations for a very long period, probably as the result of long-term geographical discontinuities. These results also suggest that the *Q. ilex* populations of the North African refugia probably did not contribute to the re-colonization of Europe after the last glaciations.

In *Q. ilex*, each phylogenetic group of chlorotypes is characterized by one or two specific chlorotypes which are distributed over large areas (i.e. number 2 in the Balkans, number 4 in the Italian Peninsula and the French region of Provence, nos 11 and 12 in the eastern and western parts of North Africa, respectively, number. 15 in western Iberia and number 21 in the eastern part of Iberia and in the two adjacent regions of France). The present distribution of these 'major' chlorotypes probably shows the distinct postglacial migration routes of the species. As indicated on the chlorotype geographical distribution map, the main chlorotypes observed in southwestern and northwestern France (i.e. numbers 21 and 25) are not closely related to those found in northwestern Spain. Chlorotype 21, which differs from chlorotype 25 by a single mutation, is predominant in Catalonia and in the Mediterranean French region of Languedoc. This unexpected result suggests that, during postglacial migration, the Pyrenees may have been crossed by *Q. ilex* exclusively in their eastern part. Moreover, in the Rhône valley the geographical boundary between chlorotypes 4 and 21 indicates the occurrence of a clear contact area between the Italian and the Iberian migration routes in the northern part of their respective Mediterranean range expansion. Except for the Balkan Peninsula, where the sampling was restricted to few populations, in each geographical group the several 'minor' chlorotypes derived from the few 'major' ones by a single mutation (or by very few), and which are restricted to local areas, are probably of more recent origin. As shown in the phylogram, chlorotype structuring is higher for the types observed in the Iberian Peninsula and in the adjacent regions of France, where several chlorotypes restricted to the northern part of the distribution were identified (e.g. number 17 in Northern Portugal and number 25 in Brittany) than for those identified in the Italian Peninsula and related islands where numerous directly unrelated chlorotypes occur very locally. Genetic specificity of the *Q. ilex* populations, which grow on the slopes of Etna (Sicilian volcano) and are characterized by chlorotype 5 (mutations 16 and F), is consistent with previous identification in the same plant material of specific alleles at several loci coding for allozymes (Michaud *et al*. 1995). The populations growing on Etna have to cope with frequent destruction and recolonization episodes leading to geographical isolation of many small populations subjected to genetic drift. As reported previously (Mindell & Thacker 1996), smaller effective population sizes can yield faster rates of changes by favouring the fixation of nuclear and cytoplasmic mutations.

In addition, few chlorotypes were distributed in several geographically distinct and distant areas. For instance, chlorotype 6 was observed both in Sicily and in the South of the Italian Peninsula, chlorotype 7 was identified in the Latium region of Italy and in a restricted area of the centre of Corsica, chlorotype 21 distributed in Catalonia and in southern France was also found in a single site of Andalusia and in a very local area of northern Morocco. The Portuguese chlorotype 17 was observed in the famous wood

('Bois de la chaise') of Noirmoutiers island (site number 90) where, according to local reports, *Q. ilex* was introduced several centuries ago and chlorotype 18, derived from it by two specific mutations, was identified exclusively in a single continental site located closely to this island (site 89). In previous reports, the existence of rare events of dispersal far away from the main colonization front was inferred to explain the cpDNA patchy geographical distribution observed in European deciduous oaks (Hewitt 1993; Ibrahim *et al*. 1996; Petit *et al*. 2001). The possibility that long-dispersal events may be at the origin of the discontinuous distribution of several chlorotypes in *Q. ilex* cannot be ruled out. However, historical events such as the long period of Spanish colonization in the extreme north of Morocco where chlorotype 21 was identified, the close historical relationships between the region of Rome and that of Corsica, where chlorotype 7 was observed, suggest that human activity may be also responsible for the disjunct distribution observed in *Q. ilex* for several of its chlorotypes.

Congruence between morphological and chlorotype variation

On the whole, a good consistency is observed between chlorotype phylogenetic organization and the geographical distribution of the two main *Q. ilex* morphotypes. The populations which show morphotype 'ilex' and have a continuous distribution along the coasts of Europe, from Greece and Crete to the French Provence region, were shown to possess chlorotypes 1–10 of which chlorotypes 3–10 are very closely related. Chlorotype 1 (see above) and chlorotype 2, both located in the eastern part of this area, may be considered as the most ancestral chlorotypes observed presently in the species. The populations which show morphotype 'rotundifolia', and are distributed over North Africa and in both the southern and the central parts of Iberia, possess chlorotypes number 11–16. These chlorotypes belong to the same clade and are divided into two groups distributed in North Africa and Iberia, respectively. From the chlorotype phylogeny observed in *Q. ilex*, evidence is obtained of early genetic differentiation between the two morphotypes which colonized distinct geographical areas characterized by different climate conditions. Moreover, the morphologically intermediate populations between the two morphotypes are distributed in northern Iberia and in adjacent French regions, and possess the chlorotypes number 17 and numbers 19–24. In the present study, evidence is shown that these chlorotypes are closely related to those identified in southern and central Iberia. In previous *Q. ilex* studies based on morphological characters (e.g. Lebreton *et al*. 2001), it was suggested that the intermediate populations may result from hybridization and/or genetic introgression between populations of the two main morphotypes. The clear-cut geographical northern limit between chlorotypes 4 and 21 observed in the present study and the *Q. ilex* allozyme geographical distribution over the whole distribution area (Michaud *et al*. 1995) do not support this assumption. We suggest, rather, the occurrence of morphological changes in the northern populations as a response to more humid climate conditions. This assumption is supported strongly by identification of chlorotypes 17, 18, 21 and 25 identical to those observed in Iberia or derived directly from them, in the French Atlantic populations which show clearly the 'ilex' morph (Michaud *et al*. 1995; Lebreton *et al*. 2001). Discrepancy between morphological and cpDNA variation may indicate the occurrence of morphological convergence as an adaptive response of *Q. ilex*, which had to face important changes in climate conditions during its northern postglacial migration. In the Atlantic area, identification of the 'ilex' morph in *Q. ilex* material possessing chlorotype 17 and very probably introduced from Iberia where the tree material possessing the same chlorotype is morphologically 'intermediate', suggests that the adaptive morphological changes may have occurred rapidly.

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The authors belong to a team that has a major interest in the study of evolutionary processes in plant polyploid complexes and long life-span plant species. A long-term research programme on genetic variation in Mediterranean forest tree species has been developed, more particularly in wild olive and oaks. This research forms a part of Henri Michaud's thesis on genetic variation in holm oak by using allozyme and DNA molecular markers. Céline Mir and Roselyne Lumaret have special interest in the evolutionary and adaptive significance of the frequent interspecific genome exchanges observed in oaks, and which are revealed by analysing genetic variation for cytoplasmic and nuclear markers. In Mediterranean oaks, a multidisciplinary study involving genetic, ecological and ecophysiological approaches, is coordinated by Roselyne Lumaret. The main objective is to improve knowledge about the evolutionary significance of interspecific genetic introgression for further conservation strategies.
