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White oaks phylogeography in the Iberian Peninsula

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Abstract

The geographic distribution of maternally inherited chloroplast DNA polymorphisms was studied to determine the phylogeographic structure of white oaks in the Iberian Peninsula. Almost 1000 mature trees from nearly 200 populations were sampled in the distribution areas of the six white oak species that are recognised in Iberia. The analysed trees roughly represent the species distribution and their respective abundance. The PCR–RFLP procedure used by all project partners revealed up to 14 cpDNA haplotypes. Seven of them had not been previously described and have not been found elsewhere in Europe. Phylogenetic relationships among the haplotypes support the existence of four maternal lineages, one of them being restricted in Europe to the Mediterranean Spain and Corsica. Genetic diversity analysis confirmed the previously described extensive sharing of haplotypes among species, but also detected some species effect in intra- and inter-specific cytoplasmic gene flow. Geographic structuration of genotypes was studied by means of autocorrelation analysis. Autocorrelation was significant for ordered and unordered alleles both when considering all populations and when analysis was restricted to lineage B. However, in both instances there was not significant autocorrelation for the contribution of the genetic divergence between haplotypes to the total coefficient of differentiation. Autocorrelograms reflect the existence of a patchy distribution of haplotypes at different scales when all lineages are considered and when only lineage B is taken into account. Finally, discussion centres on the possible role played by the Iberian Peninsula as the European westernmost refugia for the white oaks during the last glacial period. We present several lines of evidence that make us to favour the persistence of white oaks in small northern refugia during the full glacial period. © 2002 Elsevier Science B.V. All rights reserved.

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1. Introduction

Quaternary climatic changes have played a major role in the patterns of genetic polymorphism found in natural populations of plants and animals (Comes and Kadereit, 1998). During an approximated period of 100,000 years (115,000–15,000 BP), vast areas of Europe were covered by massive ice sheets, and independent centres of glaciation also occurred at low latitude in mountains such as the Alps or the

Pyrenees. Detailed pollen maps have allowed to infer the rate at which tree species recolonised northern lands, as ice sheets started to retreat after the height of the last ice age 18,000 BP (Huntley and Birks, 1983). For most temperate tree species, pollen deposits have recognised three main refugia in southern Europe: the Iberian Peninsula, Italy and the Balkans (Bennet et al., 1991).

Molecular work carried out during the last decade has also contributed to infer historical events by revealing patterns of intra-specific phylogeography in several tree species (Taberlet et al., 1998). The wide range survey of European white oaks chloroplast DNA (cpDNA) that has been lately carried out

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(Dumolin-Lapègue et al., 1997) has confirmed the existence of polymorphisms that are distributed reflecting postglacial colonisation routes from the three refugia. This study has allowed draw the recolonisation routes across Europe of different maternal lineages, enhancing the resolution offered by pollen analysis.

According to Franco (1990), there are six white oak tree species in the Iberian Peninsula; two of them being absent in the rest of Europe. The two temperate species, *Quercus robur* L. and *Quercus petraea* (Matt.) Liebl., reach their European western and southern limits in Iberia, pedunculate oaks covering westernmost areas, while sessile oaks predominate in the northeastern regions. *Quercus pyrenaica* Willd. is distributed on most of the northwestern quadrant of Iberia, being sympatric to *Q. robur*, but also extending into more continental areas. Relict isolated *Q. pyrenaica* forests can be found in Granada and Castellón. *Quercus canariensis* Willd. is the scarcest white oak species in the Iberian Peninsula, with representative forests near the Straits of Gibraltar and in the Sierra of Aracena (east Andalucía), and some dispersed stands in Monchique (southwest Portugal) and Catalonia. *Quercus pubescens* Willd. (= *Q. humilis* Miller) is distributed north of the river Ebro valley, the westernmost populations reaching the continental Basque Country. *Q. pubescens* rarely forms pure forests, but their genes can be consistently found in intermediate forms with any other sympatric oaks, mainly *Quercus faginea*. Finally, *Q. faginea* Lam., the most abundant white oak in Iberia, has a favourite Mediterranean distribution, even though some forests reach the Atlantic and Cantabric coasts. In spite of ecological similitude and abundant sympatry with the evergreen oak *Q. ilex* L., interchanging of genes is much more frequent with the white oaks complex. Gil Sánchez et al. (1996) have summarised the admitted hybridisations among the different species of the genera.

In this work, we have surveyed cpDNA polymorphism in near 200 populations and 1000 white oak individuals within the Iberian Peninsula. The importance of Iberia for the further colonisation of white oaks in western Europe has been already emphasised (Dumolin-Lapègue et al., 1997), but only a few samples from the Iberian Peninsula were included in the previous analysis and several questions remain open. First, populations from refugia commonly show

higher haplotypic diversity than those from further north (Newton et al., 1999). So, the five cpDNA polymorphisms found among the 11 samples from the Iberian Peninsula (Dumolin-Lapègue et al., 1997) might not allow to properly evaluate allelic richness in this large peninsula. Second, prolonged hybridisation opportunities in refugia areas, among these species lacking clear fertility barriers, should help to clarify the inter- and intra-specific cytoplasmic gene flow (Dumolin-Lapègue et al., 1999). Third, the near absence of common patterns in the phylogeographic structures of several European tree taxa suggests an important role of refugia (Comes and Kadereit, 1998). The particular ecogeographical conditions in the Iberian Peninsula, with several east–west range of mountains, have possibly played a major role in the distribution of refugia and the dynamics of postglacial recolonisation (Costa Tenorio et al., 1990; Blanco Castro et al., 1997).

2. Materials and Methods

2.1. Plant material

A total of 968 trees were sampled from 185 oak populations throughout the Iberian Peninsula. Sampling scheme followed the distribution range and relative frequencies of the six oak species analysed (Fig. 1). Sampling was mostly undertaken when trees were in leaf to maximise the assignment to the different species. Sampling of flushing buds in *Q. pyrenaica*, a late flushing oak at high altitudes, resulted in a relatively large frequency of poor quality DNA. As far as possible within the constraints of time, samples were taken from mixed species oak forests on an approximate 50 km grid square, although uneven distribution led to more intensive sampling in some areas and to less intense sampling in others. A total of 53 mixed and 132 pure oak stands were sampled. Whenever possible, five old trees, separated by intervals of at least 100 m, were sampled in each population. In this paper, the putative *Q. pubescens* hybrids found along their distribution range have been allocated to this species, although other choices would have been possible. Oaks from different Mediterranean origins, such as *Q. faginea* and *Q. pubescens*, often develop similar adaptations to meet particular environmental

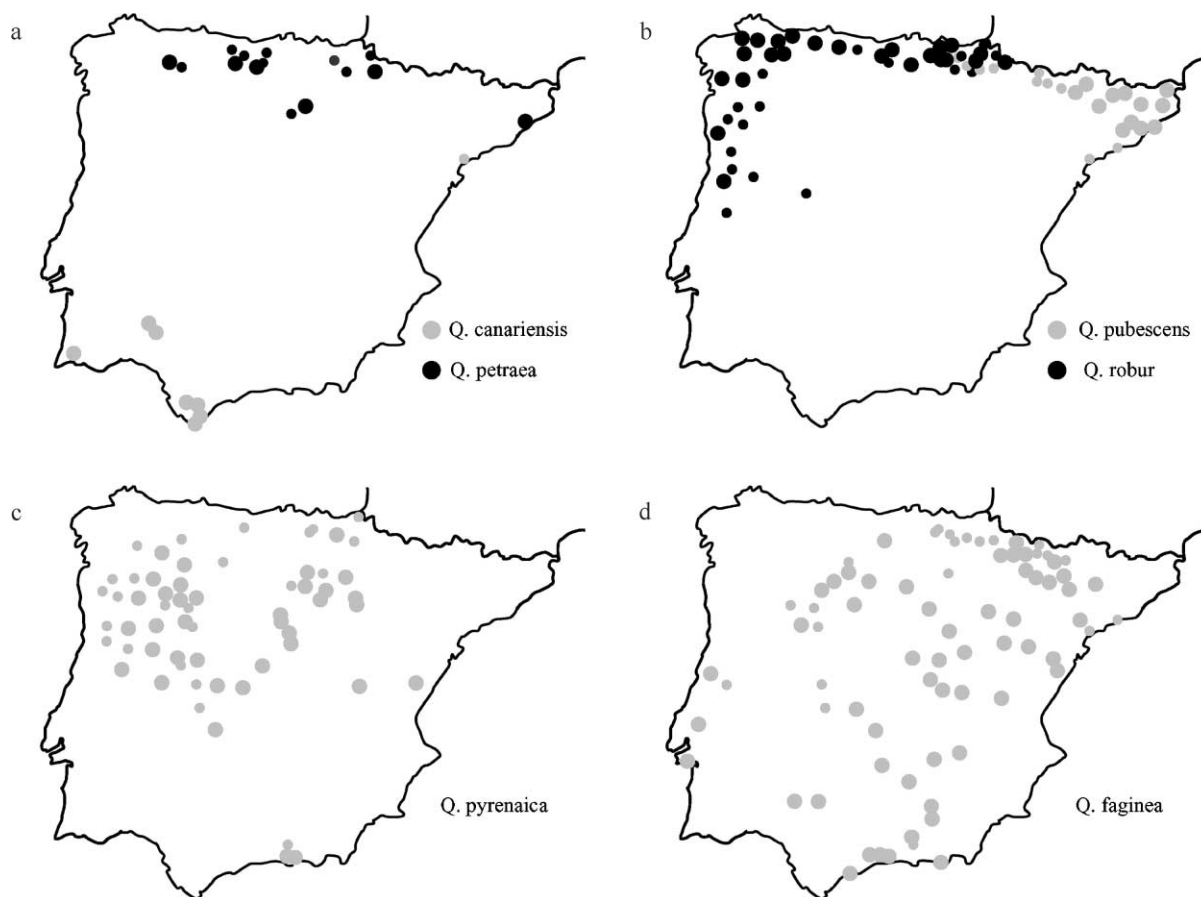


Fig. 1. Geographical location of the different white oak species sampled within the Iberian Peninsula. Large dots indicate pure species populations and small dots indicate mixed species populations.

requirements, which together with extensive introgression can easily blur the species barriers.

2.2. PCR–RFLP methods

Total DNA was extracted from leaves and buds as described by Dumolin et al. (1995). Total DNA was used as a template in PCR reactions involving four cpDNA primers that distinguished the most common polymorphisms found by Dumolin-Lapègue et al. (1997). All samples were amplified with primers *trnT/trnF* (Taberlet et al., 1991), *trnD/trnT*, *psaA/trnS* and *trnC/trnD* (Demesure et al., 1995). Amplification conditions followed Dumolin-Lapègue et al. (1997).

Five microlitres of the amplified products were digested in 20 μ l reactions with 5 U of either *TaqI* or

HinfI (see Table 1) following manufacturer's conditions (Gibco BRL). Representative individuals from each haplotype were further analysed with the DT-*AluI* combination, which detects a point mutation present in one maternal lineage, to help phylogenetic reconstruction.

Restriction fragments were separated in 8% polyacrylamide gels, using Tris Borate EDTA buffer (1 \times), at 300 V for 3–4 h. After electrophoresis, gels were stained with 0.5 μ g/ml ethidium bromide and results were recorded on a GelPrint system under UV light. Internal and external (100 bp ladder, Gibco BRL) standards were run on each electrophoresis to help the identification of the different haplotypes. Whenever a new haplotype was discovered, sample DNA was sent to project co-ordinators at INRA

Table 1

Patterns of the PCR–RFLP fragments for the 14 cpDNA haplotypes found among the white oaks from the Iberian Peninsula^a

Haplotype	DT- <i>Taq</i>			AS- <i>Hinf</i> I				TF- <i>Hinf</i> I		CD- <i>Taq</i>		DT- <i>Alu</i> I		
	1	2	3	1	2	5	6	8	9	2	3	1	2	3
H 1	9	1	2	2	3	2	2	3	1	2	2	1	1	1
H 7	1	1	5	1	4	2	3	3	1	1	1	1	2	2
H 10a	1	2	3	1	3	2	2	3	1	1	1	9	2	2
H 10b	1	2	3	1	3	2	1	3	1	1	1	9	2	2
H 11	1	2	3	1	3	2	2	1	1	1	1	9	2	2
H 12a	1	2	4	1	3	2	2	3	1	1	1	9	2	2
H 12b	1	2	4	1	3	2	1	3	1	1	1	9	2	2
H 24	1	2	3	1	4	2	2	3	1	1	1	9	2	2
H 25	1	2	2	1	3	2	2	3	1	1	1	9	2	2
H 34	1	2	2	1	3	2	2	1	1	1	1	9	2	2
H 27	1	1	3	1	2	1	4	3	2	1	1	1	2	2
H 28	1	1	3	1	2	3	4	3	2	1	1	1	2	2
H 29	1	1	3	1	1	1	4	3	2	1	1	1	2	2
H 33	1	1	2	1	2	1	4	3	2	1	1	1	2	2

^a The DT-*Alu*I combination was used only for purposes of helping the phylogenetic reconstruction.

(R. Petit and A. Kremer) to ensure standard haplotype nomenclature.

2.3. Genetic data analysis

Because a large number of new cpDNA haplotypes were found (see below), their phylogenetic relationships to the already known ones were analysed using distance programs available in Phylip 3.5 (Felsenstein, 1993). The number of different restriction fragments was used as the distance measurement.

The genetic diversity and differentiation parameters (ht, hs and G_{st}), and their standard deviations, were estimated following Pons and Petit (1995) in populations having at least three individuals. The analyses were carried out separately for each species (except *Q. canariensis*), for mixed and pure populations and over all samples.

The correlations between genetic differentiation and geographic distances were measured with the Z-statistic of Mantel (1967). The geographic distances among populations were derived from the latitude (*L*) and longitude (*G*) coordinates with the formula

$$D = 111.196811 \times \frac{180}{\pi} \times \text{Arcos}\{[\sin(L_1) \times \sin(L_2)] + [\cos(L_1) \times \cos(L_2) \times \cos(G_2 - G_1)]\}$$

(kindly provided through an internet posting by Dr. Fred Duennebier, University of Hawaii). Two genetic differentiation matrices were obtained with Arlequin 1.1 (Schneider et al., 1997) for ordered and unordered alleles. A third matrix was constructed by computing the difference between the two previous matrices. Spatial autocorrelation analysis was carried out for 50 km distance classes by replacing the geographic distance matrix with matrices of 1's (if the populations are within the desired distance class) and 0's (otherwise). (Note that this code makes nearby populations with similar haplotypes to show negative autocorrelation.) The significance of the Mantel tests was evaluated with 1000 random permutations of rows and columns of the geographic distance matrices.

3. Results

3.1. cpDNA polymorphisms

Table 1 shows the 14 different restriction fragment patterns found after digestion of products DT and CD with *Taq*I, and products AS and TF with *Hinf*I (see Annex 1 and 2 of Petit et al., 2002a, for a full description of the haplotypes). Insertion–deletions (indels) were numbered according to their molecular weights, while number 9 was assigned to new restriction sites.

Region AS detected the largest number of polymorphisms (12), followed by regions DT (8), TF (2) and CD (2). This last fragment did not reveal any discriminant fragment, as haplotype 1 could also be recognised by its DT pattern. Seven out of the 14 Iberian cpDNA haplotypes are new to the 23 previously described by Dumolin-Lapègue et al. (1997). Note that two haplotypes, 10b and 12b, have not been included in the general analysis (Petit et al., 2002a), because their distinctive mutation could not be scored by all participants.

Sampling of the different species is shown in Fig. 1, while the distribution of the cpDNA haplotypes in the Iberian Peninsula is shown in Fig. 2. Haplotypes have been colour coded respecting the conventions for other countries (see Figs. 1 and 2 of Petit et al., 2002a), except for the lineage D haplotypes (see below), which

have been coloured in green to enhance their similitudes and the distinctiveness from the other haplotypes.

3.2. Phylogenetic relationships among haplotypes

Initial tests carried out with cladistic programs showed a very large number of most parsimonious trees (>1000 before computer memory was consumed), which led us to favour phenetic ordering of the haplotypes. Analysis performed with the Phylip 3.5 distance programs Neighbor and Fitch (Felsenstein, 1993) showed similar branching patterns and identified the same maternal lineages, differences being restricted to some terminal branches of the trees. Fig. 2 also shows the tree obtained with the Neighbour-Joining algorithm used in Phylip 3.5, which should

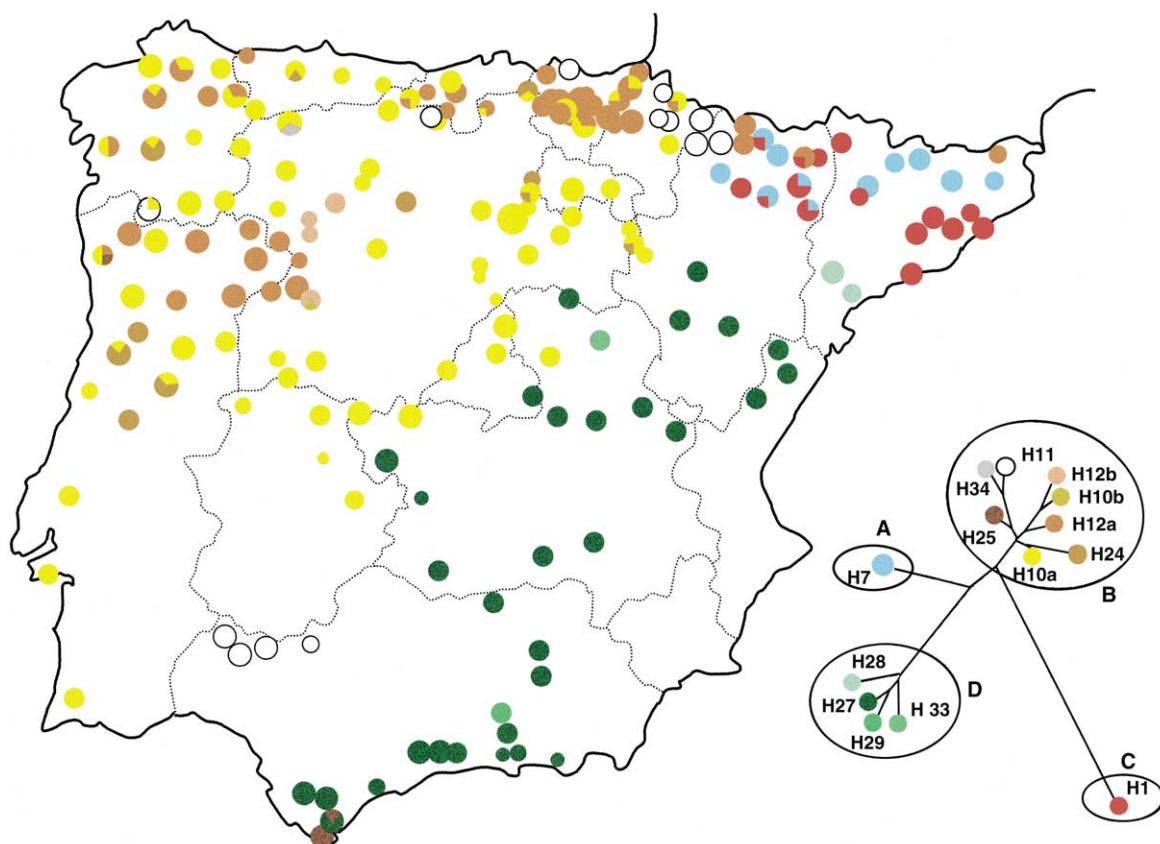


Fig. 2. Geographical distribution of the 14 cpDNA haplotypes detected during this survey in the white oaks from the Iberian Peninsula. Dot sizes indicate the number of individuals analysed in each population. Sectors within circles indicate polymorphic populations. The Neighbour-Joining tree is provided to help the interpretation of the colour-coded haplotypes and to indicate the different maternal lineages.

help the interpretation of the haplotypes distribution shown in the same figure. The phylogenetic analysis performed with all European haplotypes and the maternal lineages they belong to are indicated in Figs. 1 and 2 of Petit et al. (2002a).

The branching lengths and patterns shown by the phylogenetic analysis indicate the presence of four oak maternal lineages in the Iberian Peninsula, one of them (lineage D, haplotypes 27, 28, 29 and 33) being completely new to the three lineages described by Dumolin-Lapègue et al. (1997). Except for the four haplotypes belonging to this new lineage, all other newly discovered haplotypes are included within the B lineage (haplotypes 10a, 10b, 11, 12a, 12b, 24, 25 and 34). Lineages A and C are represented by only one haplotype each (haplotypes 7 and 1, respectively).

3.3. Genetic diversity and differentiation

The systematic sharing of haplotypes among white oaks (Dumolin-Lapègue et al., 1997) upholds in this study and is further confirmed for *Q. canariensis* (Table 2). As in previous instances, the most common haplotypes are shared by several species, while no species bears a unique haplotype.

Table 3 shows the genetic diversity and differentiation parameters estimated according to Pons and Petit (1995). In order to calculate standard deviations, only populations having more than two individuals were taken into account. This affected specially to *Q. pyrenaica* and *Q. faginea*, where 13 and 11 populations were discarded, respectively, because of the

genotyping of only two individuals. All the three parameters are species dependent. Total diversity (ht) is significantly higher for *Q. petraea* and *Q. faginea* than for the other three species. The mean intrapopulation diversity (hs) is significantly higher for *Q. robur*, with intermediate levels for *Q. pubescens* and *Q. petraea*, and very low levels for *Q. pyrenaica* and *Q. faginea*. *Q. pyrenaica* and *Q. faginea* show the highest levels of differentiation, although they are not significantly higher than the ones shown by *Q. petraea* and *Q. pubescens*. *Q. robur* shows the lowest level of populations subdivision.

The comparison between single-species and mixed oak populations does not show clear differences between the two types of forests. The level of subdivision of the cytoplasmic diversity estimated from the 170 populations is one of the highest ever-reported (90%).

3.4. Phylogeographic structure

Colour-coded haplotypes shown in Fig. 2 make clear that their geographic distribution is not random. The overall existence of a geographic structure and its significance was tested by means of a Mantel procedure comparing the matrices of geographic distances and genetic differentiations among populations (Table 4). The results show that the Z-scores are highly significant both for ordered ($Z = 0.248$, $p < 0.001$) and unordered ($Z = 0.248$, $p < 0.001$) alleles. The contribution of the genetic divergence between haplotypes to the total coefficient of differentiation (Pons and

Table 2
Frequencies of the different haplotypes among the six white oak species present in the Iberian Peninsula^a

Species	Haplotypes													Total		
	H 1	H 7	H 10a	H 10b	H 11	H 12a	H 12b	H 24	H 25	H 34	H 27	H 28	H 29			H 33
<i>Q. canariensis</i>			4		9				7		13	1			34	4.27%
<i>Q. faginea</i>	18	18	62		24	23	9	3			104	7	5	4	277	34.71%
<i>Q. petraea</i>	4		20		10	10		1		2					47	5.89%
<i>Q. pubescens</i>	31	24			2	17						1			75	9.40%
<i>Q. pyrenaica</i>			115	3	3	48	2	6			16				193	24.19%
<i>Q. robur</i>			61		11	72		16	1						161	20.18%
Others			6			2		1							9	1.13%
Total	53	42	268	3	59	172	11	27	8	2	133	9	5	4	796	

^a The table also shows the total number of trees analysed for each species. The row "Others" contains individuals that could not be assigned to any of the species, usually because of their intermediate morphology.

Table 3

Genetic diversity and differentiation statistics for white oak populations from the Iberian Peninsula. Parameters have been estimated for each species, for pure and mixed populations and for all populations altogether

Populations	Number of populations	Harmonic mean	Number of haplotypes	hs ^a	ht ^b	Gst ^c
<i>Q. faginea</i>	58	4.229	11	0.031 ± 0.018	0.783 ± 0.037	0.960 ± 0.022
<i>Q. petraea</i>	9	4.632	5	0.110 ± 0.073	0.804 ± 0.056	0.866 ± 0.097
<i>Q. pubescens</i>	16	4.286	3	0.083 ± 0.057	0.679 ± 0.043	0.877 ± 0.086
<i>Q. pyrenaica</i>	37	3.957	5	0.030 ± 0.021	0.600 ± 0.064	0.950 ± 0.035
<i>Q. robur</i>	37	3.834	5	0.205 ± 0.048	0.666 ± 0.038	0.693 ± 0.073
Pure	121	4.286	12	0.078 ± 0.020	0.792 ± 0.020	0.902 ± 0.205
Mixed	49	4.587	11	0.092 ± 0.030	0.791 ± 0.030	0.884 ± 0.038
All populations	170	4.368	14	0.082 ± 0.017	0.804 ± 0.017	0.898 ± 0.021

^a Mean intrapopulation diversity.

^b Total diversity.

^c Coefficient of genetic differentiation among populations.

Petit, 1996), failed to show any significant correlation to the geographic distance matrix ($Z = 0.009$, $p = 0.272$). More detailed analysis of the geographical structure, described as a correlogram in Fig. 3a, shows that for unordered alleles the correlations are significantly negative up to the 350 km class and become significantly positive after the 400 km class.

Finally, we have tested the existence of a geographic structure within populations that belong to the B lineage. Table 4 shows significant correlations for both ordered and unordered alleles within this lineage ($Z = 0.111$, $p < 0.001$; $Z = 0.106$, $p = 0.001$, respectively); but absence of significant contribution of the divergence among haplotypes ($Z = 0.016$, $p = 0.14$). The correlogram in Fig. 3b shows significant negative correlations for the first two classes, followed by non-significant correlations up to the

250 km class. Then, correlations become again significantly negative for the 300 and 350 km classes. Afterwards, correlations are significantly positive between the 450 and 650 km classes.

4. Discussion

The results obtained in this work highly support the general pattern described by Dumolin-Lapègue et al. (1997) in their wide range survey of European white oaks cpDNA polymorphisms. However, the systematic sampling carried out within the frame of the 50 km grid, and the sampling of new Mediterranean regions have highlighted three major issues. First, white oaks from the Iberian Peninsula show very high cpDNA diversity (for comparison to other European regions, see Petit et al., 2002a), although there is an elevated risk of extinction for some haplotypes due to small population size. Second, the Mediterranean region was recolonised with oaks from a maternal lineage that has representatives only in Corsica, Morocco and Algeria (Petit et al., 2002a). Third, communication between refugia has obviously existed as demonstrated by the presence of haplotypes 1 and 7 in the river Ebro Valley. However, the time for this horizontal transfer is dubious and several lines of evidence seem to challenge the limits of the Iberian refugia deduced on the grounds of palynological data (Brewer et al., 2002).

Table 4

Mantel test statistics for the comparison between the coefficients of genetic differentiation and the geographical distance matrices

	Z-score	p
<i>All populations</i>		
Ordered alleles	0.248	<0.001
Unordered alleles	0.248	<0.001
Difference	0.009	0.272
<i>Lineage B</i>		
Ordered alleles	0.111	<0.001
Unordered alleles	0.106	0.002
Difference	0.016	0.14

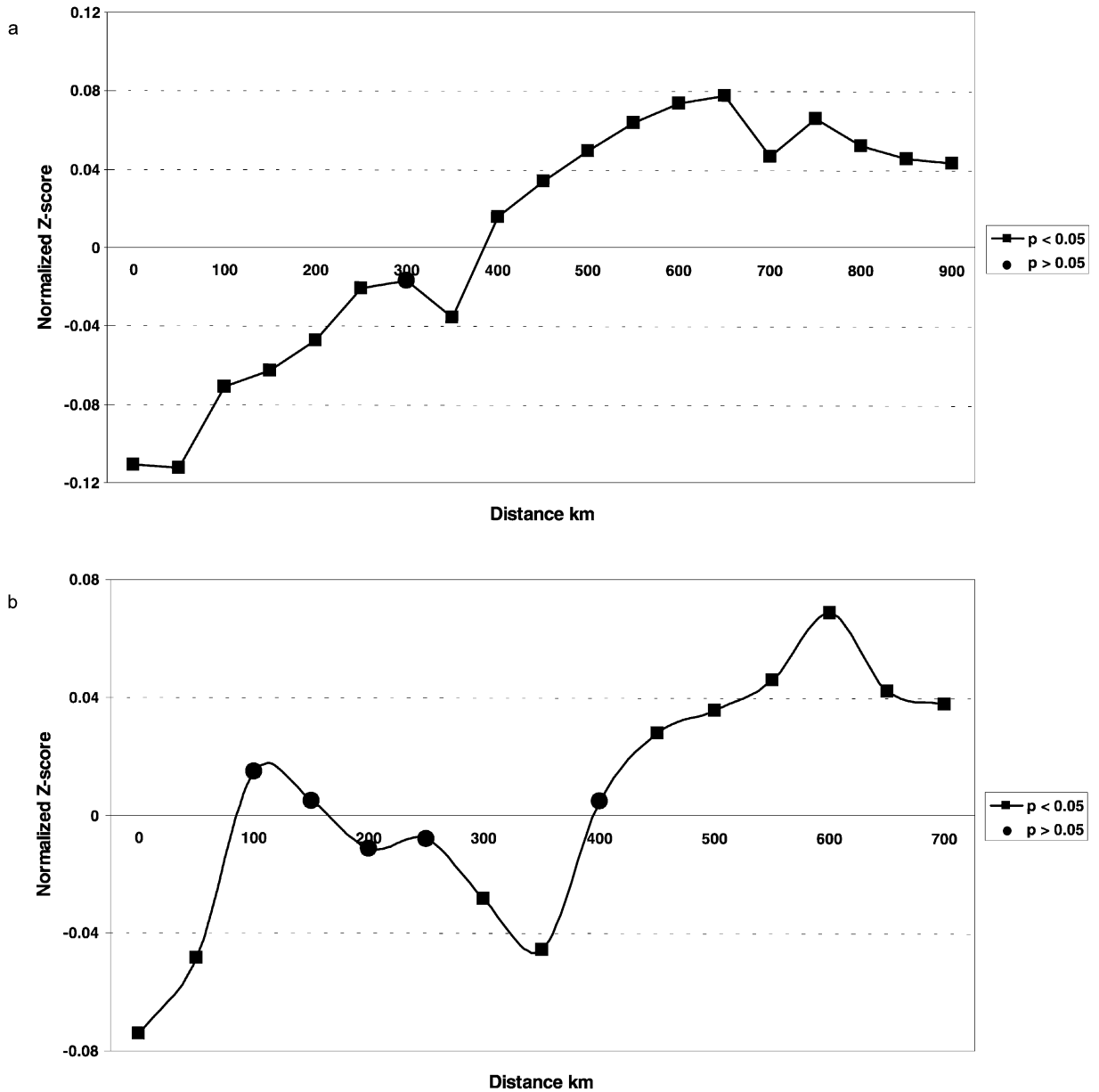


Fig. 3. Distance correlograms at 50 km spatial subdivisions when all haplotypes were considered (a) and when only lineage B haplotypes were taken into account (b). Negative Z-scores indicate that the populations within the desired distance class are more similar (as measured by F_{ST}) than populations in all other distance classes. Significant Z-values are indicated by boxes and non-significant Z-values are indicated by circles.

4.1. Genetic structure

The level of subdivision for cpDNA polymorphisms in this study (Table 3) is one of the highest ever described in plants, particularly oaks. Reasons that can

explain this strong genetic structure have been given by Dumolin-Lapègue et al. (1997) and will not be further discussed here.

In spite of the systematic sharing of haplotypes among species, species effects have been clearly

shown in the general analysis (Petit et al., 2002a) and are also obvious from Tables 2 and 3. Lineage D is very common in *Q. faginea*, rare in *Q. canariensis* and *Q. pyrenaica*, and completely absent in the pedunculate oak, *Q. robur*, and in the species originating from the east of Europe, *Q. petraea* and *Q. pubescens*. Lineages A and C, originated in the Balkans and Italy, respectively (Petit et al., 2002b), are absent from *Q. canariensis*, *Q. pyrenaica* and *Q. robur*. On the other hand, *Q. faginea* shows the greatest allelic richness with 11 out of 14 haplotypes, far from the seven haplotypes of *Q. pyrenaica*, the six haplotypes of *Q. petraea*, and the five haplotypes of *Q. robur*, *Q. pubescens* and *Q. canariensis* (Table 2). Altogether, these data confirm the species effect in intra- and inter-specific cytoplasmic gene flow observed by Dumolin-Lapègue et al. (1999), although effects due to the different ranges of the species are also probably involved (Petit et al., 2002a). The asymmetrical preferences in hybridisations among the different species, such as detected by Steinhoff (1993) in *Q. robur*–*Q. petraea*, and their different capabilities to colonise new and/or established areas, must also play an important role in both components of gene flow.

The differences in the way each species partitions diversity within and among populations could be explained, to some extent, by their biological features and by human impact. Indeed, *Q. faginea*, *Q. pyrenaica* and *Q. pubescens* are pioneer species that often forms compact and pure populations of small trees, with a great capability of response to environmentally unfavourable conditions. In addition, their ability to grow from shoots or rootings gives them great advantage against fires and grazing by large herbivores. Moreover, plantlets and leaves grazing is scarce because of abundant pilosity and/or hard sclerified leaves, which favours natural regeneration from acorns even under heavy management conditions. On the other hand, these three species have little economic importance, as compared to *Q. robur* and *Q. petraea*, which explains that plantations or human-mediated seed movements have been scarce.

In our study, there is no evidence of different levels of subdivision between mixed and pure oak populations. However, trends go into the same direction than in previous studies: larger intrapopulation diversity in mixed populations and larger level of subdivision in pure populations. Possibly, the lower levels of

intrapopulation diversity found in this study (Table 3), and the larger number of populations studied by Dumolin-Lapègue et al. (1999), could explain our ambiguous results.

4.2. Phylogeographic structure

As Fig. 2 reveals, haplotypes belonging to the same maternal lineages divide the Iberian territory into three well-defined regions. The phylogeographic structure was tested first for all populations and then was tested only for lineage B populations. In both instances, matrix correlations were significant for both ordered and unordered alleles, but failed to show significant correlations when the contribution of genetic divergence among haplotypes to the overall genetic differentiation was tested against the geographical distances. This absence of correlation in the general analysis is likely to be due to the mixture of two different lineages in Catalonia and the Pyrenees, while the small and similar interhaplotypic distances can be responsible for the lack of significance within the B lineage.

The patterns showed by autocorrelograms (Fig. 3) indicate that at least two sources are responsible for the cpDNA geographic structure. First, the spread of different maternal lineages into distinct regions has created large patches of similar populations (about 400 km) that can also be recognised in central Europe (Dumolin-Lapègue et al., 1997). Second, the haplotypes from the same maternal lineage are distributed in smaller patches (about 100 km) that have also been recognised in France and have been explained by rare long distance dispersal events during postglacial recolonisation (Petit et al., 1997). In our study, lineage sorting and drift during range expansion from several small size independent refugia seem to have played an important role in shaping these smaller patches.

4.3. Inferences about refugia and postglacial recolonisation

Since the early work by Huntley and Birks (1983), inferences based mainly in palynological and temperature grounds have restricted the glacial refugia area to the extreme south of the continent (Brewer et al., 2002). The inferred positions of refugia, in turn, have oriented inferences made about recolonisation

movements of the different oak cpDNA lineages (Dumolin-Lapègue et al., 1997; Petit et al., 2002b). However, several observations from different fields seem to challenge the extreme southern location of refugia areas and give support to the hypothesis that small refugia areas should exist in northern latitudes of the Iberian Peninsula.

Climatic reconstructions are, possibly, the major argument to support the absence of glacial refugia above any given latitude during the last ice age. However, it can hardly be expected that models at continental or planetary scale reflect conditions at southern localities of strong orography, which support temperatures and precipitations far from the means in surrounding areas. In the Iberian Peninsula, altitude and orography many times override the latitudinal climatic change, bringing moisture to southern microclimates and mild temperatures to northern ones. So, it is not difficult to imagine how small deviations from the current expectations could allow small refugia to persist in some of these areas with privileged microclimates. On the other hand, the position of the North Polar Front during the height of the last glaciation is somehow dubious, varying as much as 2° of latitude according to different sources (Ruddiman and McIntyre, 1981; CLIMAP Project Members, 1981). In addition, little is known about the location of the Gulf Stream during the last glacial period, which depressed to the south, could bring additional heat and moisture to the western coasts of the Iberian Peninsula.

Palynological evidence is the other major reason to support the proposed southern location of refugia in the Iberian Peninsula (Brewer et al., 2002; Petit et al., 2002b). However, several pollen data seem to deserve further explanation. The Padul pollen diagram shows a continuous curve for oak pollen except during the period 15–13,000 BP, coincident with the cold and dry Ancient Dryas (Pons and Reille, 1988). This should lead us to conclude that recolonisation from southern Iberia started around 13,000 BP. Such a delay to the start of recolonisation is very difficult to conciliate with the continuous presence of oak pollen found in northern Iberian localities as early as 13,700 BP (Menéndez Amor and Florschütz, 1963), and with the high oak pollen percentages found during the period 13–11,000 BP in places even northern (Montserrat Martí, 1992; Maldonado Ruiz, 1994; Peñalba, 1994; Allen et al., 1996). These elevated oak

pollen percentages (5–20%) point, instead, to the existence of nearby refugia where oaks persisted the full glacial period.

Three other palynological references deserve closer attention. A 30,000-year pollen record from Lake Banyoles, Catalonia (Pérez Obiol and Juliá, 1994), shows a tenuous but continuous presence of deciduous *Quercus* pollen along the complete ice age and Tardiglacial, till approximately 11,500 BP when the continuous curve starts. We suggest that this type of pollen record would be expected if very small oak populations did survive close to the lake area, and therefore, the data should be interpreted as indicating the presence of a full glacial refugia. Data from Quintanar de la Sierra marsh, in the Iberian Mountain Chain (Peñalba, 1989), and from five sites off and in the actual shoreline of Galicia (Ramil Rego and Gómez Orellana, 1996) point to the same direction, i.e., persistence of very little oak pollen during the whole glacial period.

Pollen records concerning other temperate tree species also support the existence of small glacial refugia in northern areas of the Iberian Peninsula. The Padul peatbog pollen record failed to reveal any presence of *Fagus* or *Corylus* in this part of the Iberian Peninsula during the complete sequence (Pons and Reille, 1988), and isochrone pollen curves from Huntley and Birks (1983) show that modern *Fagus sylvatica* extended from the Balkans into the rest of Europe during the Holocene, reaching the north Pyrenees at about 4,000 BP. However, Martínez Atienza and Morla Juaristi (1992) have demonstrated the continuous significant (2–10%) presence of *Fagus* pollen in the Cantabric Mountains and northeastern Catalonia since the Preboreal and Boreal, respectively. The fact that actual *Fagus sylvatica* populations in the Iberian Peninsula belong to a common cpDNA lineage (R.J. Petit, personal communication) can be explained in two ways that do not exclude the existence of refugia for *Fagus* in the Iberian Peninsula. First, it can be caused by the substitution of the original populations by the modern *Fagus sylvatica*. In fact, quaternary records of *Fagus* show this species as one member of a mixed temperate forest, instead of the highly successful species that actually forms monospecific forests (Blanco Castro et al., 1997). Second, the uncertainty about the evolution rate of the cpDNA sequences used to identify the haplotypes, which

could date the colonisation of Iberia back to a previous quaternary east–west movement.

Regarding the presence of *Corylus* in the pollen record of the Iberian Peninsula, Sánchez Goñi and Hannon (1999) have demonstrated that early colonisation at high altitude in the north of the Iberian Mountain Chain did not take place by the south. Comparison to nearby sequences suggests that Las Pardillas lake was colonised from populations that probably survived at northern latitudes, in deep gorges of the river Ebro basin. It could be argued that these populations correspond to the secondary refugia proposed by Brewer et al. (2002). However, the need to identify the primary refugia would still persist, as pollen record from the Mediterranean climatic region locations shows a very late expansion or its absence during the whole Holocene (Sánchez Goñi and Hannon, 1999).

In summary, all these pollen data lend support to the persistence of temperate trees in some northern areas of the Iberian Peninsula. There is still another class of evidence that points towards possible refugia areas in the Cantabric Coast and Catalonia: the environmental diversity and rich flora present today in these areas. The actual distribution area of *Q. canariensis* in the Iberian Peninsula, restricted to the southwest and northeast, cannot be easily explained without a refugia for this species in Catalonia. Other examples of relevant arcto-tertiary flora present in Catalonia and the Cantabric Coast include *Laurus nobilis*, *Prunus lusitanica*, *Viburnum tinus* and *Hypericum androsaemum* among the trees and shrubs; *Tamus communis* and *Smilax aspera* among the lianas; or *Woodwardia radicans*, *Culcita macrocarpa* and *Osmunda regalis* among the ferns (Blanco Castro et al., 1997). The presence of these taxa in actual *Quercus* forests from the Cantabric coast and Catalonia gives great support to the hypothesis that favours the existence of glacial refugia in these areas. Further support to the possibility of a gentle climate in these areas comes from reported human activity in many caves along the Cantabric coast since at least 15,000 BP. Interestingly, genome subdivision in the European grasshopper *Chortippus parallelus* has shown that populations in the Cantabric Mountains are differentiated from all other populations analysed in Spain (Cooper et al., 1995), which could also indicate the persistence of glacial refugia in this area.

The cpDNA polymorphisms data from the white oaks of the Iberian Peninsula are also easiest explained if refugia areas were not restricted to the south. First, there are nine out of 14 haplotypes absent below parallel 40° (Fig. 2). Indeed, southern extinctions might be possible (Petit et al., 2002b), but the extinction of so many Iberian haplotypes (65%) south of parallel 40° seems a very unlikely hypothesis. In fact, theoretical models and observations support extinction of pre-existing polymorphisms as the colonising front reaches further north (Hewitt, 1996). Second, some rare haplotypes are restricted to areas that could serve as refugia above parallel 40°, such as the deep gorges in the Duero Arribes (haplotypes 10b and 12b), the canyons of the river Tajo fountains (haplotype 33), or the southern Catalonia mountains (haplotype 28). At the recolonisation onset, these populations would have been unable to reach favourable routes to spread, probably due to the altitude changes involved, remaining frozen as footprints of past refugia. Third, the distribution of haplotypes 1, 7, 11 and 12 suggests that some northern refugia should also exist for these haplotypes. Finally, the colonisation of the Iberian Mountain chain with haplotypes from two different lineages, together with the *Corylus* data cited above (Sánchez Goñi and Hannon, 1999), seem to indicate bi-directional colonisation in these mountains. Lineage D haplotype 27 probably expanded to the northwest from the Maestrazgo region (Castellón), while haplotypes belonging to the B lineage probably expanded towards the southeast from the northern valleys at the river Ebro basin.

All these data lend support to the hypothesis that changes associated to altitude rather than to latitude have played the most important role in the recent history of vegetation in Iberia. According to it, vegetation would have also survived the glacial period in basal mountain valleys and/or deep gorges outside southern Iberia (Costa Tenorio et al., 1990; Montserrat Martí, 1992; Sánchez Goñi and Hannon, 1999).

The effects of repeated cycles of refugia survival and range expansion in the three southern European peninsulas during the quaternary must have a large contribution to the present pattern of subdivision in their natural forests. Geographic structuring of the different maternal lineages in the Iberian Peninsula into well-defined biogeographic regions suggests that this structure should be present before the last ice age.

Lineage D extends over the Mediterranean, while lineage B does it over the Atlantic. The river Ebro valley, which has been colonised several times since the tertiary with eastern flora, contains one rare lineage D haplotype and two haplotypes that belong to different Italian and Balkan maternal lineages. Obviously, this indicates an east–west horizontal transfer between the refugia. However, the time of this transfer is dubious, and different readings have been done. Hewitt (1999) has interpreted data from Dumolin-Lapègue et al. (1997) as indicating a Catalonian refugia, while Petit et al. (2002b) favour the hypothesis of an early arrival during the Tardiglacial of haplotypes 1 and 7 to the Iberian Peninsula.

According to our preferred hypothesis, these two haplotypes survived in Catalonia during the last glacial period, as also did haplotype 28. Data that support a Catalonian refugia have been given above, and our

reading of pollen maps shown by Brewer et al. (2002) finds no support to the very early east–west migration of haplotypes 1 and 7 during the Tardiglacial. Genetic data are also very uncertain, as the evolution rate of the cpDNA molecule is very slow (Soltis et al., 1992). So, the haplotype 7 *AS-HinfI* polymorphisms, present in the Balkans and absent from the Iberian populations, clearly indicate an east–west movement, but it could perhaps date back further than thought. The same time-scale considerations can be probably applied to the sharing of haplotypes between the south of Iberia and Morocco. Reasons to doubt a cpDNA exchange during the last glacial period are that the Mediterranean was never closed to the Atlantic and that mid-altitude mountains on both sides of Gibraltar made them appropriate places to harbour refugia. However, major rearrangements around this site occurred during the last tertiary (Hsü, 1986), coincident with oaks

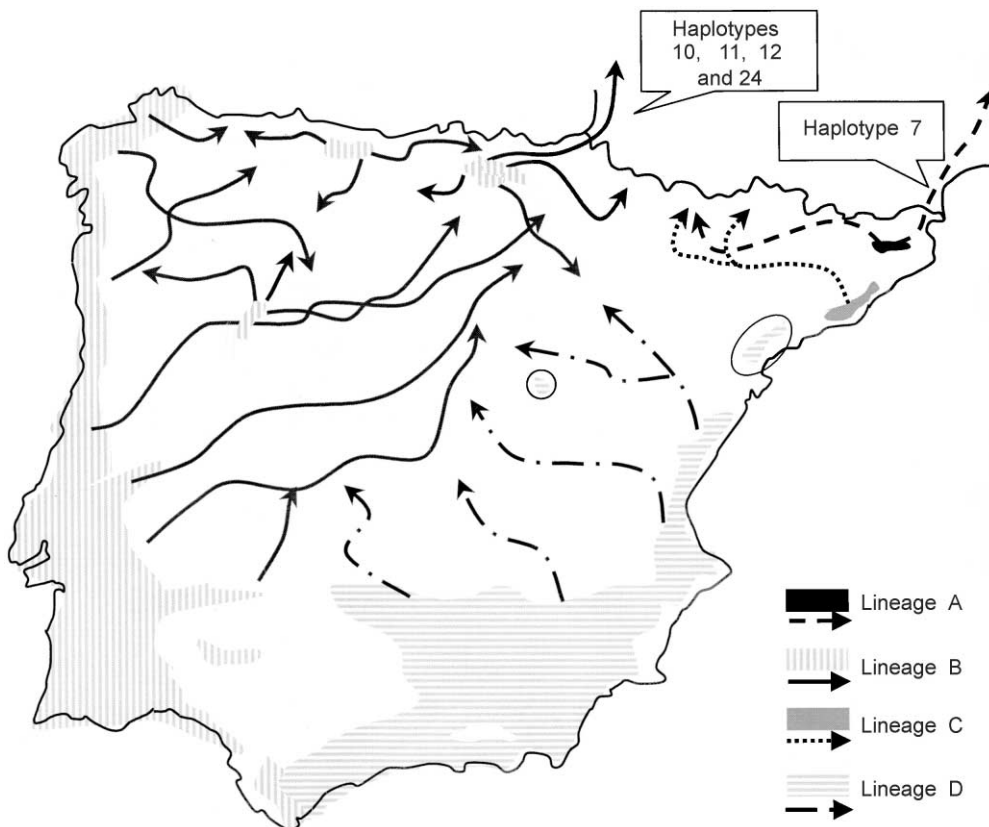


Fig. 4. Hypothesis about location of refugia and post-glacial colonisation routes in and out of Iberia followed by the white oaks after the last glacial period.

development, and other Mediterranean crisis have also occurred during the quaternary. It should not be totally unexpected that the three southern European peninsulas might still contain footprints from those early events that contributed to shape the European white oaks cpDNA phylogenetic structure. The associations between certain haplotypes and particular species that have been proposed by Petit et al. (2002b) are, probably, another blurred reminder of ancient times.

Fig. 4 summarises our preferred hypothesis about white oaks last recolonisation in the Iberian Peninsula. Possibly, the refugia extended along the Mediterranean and Atlantic coasts, with very small isolated populations surviving in several northern valleys of favourable microclimates. The geographic structuration of the different lineages should be already present, reflecting events from previous cycles of colonisation and retreat into refugia. Recolonisation of the Iberian Peninsula would have proceeded inwards following preferential routes that circumvented its pronounced orography, as river basins (i.e., river Douro) and mountain chains (i.e., Iberian Mountain Chain). Easiness to access these passages probably played a major role in the success of the different haplotypes during recolonisation, causing some of them to remain frozen at or near the refugia, while others spread out into available lands. Migration northwards of lineage D was impeded by the presence of lineages A and C in Catalonia. The Pyrenees mountains would have completely prevented oaks to migrate north, except by their sides. To the west, lineage B colonised the western half of France and extended into the Atlantic Europe. To the east, haplotype 7 proceeded in a parallel path colonising the eastern part of France, at least until the Alps, where it should merge with oaks coming north from Italy and Balkans refugia.

Note added in proofs. A new haplotype, characterised by a point mutation at band 4 of the AS-*Hinf*I pattern, has been discovered very near the river Ebro canyons at Bozoo (42°45'N, 3°7'W, approximately) by C. Collada and P. Jiménez, from the E.T.S.I. Montes, Madrid (personal communication).

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