



# Molecular and palaeoecological evidence for multiple glacial refugia for evergreen oaks on the Iberian Peninsula

U. López de Heredia<sup>1</sup>, J. S. Carrión<sup>2</sup>, P. Jiménez<sup>3</sup>, C. Collada<sup>4</sup> and L. Gil<sup>1\*</sup>

<sup>1</sup>Unidad de Anatomía, Fisiología y Genética Vegetal, ETSI Montes, Universidad Politécnica de Madrid, 28040 Madrid, Spain,

<sup>2</sup>Departamento de Biología Vegetal (Botánica), Facultad de Biología, Campus de Espinardo, Universidad de Murcia, 30100 Murcia, Spain,

<sup>3</sup>CIFOR-INIA, Ctra. de La Coruña km 7,5, 28040 Madrid, Spain, <sup>4</sup>Departamento de Biotecnología, ETSI Montes, Universidad Politécnica de Madrid, 28040 Madrid, Spain

## ABSTRACT

**Aim** A multiple glacial refugia hypothesis for Mediterranean plant species was tested with the evergreen *Quercus* complex (*Quercus suber* L., *Quercus ilex* L. and *Quercus coccifera* L.) from the Iberian Peninsula, using molecular and palaeobotanical data.

**Location** The Iberian Peninsula, which is an ecologically and physiographically complex area located on the western edge of the Mediterranean Basin.

**Methods** We sampled 1522 individuals from 164 populations of *Q. suber*, *Q. ilex* and *Q. coccifera*. A review of the recent literature on fossil pollen and charcoal records and a nested clade analysis on chloroplast DNA polymerase chain reaction-restriction fragment length polymorphism was carried out to infer demographic and historical processes.

**Results** The analysis indicates at least one glacial refugium for *Q. suber* in south-western Iberia. Extensive introgression of *Q. suber* with *Q. ilex* indicates several potential refugia in eastern Iberia. Past fragmentation was followed by a restricted range flow/range expansion, suggesting multiple refugia for *Q. ilex*–*Q. coccifera* elsewhere in central and northern Iberia and multiple areas of secondary contact. This finding is consistent with fossil records.

**Main conclusions** The predicted multiple refugia during glacial periods indicates the existence of secondary post-glaciation contact areas. These areas contained complex diversity patterns resulting mainly from range expansions followed by isolation by distance. To a lesser degree, traces of restricted and long-distance dispersal were also found.

## Keywords

cpDNA PCR-RFLPs, evergreen *Quercus*, fossil pollen, glacial refugia, Iberian Peninsula, introgression, nested clade analysis, oaks, phylogeography.

\*Correspondence: Luis Gil, Unidad de Anatomía, Fisiología y Genética Vegetal, ETSI Montes, Universidad Politécnica de Madrid, Avda. Ramiro de Maeztu 7, 28040 Madrid, Spain.  
E-mail: luis.gil@upm.es

## INTRODUCTION

Climate change is expected to be more pronounced in the Mediterranean Basin than in most other regions of the world (IPCC, 2001). Knowledge of how past climate fluctuations influenced the patterns and dynamics of Mediterranean plant taxa is of the utmost importance when developing sound conservation and climate change policies. Shifts in the ranges of the species in the Mediterranean peninsulas, where tree species remained during cold and arid glacial times, are of particular interest.

The Iberian Peninsula is a relatively isolated, physiographically complex area in the western part of the Mediterranean Basin. The Iberian mountain ranges run east–west rather than north–south, thus they are effective barriers to gene flow and recolonization along a north–south axis. There are strong climatic gradients between the Atlantic, Mediterranean and continental territories, and between the northern shaded and southern sunny exposures. In addition, the Iberian Peninsula can be subdivided into regions by two main soil types: acidic (western Iberia and some isolated areas in eastern Iberia) and calcareous (eastern Iberia). The present-day vegetation consists

of a fragmented mosaic of tree species, among which the evergreen oaks [genus *Quercus*, groups *Ilex* and *Cerris*, *sensu* Nixon (1993)] have been one of the main components since the early Pliocene (Suc, 1984; Fauquette *et al.*, 1999; Wrenn *et al.*, 1999).

*Quercus ilex* L. (holm oak), *Quercus coccifera* L. (kermes oak) and *Quercus suber* L. (cork oak) are three of the most widespread broadleaved woody species in the western Mediterranean Basin (Borelli & Varela, 2001). The three evergreen oak species form large continuous forests, sometimes merging together. However, the strict acidophilous character makes *Q. suber* grow predominantly on the siliceous side of Iberia and rarely on the small, isolated acid outcrops of the calcareous western Iberia. The greater ecological plasticity of *Q. ilex*, and to a lesser extent *Q. coccifera*, allow these species to inhabit almost the entire Iberian Peninsula.

It is widely accepted that the Pleistocene climatic changes are among the crucial determinants of the current distribution of plant species in temperate latitudes (Bennett, 1997). The spatial patterns of several tree species throughout the European continent are the long-term result of late glacial and post-glacial migration from refugial populations that were able to withstand the harsh climatic conditions of Pleistocene stadials (Bennett *et al.*, 1991; Comes & Kadereit, 1998; Hewitt, 1999). With few exceptions (Willis *et al.*, 2000; Stewart & Lister, 2001; García-Antón *et al.*, 2002; Palmé *et al.*, 2003; Petit *et al.*, 2005; Tzedakis, 2005), the locations postulated for glacial refugia of European woody angiosperms have been south of the parallel 40° N, which runs from central Portugal to Sardinia, Calabria and northern Greece. This was also the boundary between polar aridity and warmer climates during an important part of the Quaternary. In fact, traditional palaeogeographical models, inferred from scarce palynological evidence, posit a small number of refugia on the southern edges of the Iberian Peninsula (Huntley & Birks, 1983).

However, more complete palaeobotanical data sets (Willis *et al.*, 2000; Carrión *et al.*, 2003; Willis & van Andel, 2004), in combination with genetic studies, are questioning the former paradigm. Since the publication of the atlas by Huntley & Birks (1983), information from more than 50 new Iberian fossil sites has been published and the main Iberian tree species have been analysed using molecular markers. A number of chloroplast DNA (cpDNA) studies have postulated common patterns of post-glacial colonization for temperate European tree species, involving high diversity levels in southern Europe and decreasing diversity northwards (Hewitt, 1996; Petit *et al.*, 1997; Taberlet *et al.*, 1998). In contrast to the 'few southern refugia' hypothesis, under the 'multiple refugia' hypothesis, north and central Europe would have been recolonized from populations located in the north of the Iberian Peninsula. Moreover, these populations would have been barriers preventing expansions from southern refugia. If this was the case the cpDNA should show complex patterns of spatial distribution that would have resulted from the generation of multiple secondary contact zones (Petit *et al.*, 2005). In addition, evergreen oak species are less competitive with temperate

species at northern latitudes, so would have been confined to Mediterranean peninsulas or coastal areas.

Chloroplast DNA polymerase chain reaction (PCR)-restriction fragment length polymorphisms (RFLPs) analysed by Jiménez *et al.* (2004) show that the evergreen oaks have a complex pattern of variation in the western Mediterranean. Three cpDNA lineages (*suber*, *i-c I* and *i-c II*) are shared by all three species and show different haplotypic diversity levels. The central *i-c I* lineage occurs in all three species, while lineage *suber* is only present in *Q. suber* samples and lineage *i-c II* occurs only in *Q. ilex* and *Q. coccifera*. However, this analysis did not investigate the spatial patterns of genetic variation, nor did it address the genetic variation with current palaeobotanical information.

Nested clade analysis (NCA) is a widely employed phylogeographical method to separate population structure from population history (Templeton *et al.*, 1987; Templeton & Sing, 1993). It was developed to reconstruct the evolutionary history of populations by combining a cladogram of the genetic relationships among haplotypes within these populations and their geographical distribution (Templeton *et al.*, 1987). The method can discriminate between phylogeographical associations due to recurrent but restricted gene flow vs. historical events operating at the population level, such as past fragmentation, colonization or range expansion (Templeton, 1998).

The ecological and physiographical heterogeneity of the Iberian Peninsula favours the hypothesis of multiple refugia for the evergreen oaks in the last glacial period or even in prior stadials. By combining a review of the published palaeobotanical references from the last two decades with the NCA analysis of cpDNA, our aim is to provide additional support for the hypothesis of multiple glacial refugia in the Iberian Peninsula.

## MATERIALS AND METHODS

### Plant sampling and laboratory procedures

A total of 1522 individuals from 164 populations of *Q. ilex* (674 trees, 73 populations), *Q. suber* (587 trees, 60 populations) and *Q. coccifera* (261 trees, 31 populations) were sampled from the Iberian Peninsula. For each stand, adult leaves were collected from an average of nine non-adjacent trees per species. DNA was extracted using a modified protocol of Doyle & Doyle (1990), following Dumolin *et al.* (1995). PCR-RFLPs were employed using five cpDNA universal primer pairs that are widely employed for the analysis of plant taxa in phylogeography: CD, TF, DT, AS and SR (Taberlet *et al.*, 1991; Demesure *et al.*, 1995; Dumolin-Lapègue *et al.*, 1997a; Grivet *et al.*, 2001). Each amplified fragment was digested with one restriction enzyme to avoid the risk of counting the same mutation twice (Dumolin-Lapègue *et al.*, 1997b): DT-*TaqI*, CD-*TaqI*, SR-*Hinfl*, AS-*Hinfl* and TF-*Hinfl*. The procedure for assigning chlorotypes, grouping of lineages and identifying relationships between chlorotypes is described by Jiménez *et al.* (2004).

## cpDNA analysis

In the first step, a matrix of mutational differences between chlorotypes was calculated in order to yield minimum spanning networks using the R Ape 1.0 package (Paradis *et al.*, 2003). The minimum spanning networks for each species result from subtracting the chlorotypes they lack from the overall network. We performed the NCA in the network combining the chlorotypes from each species separately, as well as all the chlorotypes, because interspecific exchanges and sharing of chlorotypes is frequent between the evergreen oaks (Belahbib *et al.*, 2001). The network for a single species is obtained from the overall network by substituting the chlorotypes absent in that species by nodes that would represent unsampled or extinct chlorotypes. Chlorotype distribution maps were constructed.

The chlorotype networks were converted into a series of nested clades, using the rules defined in Templeton *et al.* (1987) and Templeton & Sing (1993). By using geographical distances among populations, two main types of distances were calculated using GeoDis 2.4 (Posada *et al.*, 2000): (1) the 'clade distance' (*D<sub>c</sub>*), which measures the geographical spread of chlorotypes within a given clade, and (2) the 'nested clade distance' (*D<sub>n</sub>*), which indicates how far apart chlorotypes within a clade are from the chlorotypes of the evolutionarily closest sister clades. An 'interior-tip' statistic (I-T; calculated separately for *D<sub>c</sub>* and *D<sub>n</sub>*) was also estimated within each nested category as the average interior distance minus the average tip distance. The significance of these statistics was estimated through a Monte Carlo procedure with 1000 random permutations (Templeton *et al.*, 1995). The interpretation of the observed distance patterns was undertaken using the most recently revised version of the inference key published by Templeton *et al.* (1995) and available at <http://darwin.uvigo.es/software/geodis.html>.

## Review of pollen and macrofossil information

A data set of Iberian *Quercus ilex-coccifera* and *Q. suber* pollen and macrofossils from three cold periods of the Pleistocene was compiled and mapped. The search of references was conducted on the following periods: pre-Würmian stadials (before *c.* 125,000 yr BP), Würmian glacial (*c.* 80,000–13,000 yr BP) and Lateglacial (*c.* 13,000–10,000 yr BP). Neither the Eemian (*c.* 125,000–80,000 yr BP) nor the Holocene (10,000–present day) interglacial periods were considered in this study. Only the most significant references containing records of evergreen oaks were considered. Pollen and macrofossil data came from studies of peatbogs, lakes, marshes, marine cores, archaeological sites (e.g. coprolites), palaeontological and palaeoanthropological sites. Pollen records can be used as supporting evidence for molecular data but they must be interpreted carefully as there are unresolved taphonomical issues for each deposit type. For example, one of the main disadvantages of pollen sequences from marine cores is that they receive pollen from river

catchment areas close to the core site, and therefore the importance of these components of pollen assemblage may be overestimated. On the other hand, one advantage of marine core sequences is that they cover a long, continuous period of time, whereas records from terrestrial sites often do not extend back into the Pleistocene. For cave sites, the presence/absence of *Quercus*, even at low percentages, may be indicative of local presence (Navarro *et al.*, 2001). For *Q. suber*, a major challenge is simply its palynological discrimination: only a few authors have considered it as a palynotype. Plausibly, *Q. suber* may have been included within both evergreen and deciduous oak pollen curves. To investigate this we have mapped the published palynological data for *Q. suber*, including charcoal records.

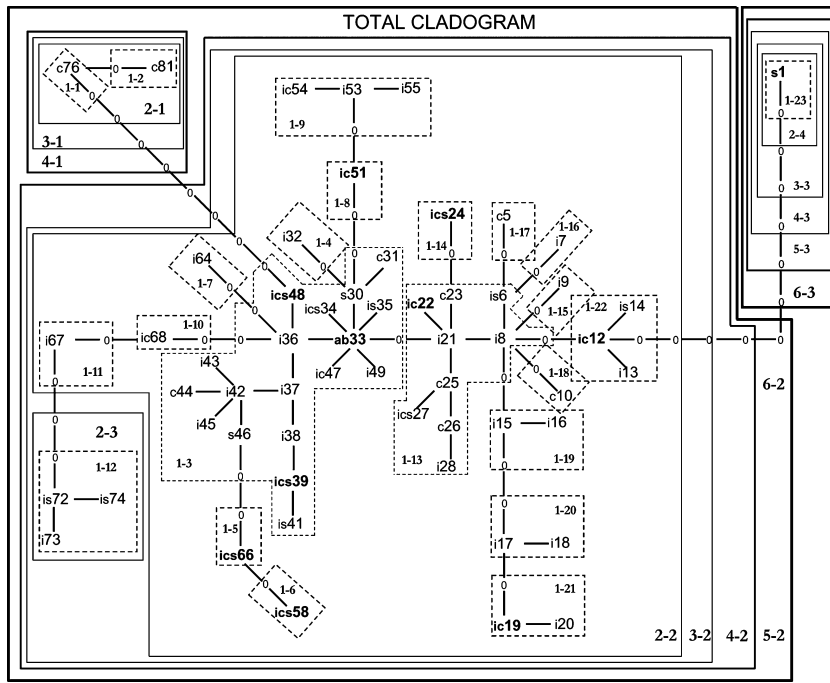
## RESULTS

### cpDNA analysis

Fifty-seven chlorotypes, belonging to three lineages, were found in the Iberian Peninsula, 17 in *Q. suber*, 23 in *Q. coccifera* and 45 in *Q. ilex*. The most diverse and widespread lineage was *i-c I*, with 54 chlorotypes, some of which were shared by all three species. In contrast, lineage *i-c II* showed only two chlorotypes in *Q. coccifera*, and lineage *suber* a single chlorotype in *Q. suber*. Fig. 1 shows the minimum spanning network between all the chlorotypes. The most frequent chlorotypes are located in the network in both interior (e.g. c12, c19, c33, c39, c48, c51, c66) and tip positions (e.g. c1, c22, c24, c58).

From the chlorotype distribution maps the extensive presence of c1 in *Q. suber* from western Iberia is clearly shown (Fig. 2a), while the remaining *Q. suber* populations show chlorotypes of *i-c I* lineage. Of the other chlorotypes, some clearly show defined geographical distributions, e.g. c22 and c24 in the Central System (Fig. 2b,c), and c25, c26, c27 and c28 on the coast of Portugal (Fig. 2b,c). Although the geographical association is strong, the chlorotype maps show occasional overlapping among chlorotype ranges [e.g. c39 and c58 in eastern Spain (Fig. 2b)]. However, other chlorotypes show a weaker geographical association and can appear in populations distant from each other: e.g. c12 (Fig. 2b) is in northern and south-eastern Spain, c66 occurs in Catalonia (north-eastern Spain), southern Spain and Galicia (north-western Spain) (Fig. 2a, c). Finally, some chlorotypes were very common in well-defined areas but also appear at very low frequencies in distant populations. For example, c19 is widespread in the Basque Country and Rioja mountains (northern Spain), but is also found in one population from Extremadura (western Spain), more than 400 km away from its main area of distribution (Fig. 2b).

The overall network was manually nested into six hierarchical levels (Fig. 1). We found 23 one-level clades (chlorotypes separated by one mutation) grouping into four two-level clades. All the chlorotypes from lineage *i-c I* are grouped at a three-level clade with two sub-clades (2-2 and 2-3). Lineages *i-c I* and *i-c II* are phylogenetically distant, therefore they group into a five-level clade still separated from lineage *suber* by one



**Figure 1** Manually nested minimum spanning network of chlorotypes. Hierarchical level clades are indicated from one-level clades to total cladogram. The most frequent chlorotypes are in bold. — = length or point mutation; 0 = unsampled or missing chlorotype; *n* = number of sampled chlorotype; *i*(*n*) = chlorotype present in *Q. ilex*; *c*(*n*) = chlorotype present in *Q. coccifera*; *s*(*n*) = chlorotype present in *Q. suber*.

level. Most one-level clades are composed of a single chlorotype (e.g. 1-1, 1-2, 1-4, 1-5, 1-6, 1-7, 1-8, 1-10, 1-11, 1-14, 1-15, 1-16, 1-17, 1-18 and 1-23). Clades 1-1, 1-2 and 1-20 showed no geographical variation.

Inferences from NCA are coincident when considering each species separately (not shown) and the overall network (Table 1; Fig. 1; a detailed list of *Dc*, *Dn* and I-T values including *P* levels can be obtained from the authors upon request). Lower-level clades showed four main types of historical–demographic processes acting on populations (Table 1): (1) restricted gene flow with isolation by distance in clades 1-9, 1-12 and 1-21, (2) restricted gene flow/dispersal but with some long-distance dispersal in clade 1-22, (3) contiguous range expansion for clade 1-19, and (4) long-distance colonization possibly coupled with subsequent fragmentation or past fragmentation followed by range expansion for clades 1-3 and 1-13. Long-distance colonization and past fragmentation followed by range expansion were the factors identified in the higher-level clades (2-2, 3-2 and 5-1). Clade 2-1 (lineage *i-c II*) showed too few chlorotypes to enable discrimination between range expansion/colonization and restricted dispersal/gene flow.

### Review of pollen and macrofossil information

We found evidence for the occurrence of evergreen oaks on the Iberian Peninsula from both pollen (*ilex-coccifera* palynotype) and charcoal from 48 fossil sites and from several pollen records, including marine cores from the Atlantic and Mediterranean (Table 2 & Fig. 3). Available information for pre-Würmian times (>125,000 yr BP) is scarce but there is evidence for evergreen oaks in full Riss glacial periods in the Sistema Central mountains (Fig. 3a). For the last glacial period (80,000–13,000 yr BP) there is clear evidence of evergreen oaks

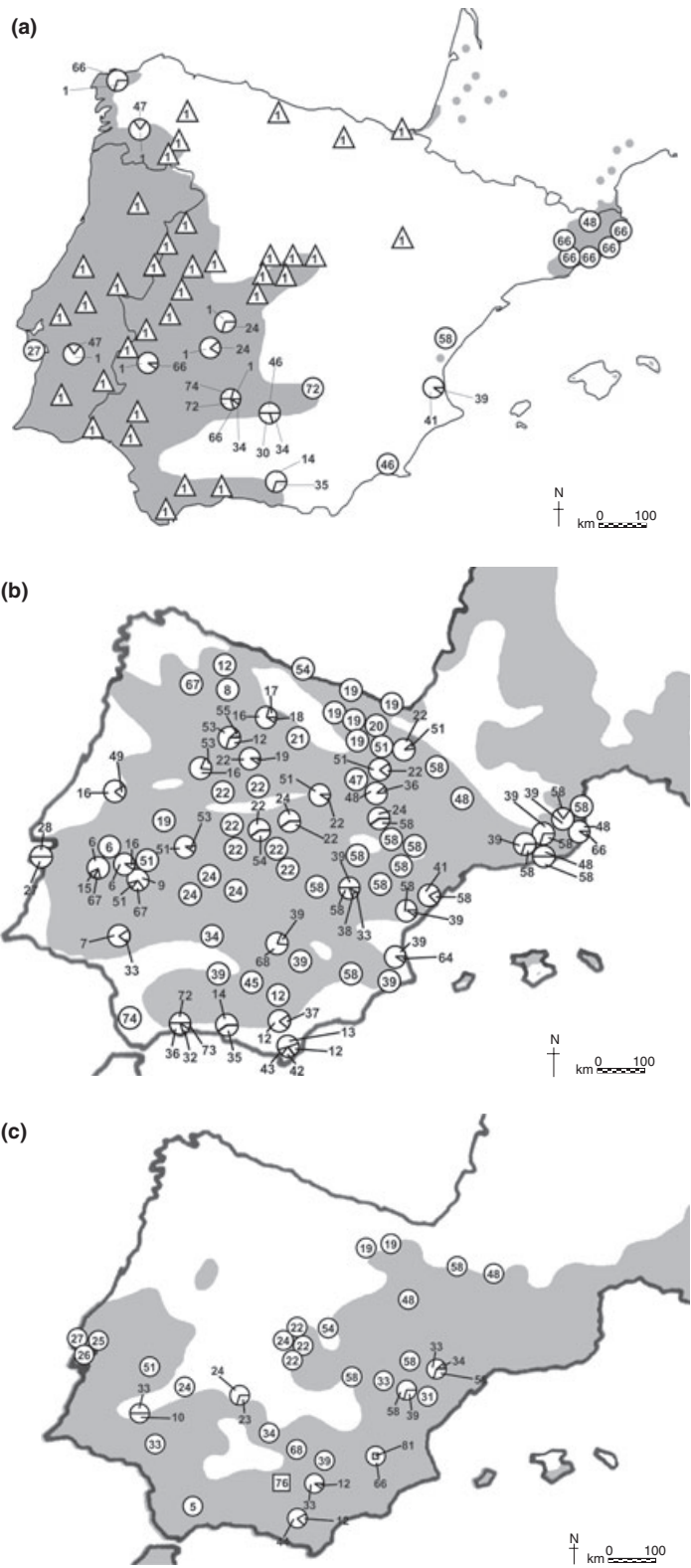
throughout the Iberian Peninsula (Fig. 3b), particularly in south-eastern Spain, Catalonia and northern areas of the peninsula and Portugal. Evergreen oaks were less abundant in central, more continental positions. Based on a number of studied pollen cores from northern Spain and Portugal (Fig. 3c) it appears that similar patterns obtained during the Lateglacial (13,000–10,000 yr BP). Plausibly, *Q. suber* may have been included within both evergreen and deciduous oak pollen curves. We have only mapped the published palynological records in which *Q. suber* was unambiguously identified and also the charcoal records from near Lisbon (Fig. 3d).

### DISCUSSION

Molecular and palaeobotanical data for white oaks indicated post-glacial recolonization of the Iberian Peninsula from two primary refugia (Brewer *et al.*, 2002) located in the south-east and south-west of the peninsula. Following initial recolonization in the Lateglacial, a second advance occurred after the start of the Holocene from refugia in Catalonia and northern Portugal. This is the accepted model, although the interpretation is based on a few palaeobotanical records from the northern latitudes and on the absence of variation in cpDNA (Lumaret *et al.*, 2002, 2005). In contrast to the hypothesis of few refugia, the results presented here support the presence of multiple refugia for the evergreen oaks on the Iberian Peninsula during, at least, the last glacial period.

### Multiple refugia for *Quercus ilex* and *Quercus coccifera*

*Quercus ilex* and *Q. coccifera* showed higher chlorotypic diversity than other closely related tree species analysed with



**Figure 2** Distribution of the chlorotypes in *Q. suber* (a), *Q. ilex* (b) and *Q. coccifera* (c). The grey shaded surface represents the distribution range of the species. *Quercus ilex* and *Q. coccifera* distribution ranges modified from Costa Tenorio *et al.* (1997). *Quercus suber* distribution range modified from the Second EUFORGEN Mediterranean Oaks Network Meeting (<http://www.montes.upm.es/Dptos/DptoSilvopascicultura/Anatomia/images/galerias/Qsubermap.jpg>):  $\Delta$ , lineage *suber*;  $\circ$ , lineage *i-c I*;  $\square$ , lineage *i-c II*.

the same set of markers (e.g. white oaks; Olalde *et al.*, 2002). Indeed, we found chlorotypes within the same lineage that were separated by 10 mutations. Usually PCR-RFLPs from other tree species show a small number of closely related

chlorotypes within lineages. Compared with cpDNA analysis by classic RFLPs (Lumaret *et al.*, 2002) we have found higher levels of polymorphism by employing the PCR-RFLP technique, namely 10 vs. 45 *Q. ilex* chlorotypes in areas common to

**Table 1** Nested contingency analysis of geographic associations and their interpretations according to the revised inference key of Templeton *et al.* (1995) for 164 populations of evergreen oaks in the Iberian Peninsula.

Clade	Chi-square	<i>P</i>	Chain of inference	Inferred demographic event
Clade 1-3	3,139,913	0.000	1-2-3-5-6-13-YES	Long distance colonization possibly coupled with subsequent fragmentation or past fragmentation followed by range expansion
Clade 1-9	34,236	0.027	1-2-3-4-NO	Restricted gene flow with isolation by distance
Clade 1-12	29,789	0.000	1-2-3-4-NO	Restricted gene flow with isolation by distance
Clade 1-13	998,593	0.000	1-2-3-11-12-13-YES	Long distance colonization possibly coupled with subsequent fragmentation or past fragmentation followed by range expansion
Clade 1-19	1000	0.080	1-2-11-12-NO	Contiguous range expansion
Clade 1-21	73,000	0.000	1-2-3-4-NO	Restricted gene flow with isolation by distance
Clade 1-22	80,667	0.000	1-2-3-5-6-7-YES	Restricted gene flow/dispersal but with some long distance dispersal
Clade 2-1	1000	0.000	1-19-20-2-3-5-6-TOO FEW CLADES	Insufficient genetic resolution to discriminate between range expansion/colonization and restricted dispersal/gene flow
Clade 2-2	12,652,619	0.000	1-2-3-5-6-13-YES	Long distance colonization possibly coupled with subsequent fragmentation or past fragmentation followed by range expansion
Clade 3-2	877,442	0.000	1-2-11-12-13-14-NO	Long distance colonization and/or past fragmentation
Clade 5-1	521,357	0.000	1-2-11-12-13-14-NO	Long distance colonization and/or past fragmentation
Total cladogram	902,366	0.000	1-2-Inconclusive outcome	Inconclusive outcome

both studies. The interpretation of such amounts of cpDNA variation is thus not straightforward.

Under a model of coalescence and isolation by distance, the frequent chlorotypes should be the most ancient, occupying central positions in the network. Conversely, infrequent chlorotypes would derive from the frequent ones and occupy the tips of the network (Watterson & Guess, 1977). However, *Q. ilex* and *Q. coccifera* have some tip chlorotypes found at high frequency in the periphery of the network and some interior chlorotypes with lower frequency in central positions of the network. Therefore, a model of fast Holocene recolonization and progressive differentiation through isolation by distance is not likely if it is assumed that it started from few refugia in the southern edge of Iberia. Even if populations increased their cpDNA mutation rates during range expansions, the time since the deglaciation (c. 13,000 BP) is far too short to acquire a degree of divergence comparable to that observed by the Iberian evergreen oaks (Petit *et al.*, 2005).

As reflected by the NCA, the spatial structure of the cpDNA of the evergreen oaks is the product of complex phenomena of past fragmentation and/or long-distance colonization, followed by restricted gene flow/dispersal or range expansion throughout the areas contiguous to the glacial refugia. Fossil information identifies several sites along the Iberian Peninsula containing *Q. ilex*–*Q. coccifera* in full glacial times, not only on the southern side but also in the northern and central mountain ranges. This is compatible with the hypothesis of past fragmentation during the glacial periods that produced the differentiation of the chlorotypes from lineage *i-c I*. Past fragmentation explains the spatial structure of the cpDNA. It identifies potential refugia in some of these areas. For example, refugia appear to have existed in the Basque–Rioja mountains in northern Spain (clade 1-21), the Sistema Central mountains (chlorotypes c22 and c24, clade 1-13), eastern Spain (clade

1-9) or multiple locations from the south of Iberia (clade 2-1; many sub-clades of clade 2-2; clade 2-3).

Past fragmentation of the populations in southern Spain was enabled by the complex orography of the Betic Cordilleras. In this area there is high population genetic differentiation. *Quercus coccifera* also shows traces of lineage *i-c II*, which is distributed in areas eastwards as far as Provence (south-western France) and the Balearics (Lopez de Heredia *et al.*, 2005). Together with the abundance of pollen records of evergreen oaks this suggests that several small isolated refugia retaining pre-Würmian diversity could have existed. The mid-elevation mountain valleys of these ranges may have provided optimal conditions allowing for altitudinal displacements of *Q. ilex*–*Q. coccifera* vegetation belts, while inter-valley isolation prevented latitudinal migration. The altitudinal range shift represents a factor of paramount importance for the maintenance of tree species against climatic or anthropogenic pressures (Gates, 1993; Peñuelas & Boada, 2003).

In a scenario of multiple refugia, contiguous range expansions (clade 1-19), restricted gene flow followed by isolation by distance (clades 1-9, 1-12, 1-21 and 1-22) and long-distance dispersal (clade 1-22) would produce an overlapping of chlorotypes and the generation of multiple secondary contact zones in the colonization of empty spaces. This colonization would have been conditioned by several factors related to the complexity of the Iberian Peninsula. Indeed, mountain systems are the most effective barriers to chlorotype recolonization in plant species with heavy seeds (Cruzan & Templeton, 2000). This is due to the abruptly changing ecological conditions between northern and southern exposures. Phenomena such as disturbance by fire, slope rejuvenation, abrupt climatic changes, herbivory, pathogens and the arrival of new competitors would also have played their role in the historical distribution of evergreen oaks, so establishing the current genetic structure of Iberian populations.

**Table 2** Pollen and charcoal deposits. Code (as in Fig. 1), location and type of deposits; species identified in the deposit, suggested chronology or period and bibliographic reference.

Code	Site name	Lat.	Long.	Site type (evidence source)	Species*	Suggested chronology (yr BP)/Period	Reference
1	Atapuerca	42°20' N	03°42' W	Archaeological, palaeontological (pollen)	QI, QC	700,000–120,000	García-Antón & Sáinz-Ollero (1991)
2	Villacastín	40°47' N	04°22' W	Palaeontological cave (pollen)	QI, QC	150,000–120,000	Carrión <i>et al.</i> (2007)
3	Sima de las Palomas	37°47' N	00°53' W	Archaeological cave (pollen)	QI, QC	150,000–40,000	Carrión <i>et al.</i> (2003)
4	Bajondillo	36°38' N	04°30' W	Archaeological cave (pollen)	QI, QC	140,000–4500	López-Sáez <i>et al.</i> (2007, in revision)
5	Perneras	37°31' N	01°25' W	Archaeological rockshelter (pollen)	QI, QC	70,000–10,000	Carrión <i>et al.</i> (1995)
6	Villena	38°39' N	00°52' W	Peatbog (pollen)	QI, QC	47,000–6000	Yll <i>et al.</i> (2003a,b)
7	Beneito	38°48' N	00°28' W	Archaeological cave (pollen)	QI, QC	38,800–16,500	Carrión & Munuera (1997)
8	Abric-Romaní	41°32' N	01°41' E	Archaeological rockshelter (pollen)	QI, QC	70,000–40,000	Burjachs & Julià (1994)
9	Arbreda	42°09' N	02°44' E	Archaeological cave (pollen and charcoal)	QI, QC	63,000–45,000	Burjachs (1993, 1994)
10	Gabasa	42°00' N	04°06' E	Archaeological cave (pollen in coprolites)	QI, QC	63,000–45,000	González-Sampérez <i>et al.</i> (2003, 2004), González-Sampérez (2004)
11	Los Torrejones	41°00' N	03°05' W	Palaeontological cave (pollen in coprolites)	QI, QC	80,000–60,000	Carrión <i>et al.</i> (2007)
12	Menorca	39°53' N	04°16' E	Marine core (pollen)	QI, QC	70,000–Present	Pérez-Obiol <i>et al.</i> (2000)
13	Malladetes	39°01' N	00°17' W	Archaeological cave (pollen)	QI, QC	29,600–16,300	Dupré (1988)
14	Navarrés	39°06' N	00°41' W	Peatbog (pollen and macroremains)	QI, QC, QS	31,000–27,000	Carrión & van Geel (1999)
15	Siles	38°24' N	02°30' W	Lake (pollen)	QI, QC	17,000–430	Carrión (2002)
16	Padul	37°00' N	03°40' W	Peatbog (pollen)	QI, QC, QS	Last interglacial–4500	Pons & Reille (1988)
17	San Rafael	36°46' N	02°36' W	Lagoon, saltmarsh (pollen)	QI, QC	16,800–Present	Pantaleón-Cano <i>et al.</i> (2003)
18	Gorham	36°07' N	05°20' W	Archaeological cave (pollen)	QI, QC	32,000–28,000	Fuentes <i>et al.</i> (2007), in press
19	Alta Garrotxa (Cueva 120)	42°17' N	02°34' E	Archaeological cave (charcoal)	QI, QC	Lower pleniglacial	Agustí <i>et al.</i> (1987)
20	Banyoles	42°07' N	02°46' E	Lake (pollen)	QI, QC	30,000–4500	Pérez-Obiol & Julià (1994)
21	Quintanar de la Sierra	42°02' N	03°01' W	Peatbog (pollen)	QI, QC	21,000–Present	Peñalba (1994)
22	8057B	37°41' N	10°05' W	Marine core (pollen)	QI, QC, QS	19,100–Present	Hooghiemstra <i>et al.</i> (1992)
23	Marine Core	36°00' N	07°40' W	Marine core (pollen)	QI, QC	19,000–Present	Parra (1994)
24	Marine Core	37°58' N	00°05' E	Marine core (pollen)	QI, QC, QS	17,000–Present	Parra (1994)
25	Les Cendres	38°43' N	00°02' E	Archaeological cave (charcoal)	QI, QC	24,000–13,000	Badal & Carrión (2001)
26	Hornillo	42°00' N	02°52' W	Lake (pollen)	QI, QC	>13,000	Gómez-Lobo (1993)
27	Las Ventanas	37°26' N	03°25' W	Palaeontological cave (pollen in coprolites)	QI, QC, QS	10,500–Present	Carrión <i>et al.</i> (2001)
28	La Pila	43°00' N	04°00' W	Archaeological site (charcoal)	QI, QC	12,160–11,710	Uzquiano (1992)
29	Los Azules	43°21' N	05°08' W	Archaeological cave (charcoal)	QI, QC	10,910–9430	Uzquiano (1992)
30	Lleguna and Sanguijuelas	41°34' N	06°54' W	Lakes (pollen)	QI, QC	>13,000	Ramil-Rego <i>et al.</i> (1998)
31	Lagoa Marinho	41°45' N	07°48' W	Lake (pollen)	QI, QC	>13,000	Ramil-Rego <i>et al.</i> (1998)

**Table 2** continued

Code	Site name	Lat.	Long.	Site type (evidence source)	Species*	Suggested chronology (yr BP)/Period	Reference
32	Salada Mediana	41°30' N	00°44' W	Salt marsh (pollen)	QI, QC	12,500–10,300	González-Sampéris (2004)
33	Santa Maira	38°45' N	00°08' W	Archaeological cave (charcoal)	QI, QC	11,600–9700	Badal & Carrión (2001)
34	Rio Maior	39°20' N	08°56' W	Archaeological site (charcoal)	QS	12,000–Present	Figueiral (1995)
35	Ekain	42°20' N	03°42' W	Archaeological cave (pollen)	QI, QC	15,400–9540	Dupré (1988)
36	Mari López	40°47' N	04°22' W	Lagoon (pollen)	QI, QC	70,000–30,000	Yll <i>et al.</i> (2003b)
37	MD95-2039	43°14' N	02°15' W	Marine core (pollen)	QI, QC	65,000–10,000	Roucoux <i>et al.</i> (unpublished data)
38	MD95-2042	37°01' N	06°19' W	Marine core (pollen)	QI, QC	48,000–28,000	Sánchez-Goñi <i>et al.</i> (2000)
39	MD95-2043	40°34' N	10°20' W	Marine core (pollen)	QI, QC	48,000–26,000	Sánchez-Goñi <i>et al.</i> (2002)
40	SO75-6kl	37°48' N	10°10' W	Marine core (pollen)	QI, QC	21,000–5570	Boessenkool <i>et al.</i> (2001)
41	ODP 976	36°08' N	02°37' W	Marine core (pollen)	QI, QC	50,000–Present	Comborieu-Nebout <i>et al.</i> (2002)
42	Pla de l' Estany	37°56' N	09°30' W	Lake (pollen)	QI, QC	30,000–1740	Burjachs (1990)
43	Tossal de la Roca	36°12' N	04°18' W	Archaeological rockshelter (pollen)	<i>Quercus</i> sp.	16,000–12,000	Cacho <i>et al.</i> (1983)
44	Castel sa Sala	42°11' N	02°29' E	Archaeological site (pollen)	QI, QC	16,000–Present	Yll (1987)
45	Charco da Candieira	38°47' N	00°14' W	Lake (pollen)	<i>Quercus</i> sp.	14,800–9525	Van der Knaap & van Leeuwen (1997)
46	Sanabria	41°57' N	02°15' E	Lake (pollen)	QC, QI	12,130–4702	Muñoz-Sobrino <i>et al.</i> (2001)
47	Lagoa de Lucenza	40°20' N	07°34' W	Lake (pollen)	QC, QI	17,390–3710	Muñoz-Sobrino <i>et al.</i> (2004)
48	Arenales de San Gregorio	42°07' N	06°43' W	Aeolian deposit (pollen)	QC, QI	22,000–23,000	Valdeolmillos <i>et al.</i> (2001)

QI, *Quercus ilex*; QC, *Quercus coccifera*; QS, *Quercus suber*.

Complementary long-distance dispersal is also identified by NCA. Birds are powerful dispersers, and are considered to be one of the main driving forces in recolonization. Some species such as the jay (*Garrulus glandarius*) act at a more local scale, rather than at long distances (Bossema, 1979; Gómez, 2003). Migratory species such as wood pigeons (*Columba palumbus*) and cranes (*Grus grus*) are the most likely dispersers to explain the distribution of chlorotypes located in distant regions. Seed transport should therefore take place in autumn, when acorns are fully developed and birds are migrating towards warmer locations in North Africa or southern Iberia. As a result, long-distance colonization would be expected to follow a north-south direction, instead of a fast recolonization towards the north.

The other main tree dispersers are people. Humans have been interacting with forest ecosystems for a long time, consuming resources and transferring species between countries (e.g. *Ulmus minor*; Gil *et al.*, 2004). Indeed, the human impact on the landscape may well have helped in the natural range expansions of the evergreen oaks throughout adjacent areas. González-Bernaldez (1990) introduced the term 'frutalization' in order to describe how humans have modelled the landscape, tending to construct forest landscapes focused on fruit production for human or cattle feeding. However,

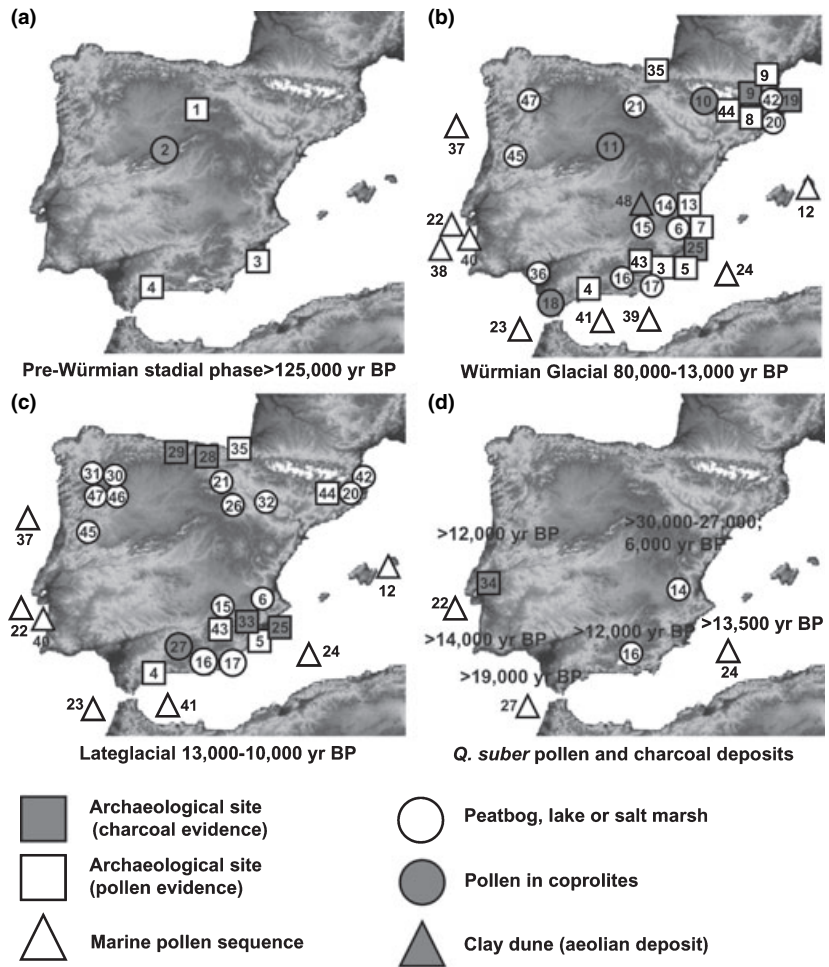
the transference of acorns over long distances is not easy, because if an acorn becomes dehydrated (below 40%) the embryo dies. The carriage of acorns over long distances and over periods of months or years would therefore seem unfeasible. Hence, while contiguous range expansions of evergreen oaks could be a result of humans, indiscriminate transference of acorns over long distances should be considered a rare event.

### Glacial refugia for *Quercus suber*

The literature reviewed herein does not indicate the presence of fossil pollen or charcoals of *Q. suber* before 30,000 yr BP and cpDNA analysis revealed fewer chlorotypes than in *Q. ilex*–*Q. coccifera*. In fact, *Q. suber* is more thermophilous and has stricter soil requirements than *Q. ilex* or *Q. coccifera*. Thus one would expect a bigger reduction of this species' range during glacial times. However, the uncertainty of palynological discrimination and the lower cpDNA variation itself could bias the identification of glacial refugia for *Q. suber*.

Nearly all the *Q. suber* populations from western Iberia are dominated by lineage *suber* (c1), indicating a glacial refugium in south-western Iberia that expanded northwards in the absence of mountain barriers and which was favoured by the





**Figure 3** *Quercus ilex* and *Q. coccifera* pollen and macrofossil citations in the Iberian Peninsula and Balearics at pre-Würmian (a), Würmian glacial (b) and Lateglacial (c) stadials. *Quercus suber* pollen and charcoal fossils are indicated when its palynotype was identified (d).

existence of siliceous outcrops. This scenario is clearly supported by both palynological (Carrión *et al.*, 2000) (Table 2) and molecular data (Lumaret *et al.*, 2005). It is worth mentioning that *Q. suber* seedlings can tolerate a certain degree of shade and therefore can displace other species, like *Q. ilex* or *Pinus* sp., on siliceous and deep substrates. There are isolated populations (siliceous outcrops surrounded by calcareous substrates) in the Cantabric Mountains showing chlorotype c1. As shown by isozyme analysis, these populations were probably the result of Holocene colonization events (Jiménez *et al.*, 1999).

In eastern Iberia, however, the cpDNA structure of *Q. suber* is completely different as it shares lineage *i-c I* with *Q. ilex* and *Q. coccifera* and lineage *suber* is absent. One of the weaknesses of NCA is that it is insensitive to the effect of processes derived from interspecific crosses. Current hybridization is still happening in central and eastern Iberia, with the first-generation hybrids (*Q. suber* × *ilex*) being easily identified in the field. In these interspecific crosses, *Q. suber* always acts as the pollen donor (Boavida *et al.*, 2001) and captures the cpDNA of *Q. ilex*. In some marginal areas of the main siliceous range of the species (western Iberia), isolated populations occur with some *Q. suber* trees of lineage *i-c I*.

This could be the result of modern hybridization and introgression.

The effect of hybridization and introgression in *Q. suber* cpDNA can produce the total replacement of the *Q. suber* chlorotype by the *i-c I* lineage (Lopez de Heredia *et al.*, 2005). This situation is common in eastern Spain, where siliceous soils are scarce and the effective population size is lower than in the distant (c. 400 km apart) continuous forests from western Iberia. It has been suggested, on the basis of the differences between *Q. ilex* and *Q. suber* chlorotypes found in sympatric populations, that hybridization and introgression in these populations may be ancient (Lumaret *et al.*, 2005). Chlorotype c66 is predominant in all *Q. suber* populations from Catalonia (north-eastern Spain), but it is very rare in *Q. ilex* (c39, c58 or c48). Therefore it cannot be categorically discounted that some populations withstood the glacial conditions in this area by hybridizing with *Q. ilex*. Likewise, a small population spread in eastern Spain could have followed the same pattern. Further research should be conducted on these sympatric populations in order to elucidate the interactions between species within and among glacial refugia.

## FINAL REMARKS AND CONCLUSIONS

Glacial and periglacial periods have had a significant effect on the modern vegetation of northern Europe (Birks, 1986). Results from research in these regions may not be directly applicable to southern latitudes, where floras may have remained relatively stationary and where biotic interactions among existing populations may be of exceptional importance (Bennett & Willis, 1995). This consideration is important if our aim is to produce parsimonious models of population genetic structure and palaeobiology. For instance, while a basic outline of Late Quaternary vegetation developments in the Eurosiberian region of Spain is available and retains explanatory power from decades ago (Allen *et al.*, 1996; Ramil-Rego *et al.*, 1998), the scenario depicted by the network of pollen diagrams from the Mediterranean region of Spain becomes more and more puzzling as we gain further information (Carrión, 2002). While some refugia for Mediterranean trees were previously identified in south-western Iberia and Catalonia, our results indicate other potential locations of refugia for Mediterranean species (e.g. Cantabric mountain ranges, south-eastern Spain or even central Spain). It is now clear, in our view, that Iberian evergreen oaks existed in multiple refugia during the Würmian stadial, and that several populations may still retain diversity from previous stages.

We conclude by highlighting the importance of identifying these multiple glacial refugial localities from which almost the whole of Iberia was recolonized. These forests, which also show high species richness for other Mediterranean plants and animals, are of the utmost importance when developing sound conservation policies or sustainable forest management strategies in the western Mediterranean countries.

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## BIOSKETCHES

**Unai López de Heredia** is an assistant lecturer in the Polytechnical University of Madrid, mainly interested in phylogeography and evolution of forest species in the Mediterranean Basin.

**José S. Carrión** teaches plant evolution, palaeoecology and global change in the Faculty of Biology, University of Murcia. His research has focused on Quaternary palaeoecology in Mediterranean and arid regions.

**Pilar Jiménez's** main interests are the study of genetic structure of forest trees, and this research is applied to the conservation of genetic resources.

**Carmen Collada's** research interests are focused on forest genomics and the development of molecular markers.

**Luis Gil** works at the Polytechnical University of Madrid, and has a long-standing interest in the forest biogeography and conservation genetics of Mediterranean forest ecosystems.

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