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Taxonomic identity of *Quercus coccifera* L. in the Iberian Peninsula is maintained in spite of widespread hybridisation, as revealed by morphological, ISSR and ITS sequence data

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Abstract

Interspecific introgression is a well-known phenomenon in oaks whose ecological and evolutionary consequences, although relevant, are still unclear. We investigated molecular variation and any evidence for hybridisation in nine natural populations of kermes oak (*Quercus coccifera* L.) from the Iberian Peninsula. Additive patterns in 59 nrDNA ITS sequences revealed that hybrid individuals showing intermediate genotypes between kermes and holm (*Q. ilex* L.) oaks are very frequent, although intermediate morphotypes are uncommon. Bayesian analysis of ISSR fingerprinting patterns indicated extensive gene-flow among *Q. coccifera* populations and neighbouring *Quercus* taxa. Introgression appeared to be an active mechanism in the pair *Q. coccifera*–*Q. ilex*, but no conclusive evidence supporting hybridisation between *Q. coccifera* and other co-occurring *Quercus* species was found. The role of canalisation in the maintenance of stable morphological characters in the face of extensive introgression is discussed.
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Introduction

Interspecific gene flow within species complexes is a key question of evolutionary biology (Arnold, 1997). The genus *Quercus* (oaks) constitutes one of the plant groups where this subject has been studied in more detail (Muir et al., 2000) since the facility with which oaks interbreed makes them a model of syngameon, where almost all crosses are possible within certain groups (Grant, 1971). The stability of oak species in the face of frequent interspecific gene exchange has long been regarded as an intriguing phenomenon, leading some authors to question the validity of the biological species concept in oaks (Van Valen, 1976). Recent research has proved the importance of interspecific introgression in *Quercus* to enable the colonisation of novel habitats and to respond to varying

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environmental selective pressures (Dodd and Afzal-Rafii, 2004; Petit et al., 2003).

In the Mediterranean region, evergreen oaks dominate most forest habitats (Takhtajan, 1986). Three species are particularly important in the Iberian vegetation: holm oak (*Quercus ilex* L.), cork oak (*Q. suber* L.) and kermes oak (*Q. coccifera* L.). Although their distributions and habitats are overlapping, ecological differences among them exist. *Q. ilex* is the most widespread and dominates forests on all kind of substrates. *Q. coccifera* and *Q. suber* are more thermophilous than *Q. ilex* and appear to have contrasting environmental requirements. Kermes oak is more frequent in arid and disturbance-prone environments, predominantly on limestone substrates, while the cork oak has strict humidity and soil requirements being only found on acidic or decarbonated soils (Costa et al., 1997; López González, 2002; Martínez-Ferri et al., 2004). Interspecific gene flow has already been detected in the crosses *Q. coccifera*–*Q. ilex* and *Q. suber*–*Q. ilex* (Belahbib et al., 2001; Lumaret et al., 2002). Between *Q. coccifera* and *Q. ilex*, gene exchange appears to be so frequent as to lead to complete sharing of cpDNA haplotypes (Jiménez et al., 2004). In spite of the apparent lack of reproductive barriers, intermediate morphological forms between both species are rarely observed in the field (although they have been reported in some studies have been reported, see for instance, Carvalho e Vasconcelos and Amaral Franco, 1954). Diagnostic characters such as glabrous leaves and spiny hooked acorn cups in *Q. coccifera* vs. densely tomentose abaxial leaf surfaces and smooth acorn cups fin *Q. ilex*, enable the unambiguous taxonomic assignment of most individuals. The fact that kermes and holm oak remain distinct species in spite of ecological similarities and frequent hybridisation raises interesting questions concerning the evolutionary relationships between the two taxa and the causes and consequences of gene exchange.

Hybridisation in oaks has been studied with several molecular tools, including both plastid and nuclear DNA. However, a most limited number of studies have used the internal transcribed spacer of ribosomal DNA (ITS) sequences, in spite of its potential as both a phylogenetic tool (Bellarosa et al., 2005; Manos et al., 1999) and to determine reticulation processes (Nieto et al., 2001; Ritz et al., 2005). Muir et al. (2001) demonstrated that the nrDNA of *Quercus* is subject to concerted evolution, although several paralog ITS copies co-occur in the genome of a single plant (Mayol and Rosselló, 2001). Nevertheless, concerted evolution is not uniform across the ITS region of angiosperms (Fuertes Aguilar et al., 1999) and failure in homogenisation among sites can supply valuable information to infer patterns of reticulated evolution (Sang et al., 1995; Vargas et al., 1999). ITS sequences are then powerful markers to reconstruct hybridisation events, but their limited variability at the infra-specific level is a drawback for their application in population genetics. Conversely, fingerprinting techniques such as RAPD, inter-simple sequence repeats (ISSR) and AFLP provide genome-wide screening of fine-scale variation. In particular, ISSR markers have proved to be a reliable molecular tool for the study of interpopulation relationships (Labra et al., 2006) and of hybrid complexes (Wolfe et al., 1998; James and Abbott, 2005).

In the present paper, we used 59 ITS sequences and ISSR banding patterns from 87 individuals of nine populations to estimate the consequences of interspecific hybridisation on the genetic structure of *Q. coccifera* populations in the Iberian Peninsula. We particularly addressed the following questions:

(1) Can ongoing hybridisation be detected in morphologically unambiguous individuals?
(2) Is hybridisation restricted to crosses with *Q. ilex*?
(3) Are phylogenetic relationships affected by interspecific gene-flow?

Material and methods

Plant material and DNA extraction

A total of 87 plants were sampled in nine populations (Fig. 1). In each population, plant material was collected in the field from nine individuals of *Q. coccifera* and from all individuals of any other *Quercus* species found within or adjacent to the kermes oak populations. As the aim of this study was to detect the influence of interspecific hybridisation in *Q. coccifera*, sampling was conducted so as to detect putative hybrid individuals only within this species. Taxonomic identification of plants was based on diagnostic vegetative and reproductive characters according to Franco (1990). DNA was extracted from ca. 0.5 g of silica dried leaf material using the Plant-DNeasy Minikit (QIAGEN Inc., Hilden, Germany) and the protocols provided by the manufacturer.

ITS amplification and sequencing

ITS region sequences from three to four samples of *Q. coccifera* from each population and from all samples of nearby *Quercus* species were analysed. Additionally, two *Q. ilex* samples from populations not included in the field survey as well as all ITS-region accessions from the GenBank from the Iberian *Q. coccifera*, *Q. ilex* and *Q. suber* were incorporated to the aligned matrix (López de Heredia et al., unpublished). Sequences deposited in GenBank were all obtained by cloning of ITS copies, and thus can be considered as haplotypes (Ritz et al., 2005). Additionally, they can also be taken as representing "pure" accessions, since cloned sequences are likely

	Name	Code	Latitude	Longitude	m.a.s.l.	*Quercus* spp.
1	Aranjuez	ARJ	40º 0' 02" N	3º 36' 27" W	579	1 [1]
2	Gargallo	GAR	40º 51' 87" N	0º 33' 38" W	1018	0
3	Cañada de Verich	CÑV	40º 52' 46" N	0º 06' 79" W	822	0
4	Cardeña	CAR	38º 21' 46" N	4º 19' 20" W	581	0
5	Facinas	FAC	36º 9' 46" N	5º 40' 09" W	118	1 [2]
6	El Saler	SAL	39º 22' 50" N	0º 19' 40" W	104	0
7	Serra da Arrabida	ARR	38º 27' 17" N	9º 00' 62" W	312	1 [3]
8	Tarazona	TAR	41º 50' 35" N	1º 38' 42" W	694	2 [1]
9	Bielsa	BIE	42º 26' 19" N	0º 05' 06" W	1549	2 [1,4]

Quercus spp.: number of individuals sampled not belonging to *Q. coccifera*. [1] Holm oak *Q. ilex* subsp. *ballota*; [2] Cork oak *Q.suber*; [3] Quejigo oak, *Q. faginea* subsp. *broteroi*; [4] Downy oak *Q. humilis*.

Fig. 1. Distribution of the kermes oak (*Quercus coccifera* L.) in Iberia with location of the studied populations and the number and species of all other *Quercus* individuals included in the analyses.

to represent the most frequent ITS copy in the genome of each plant and can thus be assumed to be the species-specific genotype. Polymerase chain reaction (PCR) amplifications were performed using the nucleotide primers 17SE (Sun et al., 1994) and C28KJ (Vargas et al., 1998). Amplifications were carried out in 25 µl reaction mixture containing 17.2 µl sterile water, 2.5 µl of 10 × PCR buffer, 1.4 µl of 50 mM $MgCl_2$, 1.6 µl of 10 mM dNTPs, 0.5 µl of each primer (0.7 µM), 0.3 µl (1.5 U) of EcoTaq *Taq* polymerase (ECOGEN, Barcelona, Spain) and 1 µl (30 ng) template DNA. A MJ Research MiniCycler™ thermal cycler was used with the following profile: 97 °C for 1 min, 25 thermal cycles (48 °C for 35 s, 72 °C for 1 min) and a final extension of 15 min at 72 °C. The amplified DNA was purified using spin filter columns (PCR Clean-Up Kit, MoBio Laboratories, Carlsbad, CA, USA). Purified products were then directly sequenced using dye terminators (Big Dye Terminator v. 2.0, PE/Applied Biosystems, Foster City, CA, USA). For cycle sequencing on forward and reverse strands the primers Leu1 (Andreasen et al., 1999) and ITS 4 (White et al., 1990) and the following conditions were used: 95 °C for 2 min followed by 25 cycles of 95 °C for 10 s, 50 °C for 5 s, and 60 °C for 4 min. Polyacrylamide gel electrophoresis of sequencing products were conducted by using a Perkin-Elmer/Applied Biosystems model 377 automated sequencer.

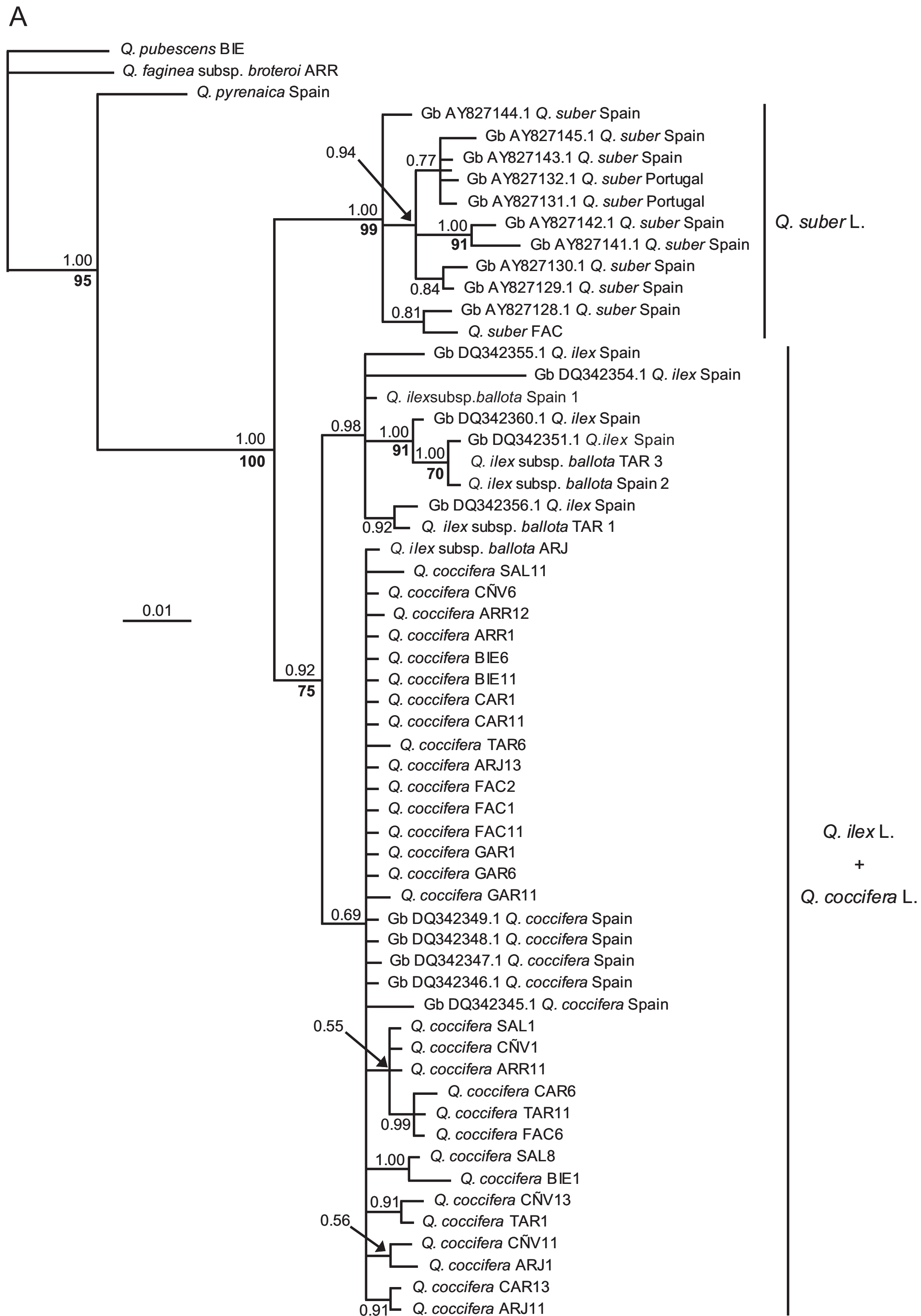

Fig. 2. Phylogeny of nrDNA ITS sequences from five *Quercus* species based on Bayesian inference. Posterior probabilities and maximum parsimony (MP) bootstrap support (bold) are indicated for each node. ITS haplotypes (clone accessions) retrieved from GenBank have the accession no. before taxon name (López de Heredia et al., unpublished): (A) phylogenetic relationships for all sequences considered. MP results based on the strict consensus of 12 most parsimonious trees (CI = 0.848). (B) Tree resulting from the exclusion of 23 sequences in which additive polymorphic sites were detected. MP results based on the strict consensus of 64 most parsimonious trees (CI = 0.795).

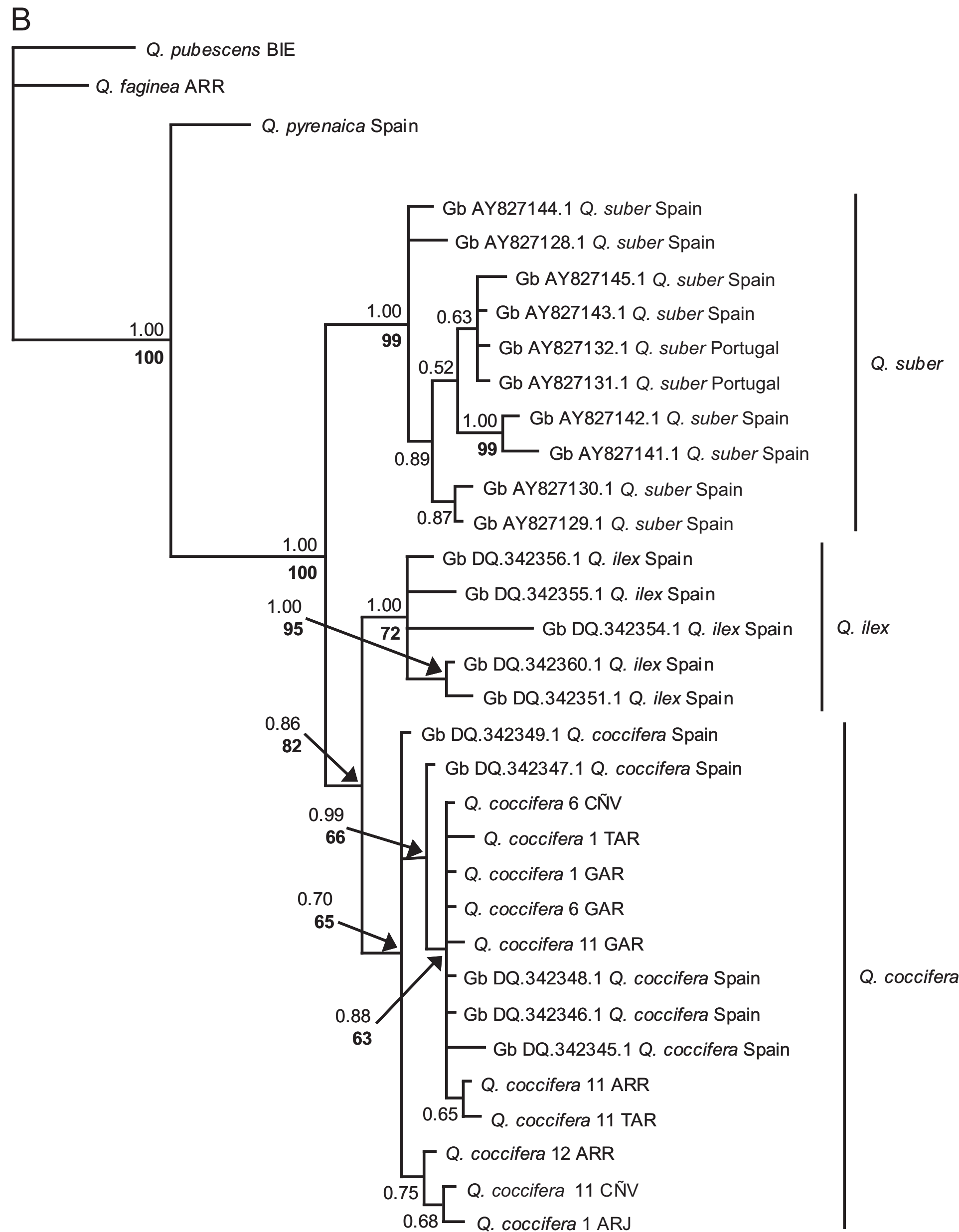

Fig. 2. (*Continued*)

ISSR amplification, electrophoresis and silver staining

Leaf material was superficially sterilised according to the protocol described by Zhang et al. (1997) to avoid amplification of fungal DNA. A set of 17 ISSR primers, commercialised by the University of British Columbia Biotechnology Laboratory, were tested in three samples to find out suitable repeats and anchors. As a result, two primers were chosen and used: 834 $(AG)_8YT$ and 818 $(CA)_8G$. PCR reaction mixtures (20 µl) contained 2 µl of 10 × PCR buffer, 0.6/1.2 µl (for the UBC-834 and 818 primers, respectively) of 25 mM $MgCl_2$, 2.7 µl of 10 mM dNTPs, 2.7 µl of the corresponding primer (1.5 µM), 0.15 µl (0.75 U) of AmpliTaq Gold® *Taq* polymerase (Applied Biosystems) and 30 ng template DNA and sterile water to a final volume of 20µl. The thermal cycler used was a Gene Amp PCR System 9700 (PE Applied Biosystems) with the following program: 94 °C for 12 min, 35 thermal cycles of 94 °C for 1.5 min, 50 °C for 1.5 min, 72 °C for 1.5 min followed by a final extension of 10 min at 72 °C. Negative controls and replicates were included to verify repeatability of results. Gel electrophoresis and visualisation of the fragments of the 87 samples was performed as in Hess et al. (2000). In the precast polyacrylamide gels (Applied Biosystems) a

Table 1. Informative variable sites and polymorphism in the generated ITS matrix of *Quercus*

	ITS1																																			
	26	37	39	43	49	54	59	60	68	69	74	76	82	84	85	88	91	100	102	108	114	131	138	152	160	163	167	168	169	171	191	197	209	212	214	222
Q. humilis BIE	C	T	C	C	G	A	T	C	T	G	C	C	C	C	T	G	T	C	C	C	C	C	C	C	G	G	A	T	G	C	C	C	C	A	G	C
Q. faginea subsp. *broteroi* ARR	C	T	C	C	G	A/G	T	C	T	G	C	C	C	C	T	G	T	C	C	C	C	C	C	C	G	G	A	T	G	C	C	C	C	A	G	C
Q. pyrenaica Spain	C	T	C	C	G	A/G	G	C	C	G	C	C	C	C/T	G	G	G	C	C	C	C	C	C	C	G	A	A	T	G	C	C	C	C	A	G	C
Gb AY827145.1 *Q. suber* Spain	C	G	T	C	G	G	–	–	C	G	C	C	C	A	C	A	C	C	C	T	T	C	C	C	G	A	G	C	G	C	C	C	C	A	G	T
Gb AY827144.1 *Q. suber* Spain	C	G	C	C	G	G	–	–	C	A	C	C	C	A	C	A	C	C	C	T	T	C	C	C	G	A	G	C	G	T	C	C	T	A	G	T
Gb AY827143.1 *Q. suber* Spain	C	G	C	C	G	G	–	–	C	G	C	C	C	A	C	A	C	C	C	T	T	C	C	C	G	A	G	C	G	C	C	C	C	A	G	T
Gb AY827142.1 *Q. suber* Spain	C	G	C	C	A	G	G	C	C	G	C	C	C	A	C	A	C	C	C	T	T	C	C	C	A	A	G	C	G	C	C	C	C	G	A	T
Gb AY827141.1 *Q. suber* Spain	C	G	C	C	A	G	G	C	C	G	C	C	C	A	C	A	C	T	C	T	T	C	C	C	A	A	G	C	G	C	C	C	C	G	A	T
Gb AY827132.1 *Q. suber* Portugal	C	G	C	C	G	G	–	–	C	G	C	C	C	A	C	A	C	C	C	T	T	C	C	C	G	A	G	C	G	T	C	C	C	A	G	T
Gb AY827131.1 *Q. suber* Portugal	C	G	C	C	A	G	–	–	C	G	C	C	C	A	C	A	C	C	C	T	T	C	C	C	G	A	G	C	G	C	C	C	C	A	G	T
Gb AY827130.1 *Q. suber* Spain	C	G	C	C	A	G	–	–	C	A	C	C	C	A	C	A	C	C	C	T	T	C	C	C	G	A	G	C	G	T	C	C	C	A	G	T
Gb AY827129.1 *Q. suber* Spain	C	G	C	C	A	G	–	–	C	A	C	C	C	A	C	A	C	C	C	T	T	C	C	C	G	A	G	C	G	T	C	C	C	A	G	T
Gb AY827128.1 *Q. suber* Spain	C	G	T	C	G	G	–	–	C	G	C	C	C	A	C	A	C	C	C	T	T	C	C	C	G	A	G	C	G	T	C	C	C	A	G	T
Q. suber FAC	C	G	C/T	C	G	G	–	–	C	A/G	C/T	C	C	A	C/G	A	C	C	C	T	T	C	C	C	G	A	G	C	G	T	C	C	T	A	G	T
Gb DQ. 342360.1 *Q. ilex* Spain	C	G	C	C	G	G	G	C	C	G	T	C	T	A	A	G	C	T	C	T	C	C	C	C	G	A	A	C	T	T	T	C	C	A	G	T
Gb DQ. 342356.1 *Q. ilex* Spain	C	G	C	C	G	G	G	C	C	G	T	C	T	A	A	G	C	C	C	T	C	C	C	C	G	A	A	C	G	T	T	C	C	A	G	T
Gb DQ. 342355.1 *Q. ilex* Spain	C	G	C	C	G	G	G	C	C	G	T	C	T	A	A	G	C	C	C	T	C	C	C	C	G	A	A	C	G	T	T	C	C	A	G	T
Gb DQ. 342354.1 *Q. ilex* Spain	C	G	C	C	A	G	G	C	C	G	T	C	T	A	A	G	C	C	C	T	C	T	C	T	G	A	A	C	G	T	T	T	C	A	G	T
Gb DQ. 342351.1 *Q. ilex* Spain	C	G	C	C	G	G	G	C	C	G	T	C	T	A	A	G	C	T	C	T	C	C	C	C	G	A	A	C	T	T	T	C	C	A	G	T
Q. ilex subsp. *ballota* ARJ	C	G	C	C	G	G	G	C	C	G	T	C	T	A	A	G	C	C	C/T	T	C	C	C/T	C/T	G	A	A	C	G	T	T	C/G	C	A	G	T
Q. ilex subsp. *ballota* TAR 1	C	G	C	C	G	G	G	C	C	G	C/T	C	T	A	A	G	C	C/T	C	T	C	C	C	C	G	A	A	C	G/T	T	T	C	C	A	G	T
Q. ilex subsp. *ballota* TAR 3	C	G	C	C	G	G	G	C	C	G	T	C	T	A	A	G	C	T	C	T	C	C	C	C	G	A	A	C	T	T	T	C	C	A	G	T
Q. ilex subsp. *ballota* Spain 1	C	G	C	C	G	G	G	C	C	G	C/T	C	T	A	A	G	C	C	C	T	C	C	C	C	G	A	A	C	G/T	T	T	C	C	A	G	T
Q. ilex subsp. *ballota* Spain 2	C	G	C	C	G	G	G	C	C	G	T	C	T	A	A	G	C	T	C	T	C	C	C	C	G	A	A	C	T	T	T	C	C	A	G	T
Q. coccifera ARJ 13	C	G	C	C	G	G	G	C/G	C	G	C	T	T	A	A	G	C	C	C	T	C	C	T	T	G	A	A	C	G	T	T	C	C	A	G	T
Q. coccifera ARJ 1	C	G	C	C	G	G	G	C	C	G	C	T	C	A	A	G	C	C	C	T	C	C	T	T	G	A	A	C/T	G	T	T	C	C	A	G	T
Q. coccifera ARJ 11	C	G	C	C	G	G	G	C	C	G	C	T	C	A	C	G	C	C	T	T	C	C	T	T	G	A	A	C	G	T	T	C	C	A	G	T
Q. coccifera GAR 1	C/T	G	C	C	G	G	G	C	C	G	C	T	C	A	C	G	C	C	T	T	C	C	T	T	G	A	A	C	G	T	T	C	C	A	G	T
Q. coccifera GAR 6	C/T	G	C	C	G	G	G	C	C	G	C	T	C	A	C	G	C	C	T	T	C	C	T	T	G	A	A	C	G	T	T	C	C	A	G	T
Q. coccifera GAR 11	C	G	C	C	G	G	G	C	C	G	C	T	C	A	C	G	C	C	T	T	C	C	T	T	G	A	A	C	G	T	T	C	C	A	G	T
Q. coccifera CÑV 13	C	G	C	C	G	G	G	C	T	G	C	T	C/T	A	C	G	C	C	T	T	C	C	T	T	G	A	A	C	G	T	T	C	C	A	G	T
Q. coccifera CÑV 6	C	G	C	C	G	G	G	C	C	G	C	T	C	A	C	G	C	C	T	T	C	C	T	T	G	A	A	C	G	T	T	C	C	A	G	T
Q. coccifera CÑV 1	C	G	C	C	G	G	G	C	C	G	C	T	C/T	A	C	G	C	C	T	T	C	C	T	T	G	A	A	C	G	T	T	C	C	A	G	T
Q. coccifera CÑV 11	C	G	C	T	G	G	G	C	C	G	C	T	T	A	A	G	C	C	C	T	C	C	T	T	G	A	A	C	G	T	T	C	C	A	G	T
Q. coccifera CAR 1	C	G	C	C	G	G	G	C	C/T	G	C	T	C/T	A	A/C	G	C	C	C/T	T	C	C	T	T	G	A	A	C	G	T	T	C	C	A	G	T
Q. coccifera CAR 11	C	G	C	C/T	G	G	G	C	C	G	C	T	T	A	A/C	G	C	C	C/T	T	C	C	T	T	G	A	A	C	G	T	T	C	C	A	G	T
Q. coccifera CAR 6	C	G	C	C	G	G	G	C	C	G	C	T	C/T	A	C	G	C	C	T	T	C	C	T	T	G	A	A	C	G	T	T	C	C	A	G	T
Q. coccifera CAR 13	C	G	C	C	G	G	G	C	C	G	C	T	C/T	A	A/C	G	C	C	C/T	T	C	C	T	T	G	A	A	C	G	T	T	C	C	A	G	T
Q. coccifera FAC 2	C	G	C	C	G	G	G	C	C	G	C	T	C/T	A	C	G	C	C	C/T	T	C	C	T	T	G	A	A	C/T	G	T	T	C	C	A	G	T
Q. coccifera FAC 1	C	G	C	C	G	G	G	C	C	G	C	T	C/T	A	A/C	G	C	C	C/T	T	C	C	T	T	G	A	A	C	G	T	T	C/G	C	A	G	T
Q. coccifera FAC 6	C	G	C	C	G	G	G	C	C	G	C	T	C	A	C	G	C	C	T	T	C	C	T	T	G	A	A	C	G	T	T	C	C	A	G	T
Q. coccifera FAC 11	C	G	C	C/T	G	G	G	C	C	G	C	T	T	A	A/C	G	C	C	C/T	T	C	C	T	T	G	A	A	C	G	T	T	C	C	A	G	T
Q. coccifera SAL 1	C	G	C	C/T	G	G	G	C	C	G	C	T	C/T	A	A/C	G	C	C	C/T	T	C	C	T	T	G	A	A	C	G	T	T	C	C	A	G	T
Q. coccifera SAL 8	C	G	C	C	G	G	G	C	T	G	C	T	C	A	C	G	C	A	T	T	C	C	T	T	G	A	A	C	G	T	T	C	C	A	G	T
Q. coccifera SAL 11	C	G	C	C	G	G	G	C	C	G	C	T	C/T	A	C	G	C	C	T	T	C	C	T	T	G	A	A	C	G	T	T	C/G	C	A	G	T
Q. coccifera ARR 12	C	G	C	T	G	G	G	C	C	G	C	T	T	A	A	G	C	C	C	T	C	C	T	T	G	A	A	C	G	T	T	C	C	A	G	T
Q. coccifera ARR 11	C	G	C	C	G	G	G	C	C	G	C	T	C	A	C	G	C	C	T	T	C	C	T	T	G	A	A	C	G	T	T	C	C	A	G	T
Q. coccifera ARR 1	C	G	C	C	G	G	G	C	C	G	C	T	C	A	C	G	C	C	T	T	C	C/T	T	T	G	A	A	C	G	T	T	C	C	A/T	G	T
Q. coccifera TAR 1	C	G	C	C	G	G	G	C	C	G	C	T	C	A	C	G	C	C	T	T	C	C	T	T	G	A	A	C	G	T	T	C	C	A	G	T
Q. coccifera TAR 11	C	G	C	C	G	G	G	C	C	G	C	T	C	A	C	G	C	C	T	T	C	C	T	T	G	A	A	C	G	T	T	C	C	A	G	T
Q. coccifera TAR 6	C	G	C	C	G	G	G	C	C	G	C	T	C	A	C	G	C	C	T	T	C	C	T	T	G	A	A	C	G	T	T	C	C	A	G	T
Q. coccifera BIE 6	C	G	C	C	G	G	G	C	C	G	C	T	C/T	A	C	G	C	C	T	T	C	C	T	T	G	A	A	C	G	T	T	C	C	A	G	T
Q. coccifera BIE 11	C	G	C	C/T	G	G	G	C	C	G	C	T	C/T	A	A/C	G	C	C	C/T	T	C	C	T	T	G	A	A	C	G	T	T	C	C	A	G	T
Q. coccifera BIE 1	C	G	C	C	G	G	G	C	T	G	C	T	C	A	C	G	C	A	T	T	C	C	T	T	G	A	A	C	G	C	T	C	C	A	G	T
Gb DQ. 342349.1 *Q. coccifera* Spain	C	G	C	C	G	G	G	C	C	G	C	T	T	A	C	G	C	C	C	T	C	C	T	T	G	A	A	C	G	T	T	C	C	A	G	T
Gb DQ. 342348.1 *Q. coccifera* Spain	C	G	C	C	G	G	G	C	C	G	C	T	C	A	C	G	C	C	T	T	C	C	T	T	G	A	A	C	G	T	T	C	C	A	G	T
Gb DQ. 342347.1 *Q. coccifera* Spain	C	G	C	C	G	G	G	C	C	G	C	T	C	A	C	G	C	C	C	T	C	C	T	T	G	A	A	C	G	T	T	C	C	A	G	T
Gb DQ. 342346.1 *Q. coccifera* Spain	C	G	C	C	G	G	G	C	C	G	C	T	C	A	C	G	C	C	T	T	C	C	T	T	G	A	A	C	G	T	T	C	C	A	G	T
Gb DQ. 342345.1 *Q. coccifera* Spain	T	G	C	C	G	G	G	C	C	G	C	T	C	A	C	G	C	C	T	T	C	C	T	T	G	A	A	C	G	T	T	C	C	A	G	T

	5.8s			ITS2																													
	352	354	370	408	417	419	441	442	443	444	517	528	532	543	546	552	554	563	565	567	568	569	570	571	572	573	575	576	577	589	599	601	602	607
Q. humilis BIE	A	T	C	C	G	G	G	C	G	T	C	T	–	T	C	C/T	C	C	G	A	C	A/T	C	G	C	A	A	C	C	C	G	G	G	C
Q. faginea subsp. *broteroi* ARR	A	T	C	C	G	G	G	C	G	T	G	C	–	T	C	C	T	C	G	A	C	A	C	A	C	A	A	C	C	C	G	G	G	C
Q. pyrenaica Spain	A	T	C	C	G	G	G	C	G	T	C	T	A	T	C	C/T	C	C	G	A	C	G	C	A/G	C	G	A	C	C	C	G	G	G	C
Gb AY827145.1 *Q. suber* Spain	T	C	C	C	G	G	G	C	A	C	T	C	A	A	C	C	C	C	G	A	C	G	T	G	C	G	A	C	T	T	G	A	G	C
Gb AY827144.1 *Q. suber* Spain	T	T	C	C	G	G	G	C	G	T	T	C	A	A	C	C	C	C	G	A	C	G	T	G	C	G	A	C	T	T	G	A	G	C
Gb AY827143.1 *Q. suber* Spain	T	T	C	C	G	G	G	C	A	C	T	C	A	A	C	C	C	C	G	A	C	G	T	G	C	G	A	C	T	T	G	A	G	C
Gb AY827142.1 *Q. suber* Spain	T	T	C	C	G	G	G	C	G	C	T	C	A	A	C	C	C	C	G	A	C	G	T	G	C	G	A	C	T	T	G	A	G	C
Gb AY827141.1 *Q. suber* Spain	T	T	C	C	G	G	G	T	G	C	T	C	A	A	C	C	C	C	G	A	C	G	T	G	C	G	A	C	T	T	G	A	G	C
Gb AY827132.1 *Q. suber* Portugal	T	T	C	C	G	G	G	C	A	C	T	C	A	A	C	C	C	C	G	A	C	G	T	G	C	G	A	C	T	T	G	A	G	C
Gb AY827131.1 *Q. suber* Portugal	T	T	C	C	G	G	G	C	A	C	T	C	A	A	C	C	C	C	G	A	C	G	T	G	C	G	A	C	T	T	G	A	G	C
Gb AY827130.1 *Q. suber* Spain	T	T	C	C	G	G	G	T	G	C	T	C	A	A	C	C	C	C	G	A	C	G	T	G	C	G	A	C	T	T	G	A	G	C
Gb AY827129.1 *Q. suber* Spain	T	T	C	C	G	G	G	C	G	C	T	C	A	A	C	C	C	C	G	A	C	G	T	G	C	G	A	C	T	T	G	A	G	C
Gb AY827128.1 *Q. suber* Spain	T	C	C	C	G	G	G	C	G	T	T	C	A	A	C	C	C	C	G	A	C	G	T	G	C	G	A	C	T	T	A	A	G	C
Q. suber FAC	T	C	C	C	G	G	G	C	G	C/T	T	C	A	A	C	C	C	C	G	A	C	G	T	G	C/T	G	A	C	T	T	A/G	A	G	C
Gb DQ. 342360.1 *Q. ilex* Spain	A	T	C	C	G	G	G	C	G	T	T	C	A	C	C	C	C	C	G	T	T	G	T	G	C	G	A	C	T	C	G	G	G	C
Gb DQ. 342356.1 *Q. ilex* Spain	A	T	C	C	G	G	G	C	G	T	T	C	A	C	C	C	C	C	G	C	C	G	T	G	C	G	A	C	T	C	G	G	G	C
Gb DQ. 342355.1 *Q. ilex* Spain	A	T	C	C	G	G	G	C	G	T	T	C	A	C	C	C	C	C	G	T	C	G	T	G	C	G	T	C	T	C	G	G	C	C
Gb DQ. 342354.1 *Q. ilex* Spain	A	T	C	C	G	G	G	C	G	T	T	C	A	C	C	C	C	C	G	T	C	G	T	G	C	G	A	T	T	C	G	G	G	C
Gb DQ. 342351.1 *Q. ilex* Spain	A	T	C	C	G	G	A	C	G	T	T	C	A	C	C	C	C	A	G	T	T	G	T	G	C	G	A	C	T	C	G	G	G	C
Q. ilex subsp. *ballota* ARJ	A	T	C/T	C/T	G	G	G	C	G	T	T	C	A	C	C	C	C	C	G/T	A	C	G	T	G	C	G	A	C	T	C	G	G	G	C
Q. ilex subsp. *ballota* TAR 1	A	T	C	C	G	G	A/G	C	G	T	T	C	A	C	C	C	C	A/C	G	C	C	G	T	G	C	G	A	C	T	C	G	G	G	–
Q. ilex subsp. *ballota* TAR 3	A	T	C	C	G	G	A/G	C	G	T	T	C	A	C	C	C	C	A	G	T	T	G	T	G	C	G	A	C	T	C	G	G	G	C
Q. ilex subsp. *ballota* Spain 1	A	T	C	C	G	G	G	C	G	T	T	C	A/G	C	C	C	C	A/C	G	T	C	G	T	G	C/T	G	A	C	T	C	G	G	G	C
Q. ilex subsp. *ballota* Spain 2	A	T	C	C	G	A/G	A	C	G	T	T	C	A	C	C	C	C	A	G	T	T	G	T	G	C	G	A	C	T	C	G	G	G	C
Q. coccifera ARJ 13	A	T	C/T	C/T	G	G	G	C	G	T	T	C	A	C	C	C	C	C	G	A	C	G	T	G	C	G	A	C	T	C	G	G	G	C
Q. coccifera ARJ 1	A	T	C	C	G	G	G	C	G	T	T	C	A	C	T	C	C	C	G	A	C	G	T	G	C	G	A	C	T	C	G	G	G	C
Q. coccifera ARJ 11	A	T	C	T	G	C/T	G	C	G	T	T	C	A	C	C	C	C	C	G	A	C	G	T	G	C	G	A	C	T	C	G	G	G	C
Q. coccifera GAR 1	A	T	C	T	G	G	G	C	G	T	T	C	A	C	C	C	C	C	G	A	C	G	T	G	C	G	A	C	T	C	G	G	G	C
Q. coccifera GAR 6	A	T	C	T	G	G	G	C	G	T	T	C	A	C	C	C	C	C	G	A	C	G	T	G	C	G	A	C	T	C	G	G	G	C
Q. coccifera GAR 11	A	T	C	T	G	G	G	n	G	T	T	C	A	C	C	C	C	C	G	A	C	G	T	G	C/T	G	A	C	T	C	G	G	T	C
Q. coccifera CÑV 13	A	T	C/G	T	A	G	G	C	G	T	T	C	A	C	C	C	C	C	G	A	C	G	T	G	T	G	A	C	T	C	G	G	G	C
Q. coccifera CÑV 6	A	T	C	T	G	G	G	C	G	T	T	C	A	C	C	C	C	C	G	A	C	G	T	G	C	G	A	C	T	C	G	G	G	C
Q. coccifera CÑV 1	A	T	C	T	A/G	G	G	C	G	T	T	C	A	C	C	C	C	C	G	A	C	G	T	G	C/T	G	A	C	T	C	G	G	G	C
Q. coccifera CÑV 11	A	T	C	C	G	G	G	C	G	T	T	C	A	C	T	C	C	C	G	A	C	G	T	G	C	G	A	C	T	C	G	G	G	C
Q. coccifera CAR 1	A	T	C	C/T	G	G	G	C	G	T	T	C	A	C	C	C	C	C	G	A	C	G	T	G	C	G	A	C	T	C	G	G	G	C
Q. coccifera CAR 11	A	T	C	C/T	G	G	G	C	G	T	T	C	A	C	C/T	C	C	C	G	A	C	G	T	G	C	G	A	C	T	C	G	G	G	C
Q. coccifera CAR 6	A	T	C	T	A	G	G	C	G	T	T	C	A	C	C	C	C	C	G	A	C	G	T	G	C/T	G	A	C	T	C	G	G	–	T
Q. coccifera CAR 13	A	T	C	T	G	T	G	C	G	T	T	C	A	C	C	C	C	C	G	A	C	G	T	G	C	G	A	C	T	C	G	G	G	A/C
Q. coccifera FAC 2	A	T	C	T	G	G	G	C	G	T	T	C	A	C	C	C	C	C	G	A	C	G	T	G	C	G	A	C	T	C	G	G	G	C
Q. coccifera FAC 1	A	T	C	C/T	G	G	G	C	G	T	T	C	A	C	C	C	C	C	G	A	C	G	T	G	C	G	A	C	T	C	G	G	G	C
Q. coccifera FAC 6	A	T	C	C/T	G	G	G	C	G	T	T	C	A	C	C	C	C	C	G	A	C	G	T	G	C	G	A	C	T	C	G	G	–	T
Q. coccifera FAC 11	A	T	C	C/T	G	G	G	C	G	T	T	C	A	C	C	C	C	C	G	A	C	G	T	G	C	G	A	C	T	C	G	G	G	C
Q. coccifera SAL 1	A	T	C	C/T	G	G	G	C	G	T	T	C	A	C	C	C	C	C	G	A	C	G	T	G	C	G	A	C	T	C	G	G	G	C
Q. coccifera SAL 8	A	T	C	C/T	G	G	G	C	G	T	T	C	A	C	C	C	C	C	G	A	C	G	T	G	C/T	G	A	C	T	C	G	G	G	C
Q. coccifera SAL 11	A	T	C/T	T	G	G/T	G	C	G	T	T	C	A	C	C	C	C	C	C/T	A	C	G	T	G	C	G	A	C	T	C	G	G	G	C
Q. coccifera ARR 12	A	T	C	C	G	G	G	C	G	T	T	C	A	C	C	C	C	C	G	A	C	G	T	G	C	G	A	C	T	C	G	G	G	C
Q. coccifera ARR 11	A	T	C	T	G	G	G	C	G	T	T	C	A	C	C	C	C	C	C/G	A	C	G	T	G	C	G	A	C	T	C	G	G	G	C
Q. coccifera ARR 1	A	T	C/T	C/T	G	G	G	C	G	T	T	C	A	C	C	C	C	C	G	A	C	G	T	G	C	G	A	C	T	C	G	G	G	C
Q. coccifera TAR 1	A	T	C	T	A	G	G	C	G	T	T	C	A	C	C	C	C	C	G	A	C	G	T	G	T	G	A	C	T	C	G	G	G	C
Q. coccifera TAR 11	A	T	C	T	G	G	G	C	G	T	T	C	A	C	C	C	C	C	G	A	C	G	T	G	C	G	A	C	T	C	G	G	–	T
Q. coccifera TAR 6	A	T	C	T	G	G	G	C	G	T	T	C	A	C	C	C/T	C	C	G	A	C	G	T	G	C	G	A	G	T	C	G	G	G	C
Q. coccifera BIE 6	A	T	C	C/T	A/G	G	G	C	G	T	T	C	A	C	C	C	C	C	G	A	C	G	T	G	C/T	G	A	C	T	C	G	G	G	C
Q. coccifera BIE 11	A	T	C	C/T	A/G	G	G	C	G	T	T	C	A	C	C/T	C	C	C	G	A	C	G	T	G	C/T	G	A	C	T	C	G	G	G	C
Q. coccifera BIE 1	T	T	C	C/T	G	G	G	C	G	T	T	C	A	C	C	C	C	C	G	A	C	G	T	G	C	G	A	C	T	C	G	G	G	C
Gb DQ. 342349.1 *Q. coccifera* Spain	A	T	C	C	G	G	G	C	G	T	T	C	A	C	C	C	C	C	G	A	C	G	T	G	C	G	A	C	T	C	G	G	G	C
Gb DQ. 342348.1 *Q. coccifera* Spain	A	T	C	T	G	G	G	C	G	T	T	C	A	C	C	C	C	C	G	A	C	G	T	G	C	G	A	C	T	C	G	G	G	C
Gb DQ. 342347.1 *Q. coccifera* Spain	A	T	C	T	G	G	G	C	G	T	T	C	A	C	C	C	C	C	G	A	C	G	T	G	C	G	A	C	T	C	G	G	G	C
Gb DQ. 342346.1 *Q. coccifera* Spain	A	T	C	T	G	G	G	C	G	T	T	C	A	C	C	C	C	C	G	A	C	G	T	G	C	G	A	C	T	C	G	G	G	C
Gb DQ. 342345.1 *Q. coccifera* Spain	A	T	C	T	G	G	G	C	G	T	T	C	A	C	C	C	T	C	G	A	C	G	T	G	C	G	A	C	T	C	G	G	G	C

Sequences retrieved from the GenBank have the accession no. before taxon name.

20-bp ladder (Ez-load™ 20 bp Molecular Ruler, BIO-RAD laboratories, Hercules, CA, USA) was included every five samples to estimate fragment size. The silver-stained gels containing the ISSR fragments were scanned and the digital images of the gels scored for presence/absence of bands with Quantity One® quantitation software (BIO-RAD laboratories). Only fragments between 180 and 1000 bp were scored, considering bands as a diallelic characters (1 = band present, 0 = band absent). The two ISSR primers generated a total of 287 bands. The basic data structure consisted of a matrix of 87 rows and 288 columns, with one column identifying the individual and 287 columns describing the presence or absence of each of the bands.

Phylogenetic and population genetic analysis

ITS sequence data were entered in a contig file and edited using the program Seqed (Applied Biosystems). A total of 55 sequences of the nuclear internal spacers of ribosomal DNA (ITS) were obtained as a result of including *Q. coccifera* samples and all other *Quercus* individuals collected in the field (35) and sequences from GenBank (20). Despite the high intragenomic variation in ITS sequences reported in *Quercus*, we consider that the ITS sequences obtained were not pseudogenes because of their length, free energy of their RNA transcripts, their G+C content and their high sequence similarity (≈99%) to *Quercus* spp. sequences considered to be functional copies (Manos et al., 1999; Bellarosa et al., 2005). Chromatograms of ITS sequences were inspected to detect any nucleotide additivity that might indicate hybridisation. Nucleotide additivity is observed in forward and reverse chromatograms as equimolar proportions of alternative nucleotide peaks and suggests the presence of different ITS copies. The aligned matrix was produced with the program Clustal X (ftp://ftp-igbmc.u-strasbg.fr/pub/ClustalX/). Additivities were coded according to IUPAC recommendations, while no coding strategy for indels (sequence gaps) was considered. We used PAUP 4.10b (Swofford, 2000) to compute pairwise sequence divergence, frequency of G+C, and maximum parsimony (MP) analysis. We performed parsimony analyses with equal weighting of all characters and of transitions : transversions. Heuristic searches were performed with random taxon-addition, retaining all best trees, tree bisection-reconnection (TBR) branch swapping and the options MULPARS and STEEPEST DESCENT in effect. Relative support for clades identified by parsimony was assessed by bootstrapping (100 re-samplings of each data set). In addition, to determine the simplest model of sequence evolution that best fits the sequence data, the hierarchical likelihood ratio test (hLRT) and Akaike information criterion (AIC) were computed with Modeltest 3.06 (Posada and Crandall, 1998) for ITS1, 5.8 s and ITS2 regions separately. Among the 56 models implemented in the software, the GTR+G model was chosen for the ITS1, the JC model was chosen for the 5.8 s and the HKY+I model was the one chosen for the ITS2. Bayesian inference (BI) was used to construct a phylogeny of the whole ITS region partitioned according to these models with MrBayes 3.1.2 (Huelsenbeck and Ronquist, 2001; Ronquist and Huelsenbeck, 2003). MrBayes analyses were performed with the following settings: sampling for ten million generations, six MCMC, chain temperature 0.2; sample frequency 100; burn-in 7000. A 50% majority-rule consensus tree was calculated from the pooled sample to yield the final Bayesian estimates of phylogeny. Similar analyses were performed on a matrix containing only the ten sequences that did not exhibit additivities and the haplotypes recovered from GenBank. In this case BI analyses were done with a burn-in period of 3000 generations.

Pairwise genetic distances among ISSR phenotypes were computed with Dice's coefficient using the expression of Nei and Li (1979) as implemented in PAUP 4.10b*. These distances were then used to perform a bi-dimensional scaling (ALSCAL) of individuals with SPSS (SPSS, Inc., Chicago, IL, USA). Bayesian Analysis of Population Structure (BAPS v. 3.1; Corander et al., 2003, 2004) was used to estimate hidden structure by clustering individuals into panmictic groups and to determine lineage admixture within individuals.

Results

Morphological identification of individuals

All *Quercus* spp. individuals sampled in the field (87) presented diagnostic morphological characters that enabled unambiguous identification. Apart from the 81 *Q. coccifera* individuals, the taxa found within the kermes oak populations were holm oak (*Q. ilex* subsp. *ballota* (Desf.) Samp.; three individuals), cork oak (*Q. suber* L.; one individual), quejigo oak (*Q. faginea* subsp. *broteroi* (Coutinho) A. Camus; one individual) and downy oak (*Q. humilis* Miller; one individual). The geographic distribution of the samples can be seen in Fig. 1. The two additional holm oak samples (labeled as *Q. ilex* subsp. *ballota* Spain1–2 in Fig. 2) included in the analyses were clearly identified as *Q. ilex* subsp. *ballota* (Desf.) Samp.

ITS sequence analysis

Total length of the ITS region considered in the analyses varied between 599 and 607 bp in *Quercus* spp.,

with length variation depending on indels. The number of variable sites was 98, of which 59 were parsimony-informative. Twenty-six of the variable sites appeared to be Additive Polymorphic Sites (APS, sensu Fuertes Aguilar and Nieto Feliner, 2003). Sixteen APS were located in the ITS1 (positions 26, 43, 60, 68, 74, 82, 85, 100, 102, 131, 138, 152, 168, 169, 197 and 212), one in the 5.8 s (pos. 370) and nine were found in the ITS2 (pos. 408, 417, 419, 441, 517, 532, 546 and 568). APS and parsimony-informative sites are shown in Table 1. All additivities except six (positions 60, 197, 212, 370, 419, 517) appeared to be due to the coexistence of two alternative nucleotides also present independently in other matrix accessions. In particular, the two alternative nucleotides were found in GenBank pure specific accesssions (haplotypes) in all but nine positions (43, 68, 131, 168, 417, 441, 532, 546 and 568). Some cases of GenBank accessions exhibiting the nucleotide considered to be specific of a different taxon were also found (82, 102, 408). In the data set obtained by direct sequencing of field-collected material, 20 of the 30 *Q. coccifera* sequences and all (five) *Q. ilex* sequences contained APS. Phylogenetic reconstructions of ITS sequences are shown in Fig. 2. Within evergreen oaks, differentiation between *Q. suber* and the group formed by *Q. ilex* and *Q. coccifera* was supported by both BI and MP. Resolution within the latter clade was inconclusive. BI showed a cluster containing all holm oak samples except one with high support (posterior probability of 0.99), but this clade was not found in MP reconstructions. Additionally, one *Q. ilex* sample (ARJ) always displayed an intermediate position between *Q. coccifera* and *Q. ilex*. Consensus-tree topologies display limited resolution mostly as a result of: (1) insufficient number of informative characters; (2) character incongruence across accessions; (3) more than one character state (additivity) at the same informative sites. To determine if lack of phylogenetic resolution was due to the widespread presence of APS, sequences displaying additivities were excluded from the analysis. When this was done, holm and kermes oak came out as sister, monophyletic clades with moderately high support (1.00 pp and 72% bs for *Q. ilex*; 0.7 pp and 65% bs for *Q. coccifera*; Fig. 2b). In no case did *Q. coccifera* samples did not cluster in a way that could be related to geographic provenance of the plants.

ISSR analysis

Bi-dimensional (ALSCAL) ordination of Dice's distances among ISSR phenotypes revealed a diffuse pattern (Fig. 3). No clear differentiation at the species or the population level was found. All samples form a single, continuous cluster, although *Q. coccifera* from the same locality tended to group together and samples

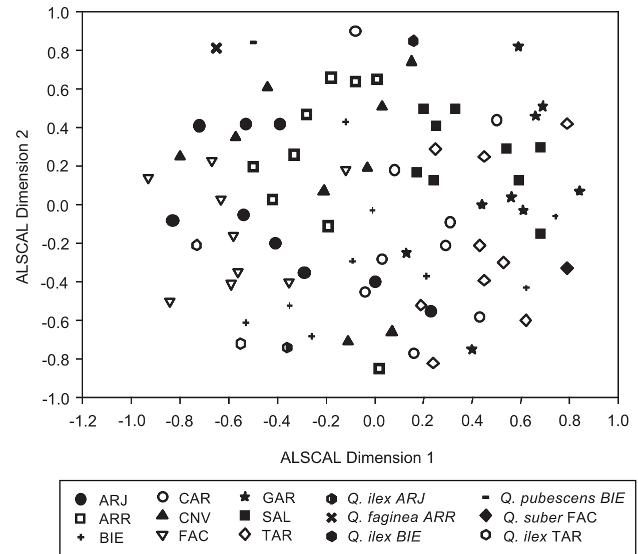

Fig. 3. Bi-dimensional scaling (ALSCAL) of Dice's distances among ISSR phenotypes from 87 *Quercus* samples.

from all other species included in the analysis were located in the periphery of the cluster. BAPS analyses revealed no differentiation either at the population or individual level (the partition with highest log marginal likelihood (−5800) was $k = 1$), indicating that all samples belong to the same panmictic group.

Discussion

Our results demonstrated extensive hybridisation in *Q. coccifera* populations of the Iberian Peninsula. Ongoing gene-flow and interspecific introgression may be responsible for limited molecular divergence of kermes oak populations and *Quercus* species. However, extensive hybridisation does not result in morphologically identifiable hybrid swarms.

Hybridisation detected in morphologically unambiguous individuals

Additive polymorphism in the ITS region was found in individuals morphologically assigned to either *Q. coccifera* or *Q. ilex* (Table 1). This additive pattern might be due to several reasons (see Fuertes Aguilar and Nieto Feliner, 2003, for a review). We regard recent and extensive hybridisation to be the most likely cause since concerted evolution of the ITS region of *Quercus* should make detection of additivities only possible in recent hybrids (Muir et al., 2001). This conclusion is in agreement with previous molecular results on interspecific introgression resulting in cpDNA lineages shared by *Q. ilex* and *Q. coccifera* (Jiménez et al., 2004; López

de Heredia et al., 2005; Lumaret et al., 2002). Our results proved that hybridisation is widespread, at least in the Iberian Peninsula: 20 out of 30 kermes and all (five) holm oak samples exhibited evidence of reticulation in the form of APS. Additionally, the results obtained with ISSR fingerprints showed that all *Quercus* individuals seem to belong to the same panmictic group (Fig. 3). In contrast, diagnostic morphological characters led to unequivocal taxonomic identification of all individuals. This phenotypic constancy in spite of genetic variations can be considered as a case of reduced phenotypic expression of genetic variance, i.e. as a case of genetic canalisation (Waddington, 1959). Even though the fixation of ITS lineages is quick in *Quercus* (Muir et al., 2001), canalisation may facilitate the expression of a specific phenotype in early stages of introgression, while genetic traces of hybridisation could still be detected. We hypothesise that the genetic variance observed in the present study is canalised to the extent that hybrids express only one of the parental phenotypes. Local environmental selective effects and topography have been found to determine the direction and ecological outcome of interspecific hybridisation in *Quercus* (Dodd and Afzal-Rafii, 2004; Morales et al., 2005). However, the similarity in habitat preferences of *Q. coccifera* and *Q. ilex* makes it difficult to foresee determinant ecological factors in the stabilisation of hybrids and their phenotypes. Alternatively, interspecific hybridisation may represent a stress factor that triggers genome remodelling to restore phenotypic stability or conservation through uniparental gene silencing or epigenetic changes (Madlung and Comai, 2004). Phenotypic resistance to the effects of genetic rearrangements (i.e. phenotypic homeostasis) might be further maintained by the environmental unpredictability of Mediterranean ecosystems, where the concourse of fluctuating selective regimes (Kawecki, 2000) seems to favour genetic canalisation (Valladares et al., 2002).

Hybridisation appears to be restricted to crosses of *Q. coccifera* with *Q. ilex*

Our results are fully congruent with gene exchange being limited to *Q. coccifera*–*Q. ilex* crosses, as no other interspecific hybridisation process was detected in ITS additivity patterns (Table 1). This was to some extent expected because both species share common habitats, have overlapping flowering periods (April–May) and taxonomic affinities (Castro-Díez and Montserrat-Martí, 1998; Manos et al., 1999; López González, 2002). *Q. coccifera* × *Q. suber* hybrids appeared to be rarer and were not detected in our study. This could be due to ecological sorting of both species, as *Q. suber* is mainly distributed in humid areas on acidic soils where *Q. coccifera* does not develop optimal populations. Additionally, direct crosses between *Q. suber* and *Q. coccifera* are hindered by a lag in flowering period (*Q. suber* flowering in May–June) and postpollination barriers (Boavida et al., 2001; Latorre and Cabezudo, 2002). Holm oak could mediate gene exchange between them since *Q. ilex* × *Q. suber* hybrids are frequent (Belahbib et al., 2001; Lumaret et al., 2002). In any case, hybridisation between kermes and cork oaks appears to be a rare event and has not been reported by any author (Franco, 1990). Conversely, morphologically intermediate forms between *Q. ilex* and *Q. coccifera* (*Q.* × *auzandrii* Gren. & Godron) are frequent enough to have been described in early botanical works (Abel, 1902), although they have always been acknowledged to be unusual in the wild (Carvalho e Vasconcelos and Amaral Franco, 1954). Unfortunately, time limitations in generating crosses appear to prevent obtaining first (F1) and second generation control hybrids, as no report of such an experiment was found.

Jiménez et al. (2004) suggested that incomplete lineage sorting between holm and kermes oaks might explain the lack of distinctive molecular characters separating both taxa. Although this explanation cannot be completely refuted at this time, evidence of cross-fertility, the existence of distinctive ITS copies in each species and the reported concerted evolution of the ITS region of *Quercus* support recurrent hybridisation as the main cause of molecular similarity.

The influence of hybrids on phylogenetic structure is small

When sequences with APS were excluded from phylogenetic analyses, support for *Q. ilex* and *Q. coccifera* as separate monophyletic groups was recovered (Fig. 2B). The resolution thus obtained did not affect relative position of the other taxa included in the analyses. White oak accessions always appeared as a clearly distinct group, while *Q. suber* sequences were resolved into a well-supported, monophyletic group sister to the *Q. ilex*–*Q. coccifera* lineage in all analyses. Fixation of nucleotides by concerted evolution together with restricted hybridisation in certain individuals may be responsible for the well-defined monophyletic groups observed after excluding ambiguous sequences. We hypothesise that processes like biased concerted evolution and recurrent introgression towards one of the parental species generate over time a strong phylogenetic signal. Such dynamics are mimicked by the exclusion of presumably recent hybrid individuals. Consequently, results obtained after exclusion of sequences with APS provide a plausible phylogenetic outcome for current reticulation patterns. The rare occurrence of intermediate morphotypes between

Q. coccifera and *Q. ilex* (*Q.* × *auzandrii* Gren. & Godron) might be due to the fact that "hybrid" morphological characters are unstable and rapidly disappear, while the apparition of nucleotides specific to another taxon in allegedly pure GenBank sequences (clone accessions) can be considered as the result of concerted evolution events.

ISSR results give additional evidence of recent and recurrent gene-flow across *Quercus* taxa; (Fig. 3). This result is congruent with a long coexistence of several *Quercus* species and highly diverse lineages within them in the Iberian Peninsula which may have enabled multiple contacts (Lumaret et al., 2002; Petit et al., 2002). ISSR fragment homoplasy makes it difficult to determine allele sharing between species, but the genetic relatedness rendered by the ALSCAL analysis together with the ITS additivity patterns and the easiness of recovering key-morphological characters suggests a rapid reestablishment of typical species features, followed by a decelerate genome stabilisation, in the sequence: morphology-nrDNA-fingerprints.

Conclusions

Extensive gene-flow among *Q. coccifera* populations of the Iberian Peninsula and between this species and *Q. ilex* resulted in limited molecular differentiation at the species level. Conversely, phylogenetic reconstructions excluding accessions of genetically ambiguous individuals resulted in monophyletic groups of species. In spite of molecular evidence for widespread hybridisation between kermes and holm oaks, morphologically identifiable hybrids are rare. We propose a recurrent evolutionary process in which canalisation of key characters of evergreen oaks contributes to early morphological stabilisation after hybridisation events, followed by genetic stabilisation of nrDNA markers by concerted evolution and introgression towards one of the parental species.

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References

Abel, A., 1902. De quelques *Quercus* hybrides ou supposés tels, de *Quercus ilex* et *coccifera*. Bull. Acad. Int. Géogr. Bot. 3, 129–131.

Andreasen, K., Baldwin, B.G., Bremer, B., 1999. Phylogenetic utility of the nuclear rDNA ITS region in subfamily *Ixoroideae* (Rubiaceae): comparisons with cpDNA rbcL sequence data. Plant Syst. Evol. 217, 119–135.

Arnold, M.L., 1997. Natural hybridization and evolution. Oxford University Press, Oxford, UK.

Belahbib, N., Pemonge, M.-H., Ouassou, A., Sbay, H., Kremer, A., Petit, R.J., 2001. Frequent cytoplasmic exchanges between oak species that are not closely related: *Quercus suber* and *Q. ilex* in Morocco. Mol. Ecol. 10, 2003–2010.

Bellarosa, R., Simeone, M.C., Papini, A., Schirone, B., 2005. Utility of ITS sequence data for phylogenetic reconstruction of Italian *Quercus* spp. Mol. Phylogenet. Evol. 34, 355–370.

Boavida, L.C., Silva, J.P., Feiji, J.A., 2001. Sexual reproduction in the cork oak (*Quercus suber* L.). II. Crossing intra- and inter-specific barriers. Sex. Plant Reprod. 14, 143–152.

Carvalho e Vasconcelos, J., Amaral Franco, J., 1954. Carvalhos de Portugal. An. Inst. Super. Agron. 21, 1–136.

Castro-Díez, P., Montserrat-Martí, G., 1998. Phenological pattern of fifteen Mediterranean phanerophytes from *Quercus ilex* communities of NE-Spain. Plant Ecol. 139, 103–112.

Corander, J., Waldmann, P., Sillanpaa, M.J., 2003. Bayesian analysis of genetic differentiation between populations. Genetics 163, 367–374.

Corander, J., Waldmann, P., Marttinen, P., Sillanpaa, M.J., 2004. BAPS 2: enhanced possibilities for the analysis of genetic population structure. Bioinformatics 20, 2363–2369.

Costa Tenorio, M., Morla Juaristi, C., Sainz Ollero, H., 1997. Los bosques ibéricos, una interpretación geobotánica.

Dodd, R.S., Afzal-Rafii, Z., 2004. Selection and dispersal in a multispecies oak hybrid zone. Evolution 58, 261–269.

Franco, J.A., 1990. *Quercus* L. In: Castroviejo, A., Laínz, M., López-González, G., Montserrat, P., Muñoz Garmendia, F., Paiva, J., Villar, L. (Eds.), Flora Iberica, vol. II. CSIC, Madrid, pp. 15–36.

Fuertes Aguilar, J., Nieto Feliner, G., 2003. Additive polymorphisms and reticulation in an ITS phylogeny of thrifts (*Armeria*, Plumbaginaceae). Mol. Phylogenet. Evol. 28, 430–447.

Fuertes Aguilar, J., Rossello, J.A., Nieto Feliner, G., 1999. Nuclear ribosomal DNA (nrDNA) concerted evolution in natural and artificial hybrids of *Armeria* (Plumbaginaceae). Mol. Ecol. 8, 1341–1346.

Grant, V., 1971. Plant Speciation. Columbia University Press, New York.

Hess, J., Kadereit, J.W., Vargas, P., 2000. The colonization history of *Olea europaea* L. in Macaronesia based on internal transcribed spacer 1 (ITS-1) sequences, randomly amplified polymorphic DNAs (RAPD), and intersimple sequence repeats (ISSR). Mol. Ecol. 9, 857–868.

Huelsenbeck, J.P., Ronquist, F., 2001. MRBAYES: Bayesian inference of phylogenetic trees. Bioinformatics 17, 754–755.

James, J.K., Abbott, R.J., 2005. Recent, allopatric, homoploid hybrid speciation: the origin of *Senecio squalidus* (Asteraceae) in the British Isles from a hybrid zone on Mount Etna, Sicily. Evolution 59, 2533–2547.

Jiménez, P., López de Heredia, U., Collada, C., Lorenzo, Z., Gil, L., 2004. High variability of chloroplast DNA in three Mediterranean evergreen oaks indicates complex evolutionary history. Heredity 93, 510–515.

Kawecki, T.J., 2000. The evolution of genetic canalization under fluctuating selection. Evolution 54, 1–12.

Labra, M., Grassi, F., Sgorbati, S., Ferrari, C., 2006. Distribution of genetic variability in southern populations of Scots pine (*Pinus sylvestris* L.) from the Alps to the Apennines. Flora 201, 468–476.

Latorre, A.V.P., Cabezudo, B., 2002. Use of monocharacteristic growth forms and phenological phases to describe and differentiate plant communities in Mediterranean-type ecosystems. Plant Ecol. 161, 231–249.

López de Heredia, U., Jiménez, P., Díaz Fernández, P., Gil, L., 2005. The Balearic Islands: a reservoir of cpDNA genetic variation for evergreen oaks. J. Biogeogr. 32, 939–949.

López González, G., 2002. Guía de los árboles y arbustos de la Península Ibérica y Baleares. Ediciones Mundi-Prensa, Madrid.

Lumaret, R., Mir, C., Michaud, H., Raynal, V., 2002. Phylogeographical variation of chloroplast DNA in holm oak (*Quercus ilex* L.). Mol. Ecol. 11, 2327–2336.

Madlung, A., Comai, L., 2004. The effect of stress on genome regulation and structure. Ann. Bot. 94, 481–495.

Manos, P.S., Doyle, J.J., Nixon, K.C., 1999. Phylogeny, biogeography and processes of molecular differentiation in *Quercus* subgenus *Quercus* (Fagaceae). Mol. Phylogenet. Evol. 12, 333–349.

Martínez-Ferri, E., Manrique, E., Valladares, F., Balaguer, L., 2004. Winter photoinhibition in the field involves different processes in four co-occurring Mediterranean tree species. Tree Physiol. 24, 981–990.

Mayol, M., Rosselló, J.A., 2001. Why nuclear ribosomal DNA spacers (ITS) tell different stories in *Quercus*. Mol. Phylogenet. Evol. 19, 167–176.

Morales, R., Vicente, J.A., Galán de Mera, A., 2005. cpDNA evidence of introgressions in *Quercus* L. (*Fagaceae*). The influence of phytotopographic position. Flora 200, 222–228.

Muir, G., Fleming, C.C., Schlötterer, C., 2000. Species status of hybridizing oaks. Nature 405, 1016.

Muir, G., Fleming, C.C., Schlötterer, C., 2001. Three divergent rDNA clusters predate the species divergence in *Quercus petraea* (Matt.) Liebl. and *Quercus robur* L. Mol. Biol. Evol. 18, 112–119.

Nei, M., Li, W.-H., 1979. Mathematical model for studying genetic variation in terms of restriction endonucleases. Proc. Natl. Acad. Sci. USA 76, 5269–5273.

Nieto Feliner, G., Fuertes, J., Roselló, J.A., 2001. Can extensive reticulation and concerted evolution result in a cladistically structured molecular data set? Cladistics 17, 301–312.

Petit, R.J., Csaikl, U.M., Bordacs, S., Burg, K., Coart, E., Cottrell, J., van-Dam, B., et al., 2002. Chloroplast DNA variation in European white oaks – phylogeography and patterns of diversity based on data from over 2600 populations. For. Ecol. Manage. 156, 5–26.

Petit, R.J., Bodénès, C., Ducousso, A., Roussel, G., Kremer, A., 2003. Hybridization as a mechanism of invasion in oaks. New Phytol. 161, 151–164.

Posada, D., Crandall, K.A., 1998. MODELTEST: testing the model of DNA substitution. Bioinformatics 14, 817–818.

Ritz, C.M., Schmuths, H., Wissemann, V., 2005. Evolution by reticulation: European dogroses originated by multiple hybridization across the genus *Rosa*. J. Hered. 96, 4–14.

Ronquist, F., Huelsenbeck, J.P., 2003. MrBayes 3: Bayesian phylogenetic inference under mixed models. Bioinformatics 19, 1572–1574.

Sang, T., Crawford, D.J., Stuessy, T.F., 1995. Documentation of reticulate evolution in peonies (*Paeonia*) using internal transcribed spacer sequences of nuclear ribosomal DNA: implications for biogeography and concerted evolution. Proc. Natl. Acad. Sci. 62, 6813–6817.

Sun, Y.D.Z., Skinner, G.H., Liang, S., Hulbert, H., 1994. Phylogenetic analysis of Sorghum and related taxa using internal transcribed spacers of nuclear ribosomal DNA. Theor. Appl. Genet. 89, 26–26.

Swofford, D.L., 2000. PAUP*, Phylogenetic analysis using Parsimony* and Other Methods. Sinauer, Sunderland, MA.

Takhtajan, A., 1986. Floristic Regions of the World. University of California Press, Berkeley, CA.

Valladares, F., Balaguer, L., Martínez-Ferri, E., Pérez-Corona, E., Manrique, E., 2002. Plasticity, instability and canalization: is the phenotypic variation in seedlings of sclerophyll oaks consistent with the environmental unpredictability of Mediterranean ecosystems? New Phytol. 156, 457–467.

Van Valen, L., 1976. Ecological species, multispecies, and oaks. Taxon 25, 233–239.

Vargas, P., Baldwin, B.G., Constance, L., 1998. Nuclear ribosomal DNA evidence for a western North American origin of Hawaiian and South American species of *Sanicula*. Proc. Natl. Acad. Sci. 95, 235–240.

Vargas, P., McAllister, H.A., Morton, C., Jury, S.L., Wilkinson, M.J., 1999. Polyploid speciation in *Hedera* (Araliaceae): phylogenetic and biogeographic insights based on chromosome counts and ITS sequences. Plant Syst. Evol. 219, 165–179.

Waddington, C.H., 1959. Canalization of development and genetic assimilation of acquired characters. Nature 183, 1654–1655.

White, T.J., Bruns, T., Lee, S., Taylor, J., 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis, M.D., Gelfand, D., Sninsky, J., White, T. (Eds.), PCR Protocols: A Guide to Methods and Applications. Academic Press, San Diego, CA, pp. 315–322.

Wolfe, A.D., Xiang, Q.Y., Kephart, S.R., 1998. Assessing hybridization in natural populations of *Penstemon* (Scrophulariaceae) using hypervariable intersimple sequence repeat (ISSR) bands. Mol. Ecol. 7, 1107–1125.

Zhang, W., Wendel, J.F., Clark, L.G., 1997. Bamboozled again! Inadvertent isolation of fungal rDNA sequences from bamboos (Poaceae: Bambusoideae). Mol. Phylogenet. Evol. 8, 205–217.