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Multiple genome relationships and a complex biogeographic history in the eastern range of *Quercus suber* L. (Fagaceae) implied by nuclear and chloroplast DNA variation

Simeone[1,*] Marco Cosimo, Alessio Papini[2], Federico Vessella[1], Rosanna Bellarosa[1], Francesco Spada[3] and Bartolomeo Schirone[1]

[1]Dipartimento di tecnologie, ingegneria e scienze dell'Ambiente e delle Foreste (D.A.F.), Università degli Studi della Tuscia, via S. Camillo de Lellis, 01100 Viterbo, Italy.
[2]Dipartimento di Biologia Vegetale, Università degli Studi di Firenze, via La Pira 4, 50121 Firenze, Italy.
[3]Dipartimento di Biologia Vegetale, Università degli Studi di Roma "La Sapienza" Piazzale Aldo Moro 5, 00185 Roma, Italy.

Abstract — The complex evolutionary history of *Quercus suber* has been throughly investigated in many recent works, but the details of its differentiation processes are still largely unknown. In addition, the geographical and evolutionary roles of the eastern parts of the species range have gained much less attention compared to other southern European areas. In order to fill this gap, new insights to infer the species diversification and range establishment of the cork oak in the east-central Mediterranean are here provided by means of inter- and intra-specific plastid DNA and nuclear ribosomal ITS phylogeographic studies. We analyzed 95 natural cork oak populations; 6 closely related, sympatric oaks were included in the study and used for comparisons.
Evidence for a clear phylogeographical structure was detected with PCR-RFLP at 5 chloroplast loci, while ITS sequence variation is apparently unrelated with the geographical distribution. Five chloroplast haplotypes and three ITS main lineages were identified. Three haplotypes and all ITS lineages occur in the Italian Peninsula, stressing the importance of these territories for the evolutionary history of the species. Two divergent "Italian" haplotypes are highly shared, and one ITS variant is basal to the ingroup, revealing sister relationships within *Cerris* taxonomic group. Hypotheses of hybridization, lineage sorting of ancient DNA polymorphisms and of reticulate evolution of the whole species group are presented and discussed.

Keywords: *Cerris* group, chloroplast DNA, cork oak, hybridization, ITS sequences, phylogeography.

INTRODUCTION

The genus *Quercus* (Fagaceae) is widespread across the northern Hemisphere with about 400-500 species in Europe, North Africa, Asia and North America (Nixon 1989; 1993). Naturally restricted to the western Mediterranean (s.l.) regions, the cork oak (*Quercus suber* L.), along with the holm oak (*Quercus ilex* L.) and the kermes oak (*Q. coccifera* L.), is the most common evergreen arboreal oak growing in the Mediterranean area, and it occurs in three out of ten biodiversity hotspots detected in the Mediterranean Basin (Medail and Quezel 1999). Evergreen oaks are suggested to have coexisted in this area since pre-Pliocene, as a result of Tertiary westwards colonisation from Peri-Balkanik areas (Palamarev 1989; Toumi and Lumaret 2001).

According to Daget (1977), the range of *Q. suber* corresponds to areas where an "oceanic Mediterranean climate" rules. The species grows along the coastlines of those European (Italy, France, Spain) and North African countries (Tunisia, Algeria, and Morocco) facing the western Mediterranean sea, on the Balearic islands and in Corsica, Sardinia and Sicily where it is also found in the interior. In the Atlantic region its main distribution is in Portugal and along the Spanish, French, and Moroccan coasts. The easternmost stands of the species belong to small enclaves scattered along the coast of Apulia (southeastern

*Corresponding author: phone: +39 0761 357393; fax: +39 0761 357413; e-mail: mcsimeone@unitus.it

Italy), whereas its past occurrence in the Balkans is historically contradictory and generally unconsidered (DE PHILIPPIS 1935, and references therein; AMIGUES 1991).

Quercus suber belongs to subgen. *Cerris* (Spach.) Öersted. [Subgen *Cerris sensu* SCHWARZ (1936-39), *Cerris* group *sensu* NIXON (1993), Sect. *Cerris* (Subsect. *Suber* and *Macrolepides*) *sensu* CAMUS (1936-54)]. This heterogeneous group of oaks does not occur far beyond the northwestern Alps, and it has not yet found either a phylogenetic or a satisfactory taxonomical definition, due to its complexity (the group prevalently occurs in Eurasia with 20-40 species, characterized by long linear styles, aborted basal ovules, an often hairy inner pericarp, annual/biennial fruit maturation, evergreen, deciduous and semi-deciduous leaves; cf. NIXON 1993). *Quercus suber* is among the few representatives of this group in western Europe, and it shares its habitat with only a few species of the *Cerris* group in France and Italy, (i.e. *Q. cerris* L. and *Q. crenata* Lam. across the whole Italian Peninsula and southeastern France, *Q. trojana* Webb. and *Q. macrolepis* Kotschy in southeastern Italy).

It is widely acknowledged that palaeogeographical processes (plate tectonics, sea transgressions-regressions), climatic fluctuations (pre- and post-glacial colonisation events), evolutionary processes (speciation, adaptation, hybridization), and human activity all account for the detected spatial patterns of genetic variation in oaks (TOUMI and LUMARET 2001; PETIT *et al*. 2002; PETIT 2004; GRIVET *et al*. 2006; LOPEZ DE HEREDIA *et al*. 2007; MAGRI *et al*. 2007). Nevertheless, several issues regarding the extent of such variation in the cork oak, and the evolutionary history that shaped its biogeography are still unknown and controversial. This is partly due to the available palaeobotanical and palaeoecological data, that are probably still insufficient to assess the species history and the importance of the geological and climatic history of the Mediterranean area on its present distribution – especially in the central eastern parts, i.e. the Italian Peninsula (KOVAR-EDER *et al*. 2006). In addition, no or often contrasting phylogenies were produced with different molecular markers and on partially incomplete range samplings (cf. LUMARET *et al*. 2005 and MAGRI *et al*. 2007), and still little is known about the roles played by species relationships on the evolution of *Q. suber*. As a point of facts, the strong attitude of oaks to interspecific hybridization (BURGER 1975) copes with an extremely limited body of investigations on those species sharing closest affinities with *Q. suber*, under the taxonomical, geographical, and ecological points of view.

The genetic variation of the cork oak was first analyzed by TOUMI and LUMARET (1998) who detected two main sets of populations from two distinct geographic areas, the Iberian Peninsula and West France at one side, North Africa, Italy, Corsica and Provence at the other. However, 20% of the populations did no fit into this framework and no description of the phylogeographic structure was provided.

More recently, several studies on organellar DNA evidenced important macro-regions of diversity and stressed the major role of introgression processes in the genetic structure of *Q. suber*. Although the new gathered information were extremely precious to depict a clearer scenario, still no exhaustive data on the phylogeography of the species were provided, and detailed information from some districts (i.e. North Africa, Central East Mediterranean) are still poor.

High haplotype sharing between *Q. suber* and *Q. ilex* (and between *Q. ilex* and *Q. coccifera*) was found in a study of 90 cork oak populations mostly from the western part of the range (71 from the Iberian Peninsula) by JIMENEZ *et al*. (2004), who recognized, however, the occurrence of 4 *Q. suber* haplotypes – one with a prevalent Iberian-Moroccan distribution and three characterising discrete areas of the central Mediterranean region. This geographical distribution of cpDNA variation was confirmed by LUMARET *et al*. (2005) by means of RFLPs of the chloroplast genome on 91 populations and with microsatellites (LUMARET and JABBOUR-ZAHAB 2009). Noteworthy, in this latter study some new and rare chlorotypes were found, and the authors postulated the most ancient ones to be located in the eastern part of the species range. On the other hand, the most recent work on the chloroplast SSR diversity in cork oak (MAGRI *et al*. 2007) demonstrated a geographical distribution of the haplotypes consistent with the Oligocene and Miocene break-up events of the European-Iberian continental margin, pointing at west-central Europe as the most probable area where the species differentiation took place some 15-35 MYA (Million Years Ago).

Our paper aims to provide a contribution towards the assessment of the genetic diversity of cork oak throughout its total range. Its speciation processes are inspected by implementing available data with new information from cpDNA variation in (a) traditionally neglected areas, (b) closely related taxa, and (c) with a molecular phylogeny of the internal transcribed spacer (ITS) region of nuclear ribosomal DNA.

Focus has been given to populations from the eastern part of the species' range. New documentation on the past and present occurrence of the species in the Balkans has been collected and data from Algerian stands of cork oak are presented for the first time. Six additional oak species (*Q. coccifera*, *Q. ilex*, *Q. cerris*, *Q. crenata*, *Q. trojana*, *Q. macrolepis*), closely related and sympatric with *Q. suber* in Italy, were included in the analyses to elucidate patterns of species- and area-relationships. With the exception of *Q. coccifera* and *Q. ilex*, all the species analysed in this study belong to subgen. *Cerris*, and no phylogeographical data on this important *taxon* have been published so far.

Although there may be other possible factors causing incongruence between the two markers (different effective population size, modes of inheritance and mutation rates among the others), congruence of cpDNA and ITS sequence variation, coupled with accuracy in the species range definition and exhaustive sampling strategies, is expected to either corroborate current assumptions or to reveal previously unnoticed evolutionary scenarios for the cork oak.

MATERIALS AND METHODS

Sampling and DNA extraction - Plant material was collected from individuals of *Q. suber* in 95 natural stands throughout the species range. Four (Tunisia) to nine (Spain) populations per geographical region were sampled, with a more exhaustive investigation (56 populations) on Italian woodland. Three to thirteen individuals per population were analysed (410 total trees), with the exception of five relic locations where only one or two samples occurred. Seventy-six trees (29 populations) belonging to 6 oak species sympatric with *Q. suber* were sampled in Italy and further East. Geographical and genetic data of the populations analized are provided as supplementary material. Total genomic DNAs were extracted individually from green tissues (leaves and buds) using the DNeasy Plant minikit (QIAGEN), following the instructions supplied by the manufacturer.

Chloroplast DNA PCR-RFLP - Chloroplast DNA was amplified with universal primers (fragments TF, CD, DT, AS, SR; TABERLET *et al.* 1991; DEMESURE *et al.* 1995). Amplification, digestion, and electrophoretic procedures followed JIMENEZ *et al.* (2004). The average within population gene diversity (h_S), the total gene diversity (h_T), the differentiation for unordered (G_{ST}) and for ordered alleles (N_{ST}) were calculated with the software PERMUT (http://www.pierroton.inra.fr/genetics/labo/Software), according to PONS and PETIT (1996). One thousand random permutations of haplotype identities were made, keeping the haplotype frequencies and the matrix of pairwise differencies. Only populations represented by three or more individuals were considered. Statistical parsimony was used to reconstruct phylogenetic relationships between haplotypes (TCS, version 1.21; CLEMENT *et al.* 2000).

Nuclear ribosomal ITS sequences - As a preliminary screening, we amplified the 5.8S nrDNA and flanking ITS1 and ITS2 regions in three cork oak trees from each of the following stands: Brindisi, Monte S. Biagio, Tuscania (peninsular Italy), Sassari (Sardinia), Ain Sobh (Tunisia), Albuquerque (Spain), and in *Q. cerris* and *Q. crenata*. Universal primers (WHITE *et al.* 1990) and the following PCR conditions were used: 1 min. at 94° (denaturation), 2 min. at 54° (annealing), and 1 min. at 72° (polimerization). PCR products were recovered, ligated in pUC8, and cloned in DH5α competent cells. Five clones were sequenced per each tree. This approach allowed the identification of two dominant ITS sequence types in every individual. One type belonged to a class of heterogeneous ITS repeats characterised by long deletions, accumulation of mutations in the 5.8S region and low GC content, whereas the second one grouped a homogeneous cluster of sequences sharing all known functional and structural ITS features of oaks (MAYOL and ROSSELLÒ 2001), congruently with all available *Quercus* ITS datasets (MANOS *et al.* 1999; 2001). This group was represented by a single sequence in every individual and was thus interpreted as a functional ITS repeat type. In order to avoid the highly divergent ITS paralogous sequences, detection of the functional repeats was then performed by use of specific primers and PCR conditions (BELLAROSA *et al.* 2005). The preferential amplification of a single repeat type by means of reaction conditions is referred to as PCR selection (WAGNER *et al.* 1994).

Three cork oak individuals per population were analysed. Sequencing and data collection were performed as in BELLAROSA *et al.* (2005). The boundaries of the internal transcribed spacers (ITS1, ITS2) and 5.8S coding region, the predicted secondary structures of the ITS1 and ITS2 RNA transcripts and their fold predictions were made as in BELLAROSA *et al.* (2005) and used to provide accuracy in sequence alignment and to test sequence orthology (MAYOL and ROSSELLÒ 2001).

Phylogenetic analysis of the ITS fragments - Optimal multiple alignment was obtained with

CLUSTALW 1.81 (THOMPSON *et al.* 1994) and checked by eye. Only the ITS alleles showing intra-population variation were included in the phylogenetic analysis (e.g. sequences sharing 100% identity within a single population were considered once). Parsimony analysis was performed with PAUP 4.0b1 (SWOFFORD 1998). All characters were weighted equally, and character state transitions were treated as unordered. Gaps were treated as "simple indel coding" after SIMMONS and OCHOTERENA (2000), coding them with the software Gapcoder (YOUNG and HEALY 2003). The maximum parsimony analysis was done with 100 replicated heuristic searches, using random stepwise addition of taxa, TBR branch swapping, and MULPARS in effect. A maximum parsimony bootstrap analysis (FELSENSTEIN 1985) was run in PAUP with default options: branch support was estimated using bootstrap sampling with 1000 pseudoreplicates and 10 random-addition replicates with a full heuristic search.

A neighbor-joining analysis (SAITOU and NEI 1987) was done with PAUP, with Kimura's 2 parameters distance (KIMURA 1980) with equal base frequencies and unequal Transition/transversion ratio.

A maximum likelihood (FELSENSTEIN 1981) search approach was performed as follows: we deleted gaps data from the matrix and used Modeltest 3.06 (POSADA and CRANDALL 1998) to evaluate the likelihood of 56 different models of sequence evolution on the basis of our data. The likelihood ratio test option in Modeltest 3.06 was used to compare likelihood scores in a nested design. Also MrModeltest 2.0 (NYLANDER 2004) was used under the Akaike Information Criterion to evaluate the best models. We used the most likely model of evolution from Modeltest 3.06 as settings in a maximum likelihood (ML) phylogenetic analysis in PAUP. Also the Bayesian analysis was done using the model of sequence evolution obtained with Modeltest and MrModeltest. Five independent Bayesian analyses were performed on the combined data set using the software MrBayes 3.0b4 (HUELSENBECK and RONQUIST 2001). The analysis was initiated from a random tree and run in a Markov chain for 10^6 cycles with tree sampling every 100th cycle in the chain. Four chains were run simultaneously. Following the analysis, the posterior probabilities were checked in the output of Mrbayes to estimate the number of trees that should be discarded as "burn-in." After the "burn-in" trees were removed from the data set, trees were used to produce a majority-rule consensus in which the percentage support is equivalent to Bayesian posterior probabilities.

To test the significance of the difference of the maximum likelihood tree with parsimony criterion, the Templeton test (TEMPLETON 1983) was used as implemented in PAUP. To test the maximum parsimony trees under the likelihood criterion we used the Kishino-Hasegawa test (KISHINO and HASEGAWA 1989) with RELL option on.

RESULTS

CpDNA variation in Quercus suber - A total number of 52 fragments were generated by PCR-RFLP. All primer-enzyme combinations were polymorphic within *Q. suber*, and led to the identification of five haplotypes. The observed total genetic diversity (h_T) was very high (0.675, se = 0.0338) and the average differentiation within populations (h_S) particularly low (0.031, se = 0.0152). The level of population subdivision, as expressed by the values of G_{ST} and N_{ST} statistics, is significantly high and the two coefficients were quite similar: G_{ST} was 0.954 (se = 0.0226), and N_{ST} (0.956, se = 0.0254) was slightly but not significantly higher than G_{ST} when tested with 1,000 permutations.

A geographical organization of genetic variation is evident from the haplotype frequency map (Fig. 1a).

No new haplotypes were found in the newly investigated areas (Algeria and Croatia); nevertheless, occurrence of haplotypes in these 2 regions is consistent with that of neighbouring areas. Besides H2, one single haplotype (H1) appears to be dominant in Italy; these 2 haplotypes are closely related and differ by only 2 mutations evidenced with TF/Hinf I and SR/Hinf I primer/enzyme combinations. Haplotype H3, previously known only for Provence-Corse-Sardinia-Tunisia, was detected in northwestern Italy, as well. The haplotypes were fixed in every population, with the exception of Ostuni (Italy), Oulmes (Morocco), Chlef (Algeria) where we detected the presence of neighbouring haplotypes H1-H2 and H4-H5, and Massa Marittima, (Italy) and Erbalunga (Corse) where long distance dispersal of haplotypes H1 and H3 was found.

Data from sympatric oaks (Fig. 1b) show that H5 belongs to the *Q. ilex* lineage (JIMENEZ *et al.* 2004) with 11 evolutionary events from the closest *Suber* haplotype (H4), H1 is fully shared with *Q. cerris* (8 populations from Italy, 2 from Croatia, 1 from Albania and 1 from Macedonia) and *Q. crenata* (6 populations), while H2 was found also

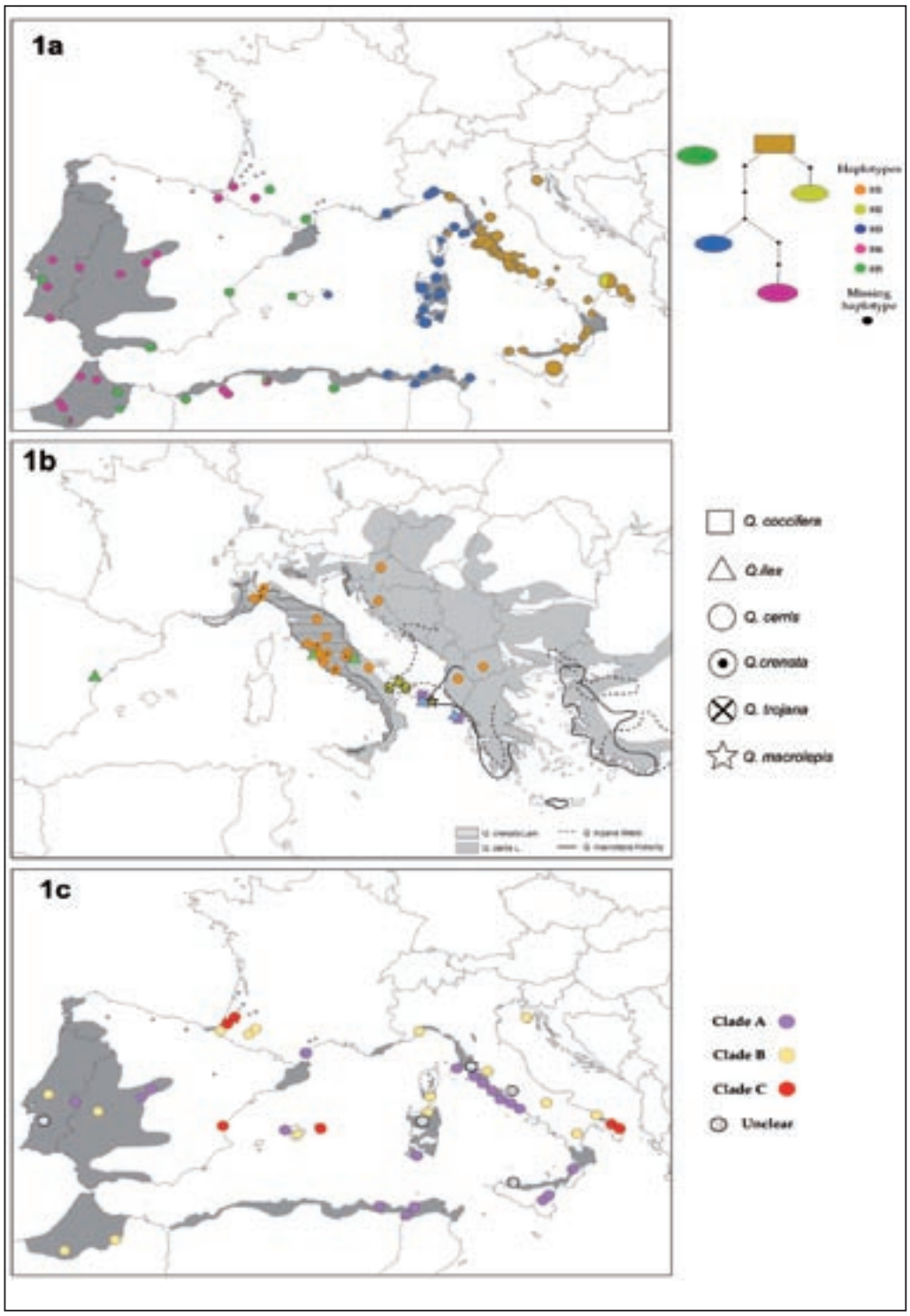

Fig. 1a — Distribution of cpDNA haplotypes within *Q. suber* populations throughout its natural range and phylogenetic reconstruction of the relationships among haplotypes using statistical parsimony. Size of pies indicates the number of individuals for each population (from 1 to 13).
Fig. 1b — Map of cpDNA haplotypes in 6 oak taxa sympatric with *Q. suber*. Distibution of *Cerris* group taxa are reported after Jalas and Suominen (1976) and Pignatti (1982). Shared haplotypes are indicated with the same colours as in Fig. 1a.
Fig. 1c — Distribution of *Quercus suber* ITS variants throughout its natural range. Symbols are to be referred to the main branches of cladogram in Fig. 3 (see text for explanations).

in *Q. macrolepis* (1 population) and *Q. trojana* (3 populations). Besides the haplotype shared with the Spanish *Q. ilex* population, highly divergent haplotypes were those produced by 3 Italian and 1 Greek *Q. ilex* populations (2 haplotypes) and by *Q. coccifera* (the same haplotype in 2 populations from Greece and southeastern Italy).

ITS region in Quercus suber - Nucleotide sequences of the internal transcribed spacers (ITS1 and ITS2) and the 5.8S coding region of nuclear ribosomal DNA repeats were obtained from cork oak individuals representing 39 provenances across the total species range.

In the majority of the stands, the three analyzed individuals showed 100% identical ITS sequences. Exceptions were the populations of Tuscania, Sassari, Brindisi, Catania (Italy), Castelljaloux, Seignosse (France), and Mallorca (Spain), in which two different ITS variants were detected. Consequently, the phylogenetic analysis included 46 ITS sequences. Of these, some from different locations were 100% identical; in total, 32 ITS sequence variants were found. All sequences are deposited in the Genbank under the accession numbers AY827107-AY827145, and EF581307-EF581312.

The cork oak ITS sequences displayed only 2 length differences and could be unambiguously aligned, producing a consensus sequence of 592 bp. Two indels were found: a stretch of 7 bp at position 56-63 (ITS1) in the samples of Minorca, Valencia, Brindisi, Seignosse, Seignosse2, and in all outgroups used in the analysis; a 1bp at position 402 (ITS2) in the samples of Minorca, Valencia, Brindisi, Brindisi2, Seignosse, Seignosse2, and only in *Q. coccifera* and *Q. cerris* among the outgroups. Indels were confirmed with sequence analysis of two additional individuals from Brindisi and two from Seignosse. The sequences alignment (synonymous sites omitted) is shown in Fig. 4.

Thermodynamically, the secondary structures of ITS1 and ITS2 RNA transcripts showed a fairly uniform stability, as inferred from the lower free energy values, ranging from -89.4 to -93.6 (kcal/mol) and from -101.0 to -102.9 (kcal/mol), for the two spacers, respectively. These data agree with the accuracy tests of secondary structures of rRNAs (MATHEWS *et al*. 1999), as well as with previous studies involving ITS transcripts analysis of oak species (MAYOL and ROSSELLÒ 2001).

Every ITS model yielded absolutely uniform fold predictions, well in line with the known substructural features of vascular plants ITS transcripts (HERSHKOWITZ and ZIMMER 1996; MAI and COLEMAN 1997; COLEMAN 2003). The nucleotide substitutions did not affect the uniformity of the models (compensatory or hemi-compensatory base change). The 7 bp stretch detected in the ITS1 transcripts of Brindisi, Seignosse, Valencia, and Minorca sequences caused a slightly longer helix III, which made the RNA foldings more similar to those predicted for other *Quercus* species (e.g. *Q. ilex*, *Q. coccifera, Q. cerris*, *Q. macrolepis*, *Q. trojana*).

HERSHKOWITZ and ZIMMER (1996) showed that among flowering plants the ITS2 region is characterised by the presence of a complex pattern of conserved (c1-c6) and variable (v1-v6) domains. Accordingly, the substitutions detected across all samples were located in the variable regions v2 and v6. All these data indicate high probabilities that only functional ITS sequences were included in the present study. The necessity of showing that sequenced nrDNA fragments are functional is important to suppose that those fragments are really orthologous and not paralogous. This question is crucial in systematic studies employing nrDNA sequences (Bailey et al. 2003). (BAILEY *et al*. 2003).

Phylogenetic analysis - The data matrix listed 53 taxa (with four outgroups) and 595 characters (545 constant, 21 variable but parsimony-uninformative and 29 parsimony-informative). Six characters (four parsimony-informative) deriving from indel coding were added in the maximum parsimony analysis (heuristic search and bootstrap).

The maximum parsimony analysis produced 13320 most parsimonious trees 85 steps long, consistency index (CI) = 0.682 and retention index (RI) = 0.866. The strict consensus tree reported in Fig. 2 shows all the analyzed sequences of *Q. suber* clustering together in a monophyletic clade with *Q. coccifera* (AY226834), *Q. ilex* (AY226837), and *Q. cerris* (accession 1: AY226832, accession 2 AY226833) as outgroups. We tested the position of *Q. trojana, Q. crenata and Q. macrolepis* (GenBank acc. AY226843, AY226844 and AY226845), also. The strict consensus tree (parsimony) maintained *Q. coccifera, Q. ilex*, and *Q. cerris* as outgroups, but showed an unresolved polytomy formed by three monophyletic group (Fig. 2): *Q. trojana*, *Q. suber* group C and *Q. macrolepis* (basal position) + group A and B with *Q. crenata* nested within.

Two groups of *Q. suber* provenances appeared clearly separated from the bulk. These groups of provenances belong (1) to the Italian Tyrrhenian coast and (2) to a group of geographically unrelated stands.

Intraspecific genetic analysis was further evaluated using the Neighbor Joining method, with Maximum Likelihood and Bayesian analysis. Both Modeltest 3.06 and MrModeltest 2.0 (Akaike Information Criterion) individuated the same best-fit model: HKY+I+G (HASEGAWA *et al.* 1985). Parameters estimated for this model were: Ti/Tv = 7.6009, gamma shape parameter = 0.9307, proportion of invariable sites (I) = 0.8361, base frequencies A = 0.1833, C = 0.3366, G = 0.3071, T = 0.1730.

This evolution model was used both for Maximum likelihood and for Bayesian inference. Maximum likelihood analysis produced one tree two steps longer than the most parsimonious trees (CI = 0.649 and RI = 0.856) under parsimony criterion including indels data (no significant difference after the Wilcoxon test). Four of the maximum parsimony trees displayed the highest likelihood values with the same settings and matrices used for the maximum likelihood analysis. The difference was not statistically significant with the Kishino-Hasegawa test.

In the Bayesian analysis, we omitted the first 25000 generations/cycles produced by MrBayes as "burn in" trees. With the remaining trees we produced a majority rule consensus tree, obtaining a measure of Bayesian support for the branches (reported in Fig. 3).

The phylogeographic structure of the provenances of cork oak is presented in the neighbor-joining tree (Fig. 3) with both neighbor-joining bootstrap and Bayesian support reported above branches (if higher than 50%). A monophyletic clade for *Q. suber*, *Q. trojana*, *Q. macrolepis* and *Q. crenata* was produced with 94% NJ bootstrap. Within the clade, three main groups (A, B and C) of *Q. suber* were recognized. The provenances in group A (Fig. 1c) are predominantly located in the eastern part of the species' range, along the Tyrrhenian coast of Italy (8 populations), in S Sardinia, Sicily (2 populations), Algeria, and Tunisia; group A includes a highly supported subclade (this clade resulted also in all Maximum Parsimony trees and in the maximum Likelihood tree) where populations from the entire Italian Tyrrhenian coast and Tunisia take place. Exceptions to a "fully" eastern clade are three populations in continental Spain, one from Mallorca and one from eastern Pyrenees, as a western branch of this group of genotypes.

Group B displays provenances located rather uniformly in the western (Morocco, Portugal, central Spain, western France), central (Mallorca, northern Sardinia, Corsica), and eastern districts (Italian Peninsula, Croatia) of the range. Few sequences of the ingroup (in Sicily and the Italian Peninsula) cluster separately from clades A and B.

Group C is sister to A and B clades, with six sequences (Minorca, Brindisi, Brindisi2, Seignosse, Seignosse2, and Valencia). As illustrated by the strict consensus Maximum Parsimony tree, group C is characterized by a relatively long branch, 92% NJ bootstrap and 97% Bayesian support. All members of clade C but one of the two accessions from Brindisi were characterized by the 7 bp longer ITS1; the 1 bp indel in ITS2 was common for all the members of the clade (Fig. 4). These two features were present also in the outgroup species: the 7 bp extra-sequence in ITS1 was found in all other *Quercus* species previously investigated and available in the GenBank. The extra-C in ITS2 belongs to an array of cytosines which is 7 bp long in all accessions of *Q. suber* with the exception of the six sequences in the C group where it is 8 bp long, as in *Q. coccifera*, *Q. cerris*, *Q. trojana*, and other non-Mediterranean oaks. With some substitution, the c-array arrives to 9 bp in *Cyclobalanopsis* and 11 in *Colombobalanus* and *Trigonobalanus* (Fagaceae, suggested to be basal to *Quercus*). It has to be pointed out that the populations in group C belong to disjunct stands, far from the bulk range of the species.

DISCUSSION

Consistent with previous studies, the following lineage-area relationships can be outlined for the cork oak, based on cpDNA variation: the Atlantic coasts (France, Portugal, Spain, Morocco), the western Mediterranean (southwestern France, eastern Spain, central eastern Morocco, Algeria), the central Mediterranean (southeastern France, northwestern Italy, Corse, Sardinia, Tunisia, eastern Algeria), and the eastern Mediterranen range (Italian Peninsula, Croatia, Sicily). Moreover, the following species relationships have been documented: *Q. suber-Q. ilex* at West, *Q. suber-Q. crenata-Q. cerris*, and *Q. suber-Q. trojana-Q. macrolepis* (i.e. the *Cerris* group) at East.

The ITS nucleotide sequence variation, as shown by the low consistency index calculated, indicates a high degree of homoplasy, and it did not identify any clear correlation with the observed pattern of cpDNA genetic structure. Nevertheless, two main groups of repeat types could be evidenced, although apparently unrelated with a clear geographical distribution; a third group deserves a deeper inspection due to its highly

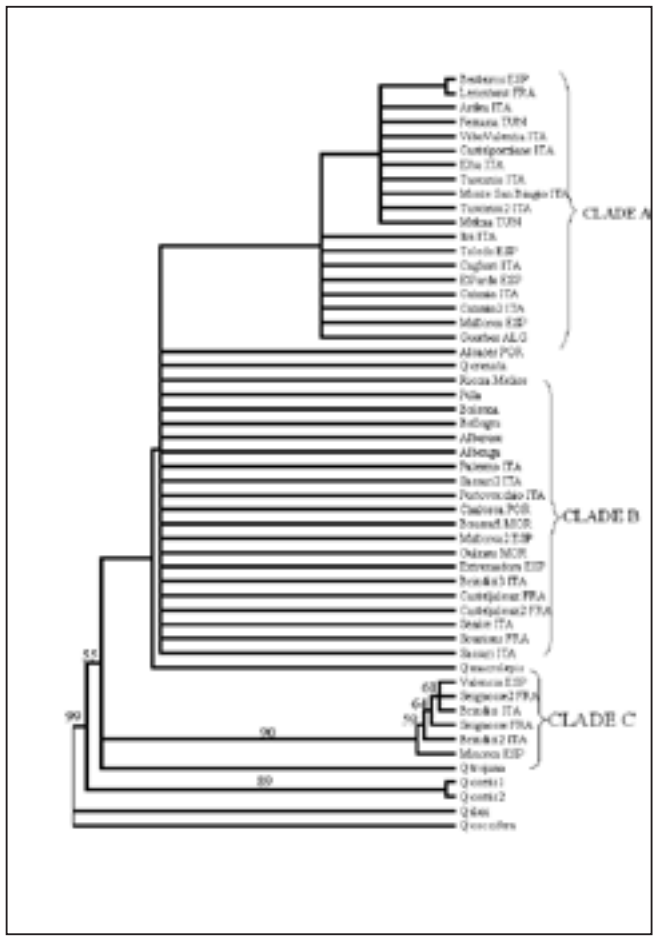

Fig. 2 — Strict consensus tree of 13320 maximum parsimony trees. Four of the maximum parsimony trees (those with the best maximum likelihood scores with the model here used) had a not significant difference with respect to the maximum likelihood tree after the Kishino-Hasegawa test. Bootstrap (parsimony) support is indicated above branches.

scattered distribution (islands and border-areas of the total range) and to its peculiar sequence characteristics.

CpDNA variation in Quercus suber - The haplotype network in Fig. 1a indicates the minimum numbers of evolutionary events separating the haplotypes.

The geographic distribution of the haplotypes matches perfectly with that presented by Jimenez et al. (2004), with the exception of a new haplotype (H2) discovered in southeastern Italy, and of an incongruence related to the Sicilian haplotypes, which exhibited no difference from those in peninsular Italy. Noteworthy, LUMARET *et al.* (2005) identified a rare haplotype in southeastern Italy, and detected no difference between Sicily and South Italy. Finally, MAGRI *et al.* (2007) detected 2 haplotypes with a clear divide in central Italy, probably included in our H1 haplotype, whereas no additional variation was found in southeastern Italy and between Sicily and South Italy.

The haplotypes are all related by two or more mutations, and six putative haplotypes, corresponding to intermediate evolutionary steps, were not detected in our dataset (black circles). One haplotype appears unrelated, and it is the one belonging to *Ilex* lineage (JIMENEZ *et al.* 2004), whereas haplotype H1 (squared) has the biggest outgroup weight. The missing haplotypes might better represent extinct lineages rather than unsampled variation, since combination of our data with those of previous studies now provide an exhaustive view of cork oak genetic structure across the entire species range (as investigated by PCR-RFLP of the 5 primer/enzyme combinations used).

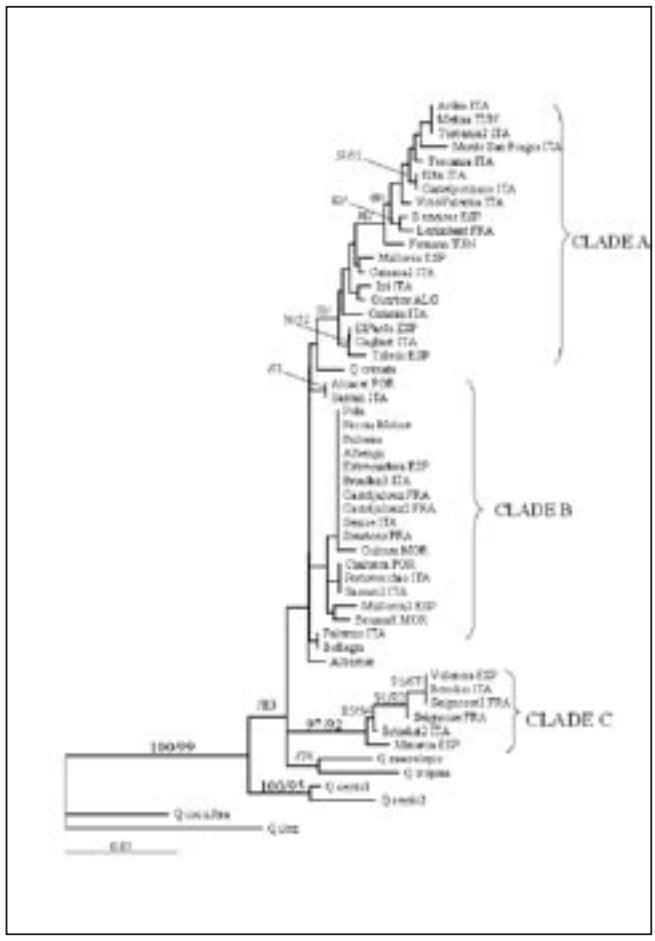

Fig. 3 — Neighbor joining tree with Bayesian Inference support and Bootstrap (Neighbor Joining) indicated above branches (BP/BI). Main clades are designated with capital letters.

Consideration of the chloroplast haplotype network and of the geographical distribution of the haplotypes evidences two chloroplast lineages (H1-H2 and H3-H4, separated by at least four mutations), corresponding to the Italian Peninsula and the Balkans, and to the remaining central and western parts of the species range. This finding might be interpreted in the light of an early separation of the two lineages favoured by a strong geographical disjunction, unless hybridization and introgression processes are taken into account.

The distribution of the haplotypes demonstrates 3 clear longitudinal divides: (1) along western and eastern Iberia, (2) from Provence to Tunisia, and (3) along the Italian Peninsula. The phylogeographical importance of the Provencal-North African axis was described in MAGRI *et al.* (2007), whereas establishment of haplotypes H4-H5 in the western areas of the species range has been fully described elsewhere (LUMARET *et al.* 2005; LOPEZ DE HEREDIA *et al.* 2005). Three of the five cpDNA cork oak haplotypes have been found in the Italian Peninsula (H1, H2, H3); two are exclusive (H1, H2), one has been newly identified (H2). Three haplotypes occur in North Africa; they all gather in Algeria, where mountain chains and deserts consitute interesting suture zones deserving a deeper attention in future studies. Haplotypes H4 and H5, which colonised most of the Atlantic regions and of the western Mediterranean are absent from Italy. In this sense, no colonisation of the Mediterranean seems to have occurred from West to East, at least in recent times.

Although the Italian Peninsula is traditionally considered the easternmost border of *Q. suber* range, a natural Croatian stand in Istria (Pula) was recently documented (TRINAJSTIĆ 2006), to-

```
                                                                      4
                     5       6                                        0
            6        3                                  2
Q_cerris1            GCGGGGGGCGCTCGTCGTCC CC-TCACC-T CCCGGGCCGC CAGGATTCCC TGCGTCG-AG ATGA
Q_cerris2            GCGGGGGGCGCTCGTCGTCC CCCTCACC-T CCCCGGCTGC CACGATTCCC TGCGTCG-AG ATGA
Itri_ITA             GCA-------CTCGTCACCC CC-CCACC-T CCCGGGCTGC TAGGGTTCC- TGCACCG-AC ATGA
Guerbes_ALG          GCA-------CTCGTCACCC CC-CCACC-T CCCGGGCTGC TAGGGTTCC- TGCGCCG-AC ATGA
Mallorca_ESP         GCG-------CTCGTCACCC CC-CCACC-T CCCGGGCTGC TAGGGTTCC- TGCGTCG-AC ATGA
Catania2_ITA         GCG-------CTCGTCACCC CC-CCACC-T CCCGGGCTGC TAGGGTTCC- TGCGCCG-AC ATGA
Ardea_ITA            GTA-------CTCGTCACCC CC-CCACC-T CCCGGGCTGC TAGGGTCCC- TGCGTCG-AC ATAA
Mekna_TUN            GTA-------CTCGTCACCC CC-CCACC-T CCCGGGCTGC TAGGGTCCC- TGCGTCG-AC ATAA
Tuscania2_ITA        GTA-------CTCGTCACCC CC-CCACC-T CCCGGGCTGC TAGGGTCCC- TGCGTCG-AC ATAA
Monte_San_Biagio_ITA ATG-------CTCGTCACCC CC-CCACC-T CCCGGGCTGC TAGGGTCCC- TGCGTCG-AC ATAA
Tuscania_ITA         GTA-------CTCGTCACCC CC-CCACC-T CCCGGGCTGC TAGGGTCCC- TGCGCCG-AC ATAA
Elba_ITA             GTA-------CTCGTCACCC CC-CCACC-T CCCGGGCTGC TAGGGTCCC- TGCGTCG-AC ATGA
Castelporziano_ITA   GTA-------CTCGTCACCC CC-CCACC-T CCCGGGCTGC TAGGGTCCC- TGCGTCG-AC ATGA
ViboValentia_ITA     GTG-------CTCGTCACCC CC-CCACC-T CCCGGGCTGC TAGGGTCCC- TGCGTCG-AC ATGA
Besteiros_ESP        GTG-------CTCGTCGCCC CC-CCACC-T CCCGGGCTGC CAGGGTCCC- TGCGTCG-AC ATAA
Lerimbaut_FRA        GTG-------CTCGTCACCC CC-CCACC-T CCCGGGCTGC CAGGGTCCC- TGCGTCG-AC ATAA
Fernana_TUN          GTA-------CTCGTCACCC CC-CCACC-T CCCGGGCTGC TAGGGTTCC- TGCGCCG-AC ATAA
Catania_ITA          GCG-------CTCGTCACCC CC-CCACC-T CCCGGGCTGC TAGGGTTCC- TGCACCG-AC ATGA
ElPardo_ESP          GCA-------CTCGTCACCC CC-CCACC-T CCCGGGCTGC CAGGGTTCC- TGCGCCG-AC ATGA
Cagliari_ITA         GCA-------CTCGTCACCC CC-CCACC-T CCCGGGCTGC CAGGGTTCC- TGCGCCG-AC ATGA
Toledo_ESP           GCA-------CTCGTCACCC CC-CCACC-T CCCGGGCTGC CAGGGTTCC- TGTGCCG-AC ATGA
Alcacer_POR          GCG-------CTCGTCGCCC CC-CCACC-T CCCGGGCTGC CAGGGTTCC- TGCACCG-AC ATGA
Sassari_ITA          GCG-------CTCGTCGCCC CC-CCACC-T CCCGGGCTGC CAGGGTTCC- TGCACCG-AC ATGA
Soustons_FRA         GCG-------CTCGTCGCCC CC-CCACC-T CCCGGGCCGC CAGGGTTCC- TGCACCG-AC ATGA
Senise_ITA           GCG-------CTCGTCGCCC CC-CCACC-T CCCGGGCCGC CAGGGTTCC- TGCACCG-AC ATGA
Casteljaloux2_FRA    GCG-------CTCGTCGCCC CC-CCACC-T CCCGGGCCGC CAGGGTTCC- TGCACCG-AC ATGA
Casteljaloux_FRA     GCG-------CTCGTCGCCC CC-CCACC-T CCCGGGCCGC CAGGGTTCC- TGCACCG-AC ATGA
Brindisi3_ITA        GCG-------CTCGTCGCCC CC-CCACC-T CCCGGGCCGC CAGGGTTCC- TGCACCG-AC ATGA
Extremadura_ESP      GCG-------CTCGTCGCCC CC-CCACC-T CCCGGGCCGC CAGGGTTCC- TGCACCG-AC ATGA
Oulmes_MOR           GCG-------CTCGTCGCCC CC-CCACC-T CCCGGGTCGC CAGGGTTCC- TGCACCG-AC ATGA
Mallorca2_ESP        GTG-------CTCGTCGCCC CC-CCACC-T CCCGGGCCGC CAGGGTCCC- TGCACCG-AC ATGA
Boussafi_MOR         GCA-------CTCGTCGCCC CC-CCACC-T CCCGGGCCGC CAGGGTCCC- TGCACCG-AC ATGA
Chalusca_POR         GCA-------CTCGTCGCCC CC-CCACC-T CCCGGGCCGC CAGGGTTCC- TGCACCG-AC ATGA
Portovecchio_ITA     GCA-------CTCGTCGCCC CC-CCACC-T CCCGGGCCGC CAGGGTTCC- TGCACCG-AC ATGA
Sassari2_ITA         GCA-------CTCGTCGCCC CC-CCACC-T CCCGGGCCGC CAGGGTTCC- TGCACCG-AC ATGA
Valencia_ESP         GCAGGGGGCGCTCGTCGCCC TC-CCATC-T CCCGAGCCGC CGAGGTTCCC TGTGCCG-AC ATGA
Brindisi_ITA         GCAGGGGGCGCTCGTCGCCC TC-CCATC-T CCCGAGCCGC CGAGGTTCCC TGTGCCG-AC ATGA
Seignosse2_FRA       GCAGGGGGCGCTCGTCGCCC TC-CCATC-T CCCGAGCCGC CGAGGTTCCC TGTGCCG-AC ATGA
Seignosse_FRA        GCAGGGGGCGCTCGTCGCCC TC-CCACC-T CCCGAGCCGC CGAGGTTCCC TGTGCCG-AC ATGA
Brindisi2_ITA        GCA-------CTCGTCGCCC TC-CCACC-T CCCGAGCCGC CGAGGTTCCC TGCGCCG-AC ATGA
Minorca_ESP          GCAGGGGGCGCTCGTCGCCC CC-CCACC-T CCCGAGTCGC CGAGGTTCCC TGCGCCG-AC ATGA
Palermo_ITA          GCG-------CTCGTCGCCC CC-CCACC-T CCCGGGCCGC CAGGGTTCC- TGCGCCG-AC ATGA
Albenga              GCG-------CTCGTCGCCC CC-CCACC-T CCCGGGCCGC CAGGGTTCC- TGCACCG-AC ATGA
Alberese             GCG-------CTCGTCGCCC CC-CCACC-T CCCGGGCCGC CAGGGTTCC- TGCGTCG-AC ATGA
Bellegra             GCG-------CTCGTCGCCC CC-CCACC-T CCCGGGCCGC CAGGGTTCC- TGCGCCG-AC ATGA
Bolsena              GCG-------CTCGTCGCCC CC-CCACC-T CCCGGGCCGC CAGGGTTCC- TGCACCG-AC ATGA
Pola_3               GCG-------CTCGTCGCCC CC-CCACC-T CCCGGGCCGC CAGGGTTCC- TGCACCG-AC ATGA
Riccia_Molise        GCG-------CTCGTCGCCC CC-CCACC-T CCCGGGCCGC CAGGGTTCC- TGCACCG-AC ATGA
Q_crenata            GCG-------CTCGTCGCCC CC-CCACG-T CCCGGGCTGC CAGGGTTCC- TGCGCCG-AC ATGA
Q_macrolepis         GCGGGGGGCGGTGGTTGCCC CC-CCATC-T CCCGGGCCGC CAGGGTTCC- TGCGCCG-AC ATGA
Q_trojana            GCGGGGGGCG-------CCC CC-CCACC-T CCCGGGCCGC CAGAGTTTTC TACGCCG-AC ATGA
Q_coccifera          GCGGGGGGCGCTCGTCACCT CC-CCGCTTC CTTGGACTGT CAGGGATCCC CGCGTCGTCC ACGG
Q_ilex               GCGGGGGGCGCTCGTCGCTC CG-TAGCCTC TCTGGACTAT CAGGAATCC- TGCGTGATCC TCGG
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Fig. 4 — Alignment of ITS sequences. Synonymous sites have been omitted. Numbers above the alignment indicate the position (in the complete alignment) of the two indels characterizing the C Group.

gether with the outstanding recovery of a cork oak macrofossil (5800 years BP) in Greece (www.unep-wcmc.org/sites/pa/1579v.htm). More interestingly, the fossil was found together with remnants of *Pinus pinaster* Aiton, a palaeo-endemism of the Mediterranean area with a present range and a geographical distribution of the cytoplasmic DNA variation very similar to that of *Q. suber* (MAGRI *et al.* 2007). Although indigenate of the Croatian stand has yet to be fully inspected (few isolated trees in the Croatian islands of Mljet, Korĉula, Rab, and in the Aegean island of Ikaria

possess a documented XIX-XX century anthropic origin), these findings might be indicative of an early occurrence of the species in the Balkans, and of its historical withdrawal in recent times, a hypothesis that was not taken into consideration in previous studies.

The presence of haplotype H1 both in the Italian and the Balkan Peninsulas supports the hypothesis of exchange between the two areas. The opening of vast connections across the Adriatic sea during the Quaternary pleniglacials is likely to account for early and reiterated contacts between Balkan and Italian species/populations (TABERLET *et al.* 1998; FINESCHI *et al.* 2002). The Adriatic connection between Italian and Balkan flora is particularly evident in Apulia, the southeastern-most Italian region, where the highest number of native oak species in Italy as well as southern Europe occurs. The only western European stands of *Q. macrolepis* Kotschy and *Q. trojana* Webb. occur in Apulia (SCHIRONE and SPADA 1995): these oaks belong to the tree flora which is likely to have survived in scattered refugia in southern Italy even during the dominance of a steppe-like vegetation during the Last Glacial Maximum.

Common biogeographical patterns are particular evident in deciduous and evergreen oaks, for which genetic structure and post-glacial recolonization routes have been extremely well documented by means of chloroplast DNA variation (PETIT *et al.* 2002; FINESCHI *et al.* 2002; LUMARET *et al.* 2002; LOPEZ DE HEREDIA *et al.* 2007). Besides showing a reduced diversity and high levels of fixation, those results revealed a geographical structure characterised by a loss of haplotype richness northward and a longitudinal partitioning of the lineages with discrete western, central, and eastern groups. Clearly, this biogeographic pattern describes three areas where (multiple) glacial refugia also for the cork oak were located. Accordingly, fossil records suggest that cork oak was distributed in approximately the same areas as today even before the Neolithic (MAGRI *et al.* 2007).

Furthermore, it has been shown that European oak populations experienced severe bottlenecks, based on low genetic variation (PETIT *et al.* 2002). Haplotypes are shared among sympatric species, and only the very rare ones are restricted to one species. The consequence is that each species shows a very low haplotype diversity. In agreement, our data show samples splitting according to geographical macro-regions rather than grouping according to species. Especially in the Italian Peninsula, the shared polymorphisms involve four oak species and *Q. suber*. This group of 5 species belongs to the subg. *Cerris*, and it is sympatric uniquely in southeastern Italy.

Potential reasons for shared cpDNA polymorphisms - The scientific community has recently come to debate the meaning of shared DNA components in some white oaks (see for instance MUIR and SCHLOTTERER 2006): prints of recent gene flow, distant introgression phenomena, reticulated evolution, and sharing of ancestral traits can all be invoked to support the obtained data.

The strongest evidence for gene flow between two species is the phylogeographical pattern observed for cytoplasmic DNA (e.g. cytoplasmic capture, RIESEBERG and SOLTIS 1991). PETIT (2004) suggested inter-specific hybridization as a mechanism for natural plant invasions following the last pleniglacial, with special emphasis on *Quercus*. Interspecific geneflow may cause hybrid speciation, when two otherwise indipendent lineages recombine sexually to create a new species. Speciation also requires hybrids to be isolated from parental species by new competition or adaptation traits in a novel environment. Conversely, during introgression, one species repeatedly pollinates a second species unidirectionally, until most of the backcrossed species genome is replaced by the pollinator's one. The unique direction of the pollination is usually accomplished by biological/phenological barriers, as in the case of *Q. ilex*-*Q. suber* (ELENA-ROSSELLÒ *et al.* 1992), or may be favoured by demographical factors under specific stress conditions (PETIT 2004).

Nevertheless, gene flow is difficult to quantify if only cpDNA is investigated. In fact, regardless of the sites assayed, all polymorphisms concern only the clonally inherited chloroplast, and as such, they may reflect a particular evolutionary force, like selection or drift. Moreover, gene flow is notoriously difficult to distinguish from shared ancestral variation as both scenarios produce a similar pattern of allele sharing (see for instance MUIR and SCHLOTTERER 2005). Allele sharing between two (or more) species resulting from shared ancestral variation has been extensively documented for closely related and for recently diverged species, and it could be emphasized by stochastic processes of lineage sorting which followed to genetic drift after strong climatic oscillations (MUIR and SCHLOTTERER 2005; RAN *et al.* 2006). As well, sharing of cpDNA polimorphisms between divergent taxa (in phenology, ecology, range, etc.) may account for reticulation during recent species radiation, as well as in the early diversification of taxonomic groups (SOLTIS and KUZOFF 1995; GUO *et al.* 2004; CRONN and WENDEL. 2004).

Consideration on the utility of ITS variation - Besides cpDNA sharing, the ITS region can provide evidence of inter-specific geneflow when the hybrids retain the repeat types that each parental species contributed (HERSHKOWITZ *et al*. 2006). However, in diploid hybrids one of the parental ribosomal DNA loci may be lost quickly through recombination and segregation. At the same time, concerted evolution can act to homogenize variation between repeat types. The roles played by timing of the hybridization events and long generation periods are still unclear, as they may be both correlated with one fate or another: some woody groups have shown partial to little homogenization (SANG *et al*. 1995; CAMPBELL *et al*. 1997), whereas in other cases interlocus homogenization may be complete (AGUILAR *et al*. 1999) or even absent (RITZ *et al*. 2005). If hybrids are very recent, both parental types are almost always present (BELLAROSA *et al*. 2005) and direct sequencing can quickly reveal hybridization through an additive pattern of sequence variation. When direct sequencing fails to find a second parental repeat in a suspected hybrid, cloning and search for the second repeat is the next step, but if the missing repeat is relatively rare, it may be necessary to screen a large number of clones, which might cost a lot in terms of labour and time (RFLP techniques and specific primer sequences are not always available, if the parental species are not very close).

Hypotheses for a phylogenetic reconstruction - Therefore, identifying inter-taxa gene flow and determining whether a particular species has a hybrid origin is not easy. Our genetic dataset shows three main results: (1) two *Q. suber* Italian haplotypes are highly shared within subg. *Cerris*; (2) a group of ITS sequences from disjoint cork oak populations cluster in a clade (C) that is basal to *Q. suber*, sister to other species of the same subgenus, and it shares some structural features with the outgroups; (3) the ITS sequence of *Q. crenata*, a species of presumed hybrid origin (*Q. cerris* x *Q. suber*) clusters within *Q. suber* clades.

In the light for hybridization, these results might indicate, respectively: (1) introgression of *Q. suber* in sympatric oak species in the Italian territories; (2) ITS repeat homogenization following hybridization between disjoint *Q. suber* populations and sympatric oaks; (3) replacement of the ITS loci of *Q. cerris* with those of *Q. suber*, in *Q. crenata*.

In Italy, the occurrence of hybrids between *Q. cerris* and *Q. suber* (*Q. crenata*), as well as between *Q. cerris* and *Q. trojana* (*Q. x schneideri* Vierh.), is traditionally acknowledged, based on intermediate morphology (CAMUS 1936-54). Conversely, hybrids between *Q. suber* and *Q. trojana* or *Q. macrolepis*, and between these two latter species, have never been described. Individuals of *Q. x schneideri* are very rare (few individuals in the Balkans and in Apulia); *Q. crenata* is a scattered species with a bulk range centered in Italy, and extending across the Adriatic sea; it is rarer than the two parental species, and its ecology seems to join *Q. suber* and *Q. cerris* self-ecologies to assure competition in new niches. However, morpho-ecological intermediacy may be useful but also potentially misleading (RIESEBERG 1995).

If we explain the sharing of H1 haplotype sharing with the possibility of a recent (Holocene) introgression of *Q. suber* into *Q. cerris*, the large distribution of this haplotype would imply that almost every single *Q. suber* population in Italy is an introgressed one, originating during survival in glacial refugia and/or along common recolonization routes. This model has been proposed to explain *Q. suber-Q. ilex* sharing of cpDNA variation in Morocco and in the Iberian Peninsula (BEHLABIB *et al*. 2001; JIMENEZ *et al*. 2004). Along with this assumption, only haplotypes H3 and H4 would be exclusive of the cork oak, and the areas where they occur would have preserved the species purity across time; as a consequence, *Q. crenata* would be just an intemediate step, originating from still ongoing introgression phenomena and susceptible to revert to either parent, except where one or both are missing.

Conversely, another feasible explanation would involve prints of more distant reticulation events betwen *Q. suber* and *Q. cerris* and an early, occasional formation of a hybrispecies, which experienced an evolutionary success thanks to acquisition and mantainance of an own species identity across space and time. The likeliness of these conclusions cannot be extensively supported by our and currently available data, and it would require larger samplings and the use of more powerful molecular tools. On the other hand, if we look at the very limited spatial distribution of haplotype H2, *Q. suber*, *Q. trojana*, and *Q. macrolepis* might have indeed experienced cytoplasmic capture or pollen swamp phenomena in more recent times. In this case, long-term sympatry, and/or ecological instability/extreme climatic conditions of surrounding areas should be taken into account; interfertility and direction of the (multiple) crosses need to be demonstrated and assessed, as well. However, the presumed hybrids had no evolutionary importance, as they were completely introgressed in one species or in the other.

Remarkably, the observed shared haplotypes between the group of species in the Italian Peninsula may also result from shared ancestral variation. This hypothesis would be supported by the ITS sequence data suggesting a possible plesiomorphic trait within *Quercus*, and by some palaeogeographic considerations. In this case, fixation of the ancestral cytotype(s) in the eastern areas of the *Q. suber* range and extinction in the central and western parts might be related to a closer vicinity of Peninsular Italy to the differentiation center of the species group, to stochastic processes (or a vicariant event), and to the higher permissivness of southern Italy as a refugial area. As a consequence, relationships among ITS lineages may reflect stochastic sorting processes rather than species relationships, and incomplete lineage sorting of slowly evolving cpDNA loci, together with the severe climatic bottlenecks of the past, may account for local extinction of variants and their preservation in long-permissive areas.

Reconstruction of a phylogeographic model - As noted above, following hybridization and/or reticulation, the first coexistence of divergent rDNA types may follow different ways towards interlocus omogenization. We didn't find any evidence for the divergent copies to be mantained and evolve indipendently; contrarily, *Q. crenata* as an example of one repeat type that comes to dominate the rDNA repeat population, with the other one being lost through segregation/introgression, might be a convincing possibility. On the other hand, the origin of ITS C group from an hybridization event and a subsequent process of concerted evolution could represent a contrasting evidence of chimeric molecules that in phylogenetic analyses behave erratically and resolve basally to either parental lineage.

Therefore, a hypothesys of ancestrality cannot be rejected easily, especially considering that the present-day distribution of *Q. suber* stands in Apulia, Landes, and eastern Spain overlaps only with that of *Q. ilex,* and *Q. suber-Q. ilex* hybridization is uni-directional, with the cork oak exclusively acting as pollinator (as shown by JIMENEZ *et al*. 2004); in agreement with this, the ITS sequences detected in populations with haplotype H5 grouped in clades A (Mallorca, Lerimbaut) and B (Oulmes), whereas we detected only haplotypes exclusive of the *suber* lineage in 3 out of four stands exhibiting the C group sequences: H4 in Seignosse, H3 in Minorca, and H1 in Brindisi.

If it is reasonable to assume (1) *Q. crenata* a "true" hybrispecies, (2) group C sequences possessing plesiomorphic traits within the genus *Quercus*, and (3) H1 (at least) haplotype sharing as potentially consistent with ancestrality, at the same time the Italian peninsula and the sites where group C was detected could be interpreted as relics of an older range of the species. However, it is difficult to explain the observed spatial distribution of such populations, and to delineate their temporal location.

Macrofossil evidences show that a possible ancestor of *Q. suber*-(*cerris*) (*Q. sosnowskyi* Kolak.) was present in the area of the Parathetys corresponding to present day central Europe up to southwestern Asia, during the Middle Miocene (PALAMAREV 1989; KVAČEK *et al*. 2002; KHONDKARIAN *et al*. 2004; KOVAR-EDER *et al*. 2006). We can only presume that the species differentiated in a yet unidentified thereabout area and then spread into southern Europe and North Africa, where proofs of a late Miocene-early Pliocene presence of the species have been collected (DEPAPE 1932; CARVALHO 1957; QUEZEL 1995). Its range probably underwent several and reiterated fluctuations, due to the severe climatic oscillations of the Late Tertiary and throughout Quaternary (SUC 1984; HEWITT 2004; PETIT and VENDRAMIN 2005), when the modern range established (PONS and REILLE 1988; FAUQUETTE et al. 1999). In support of this hypothesis, the prevalently eastern European-Asian range of the whole subg. *Cerris* (SCHWARZ 1936-39; CAMUS 1936-54; NIXON 1993) and of most of its ancestors (including those coeval of *Q. trojana* and *Q. macrolepis*) that have been scored in central Europe and in the peri-Caucasian area (ZOHARY 1973; TAKHTAJAN 1982; PALAMAREV and TSENOV 2004, and references therein). *Quercus ilex* and *Q. coccifera* ancestors (*Q. mediterranea* Ung.) also have been scored in the same areas. Noteworthy, both these species display interesting vicariance phenomena, with a clear eastern/western pattern at the subspecific level (LOPEZ DE HEREDIA *et al*. 2007).

Therefore, the peculiar isolates of older genetic prints in southeastern Italy (where haplotypes H1-H2 and the C group sequences have been found), and their disjunct analogues in Aquitania and eastern Spain (where the original haplotypes would have presumably gone extinct) could even represent the remnants of the late Miocene-early Pliocene palaeo-European range, in a scenario mostly characterised by an environment with moist, warm climate and subtropical flora (SUC 1984; MAI 1989; THOMPSON 2005). More recent colonization events (Pleistocene) or postglacial re-colonization may be two alternatives. However, migrates should have necessarily moved from close refugia. Interestingly, Apulian popula-

tions of most of the local oaks have been repeatedly suggested to be very ancient due to recurrent pheno-atavism phenomena, e.g. anomalies in the reproductive cycle typical of a warm and moist environment, and general relictuality of the associated flora, which are likely to have been maintained due to long-lasting and highly permissive climatic conditions (SCHARFETTER 1953; SCHIRONE and SPADA 1995). Proofs of the past (in Greece) and recent (in Croatia) occurrence of the species in the Balkans might eventually speak in favour of this hypothesis. This scenario would be consistent with the refugial character of the Iberian Atlantic (CARRION *et al*. 2000) and eastern (LOPEZ-DE-HEREDIA *et al*. 2005) coastlines.

Conclusions - Late and post-Miocene ecological/climatic changes in the Mediterranean region seemingly triggered great changes in species distribution and population structure. Species went extinct over large parts of their range (THOMPSON 2005), and some populations were dispersed to new locations, or survived in refugia and then expanded again (BREWER *et al.* 2002). Allopatric speciation as well as hybridization were indeed stimulated by these repeated processes, and the geographic departure of a species into separate lineages may represent the legacy of a complex amount of interacting phenomena (including vicariance and ecological specialization) in the distant past (WIENS 2004).

In the light for a distant sympatry, and a general interfertility observed among *Q. suber-cerris-macrolepis-trojana*, reticulate evolution could be considered highly likely in the early diversification of this group. As well, more recent and local events of cytoplasmic capture might have happened during the climatic fluctuations that followed until recent times.

Nevertheless, sharing of ancestral polimorphisms within the *Cerris* group is highly probable, also. In this case, divergence of the lineages would suggest vicariance between western and eastern haplotypes, with one haplotype with Balkanik-Italian distribution compatible with an early colonization/closer vicinity of Italian Peninsula to the differentiation center of *Cerris* group; with regard to the ITS phylogeny also, the pattern of the fragmentary, discontinuous distribution of the Apulian, Aquitanian, and eastern Iberian populations across the total range of *Q. suber* seems to be consistent with the heritage of a remote colonization event prior to the Plio-Pleistocene reassessment of the Mediterranean geography and climate, leading back to the Miocene palaeo-range of the species. The possibility that cpDNA variation in *Q. suber* might reflect extremely ancient evolutionary and colonisation events was recently discussed; MAGRI *et al*. (2007) presented a phylogenetic hypothesys tracing back the origin of cork oak to the Oligocene-Miocene palaeogeographic history of the Mediterraean basin. In this sense, the detected patterns of cpDNA sharing would address the earliest events of the subgenus diversification, thus strenghtening the hypothesis of ancestrality.

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