The distribution of *Quercus suber* chloroplast haplotypes matches the palaeogeographical history of the western Mediterranean

D. MAGRI, * S. FINESCHI, † R. BELLAROSA, ‡ A. BUONAMICI, § F. SEBASTIANI, § B. SCHIRONE, ‡ M. C. SIMEONE ‡ and G. G. VENDRAMIN ¶

* Dipartimento di Biologia Vegetale, Università di Roma ‘La Sapienza’, Piazzale Aldo Moro 5, 00185 Roma, Italy, † Consiglio Nazionale delle Ricerche, Istituto per la Protezione delle Piante, via Madonna del Piano 10, 50019 Sesto Fiorentino (Firenze), Italy, ‡ Dipartimento di Tecnologie, Ingegneria e Scienze dell’Ambiente e delle Foreste, Università della Tuscia, Via S. Camillo de Lellis, 01100 Viterbo, Italy, § Dipartimento di Biotecnologie Agrarie, Università degli Studi di Firenze, Via della Lastruccia 14, 50019, Sesto Fiorentino (Firenze), Italy, ¶ Consiglio Nazionale delle Ricerche, Istituto di Genetica Vegetale, Via Madonna del Piano 10, 50019 Sesto Fiorentino (Firenze), Italy

Abstract

Combining molecular analyses with geological and palaeontological data may reveal timing and modes for the divergence of lineages within species. The Mediterranean Basin is particularly appropriate for this kind of multidisciplinary studies, because of its complex geological history and biological diversity. Here, we investigated chloroplast DNA of *Quercus suber* populations in order to detect possible relationships between their geographical distribution and the palaeogeographical history of the western Mediterranean domain. We analysed 110 cork oak populations, covering the whole distribution range of the species, by 14 chloroplast microsatellite markers, among which eight displayed variation among populations. We identified five haplotypes whose distribution is clearly geographically structured. Results demonstrated that cork oak populations have undergone a genetic drift geographically consistent with the Oligocene and Miocene break-up events of the European–Iberian continental margin and suggested that they have persisted in a number of separate microplates, currently found in Tunisia, Sardinia, Corsica, and Provence, without detectable chloroplast DNA modifications for a time span of over 15 million years. A similar distribution pattern of mitochondrial DNA of *Pinus pinaster* supports the hypothesis of such long-term persistence, in spite of Quaternary climate oscillations and of isolation due to insularity, and suggests that part of the modern geographical structure of Mediterranean populations may be traced back to the Tertiary history of taxa.

Keywords: chloroplast DNA, genetic drift, geographical structure, Neogene, *Quercus suber*, western Mediterranean

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Introduction

The Mediterranean Basin is a hotspot for biogeographical and evolutionary studies, showing an exceptional level of biodiversity (Greuter 1995; Quézel 1995; Blondel & Aronson 1999; Comes 2004; Thompson 2005). A complex geological history, characterized by orogenic processes and widespread extensional tectonics, a considerable physiographic and climatic heterogeneity and a long-term impact of human activities have caused repeated isolations of individual plant populations and admixtures of populations of different origins. Another prominent feature of the Mediterranean flora is its high rate of regional endemicism, due partly to a recent diversification (neo-endemics), and partly to the conservation of taxa with relictual distribution, resulting from a long-term regional permanence without evolutionary changes (palaeo-endemics) (Comes 2004; Thompson 2005).

Correspondence: Giovanni G. Vendramin, Fax: +39-055-5225729; E-mail: giovanni.vendramin@igv.cnr.it

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In addition to a very high level of species diversity, the Mediterranean region shows a considerable diversity among and within plant populations, documented by a large number of studies on different genetic markers (Fady-Welterlen 2005). The main factor for the differentiation of European gene resources is generally found in Quaternary climate oscillations, causing repeated retreats and re-advances of plant populations into and from refuge areas (Comes & Kadereit 1998; Taberlet et al. 1998; Hewitt 1999, 2000). Recently, combined phylogeographical and palaeobotanical studies have shown that within-species diversity for the European beech was shaped over times much longer than the last interglacial–glacial cycle (Magri et al. 2006). However, a possible age for the genetic divergence of tree populations remains a challenge and urges new investigations. The Mediterranean flora is especially appropriate for this purpose, as many species with a Tertiary origin have persisted through the Quaternary up to present, and so may bear witness of long-term changes of genetic characters.

With this purpose in mind, we have focused our attention on cork oak (*Quercus suber* L), an emblematic Mediterranean evergreen sclerophyllous tree. Cork oak is a slow growing, extremely long-lived evergreen tree. It is a monoecious wind-pollinated species with a protandrous system to ensure cross-pollination. Reproductive system (mostly sexual) and dissemination (gravity and zoochory) of cork oak are coherent with those of most oak species.

It may reach about 20 m in height, with massive branches forming a round crown. Its thick and soft bark is the source of cork, which is stripped every 10–12 years from the outer layer of the bark along the lower portion of the trunk (Gellini & Grossoni 1997). The modern distribution of *Q. suber*, rather discontinuous, ranges from the Atlantic coasts of North Africa and Iberian Peninsula to the southeastern regions of Italy, and includes the main west Mediterranean islands as well as the coastal belts of Maghreb (Algeria and Tunisia), Provence (France) and Catalonia (Spain) (Fig. 1).

*Quercus suber* is widely cultivated within its natural range for the production of cork. According to Carrión et al. (2000a), without human activities *Q. suber* would never develop pure stands in the Iberian Peninsula, and would form mixed forests with xerophyllous and deciduous oaks together with *Pinus pinaster* instead.

In recent years, cork oak has been the subject of intensive genetic studies by means of different markers, and a strong differentiation among populations has been detected around the Mediterranean Basin by investigating both chloroplast and mitochondrial DNA (which are maternally inherited in oaks, as demonstrated by Dumolin et al. 1995), as well as allozyme variations; moreover, evidence of cytoplasmic introgression of *Q. suber* by *Quercus ilex* genes has been reported (Toumi & Lumaret 1998, 2001; Belahbib et al. 2001; Lumaret et al. 2002, 2005; López de Heredia et al. 2005). Although both *Q. suber* and *Q. ilex* are evergreen oaks, they belong to different subgenera (*Q. suber*, subgenus *Cerris*; *Q. ilex*, subgenus *Sclerophyllodrys*) according to classical oak taxonomy (Schwarz 1993). However, a more recent classification includes both species within the same Eurasian section *Cerris* (Manos et al. 1999). In their survey on the Mediterranean evergreen oak species, Jiménez et al. (2004) and López de Heredia et al. (2005) detected three lineages of chloroplast DNA haplotypes, one of them (*suber* lineage) being specific of cork oak populations from peninsular Italy, Sardinia, Sicily, Corsica, northern Africa and the island of Minorca, whereas cork oak populations from the Spanish mainland and from the island of Majorca were characterized by one maternal lineage also shared by *Q. ilex* and *Quercus coccifera*.
Several hypotheses have been advanced concerning the evolutionary history of cork oak as well as the geographical location of its centre of origin, which has been suggested to be in the eastern countries of the western Mediterranean Basin (Bellarosa et al. 2005; Lumaret et al. 2005). However, the details of its differentiation processes are still largely unknown.

We have analysed cork oak populations throughout the species distribution range using chloroplast DNA (cpDNA) markers (microsatellites) and have combined the genetic results with palaeobotanical data and geodynamic models to suggest new insights on the modes and timing of the genetic divergence of cork oak populations.

Materials and methods

Genomic DNA was extracted from fresh leaves of 110 populations from Italy (including Sicily, Sardinia and the Tuscan Islands), France and Corsica, Spain and Balearics, Portugal, Morocco, Algeria and Tunisia. At least three individuals were analysed for each population. Invitrek (Invisorb Spin Plant Mini Kit) protocol was utilized for DNA extraction. List of populations, number of individuals per population, and geographical coordinates are available as Supplementary material (S1). Fourteen chloroplast microsatellite regions were amplified using primers: ccmp 2, ccmp 4, ccmp 6, ccmp 7, ccmp 10 (Weising & Gardner 1999); and cmcs 1, cmcs 2, cmcs 3, cmcs 6, cmcs 7, cmcs 8, cmcs 9, cmcs 13, cmcs 14 (Sebastiani et al. 2004). Polymerase chain reaction (PCR) amplification and sizing of the fragments followed procedures described by Fineschi et al. (2005). Amplification conditions were: 4 min at 95 °C, 25 cycles each consisting of 95 °C for 30 s, 50 °C for 30 s, 72 °C for 30 s; then 72 °C for 8 min. PCR products were loaded on a capillary automatic sequencer MegaBACE 1000 (GE Healthcare). MegaBACE ET400 (GE Healthcare) was used as size standard. Fragment lengths were determined using MegaBACE fragment profiler software version 1.2 (GE Healthcare).

In order to check for introgression between Quercus suber and Quercus ilex, microsatellite fragments polymorphic in Q. suber were also analysed in Q. ilex, choosing individuals previously characterized as having different haplotypes (Fineschi et al. 2005). Amplified fragments of the polymorphic microsatellites were cloned into plasmid vectors using the Invitrogen TOPO cloning kit and then sequenced from both ends using an automatic sequencer MegaBACE 1000 (GE Healthcare). Each fragment was sequenced twice. The software PERMUT (Pons & Petit 1996) (www.pierroton.inra.fr/genetics/lab0/Software) was used to measure differentiation for unordered alleles (G_{ST}) and for ordered alleles (N_{ST}). One thousand random permutations of haplotype identities were made, keeping the haplotype frequencies and the matrix of pairwise haplotype differences as in the original study (Burban et al. 1999). If N_{ST}, which takes the genetic differences between the haplotypes into account, is higher than G_{ST}, this indicates the presence of a phylogeographical structure (Pons & Petit 1996), that is closely related haplotypes are more often found in the same geographical area than would be expected by chance.

Phylogenetic relationships among haplotypes were inferred with NETWORK 4.2.0.1 (Fluxus Technology Ltd. at www.fluxus-engineering.com) using the median-joining method (Bandelt et al. 1999).

Results

Polymorphisms at eight (ccmp4, ccmp6, cmcs1, cmcs6, cmcs7, cmcs8, cmcs9, cmcs14) of 14 microsatellite loci identified only five different haplotypes (S2) with a strong geographical structure (Fig. 1), as demonstrated by the high value of genetic differentiation between populations for unordered alleles: G_{ST} = 0.965 (SE 0.016) as well as for ordered alleles N_{ST} = 0.962 (SE 0.018). Most populations are fixed for one haplotype. Only five populations (one per region in Spain, Morocco, and Corsica, and two in Italy) contain two different haplotypes. Sequence data confirmed that the detected variation is due to differences in the number of repeats within the microsatellite stretches. In addition, an insertion/deletion of 25 bp was detected in the flanking regions of the microsatellite CmCs9 (see GenBank Accession nos EU088193 and EU088194).

In the Italian peninsula and Sicily, two closely related haplotypes (H1 and H2) of cork oak are present. Haplotype H3 is found along the Mediterranean coast of Provence and Liguria, in the islands of Corsica, Sardinia, and northern Tuscany, as well as in the northern part of Tunisia and Algeria. Haplotype H4 is distributed in the westernmost part of the range (southwest France, Portugal, southwest Spain and northern Morocco). Haplotype H5 shows a discontinuous distribution in Catalonia, the Balearic Islands and the Rif mountain range in Morocco.

The haplotype network (Fig. 1) indicates that the two Italian haplotypes, H1 and H2, differ from each other by only one mutation, and are very divergent from haplotypes H4 (Portugal–western Spain–southwest France–northern Morocco) and H3 (north Africa–Sardinia–Corsica–Provence). H5 corresponds to a cpDNA lineage shared with Quercus ilex, interpreted as the result of multiple and mainly unidirectional cytoplasmic introgression of Quercus suber by Q. ilex (Belahbib et al. 2001; López de Heredia et al. 2005; Lumaret et al. 2005) resulting in populations morphologically identified as cork oak, which maintain the chloroplast genome of the seed parent species Q. ilex. Belahbib et al. (2001), López de Heredia et al. (2005) and Lumaret et al. (2005) suggest that hybridization between holm oak and cork oak must be an ancient event (repeated hybridization events and backcrosses with the pollen parent). Similarly,
haplotypes H1 and H2 may be the result of unidirectional cytoplasmic introgression by *Quercus cerris*, an eastern European deciduous oak that is widespread in the Italian peninsula. In fact, our unpublished data (not shown) indicate a sharing of haplotypes H1 and H2 between *Q. cerris* and *Q. suber* in Italian populations.

**Discussion**

The modern history of *Quercus suber* is closely related to human activity for cork production. For this reason, humans have been considered responsible for a reduction in genetic variation in some stands of cork oak, as well as for hybridization with congeners (Blondel & Aronson 1999; Thompson 2005). However, the geographical distribution of the cork oak haplotypes does not appear to be related to cultivation. In fact, fossil pollen and wood records suggest that cork oak was distributed in approximately the same areas as today even before the Neolithic (Fig. 2). In the Iberian Peninsula and North Africa, there is palynological evidence for a long-term persistence of cork oak dating back to the last glacial, indicating that at least some of the areas where cork oak is presently found had glacial climate conditions suitable for its survival. The pollen records of cork-oak of postglacial age do not exclude the presence of *Q. suber* in much earlier times at a level not detected by pollen analysis. In Italy, the distributions of *Q. suber* and *Quercus cerris* overlap. As the pollen of these two oak species cannot be distinguished, it is not possible to use fossil pollen to reconstruct the history of cork oak from the Italian peninsula.

Other cultivated tree species in the Mediterranean display low geographical structure in genetic variation, arguing for a multidirectional diffusion of the cultivated taxa because of human activity. For example, in *Castanea sativa* (Fineschi et al. 2000), the low geographical structure of the chloroplast diversity may be explained with a strong human impact, documented by a wealth of fossil data during the last few thousands of years (Conedera et al. 2004). The phylogeographical pattern of *Olea europaea* (Besnard et al. 2002; Besnard & Bervillé 2002; Besnard et al. 2007), although slightly different from that of chestnut, confirms the important role played by humans in transferring propagation material throughout the Mediterranean, mainly following an east–west direction. Contrary to this pattern, distinct cork oak haplotypes are found even in neighbour geographical areas such as Corsica and Italy, Tunisia and Sicily, and western and eastern Morocco, indicating that human activity did not blur the original genetic structure.

Another possible hypothesis to explain the modern distribution of the cpDNA haplotypes of cork oak is its post-glacial population expansion from potential glacial refuges in Italy, North Africa and Iberia (Lumaret et al. 2005). Cork oak populations are expected to migrate from one region to the other along continental areas. Excluding exceptional and unproven cases of long-distance dissemination, the modern distribution of haplotype H3 cannot be ascribed to a postglacial colonization route starting from Tunisia, crossing the Mediterranean Sea and reaching southern France through Sardinia and Corsica. In fact, the geological record suggests that there have been no land connections between northern Africa and Europe, or between Sardinia and Provence during the Quaternary, in spite of substantial sea-level oscillations.

Only during a short time interval of the Messinian salinity crisis, between 5.59 and 5.50 million years ago (Krijgsman et al. 1999), might there have been such connections, but the Mediterranean Basin may not have been completely dry even during this time (Cipollari et al. 1999; Manzi et al. 2005) and the exposed land may not have been suitable for plant life (Quézel 1995; Thompson 2005). More importantly, our data are not consistent with a Messinian migration of
cork oak. In fact, Tunisia and Sicily, which could have been connected to each other, show different haplotypes (H3 and H1, respectively), while Tunisia and Sardinia, which would have remained separated even in the event of a sea-level drop of 1000 m, share the same haplotype (H3).

Mediterranean biogeographers agree that the present distributional patterns of many taxa in the region are well explained by the late Cenozoic palaeogeographical evolution of the Mediterranean (Greuter 1995; Quézel 1995; Blondel & Aronson 1999; Comes 2004; Thompson 2005). In the last 40 years, a number of geodynamic reconstructions of the Mediterranean basin have been advanced (Rosenbaum et al. 2002). Although some aspects of these models are still controversial, there is a general agreement that during the Oligocene, the European–Iberian continental margin assembled continental terranes that are now found in Calabria, Sicily, Corsica, Sardinia, Kabylies (Algeria), Balearic Islands and Rif range in Morocco (Fig. 3). Starting in the late Oligocene (30–25 million years) and during the Miocene, these microplates underwent separation from the mainland and drifted towards south or southeast reaching their current positions (Gueguen et al. 1998; Roca et al. 1999; Gelabert et al. 2002; Rosenbaum et al. 2002; Cavazza & Wezel 2003; Carminati & Doglioni 2005).

Cork oak is presently found on all these plates together with many other Mediterranean palaeo-endemics. Thus, it seems reasonable to infer an early Cenozoic origin for this species in the Iberian continent and subsequent displacement on the drifted microterranes. This is consistent with two fossil records of cork oak of Miocene age in Portugal (de Carvalho 1958; Mai 1995) and two Pliocene finds in Tunisia and Galicia, respectively (Losa Quintana 1978; Quézel 1995).

If we assign the colours of the modern cork oak haplotypes (Fig. 1) to the corresponding microplates rifted off the European continent during the Miocene (Fig. 3), we find a remarkable conformity between the position of the drifting blocks and the distribution of haplotypes. Cork oak populations, isolated from the Iberian population and island-rafted across the Mediterranean to their present position, appear to have undergone a genetic drift consistent with the break-up of the main continent.

The distribution of haplotypes suggests a western Mediterranean origin for cork oak, according to the following palaeogeographical history. Provence, Corsica, Sardinia and Kabylies, which were connected to each other until the early Miocene (Gueguen et al. 1998; Roca et al. 1999; Rosenbaum et al. 2002), share haplotype H3 that differs from the Iberian haplotype (H4) by two mutations. Therefore haplotype H3 presumably differentiated when Provence, Corsica, Sardinia and Tunisia were still in connection with each other, but already separated from Iberia (H4). The Calabrian haplotype H1, very different from both H4 and H3, indicates an early isolation of the Calabrian microplate from Iberia and the Corsica–Sardinia–Kabylies block. This scenario is supported by the observation that Corsica and Sardinia have closer floristic affinities to the Balearics than to Calabria (Gamisans & Jeanmonod 1995). Alternatively, the differences between H3 and H1 might be due to a possible cytoplasmic introgression with Q. cerris in the Italian peninsula and Sicily, which may have occurred rather late, after the detachment of Calabria from the Corsican–Sardinian block. This second hypothesis is supported by: (i) a late (10–8 million years ago) detachment of Calabria and Sicily (Peloritani mountains) from the Corsica–Sardinian block (Rosenbaum et al. 2002), and (ii) geological studies indicating that Sicily moves together with the African Plate in its collision against Calabria (Goes et al. 2004), and that Sicily and Tunisia may have shared a geological connection until
at least the Late Pliocene–Pleistocene across the Magrebides (Rosenbaum et al. 2002).

The Balearic Islands and Rif (sharing haplotype H5) underwent a break-up leading to the displacement of the Rif chain from the Iberian mainland to Morocco, which must have occurred after the introgression of Quercus ilex by Q. suber. Our hypothesis still holds if we assume that only H3 and H4 are the ancestral Q. suber haplotypes, with H1, H2 and H5 originating through either ancient or recent introgression with Q. cerris (H1 and H2) and Q. ilex (H5).

The geodynamic models for the western Mediterranean do not provide detailed ages for break-up events, which are believed to have occurred between 25 million years and 15 million years (Gueguen et al. 1998; Roca et al. 1999; Gelabert et al. 2002; Rosenbaum et al. 2002; Cavazza & Wezel 2003; Carminati & Doglioni 2005). Even with this large age uncertainty, it is possible to highlight some evolutionary patterns. In particular, the broken distribution of haplotype H3 suggests that cpDNA differentiation occurred at a time of intense tectonic activity along the Iberian margin, which produced the detachment of the Corsica–Sardinia–Kabylies block from the mainland during the late Oligocene, and was followed by the fragmentation of Corsica, Sardinia and Kabylies in separate microplates after 20 million years (Gueguen et al. 1998; Roca et al. 1999; Gelabert et al. 2002; Rosenbaum et al. 2002; Cavazza & Wezel 2003; Carminati & Doglioni 2005). After the fragmentation of the Corsica–Sardinia–Kabylies block, for a time span of at least 15 million years, cork oak populations appear to have persisted within each microplate without detectable cpDNA modifications.

Long-term permanence in situ and prolonged evolutionary standstill of Mediterranean palaeo-endemic species, implying long periods without speciation, are well known to biogeographers and evolutionists (Greuter 1995; Quézel 1995; Blondel & Aronson 1999; Comes 2004). Our data suggest the possibility of a long-lasting stability not only at the species level, but also at the level of cpDNA microsatellites, in spite of isolation due to insularity conditions and of population responses to Quaternary climate oscillations. Low rates of evolution of tree species were highlighted and discussed by Petit & Hampe (2006). Indeed, perennials evolve more slowly than other plants at the DNA sequence level, for chloroplast, mitochondrial, and nuclear genes, particularly at silent sites, as reported by Petit & Hampe (2006).

This issue raises the question whether other genetic markers, evolving at faster rates than cpDNA, would provide a different microevolutionary scenario and whether other Mediterranean palaeo-endemics have similar evolutionary histories. Only few published phylogeographical studies refer to such taxa. Among them, Pinus pinaster Aiton has a modern distribution similar to Q. suber, as it is limited to the western Mediterranean basin, where it occurs as scattered nuclei in Portugal, Spain, southern France, Italy, Morocco, Tunisia, Algeria, and in several islands, such as Sardinia, Corsica, Pantelleria and Minorca (Fig. 4). Pinus sect. Pinaster is considered to be a palaeomediterranean element, being found with many other evergreen taxa in early Cenozoic deposits (Mai 1995). Although it is often described as an invasive species, P. pinaster is certainly native in the Iberian Peninsula, where both fossil wood (Figueiral 1995) and pollen (Carrión et al. 2000b) indicate that it survived during the last glacial, sharing its refugia with Q. suber.

Molecular analysis of mitochondrial (maternally inherited) markers of P. pinaster provides a clear picture of three nonoverlapping mitochondrial haplotypes (Burban & Petit 2003). In particular, one mitotype is found in Morocco, a second mitotype is present in the Iberian peninsula, and a third one is found in all populations from southeastern France, Corsica, Italy, Pantelleria Island, Tunisia and Algeria (Fig. 4). The similarity of the distribution of the second mitotype of P. pinaster with the distribution of haplotype...
H3 of Q. suber strongly supports our hypothesis that: (i) a genetic differentiation occurred when Corsica, Sardinia and the Kabylie blocks were already separated from the Iberian mainland, but still in connection among them and with southern France; (ii) survival of trees in the rifted microterranes persisted until present without significant genetic modifications, even at the population level; and (iii) Neogene tectonic events may leave their footprint on modern population genetic diversity/differentiation. It is remarkable that such scenario is supported by the distribution of diversity detected using two different molecular markers (cpDNA for Q. suber and mitochondrial DNA for P. pinaster).

This evidence puts forward the question of how much of the modern genetic diversification of Mediterranean populations is due to isolation in glacial refugia, as generally assumed, and how much is to be traced back to the Tertiary history of taxa, as suggested by the distributions of Q. suber and P. pinaster. There is therefore a need for new analyses on different taxa and molecular markers. This might lead to a reassessment of the age of processes shaping the phylo-geographical structure of tree species.

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Donatella Magri is a palaeoecologist interested in past Mediterranean vegetation. Rosanna Bellarosa and Bartolomeo Schirone are molecular botanists interested in oak phylogenesis. This work represents part of the PhD thesis of Marco C. Simeone. A. Buonamici is a PhD student working on the phylogeography of Angiosperms. F. Sebastiani is experienced in the development and application of molecular markers for the analysis of diversity in trees. G. G. Vendramin’s and Silvia Fineschi’s studies focus on conservation and population genetics, with particular emphasis on range-wide phylogeography and fine-scale population gene dynamics.

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## Supplementary material

The following supplementary material is available for this article:

**Table S1** Location and frequency of SSR haplotypes within Quercus suber populations

**Table S2** Description of SSR haplotypes identified in Quercus suber (in bold polymorphic fragments)

**Fig S1** Location and references of palaeobotanical sites reported in Fig. 2

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