RELICT POPULATIONS OF *QUERCUS CALLIPRINOS* WEBB ON SARDINIA ISLAND IDENTIFIED BY CHLOROPLAST DNA SEQUENCES

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Received June 7, 2000, accepted October 26, 2000

ABSTRACT

Among species of the genus *Quercus* L. in temperate regions, the horny oak is indigenous in Southern Europe and from Northern Africa to the Middle East. Several authors have classified the "horny oak complex" in different way, taking into account the variability of different morphological traits. Horny oak shows great polymorphism, and the exact geographic distribution of the populations is not easily defined. Moreover, another complication is the unclear systematics, and some authors suggest that the horny oak complex split in two different species: *Quercus calliprinos* Webb, which has an eastern distribution (the Mediterranean basin), and *Quercus coccifera* L., which has a more western distribution.

To contribute to the systematics of three relict populations of horny oak on Sardinia, we studied the *trn*L-*trn*F region of chloroplast DNA (cpDNA). We evaluated eleven horny oak populations sampled throughout the geographic distribution of the species, as well as six additional species of *Quercus* and *Fagus sylvatica* L. for comparison. In the *Fagaceae* family, the variable and informative sites at the interspecific level are localised in the sequences of the introns, whereas the intergenic region has a higher variability at the intraspecific level, indicating that the intergenic region evolves at a faster mutation rate than the intron. It was possible to distinguish between the different species of *Fagaceae* and the two systematic entities of horny oaks studied with cpDNA analysis. Moreover, it was possible to identify the relict horny oak populations on Sardinia as *Q. calliprinos*. These populations represent an isolated group of *Q. calliprinos* in the western Mediterranean basin. The geographic isolation permitted a mutation to arise in the intergenic region near a microsatellite, and this mutation was fixed in the group and identifies the relict Sardinia populations.

Key words: oak, *Quercus calliprinos* Webb, chloroplast DNA, *trn*L intron, *trn*L-*trn*F region

INTRODUCTION

To preserve biological diversity, conservation programs must be guided by the biology of the species or systems that they seek to preserve. This is the basic axiom of conservation biology (FALK & HOLSINGER 1991). The current geographical distribution of living organisms depends upon both ecological and historical parameters. The influence of historical parameters can be assessed *via* historical biogeographic studies (WILEY 1988), and most of these have compared the geographical distribution of taxa at or above the species level.

With the development of molecular methods, it is now possible to investigate the geographic variation using molecular markers and to deduce intraspecific phylogeographic structures (AVISE *et al.* 1987). Intraspecific phylogeography, defined by AVISE *et al.* (1987) as the study of the relationship between the phylogeny of variants and their geographic distribution, is more and more becoming of interest in evolutionary science (DUMOLIN-LAPÈGUE *et al.* 1997).

Several studies of geographical variation in the *Fagaceae* family have been done using molecular markers of chloroplast DNA (cpDNA) (DEMESURE *et al.* 1996; DUMOLIN-LAPÈGUE *et al.* 1997; DUMOLIN-LAPÈGUE *et al.* 1998, TARBELET *et al.* 1998; WHITTEMORE and SCHAAL 1991), but very few analyses of cpDNA or nuclear sequences at both the inter- and intraspecific level have been performed (FERRIS *et al.* 1995; FERRIS *et al.* 1998; MANOS *et al.* 1999).

The oaks belong to the most widely distributed genus of the *Fagaceae* family; they are dominant trees and shrubs in a broad range of habitats in the Northern Hemisphere. Among the species of *Quercus* L. distributed in the temperate regions, the horny oak is indigenous in Southern Europe and from Northern Africa to the Middle East (TUTIN 1964–1980).

Several authors have classified the "horny oak complex", taking into account the variability of different morphological traits, either into *Quercus coccifera*

L. and *Quercus calliprinos* Webb or only within *Q. coccifera* with several subspecies and varieties (WEBB 1838; DE CANDOLLE 1864; BOISSIER 1879; CAMUS 1936–1938; NAHAL 1962; ZOHARY 1966; TUTIN 1964–1980). Moreover, the geographic distribution of the two entities is not clear. CAMUS (1936–1938) indicated the western Mediterranean basin, probably excluding the island of Sardinia, as the distribution range for *Q. coccifera* and the eastern zone as the distribution range of *Q. calliprinos*. Both species could be present in the Balkan and Italian Peninsulas. However, the exact distribution of *Q. coccifera* on a regional scale and on national Italian territory does not appear to be easily delimited, as it shows great polymorphism (PIGNATTI 1982). Several researchers have included the southwest of Sardinia in the distribution range of *Q. coccifera* (MARTINOLI 1953; ARRIGONI 1972; MARIANI COLOMBO *et al.* 1983; MOSSA 1990), and CAMARDA and VALSECCHI (1982), in particular, excluded the presence of *Q. calliprinos* from the island. In contrast, GENTILE and GASTALDO (1976) indicated that the relict populations on Sardinia belong to *Q. calliprinos* based on morphological and anatomic-micrometrical studies of leaf samples. Therefore, it is of interest to study the phylogenetic problem of horny oak by molecular methods and, in particular, to investigate the chloroplast genome.

The cpDNA molecule has a lower mutation rate than the nuclear genome, and it is possible that changes in the structure of the chloroplast genome are rare because transposition, recombination, and transfer of sequences between different genomes are rare. Therefore, some authors consider it as a slowly evolving genome that is not suited for fine phylogenetic resolution because it will not have enough changes (BIRKY 1988). Moreover, inheritance of cpDNA in *Quercus* genus is known to be maternal (DUMOLIN *et al.* 1995).

The main goal of this study was to: (i) analyse the sequence of the chloroplast *trn*L-*trn*F region among and within species of the genus *Quercus*; (ii) study polymorphism among and within populations of the horny oak complex; and (iii) provide a systematic position for the relict Sardinia populations.

MATERIALS AND METHODS

Taxa and populations analysed

Eleven horny oak populations and six additional *Quercus* species for comparison were sampled, with a total of 110 and 60 individuals, respectively. Ten individuals of *Fagus sylvatica* L. were sampled and used as the outgroup species (Table 1).

DNA extraction

Total DNA was extracted from young leaves or dormant buds using the DNeasy plant kit (QiAgen, Germany) following the manufacturer specifications.

PCR amplifications

The *trn*L-*trn*F cpDNA region was amplified using the universal primers (c and d) described by TARBELET *et al.* (1991). Each 20 µl of amplification reaction mixture contained: 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.001% (w/v) gelatin, 250 µM of each deoxynucleoside triphosphate, 1 µM of each primer, 1 U of *AmpliTaq* DNA polymerase (Perkin-Elmer, USA), and 10 ng of genomic DNA. After incubation for 60 s at 90°C and for 90 s at 95°C, the reaction mixtures were subjected to different cycles by using the following temperature profiles: (1) 95 °C for 30 s, 60 °C for 30 s, 72 °C for 4 min, for 5 cycles; (2) 95 °C for 30 s, 55 °C for 30 s, 72 °C for 4 min, for 5 cycles; and (3) 95 °C for 30 s, 50 °C for 30 s, 72 °C for 4 min, for 25 cycles. Amplification products were then incubated at 72 °C for 10 min. A Perkin Elmer 9700 thermocycler was used. 5 µl of each amplification product was analyzed by gel electrophoresis on 1% (w/v) agarose gel (GIBCO BRL, USA) at 10 $V \cdot cm^{-1}$ for 2 h in Tris acetate-EDTA buffer containing 0.5 $\mu g \cdot ml^{-1}$ of ethidium bromide (SAMBROOK *et al.* 1989). The gels were photographed and analyzed with an UVP scanner (Photo-Capt, Vilbert Coormat, France).

Restriction fragment length polymorphism (RFLP) analysis

15 µl of each PCR mixture (1 µg of DNA) was treated with 5 U of the restriction enzymes *Alu*I or *Hae*III (Takara, Japan) in a total volume of 20 µl at 37 °C for 4 h. The mixtures (20 µl per lane) were analyzed by electrophoresis on 4 % (w/v) Separide gel (GIBCO BRL, USA) as above, except that 12 h were used. The gels were photographed and analyzed with an UVP scanner (Photo-Capt, Vilbert Coormat, France).

DNA sequencing

The amplification products were gel purified using the Agarose Gel DNA Extraction Kit (Boehringer-Mannheim, Germany) following the manufacture specifications. Direct sequencing of amplified DNA was done in both directions from independent amplification reactions using the dideoxy-chain termination method (SANGER *et al.* 1977) and the Sequenase kit (USB) with the necessary modifications for direct PCR sequencing.

Table 1. Taxonomic position (classification by TUTIN *et al.*1993) and geographic origin of plant materials *(Fagaceae* family).

Taxa	Number of individuals	Geographic origin
Subfamily *Fagoideae* *Fagus sylvatica L.*	10	San Boldo (Friuli Venezia Giulia, Italy)
Subfamily *Quercoideae* Subgenus *Quercus*		
Quercus robur L.	10	Münden–Low Saxony (Germany)
Quercus pubescens Willd.	10	Radda in Chianti (Tuscany, Italy)
Subgenus *Cerris*		
Quercus suber L.	10	Tempio Pausania (Sardinia, Italy)
Quercus trojana Webb	10	Cassano Murge (Apulia, Italy)
Quercus macrolepis Kotschy	10	Tricase (Apulia, Italy)
Subgenus *Sclerophyllodrys*		
Quercus ilex L.	10	Tempio Pausania (Sardinia, Italy)
Quercus coccifera L.	10	Avignon (France)
Quercus calliprinos Webb	10	Haifa (Israel)
Horny oak populations [a]		
Horny oak (CPMG)	10	Cassano Murge (Apulia, Italy)
Horny oak (LPG)	10	Lecce (Apulia, Italy)
Horny oak (RODI)	10	Rodi (Greece)
Horny oak (CYPRUS)	10	Cyprus (Greece)
Horny oak (TUR)	10	Antalya (Turkey)
Horny oak (PORT)	10	Portixeddu (Sardinia, Italy)
Horny oak (PINO)	10	Porto Pino (Sardinia, Italy)
Horny oak (MON)	10	Castello di Monreale (Sardinia, Italy)
Horny oak (SIC)	10	Ragusa (Sicily, Italy)

[a)] CMPG: Cassano Murge (Apulia, Italy), LPG: Lecce (Apulia, Italy), RODI: Rodi (Greece), CYPRUS: Cyprus (Greece), TUR: Antalya (Turkey), PORT: Portixeddu (Sardinia, Italy), PINO: Porto Pino (Sardinia, Italy), MON: Castello di Monreale (Sardinia, Italy), SIC: Ragusa (Sicily, Italy).

Table 2. Sequence variations in the *trn*L intron region, second *trnL* exon, and *trnL–trn*F intergenic region of 9 *Fagaceae* species considered in this study.

Region	*trn*L intron	*trn*L exon	*trn*L-*trn*L intergenic	Total
Length range (bp)	479–537	50	197–383	784–923
Length mean (bp)	485.4	50	369.7	885.6
Aligned length (bp)	565	50	416	1031
G + C content range (%)	31.4–32.5	46–48	29.9–31.2	31.7–32.3
G + C content mean (%)	32.2	47	30.7	32.3
Constant sites	419 (74.2 %)	49 (98 %)	93 (22.4 %)	561 (54.4%)
Variable sites (F)	146 (25.8 %)	1 (2 %)	323 (77.6 %)	470 (45.6%)
Informative sites (F)	141 (96.6 %)	1 (100 %)	276 (85.4 %)	418 (88.9%)
Variable sites (Q)	17 (3.1 %)	0	66 (18.9 %)	83 (8.7%)
Informative sites (Q)	15 (88.2 %)	0	25 (37.9 %)	40 (48.2%)
In/del	109	0	258	367
Transitions	15	1	20	36
Transversions	22	0	45	67
Transitions/transversions	0.7		0.4	0. 5

[a)] (F): sites among all species;
[b)] (Q): sites among *Quercus* species;
[c)] In/del: insertion/deletion.

The *Quercus* and *F. sylvatica* sequences generated in this study have been submitted to GenBank with the following accession numbers: *Quercus macrolepis* Kotschy (AF133647), *Quercus trojana* Webb (AF133648), *Quercus calliprinos* (AF133649), *Quercus coccifera* (AF133650), horny oak of Sardinia (AF133651), *Quercus pubescens* Willd. (AF133652), *Quercus robur* L. (AF268937), *Quercus suber* L. (AF-268938), *Quercus ilex* L. (AF268939), and *F. sylvatica* (AF133654).

Data analysis

DNA sequences were multiply aligned, using the Clustal X program (THOMPSON *et al.* 1997), among them and with sequences of *Q. robur* (X75707, X75714), *Q. suber* (AJ002058, AJ002163), *Q. pubescens* (Z48964, AJ002161), and *Q. coccifera* (AJ00-2060, AJ002160) present in GenBank, and these alignments were used for further analysis. Phylogenetic analysis was performed as maximum-likelihood phylogeny estimation using the DNAML program in PHYLIP version 3.5c (FELSENSTEIN 1993). A maximum likelihood tree was inferred using a transition /transversion ratio of 0.5, global rearrangement, and randomizing the sequence addition order (JUMBLE).

RESULTS

RFLP analysis

To investigate the genetic variability among the horny oak populations, the cpDNA *trn*L-*trn*F region was analyzed by restriction analysis. The restriction profiles of the horny oak population (Table 1), ten individuals for each population, were compared with those obtained from populations of *Q. macrolepis*, *Q. trojana*, *Q. ilex*, *Q. suber*, *Q. pubescens*, *Q. robur*, and *F. sylvatica* (ten individuals for each population). The length of the amplified fragment was about 923 bp for *Quercus* species and 784 bp for *F. sylvatica*. The amplification products were digested with *Hae*III and *Alu*I endonucleases (the restriction sites are reported in Fig. 1). The restriction fragments of the *trn*L-*trn*F region did not show polymorphism among individuals within any population analysed. In contrast, between the horny oak populations from Avignon (France) and from Haifa (Israel), classified by CAMUS (1936–1938) as *Q. coccifera* and *Q. calliprinos*, respectively, one fragment of different length (370 bp and 366 bp, respectively) was found. The same fragment of *Q. calliprinos* (366 bp) was present in the horny oak populations of Sardinia and Sicily, and the same fragment of *Q. coccifera* (370 bp) was found in populations from Apulia, Cyprus, and Rodi.

Sequence analysis

To corroborate the results of the restriction analysis, the cpDNA *trn*L-*trn*F region of one individual for each species and population was sequenced.

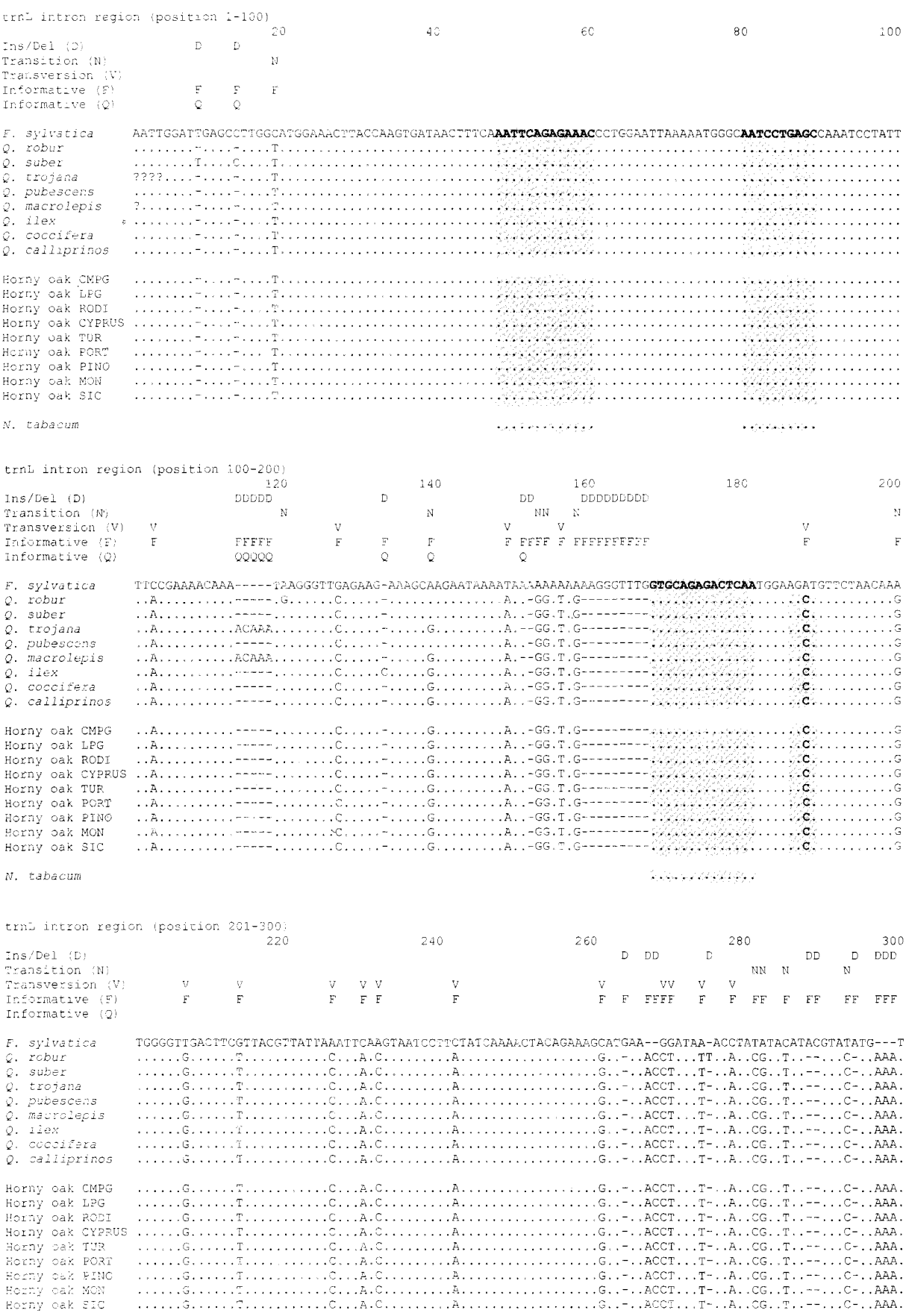
The DNA sequence of the *trn*L-*trn*F region is summarized in Table 2, and aligned sequences are shown in Fig. 1. The entire region, including the *trn*L intron, the second *trn*L exon, and the *trn*L-*trn*F intergenic spacer, ranged from 784 bp in *F. sylvatica* to 923 bp in *Q. macrolepis*.

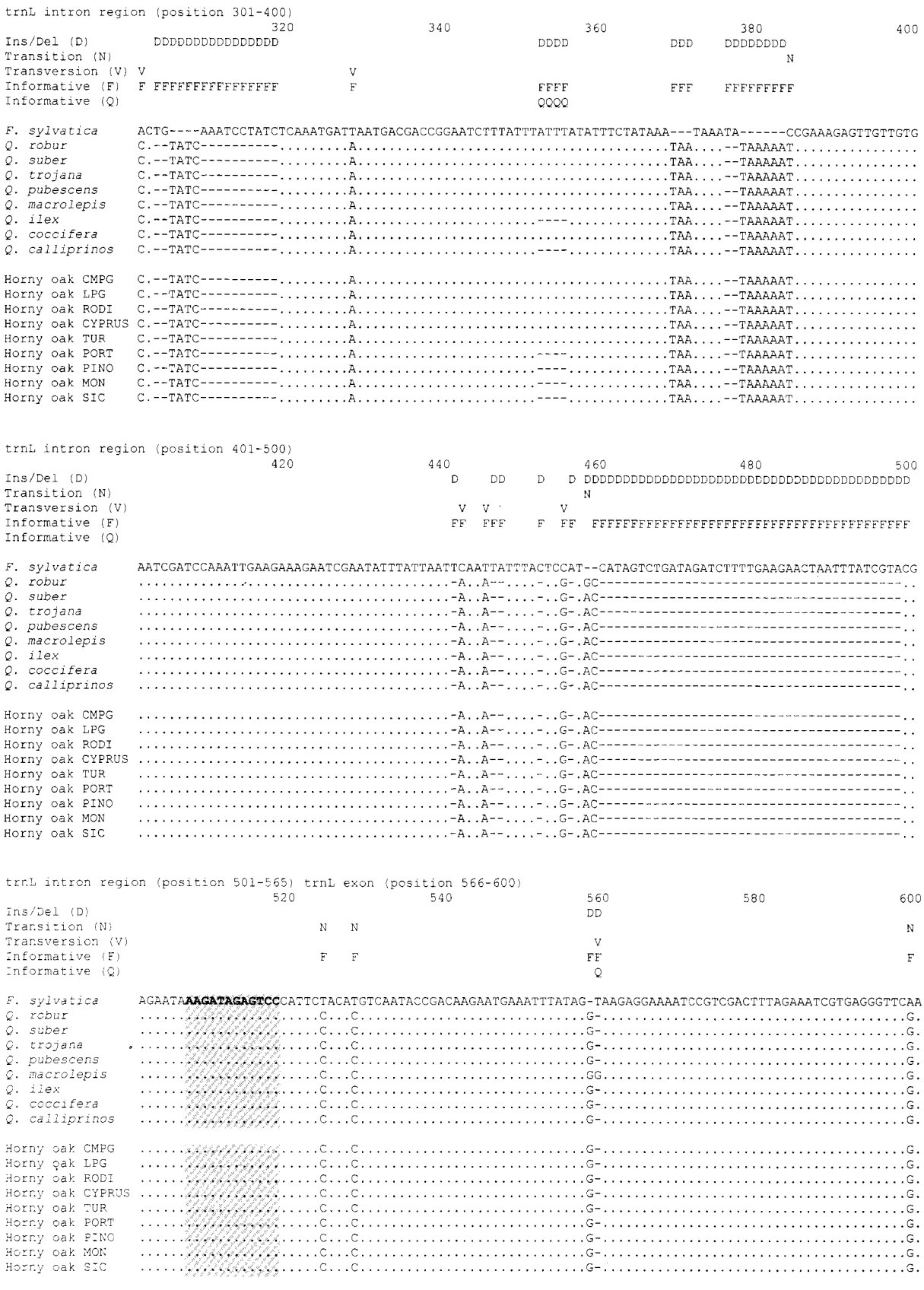
After multiple alignment of the *trn*L-*trn*F region, the *trn*L intron alignment had a total length of 565 bp, the second *trn*L exon a length of 50 bp, and the *trn*L-*trn*F intergenic spacer a length of 416 bp. There were less variable sites (146 bp; 25.8%) and informative sites (141 bp; 96.6%) in the intron than in the intergenic region (Table 2).

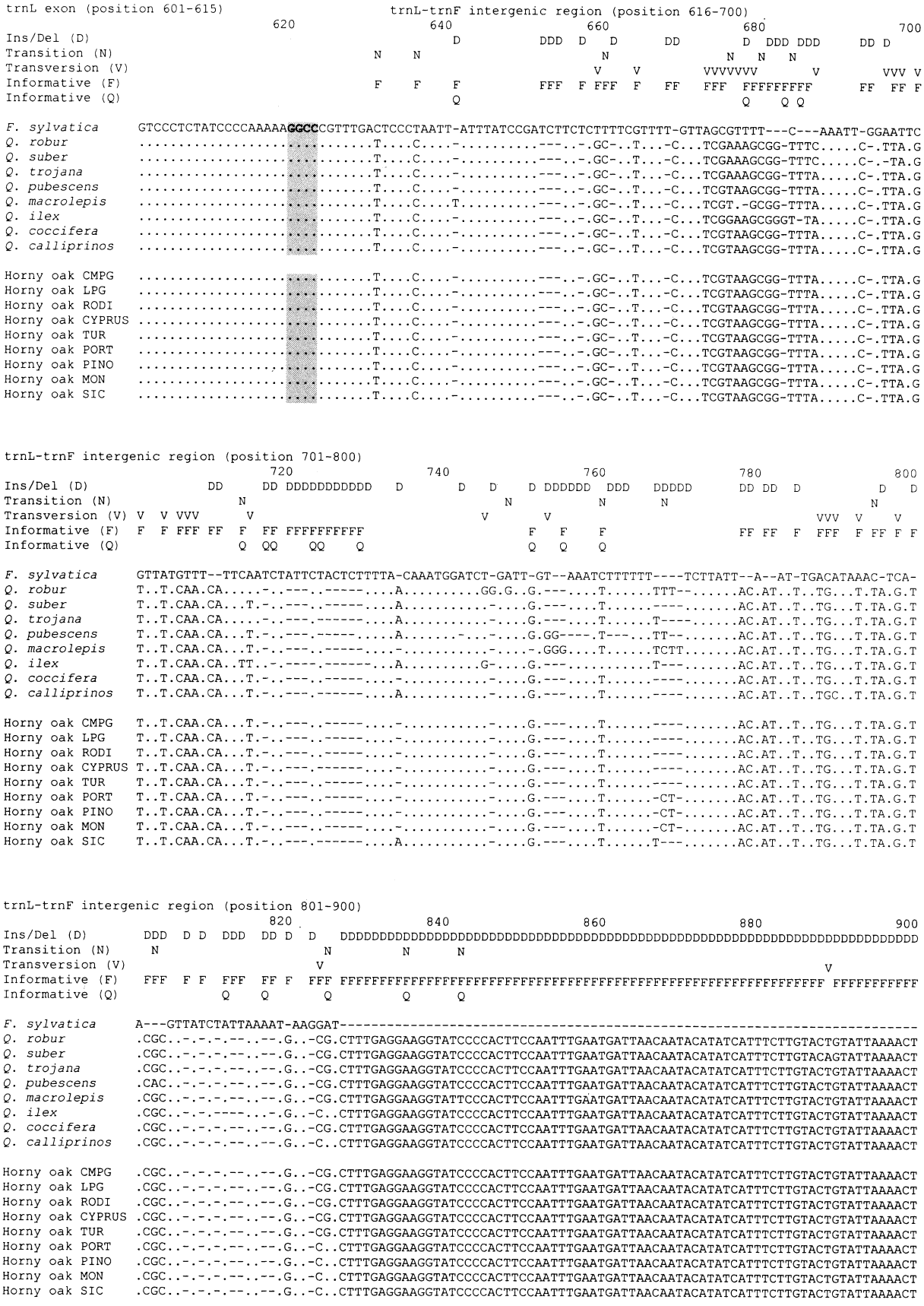
Most of the conserved nucleotides are contained within the four short sequences designated P, Q, R, and S (CECH 1988). The P, Q, R, and S zones are indicated in the alignment, and these short sequences are identical in the species analyzed and in the *Nicotiana tabacum* L. ones (GenBank Accession Number: Z00044) (Fig. 1).

The variable and informative sites are localised among the intron zones and, in particular, between R and S. These sites seem to show differences between subfamilies of the *Fagaceae* family: (i) *F. sylvatica*, a species of the *Fagoideae* subfamily, shows an insertion between positions 159 and 167 and a large insertion between positions 460 and 498, and (ii) the *Quercoideae* subfamily shows differences among species (17; 3.1% variable sites of which 15; 88.2% are informative sites), but intraspecific variability was only found (in positions 120 and 275) in *Q. robur* (Fig. 1 and Table 2). In contrast, there are high levels of sequence divergence in the intergenic region between subfamilies. *F. sylvatica* shows a large deletion between positions 826 and 1003. The *Quercoideae* subfamily shows differences among and within species; in fact, 25 sites of the 66 variable sites show intraspecific variability in most species (Fig. 1).

A dendrogram of 8 *Quercus* species and of *F. sylvatica* as the outgroup was established by maximum-likelihood (the intraspecific variable sites were excluded from the analysis as uninformative at a phylogenetic level), according to the assumptions of the model. In particular, each site in the sequence is assumed to have evolved independently, and all relevant sites are included in the sequence, not just those that have changed or that are "phylogenetically informative". The deletion and insertion events, which usually


trnL intron region (position 1-100)
20
40
60
80
100
Ins/Del (D)
Transition (N)
Transversion (V)
Informative (P)
Informative (Q)
F. sylvatica
Q. robur
Q. suber
Q. trojana
Q. pubescens
Q. macrolepis
Q. ilex
Q. coccifera
Q. calliprinos
Horny oak CMPG
Horny oak LPG
Horny oak RODI
Horny oak CYPRUS
Horny oak TUR
Horny oak PORT
Horny oak PINO
Horny oak MON
Horny oak SIC
N. tabacum
trnL intron region (position 100-200)
120
140
160
180
200
Ins/Del (D)
Transition (N)
Transversion (V)
Informative (P)
Informative (Q)
F. sylvatica
Q. robur
Q. suber
Q. trojana
Q. pubescens
Q. macrolepis
Q. ilex
Q. coccifera
Q. calliprinos
Horny oak CMPG
Horny oak LPG
Horny oak RODI
Horny oak CYPRUS
Horny oak TUR
Horny oak PORT
Horny oak PINO
Horny oak MON
Horny oak SIC
N. tabacum
trnL intron region (position 201-300)
220
240
260
280
300
Ins/Del (D)
Transition (N)
Transversion (V)
Informative (P)
Informative (Q)
F. sylvatica
Q. robur
Q. suber
Q. trojana
Q. pubescens
Q. macrolepis
Q. ilex
Q. coccifera
Q. calliprinos
Horny oak CMPG
Horny oak LPG
Horny oak RODI
Horny oak CYPRUS
Horny oak TUR
Horny oak PORT
Horny oak PINO
Horny oak MON
Horny oak SIC


trnL intron region (position 301-400)
320
340
360
380
400
Ins/Del (D)
Transition (N)
Transversion (V)
Informative (F)
Informative (Q)
F. sylvatica
Q. robur
Q. suber
Q. trojana
Q. pubescens
Q. macrolepis
Q. ilex
Q. coccifera
Q. calliprinos
Horny oak CMPG
Horny oak LPG
Horny oak RODI
Horny oak CYPRUS
Horny oak TUR
Horny oak PORT
Horny oak PINO
Horny oak MON
Horny oak SIC
trnL intron region (position 401-500)
420
440
460
480
500
trnL intron region (position 501-565) trnL exon (position 566-600)
520
540
560
580
600
N. tabacum


trnL exon (position 601-615)
trnL-trnF intergenic region (position 616-700)
trnL-trnF intergenic region (position 701-800)
trnL-trnF intergenic region (position 801-900)
F. sylvatica
Q. robur
Q. suber
Q. trojana
Q. pubescens
Q. macrolepis
Q. ilex
Q. coccifera
Q. calliprinos
Horny oak CMPG
Horny oak LPG
Horny oak RODI
Horny oak CYPRUS
Horny oak TUR
Horny oak PORT
Horny oak PINO
Horny oak MON
Horny oak SIC

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trnL-trnF intergenic region (position 901-1000)
                                          920                 940                 960                 980                1000
Ins/Del (D)           DDDDDDDDDDDDDDDDDDDDDDDDDDDDDDDDDDDDDDDDDDDDDDDDDDDDDDDDDDDDDDDDDDDDDDDDDDDDDDDDDDDDDDDDDDDDDDDDDDDD
Transition (N)
Transversion (V)                              V                            V            V           V           V
Informative (F)       FFFFFFFFFFFFFFFFFFFFFFFF FFFFFFFFFFFFFFFFFFFFFFFFFFFF FFFFFFFFFFFF  FFFFFFFFFFFFFFFFFFFFFFFFFFFFFFFF
Informative (Q)                                                                                     Q           Q

F. sylvatica          ----------------------------------------------------------------------------------------------------
Q. robur              TATAAAGTTTCTTTTTGAAGATCGAAGAAATTACAGGGTCTGGATAAAGCTTTGTAAGACTTTTTTT-GTCTTTTTAATTGACATAAACTCAAGTTATC
Q. suber              TATAAAGTTTCTTTTTGAAGATCCAAGAAATTACAGGGTCTGGATAAAGCTTAGTAAGACTTTTTTTTGTCTTTTTAATTGACATAAACTCAAGTTATC
Q. trojana            TATAAAGTTTCTTTTTGAAGATCGAAGAAATTACAGGGTCTGGATAAAGCTTTGTAAGACTTTTTTT-GTCTTTTTAATTGACATAAACTCAAGTTATC
Q. pubescens          TATAAAGTTTCTTTTTGAAGATCGAAGAAATTACAGGGTCTGGATAAAGCTTTGTAAGACTTTTTTT-GTCTTTTTAATTGACATAAACTCAAGTTATC
Q. macrolepis         TATAAAGTTTCTTTTTGAAGATCGAAGAAATTACAGGGTCTGGATAAAGCTTTGTAAGACTTTTTTT-GTCTTTTTAATTGACATAAACTCAAGTTATC
Q. ilex               TATAAAGTTTCTTTTTGAAGATCCAAGAAATTACAGGGTCTGGATAAAGCTTTGTAAGACTTTTTT-GGTCTTTTTAATGGACATAAACTCAAGTTATC
Q. coccifera          TATAAAGTTTCTTTTTGAAGATCGAAGAAATTACAGGGTCTGGATAAAGCTTTGTAAGACTTTTTTT-GTCTTTTTAATTGACATAAACTCAAGTTATC
Q. calliprinos        TATAAAGTTTCTTTTTGAAGATCCAAGAAATTACAGGGTCTGGATAAAGCTTTGTAAGACTTTTTT-GGTCTTTTTAATTGACATAAACTCCAGTTATC

Horny oak CMPG        TATAAAGTTTCTTTTTGAAGATCGAAGAAATTACAGGGTCTGGATAAAGCTTTGTAAGACTTTTTTT-GTCTTTTTAATTGACATAAACTCAAGTTATC
Horny oak LPG         TATAAAGTTTCTTTTTGAAGATCGAAGAAATTACAGGGTCTGGATAAAGCTTTGTAAGACTTTTTTT-GTCTTTTTAATTGACATAAACTCAAGTTATC
Horny oak RODI        TATAAAGTTTCTTTTTGAAGATCGAAGAAATTACAGGGTCTGGATAAAGCTTTGTAAGACTTTTTTT-GTCTTTTTAATTGACATAAACTCAAGTTATC
Horny oak CYPRUS      TATAAAGTTTCTTTTTGAAGATCCAAGAAATTACAGGGTCTGGATAAAGCTTTGTAAGACTTTTTTT-GTCTTTTTAATTGACATAAACTCAAGTTATC
Horny oak TUR         TATAAAGTTTCTTTTTGAAGATCGAAGAAATTACAGGGTCTGGATAAAGCTTTGTAAGACTTTTTTT-GTCTTTTTAATTGACATAAACTCAAGTTATC
Horny oak PORT        TATAAAGTTTCTTTTTGAAGATCCAAGAAATTACAGGGTCTGGATAAAGCTTTGTAAGACTTTTTTT-GTCTTTTTAATTGACATAAACTCCAGTTATC
Horny oak PINO        TATAAAGTTTCTTTTTGAAGATCCAAGAAATTACAGGGTCTGGATAAAGCTTTGTAAGACTTTTTTT-GTCTTTTTAATTGACATAAACTCCAGTTATC
Horny oak MON         TATAAAGTTTCTTTTTGAAGATCCAAGAAATTACAGGGTCTGGATAAAGCTTTGTAAGACTTTTTTT-GTCTTTTTAATTGACATAAACTCCAGTTATC
Horny oak SIC         TATAAAGTTTCTTTTTGAAGATCCAAGAAATTACAGGGTCTGGATAAAGCTTTGTAAGACTTTTTTT-GTCTTTTTAATTGACATAAACTCCAGTTATC
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trnL-trnF intergenic region (position 1001-1031)
                                         1020       1031
Ins/Del (D)         DDD        D  DDDD  D  D  DDD D
Transition (N)            N  N         N  N  N
Transversion (V)      VVV VVVV            VV    VV
Informative (F)     FFFF FFFFFFF  FFFF FF    FFFF F
Informative (Q)     Q       QQ Q              Q

F. sylvatica        ---GATGCACC-GG----TA-AT-GG---T-
Q. robur            TATT.AAATAA-..ACGA.GC.CG.ATAA.G
Q. suber            TATT.AAATAA-..ACGA.GC.CG.ATAA.G
Q. trojana          TATT.AAATAA-..ACGA.GC.CG.ATAA.G
Q. pubescens        TATT.AAATAA-..ACGA.GC.CG.ATAA.G
Q. macrolepis       TATT.AAATAA-..ACGA.GC.CG.ATAA.G
Q. ilex             -ATTCAAA.TAT..ACGA.GC.CG.ATAT.G
Q. coccifera        TATT.AAATAA-..ACGA.GC.CG.ATAA.G
Q. calliprinos      TATTCAAATAA-..ACGA.GC.CG.ATAA.G

Horny oak CMPG      TATT.AAATAA-..ACGA.GC.CG.ATAA.G
Horny oak LPG       TATT.AAATAA-..ACGA.GC.CG.ATAA.G
Horny oak RODI      TATT.AAATAA-..ACGA.GC.CG.ATAA.G
Horny oak CYPRUS    TATTCAAATAA-..ACGA.GC.CG.ATAAGG
Horny oak TUR       TATT.AAATAA-..ACGA.GC.CG.ATAA.G
Horny oak PORT      TATTCAAATAA-..ACGA.GC.CG.ATAA.G
Horny oak PINO      TATTCAAATAA-..ACGA.GC.CG.ATAA.G
Horny oak MON       TATTCAAATAA-..ACGA.GC.CG.ATAA.G
Horny oak SIC       TATTCAAATAA-..ACGA.GC.CG.ATAA.G
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Fig. 1. Aligned DNA sequences of the *trn*L-*trn*F chloroplast region of 8 *Quercus* species, 9 horny oak populations, and *F. sylvatica.* Nucleotide sites are numbered 5' to 3'. Informative (F): informative sites among all species. Informative (Q): informative sites among *Quercus* species. .... : P, Q, R, and S zones. ***AGCT*** or ***GGCC*** : *Alu*I or *Hae*III sites, respectively. CMPG: Cassano Murge (Apulia, Italy), LPG: Lecce (Apulia, Italy), RODI: Rodi (Greece), CYPRUS: Cyprus (Greece), TUR: Antalya (Turkey), PORT: Portixeddu (Sardinia, Italy), PINO: Porto Pino (Sardinia, Italy), MON: Castello di Monreale (Sardinia, Italy), SIC: Ragusa (Sicily, Italy). Only the P, Q, R, and S regions of the *N. tabacum* sequence are reported.

involve adjacent sites, cannot be adequately modelled by assuming independence of events at different sites (FELSENSTEIN 1993). The dendrogram using a transition/transversion ratio of 0.5 had a ln-likelihood of –1704.5 (Fig. 2), and 13,639 dendrograms were examined. The dendrogram indicated that this cpDNA region enables: (i) distinguishing between the *Quercus* species analyzed; and (ii) identifying *Q. calliprinos* and *Q. coccifera* as different entities.

The horny oak populations

The two populations of *Q. coccifera* (Avignon) and *Q. calliprinos* (Haifa) species, indicated by CAMUS (1936–1938) as two different entities, were distinguished by: (i) a deletion of 4 bp from positions 352 to 355 in the intron region; (ii) one transition in position 825; and (iii) one transversion in position 993. The same deletion of 4 bp in the intron region of *Q. calliprinos* was present in the sequence of *Q. ilex*, but this species is further distinguished from the first by five

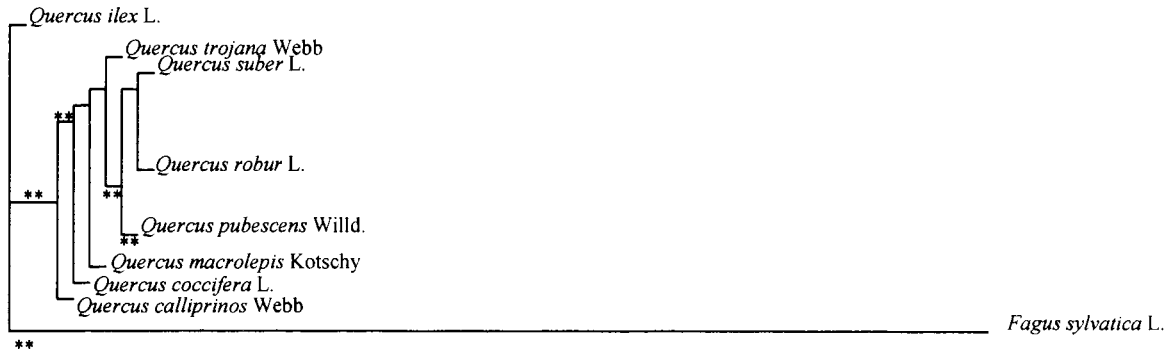

Fig. 2. Maximum-likelihood dendrogram of 8 *Quercus* species and *Fagus sylvatica*. ** = significantly positive, $p < 0.01$.

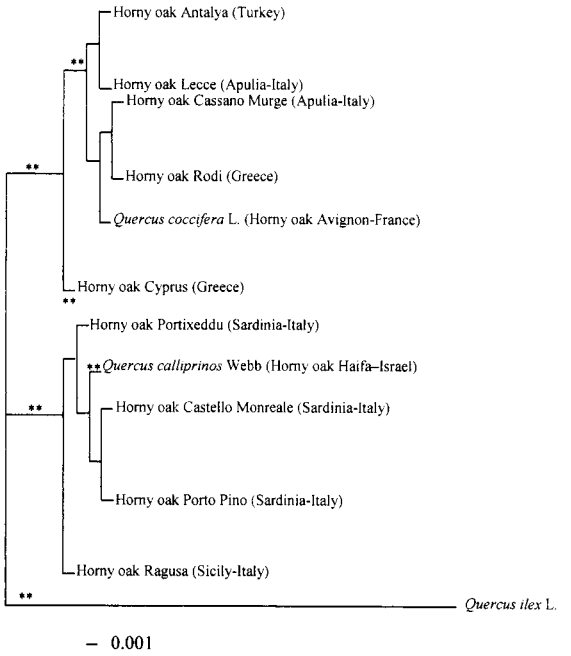

Fig. 3. Maximum-likelihood dendrogram of horny oak populations and *Quercus ilex*. ** = significantly positive, $p < 0.01$. CMPG: Cassano Murge (Apulia, Italy), LPG: Lecce (Apulia, Italy), RODI: Rodi (Greece), CYPRUS: Cyprus (Greece), TUR: Antalya (Turkey), PORT: Portixeddu (Sardinia, Italy), PINO: Porto Pino (Sardinia, Italy), MON: Castello di Monreale (Sardinia, Italy), SIC: Ragusa (Sicily, Italy).

transversions and two transitions (Fig. 1). The deletion, transition, and transversion mutations have enabled subdividing the nine horny oak populations, sampled along their natural area of occurence, into species of *Q. coccifera* and *Q. calliprinos*. Two Apulia, Italy (Cassano Murge and Lecce), two Greek (Rodi and Cyprus), and one Turkish (Antalya) populations belong to *Q. coccifera*, while the Sicily, Italy (Ragusa) and the Sardinia – Italy relict populations (Portixeddu, Porto Pino, and Castello di Monreale) cluster with *Q. calliprinos*.

A dendogram of horny oak populations and *Q. ilex* as the outgroup was established as above, except that all the variable sites were used to evaluate the differences among populations. The dendrogram obtained by maximum likelihood using a transition/transversion ratio of 0.5 had a ln-likelihood of –1704.5 (Fig. 3), and 13,639 dendrograms were examined. The dendogram shows two separated clusters of the horny oak populations, one clustering with the *Q. coccifera* entity and the other with *Q. calliprinos*. This result suggests that the two entities can be distinguished using the cpDNA *trn*L-*trn*F region, even if it is not possible to establish that they are two species.

The *Q. calliprinos* populations were more polymorphic than the *Q. coccifera* populations. In particular, the intergenic region shows intraspecific differences among populations. The insertions between positions 767 and 768 (CT) identify the Sardinia populations (Fig.1), and one insertion in the 766 position (T) identifies the Sicily population. Moreover, the Cyprus population presents two transversions common to some populations of *Q. calliprinos*.

DISCUSSION

It is well known that oak species are difficult to identify and classify taxonomically because of their inter and intraspecific morphological variations, which may be the result, in part, of hybridization and introgression (MULLER 1952; RUSHTON 1993; BACILLIERI *et al.* 1996). Moreover, hybridization in the oaks has been used to argue for alternatives to the biological species concept, as well as to suggest a mechanism by which species can adapt genotypically to a change in the ecological landscape (VAN VALEN 1976). DNA-based evidence of gene flow between species was first reported on the basis of shared patterns of cpDNA haplotypes in sympatric populations of white oak

species (WHITTEMORE & SCHAAL 1991). Additional studies of cpDNA from a broad sampling of populations of two western European white oak species suggested that geographical patterning and discontinuities among haplotypes could also be explained by geological barriers (FERRIS *et al.* 1993) and postglacial migration via long-distance dispersal (PETIT *et al.* 1993; PETIT *et al.* 1997). Although sterility barriers between oak species are poorly developed, hybridization appears to be limited to species that belong to the same major group or section within the genus (STEBBINS 1950). Nevertheless, analyses of the cpDNA *trn*L-*trn*F region enabled distinguishing all the systematic entities in this study.

The RFLP analysis showed the presence of the same fragment of *Q. calliprinos* (345 bp) in the relict horny oak populations of Sardinia and Sicily, indicating that these populations belong to the same species.
The analysis sequence confirmed the above conclusion. The intron region showed less variable sites than the intergenic sequence, both at the intra- and interspecific level.

The stability and conservation of the intron sequence could explain its lesser variability. The chloroplast *trn*L intron belongs to the group I introns that are particularly interesting for RNA structure, because they fold to form active sites that accomplish their own splicing. These introns, although quite different in primary sequence, could all be folded into a secondary structure that has some common short regions. The conservation of short sequences among group I introns has been noted in fungal mitochondria, nuclear rRNA of *Tetrahymena*, *Physarum*, *Pneumocystis*, mRNA introns of bacteriophages T4 and SPO1, chloroplast mRNA and rRNA introns of *Chlamydomonas*, and chloroplast tRNA introns of higher plants. Most of the conserved nucleotides are contained within the four short sequences designated P, Q, R, and S. These sequences always occur in the same order along the intron (5'- P-Q-R-S -3'), but the distance between adjacent sequence elements can vary from 20 to many hundreds of nucleotides (CECH 1988). In fact, the variable and informative sites in the *Fagaceae* family are localised between the R and S zones of the intron sequence. The intergenic region has a higher variability at the intraspecific level, indicating that it probably evolves at a faster mutation rate than the intron region. The sequence analysis of the *trn*L-*trn*F cpDNA region indicated that:

1) the three relict Sardinia populations studied can be classified as *Q. calliprinos*, confirming the morphological and anatomic-micrometrical studies by GENTILE and GESTALDO (1976). Therefore, these populations represent an isolated group of *Q. calliprinos* in the western Mediterranean basin. GENTILE and GESTALDO (1976) indicated that using these markers, both entities are present in the populations of Sicily, Apulia, Cyprus and Greece, whereas the Avignon and Israel populations are represented by only *Q. coccifera* and *Q. calliprinos*, respectively;

2) the geographic isolation permitted a mutation to arise in the intergenic region close to a microsatellite (mutation CT in positions 767 and 768), which was restricted to the relict Sardinia populations. The implicated zone is a microsatellite length variation in a mononucleotide T repeat (from position 757 to 770), which identifies four cytotypes in European samples of *Q. robur* and *Q. petraea*, as reported by FERRIS *et al.* (1998); and

3) this mutation (CT) identifies the three relict Sardinia populations.

We can say that the insertion/deletion mutations in the microsatellite permit to determine genetic variability at the interpopulations level, and to identify different cytotypes in each species but not the species. Therefore, any investigation that would rely on sequence length only in such a situation may produce wrong conclusions. On the contrary, the study of the entire sequence permits to discriminate among species, as the presence of transversions/transitions gives the possibility to identify each entities.

ACKNOWLEDGEMENTS

We thank M. Puxeddu, G. Giaimi, N. Luisi, Y. Reves, M. Bariteau, Z. Kaya, and F. Isik for helpful sampling. We thank G. Stotzky for critically reviewing the manuscript. This work was supported by a research grant from P.O. CNR/MURST "Interventi per la salvaguardia e valorizzazione della biodiversità".

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