Research Article

Taxonomic and ecological discrimination of Fagaceae species based on internal transcribed spacer polymerase chain reaction–restriction fragment length polymorphism

João Paulo Coutinho, Ana Carvalho and José Lima-Brito*

Institute of Biotechnology and Bioengineering (IBB), Centre of Genomics and Biotechnology (CGB), University of Tras-os-Montes and Alto Douro, 5001-801 Vila Real, Portugal

Received: 30 August 2014; Accepted: 15 November 2014; Published: 26 November 2014

Associate Editor: Kermit Ritland

Citation: Coutinho JP, Carvalho A, Lima-Brito J. 2015. Taxonomic and ecological discrimination of Fagaceae species based on internal transcribed spacer polymerase chain reaction–restriction fragment length polymorphism. AoB PLANTS 7: plu079; doi:10.1093/aobpla/plu079

Abstract. The internal transcribed spacer (ITS) of ribosomal DNA has been used to confirm taxonomic classifications and define phylogenies in several plant species following sequencing or polymerase chain reaction–restriction fragment length polymorphism (PCR–RFLP) techniques. In this study, co-dominant ITS PCR–RFLP molecular markers were produced in 30 Fagaceae individuals belonging to the Castanea, Fagus and Quercus genera in order to assess the potential of this technique for taxonomic discrimination and determination of phylogenies. The complete ITS region (ITS1-5.8S rRNA-ITS2) was amplified in most of the Fagaceae individuals as a single fragment of ~700 bp. The ITS amplified products were digested with nine restriction enzymes, but only four (HaeIII, HpaII, TaqI and Sau96I) produced polymorphic/discriminative patterns. The total expected heterozygosity (\(H_E\)) was 20.31 % and the gene diversity (\(I\)), 32.97 %. The ITS polymorphism was higher within the Quercus genus (85.3 %). The ITS PCR–RFLP markers clustered the Fagaceae species according to genus or infrageneric group (in the case of Quercus sp. individuals). Five oaks did not cluster in line with the adopted infrageneric classification, but three of these were grouped according to their actual ecological distributions. The ITS PCR–RFLP markers indicated their potential for phylogenetic studies since all Fagaceae individuals were discriminated according to genus, and most of the oaks were clustered according to infrageneric group or ecological area.

Keywords: Castanea; Fagus; internal transcribed spacer (ITS); Quercus; restriction fragment length polymorphism (RFLP).

Introduction

Ribosomal DNA (rDNA) is a nuclear multigene family with copies arranged in tandem arrays within the nucleolar organizer regions (NORs) that form the secondary chromosomal constriction, with the centromere being the primary constriction (McClintock 1934; Navashin 1934). Each rDNA unit is composed of the conserved coding regions of the 18S-5.8S-26S rRNA genes, including the variable non-coding intergenic spacer (IGS). The IGS itself consists of a non-transcribed spacer region containing motifs that are designated as sub-repeats and is flanked by external transcribed spacers. The 5.8S rRNA gene is flanked by two non-coding sequences referred to as the...
internal transcribed spacers (ITS), ITS1 and ITS2, constituting the entire ITS region. Despite being fast-evolving regions, the rDNA spacers experience concerted evolution, and low diversity is expected within and among the rDNA loci. Such sequence uniformity allows the numerous rDNA copies in a genome to be treated as a single gene. Furthermore, the biparental inheritance, universality and orthology/paralogy of the rDNA sequences allow the use of ITS in taxonomic inferences and phylogenetics among diverged taxa (Baldwin et al. 1995).

As an alternative to sequencing or studies involving the laborious Southern blotting technique (Carvalho et al. 2010, 2013), the polymerase chain reaction–restriction fragment length polymorphism (PCR–RFLP) technique has been applied to the ITS or IGS rDNA spacers, resulting in co-dominant molecular marker systems that have been applied to the ITS or IGS rDNA spacers, resulting in co-dominant molecular marker systems that have proved useful for the detection of length variants, establishment of genetic relationships and assessment of genetic diversity in different plant species (Quijada et al. 1998; Nwakanma et al. 2003; Nalini et al. 2007; Saini et al. 2008; Carvalho et al. 2009, 2011a, b; Lima-Brito et al. 2011). Due to their repetitive nature, the spacers tend to be more variable than the coding regions of the rDNA, intra-individual variability may be found in Faga-
cese species when sequence homogenization among paralogues has not yet achieved completion (Mayol and Rosselló 2001; Muir et al. 2001). However, the identification of paralogues in an individual does not always seem to imply the presence of pseudogenes (Denk et al. 2002).

Several studies have resorted to ITS sequencing for taxonomic clarification in this family, particularly in the assessment of phylogenetic relations in Quercus (Manos et al. 1999, 2001; Bellarosa et al. 2005; Casas et al. 2007; Denk and Grimm 2010), Castanea (Manos et al. 2001) and Fagus (Denk et al. 2002). The fact that the RFLP technique is rapid and able to analyse a large number of samples simultaneously has made it useful for studying the chloroplastidial DNA (cpDNA) or mitochondrial DNA in the Quercus genus, and has enabled the following: evaluation of the cpDNA variation of Quercus suber L. in the Mediterranean basin (Lumaret et al. 2005); study of the level of cytoplasmic exchange between Q. suber and Q. ilex L. in Morocco (Belahbib et al. 2001); investigation of the dynamics of the spread of Q. robur L. and Q. petraea (Mattuschka) Liebl. throughout England (Cottrell et al. 2002); retracing of the re-colonization routes of white oaks in the Alps (Csaikl et al. 2002); and inference of the structure of the Q. affinis Scheidweiler— Q. laurina Humboldt complex in Mexico (González-Rodriguez et al. 2004).

Subjecting the amplified ITS1-5.8S-ITS2 region to the RFLP technique may allow the evaluation of the ITS polymorphism. Instead of searching for dissimilarities in a nucleotide sequence, the RFLP methodology identifies only the differences associated with the presence/absence of endonuclease recognition sites due to mutations, insertions or deletions. As a result, only highly prevalent ITS differences present in the large rDNA multigene family of an organism will allow the development of a visible band in an agarose gel, thus producing a specific band pattern.

In the current study, our aim was to evaluate the ITS polymorphism in individuals from the genera Castanea Mill., Fagus Tourn and Quercus L. using PCR–RFLP performed with nine restriction enzymes. We also aimed to investigate the potential of the ITS PCR–RFLP markers for taxonomic discrimination and determination of phylogenies, with a particular focus on the genus Quercus.

**Methods**

**Plant material**

The plant material used in this study is listed in Table 1 and comprised 30 species belonging to the Fagaceae family: 3 chestnuts (Castanea Mill.), 3 beeches (Fagus Tourn) and 24 oaks (Quercus L.).

Regarding the taxonomy of the genus Quercus, we adopted (except for Q. serrata) the informally unranked infrageneric ‘groups’ proposed by Denk and Grimm (2009, 2010). Thus, the oak species used in this study were classified as Cerris (cerris oaks; ecologically confined to Eurasia), Ilex (ilex oaks; Eurasia and North America) and Lobatae (red oaks; Americas). We assumed that the hybrid Q. crenata would belong to the Cerris group along with the parental Q. cerris and Q. suber. All the studied species have ecological origins that are representative of the natural distributions of the Fagaceae across the northern hemisphere.

To rule out hybridization issues between the mother trees of the plants used for DNA extraction, the seeds were harvested in different botanical gardens (Table 1), ensuring that the plants had morphological characters that enabled unambiguous classification. Dormancy was broken at 4 °C, and germination occurred at 7 °C in...
the dark on moistened cotton. Plantlets were grown in peat moss under greenhouse conditions at the University of Tras-os-Montes and Alto Douro (Vila Real, Portugal).

### Genomic DNA extraction and ITS amplification
To avoid fungal contamination in the ITS amplification, young, healthy leaves from one individual per species were used.

| Genus   | Infrageneric group | Species                      | Ecological distribution          | Seed origin* | Voucher number |
|---------|--------------------|------------------------------|----------------------------------|--------------|----------------|
| Quercus | Q. acutissima Carruth. | East of Asia                 | Vila Real, Portugal              | HVR20134     |
|         | Q. castaneifolia C.A. Mey. | SW Asia                      | Vila Real, Portugal              | HVR20135     |
|         | Q. cerris L.          | Europe and SW Asia           | Siena, Italy                     | HVR20136     |
|         | Q. crenata Lam. (Q. cerris × Q. suber) | Iberian Peninsula           | Vila Real, Portugal              | HVR20139     |
|         | Q. ibani G. Oliver    | E Medit. and Asia Minor      | Berlin, Germany                  | HVR20658     |
|         | Q. suber L.           | SW Europe and NW Africa      | Vila Real, Portugal              | HVR20153     |
| Ilex    | Q. cocifera L.        | Mediterranean                | Vila Real, Portugal              | HVR20137     |
|         | Q. ilex L.            | Mediterranean                | Münster, Germany                 | HVR20141     |
|         | Q. ilex subsp. rautundifolia Lam. | Mediterranean             | Vila Real, Portugal              | HVR20142     |
|         | Q. phillyraeoides A. Gray | SW Asia                    | Kyoto, Japan                     | HVR20148     |
| Lobatae | Q. coccinea Muenchh.  | North America                | Vila Real, Portugal              | HVR20138     |
|         | Q. kellogii Newb.     | California and SW Oregon    | Lyon, France                     | HVR20143     |
|         | Q. palustris Münchh.  | NE America                   | Berlin, Germany                  | HVR20145     |
|         | Q. phellos L.         | NE America                   | Vila Real, Portugal              | HVR20147     |
|         | Q. rubra L.           | NE America                   | Hunedoara, Romania               | HVR20152     |
|         | Q. velutina Lamb.     | NE America                   | Berlin, Germany                  | HVR20657     |
| Quercus | Q. faginea Lam.       | SW Europe                    | Vila Real, Portugal              | HVR20666     |
|         | Q. fraineto Ten.      | SE Europe and SW Asia       | Vila Real, Portugal              | HVR20140     |
|         | Q. mongolica Fisch. Ex Turcz. | East of Asia             | Tübingen, Germany                | HVR20144     |
|         | Q. petrea (Mottuschka) Liebl. | Europe and SW Asia          | Tallinn, Estonia                 | HVR20146     |
|         | Q. pubescens Willd.   | Europe and SW Asia          | Lyon, France                     | HVR20149     |
|         | Q. pyrenaica L.       | SW Europe and N Africa      | Bayreuth, Germany                | HVR20150     |
|         | Q. robur L.           | Europe and SW Asia          | Riga, Latvia                     | HVR20151     |
|         | Q. serrata Thunb. ex Murray | East of Asia             | Kanagawa, Japan                  | HVR20659     |
| Castanea| C. crenata Siebold & Zucc. | Japan and South Korea     | Kyoto, Japan                      | HVR20128     |
|         | C. mollissima Blume   | China                        | Vila Real, Portugal              | HVR20129     |
|         | C. sativa Mill.       | Europe and SW Asia          | Deurne, Belgium                  | HVR20130     |
| Fagus   | F. japonica Maxim.    | Japan                        | Vila Real, Portugal              | HVR20133     |
|         | F. sylvatica L.       | Europe and SW Asia          | Kaunas, Lithuania                | HVR20131     |
|         | F. sylvatica (Atropurpurea Group) ‘Riversii’ AGM | Europe                    | Duisburg, Germany                | HVR20132     |

The Authors 2014
were collected and thoroughly cleaned with 70 % v/v alcohol and deionized water for further genomic DNA extraction using the cetyl trimethylammonium bromide method (Doyle and Doyle 1987). The integrity evaluation was done by electrophoresis performed in 0.8 % w/v agarose gels and by DNA quantification in a Nanodrop ND-1000 spectrophotometer. DNA stocks were diluted to a concentration of 50 ng µL⁻¹.

The complete ITS region (ITS1-5.8S-ITS2) was amplified using the primers ITS-5 (5′-GGAAGTAAATACGACTCACTATAGG-3′) as forward and ITS-4 (5′-TCCTCCGCTTATTGATATGC-3′) as reverse, both from White et al. (1990). These primers were designed for the highly conserved 5′-end of the 26S rRNA gene and for the 3′-end of the 18S rRNA gene, respectively (White et al. 1990). Each PCR (final volume of 50 µL) consisted of 50 ng of template DNA, 500 µM KCl reaction buffer, 2.5 mM of each dNTP, 62 µM of each primer and 4 units of Dream Taq DNA polymerase (Thermo Fisher Scientific, Waltham, MA, USA). Ten microlitres of each amplified product were loaded on 1.5 % w/v agarose gels stained with ethidium bromide in order to confirm the ITS amplification.

**ITS PCR–RFLP markers**

Nine restriction enzymes were tested for the production of ITS PCR–RFLP markers: AluI, BamHI, DpnI, EcoRI, HaeIII, HpaII, Rsal, Sou96I and TaqI (Thermo Fisher Scientific). The digestion reactions (final volume of 30 µL) followed the manufacturer’s instructions. In each digestion reaction, 10 µL of the amplified product were used. The enzymatic reaction was thermally interrupted, and the digested products were separated by electrophoresis performed in 2 % w/v agarose gels stained with ethidium bromide. The sizes of the fragments were estimated by comparison with the molecular weight marker Gene Ruler DNA Ladder 100 bp Plus (Thermo Fisher Scientific) loaded in each agarose gel.

**Statistical analysis**

Despite being co-dominant markers, the ITS fragments were scored as presence (1) or absence (0) in order to construct binary matrices for statistical analysis. For each genus and the Quercus infrageneric groups, the software POPGENE 1.32 (Yeh et al. 1999) was used to calculate Nei’s expected heterozygosity (Hₑ; Nei 1973) and Shannon’s information index (I; Lewontin 1972) to measure the level of gene diversity, and the same software was used to estimate Nei’s original measures of genetic identity and genetic distance (Nei 1973). To evaluate the phylogenetic relationships, a radial tree and a phylogram were generated with TreeView (Page 1996) based on the genetic distance (Nei 1987) and neighbour-joining method (Saitou and Nei 1987), calculated using POPGENE. A pairwise similarity matrix was calculated using Dice’s coefficient, and a dendrogram of genetic similarity was constructed using the unweighted pair group method with arithmetic averages (UPGMA) and the Numerical Taxonomy and Multivariate Analysis System software (NTSYS-PC 2.02) (Rohlf 1998). A bootstrap analysis based on 1000 replications was performed with Winboot (Yap and Nelson 1996) to test the clustering reliability. Only bootstrap values >50 % and common to the consensus trees generated either by Winboot and UPGMA are shown (Felsenstein 1985). The adjustment fitness of the UPGMA dendrogram to the binary matrices was verified using the COPH and MXCOMP modules from the NTSYS software. To certify the NTSYS UPGMA clustering and estimate the genetic structure of the individuals under study, we used STRUCTURE 2.3.4 software (Pritchard et al. 2000), where several clusters (K) were tested with 50 000 generations of burn-in and 100 000 Markov chain Monte Carlo iterations.

**Results and Discussion**

**ITS amplification**

The ITS1-5.8S-ITS2 length usually varies from 500 to 750 bp in angiosperms (Baldwin et al. 1995). Phylogenetic studies based on ITS sequencing often detect, beyond point mutations, short nucleotide insertions/deletions responsible for the variable length of the ITS1-5.8S-ITS2 region. Such an event is related to dissimilarities in the spacers, since the 5.8S gene exhibits high sequence conservation and minimal variation in the length of ~163–165 bp in several species (Baldwin et al. 1995; Manos et al. 2001; Denk et al. 2002; Papini et al. 2011).

Regarding the results in the genus Quercus, a single band of ~700 bp was amplified in 19 species (data not shown). However, in five oak species, two ITS bands of ~600 and 700 bp in length were detected. In Q. cerris and Q. friaineto, the two bands exhibited the same intensity in the agarose gel, while in the other three species (Q. cerrata, Q. castaneifolia and Q. ilex subsp. rotundifolia), the 700-bp band was more intense. Several authors have reported variable ITS lengths for the same oak species. Some examples are described below, and may be attributed to different amplification conditions or primers. Manos et al. (2001) reported an alignment of 635 bp for 179 Fagaceae taxa. Muir et al. (2001) detected a single band of ~720 bp in Q. petraea and Q. robur. Bellarosa et al. (2005) amplified a single band of ~600 bp in 11 oak species (some common to this study: Q. cerris, Q. suber, Q. friaineto, Q. petraea, Q. pubescens, Q. robur, Q. cocceifera and Q. ilex). Casas et al. (2007) reported an ITS length ranging from 599 to 607 bp in six oak species (Q. pubescens, Q. faginea, Q. pyrenaica, Q. suber, Q. ilex and Q. cocceifera). Regarding the detailed lengths, Manos et al. (1999)
assumed a size of 614 bp in 44 Quercus species after detecting different lengths for ITS1 (223–234 bp) and ITS2 (206–214 bp), while 5.8S exhibited a rather stable length of 164 bp. Papini et al. (2011) reported a 615-bp matrix (indels included), where ITS1 represented 212 bp in Q. iberica and 222 bp in Q. macranthera, while the ITS2 and the 5.8S coding region were 211 and 163 bp, respectively, in both species.

The three Castanea species showed bands ~600 bp in length, but an additional band of ~700 bp was amplified in Castanea mollissima and C. sativa (data not shown). Interestingly, the 600-bp band was more intense in C. mollissima, and the 700-bp band was more intense in C. sativa. According to Samuel et al. (1998), the lengths in Castanea are 231 bp for ITS1 (U93097) and 183 bp for ITS2 (U93098).

Denk and Grimm (2010) suggested that the dissimilarities in Fagus ITS1 to other Fagaceae may discourage comparisons in phylogenetic studies. Indeed, Manos et al. (2001) found a 635-bp alignment for 179 Fagaceae taxa, but, in Fagus, an additional 40-bp insertion occurred in ITS1. Despite this, we decided to include our results in the pooled data to understand how they would stand out from the remaining genera and to survey for intra-specific differences. In the three Fagus species studied here, a single band ~750 bp long was amplified (data not shown), confirming its longer length. Denk et al. (2002) encountered a total length varying from 644 to 660 bp, average lengths of 259 bp for ITS1, 229 bp for ITS2 and a stable length of 165 bp for the 5.8S gene.

The ITS length variants and ITS sequence variants (amplified from different loci) can be found in one organism. The former diverge in size due to insertion or deletion events, and the latter (while having the same length) exhibit sequence polymorphisms. The fact that length variants were amplified in five oaks and two chestnuts suggests either intra-individual rDNA polymorphism (due to the lack of homogenization within rDNA paralogues) or amplification of pseudogenes (non-functional paralogues that are transcriptionally inactive).

Figure 1. Unweighted pair group method with arithmetical averages dendrogram of genetic similarity based on the pool of ITS PCR–RFLP data achieved with the restriction enzymes HaeIII, HpaII, Sau96I and TaqI. Only bootstrap values >50 are represented. The ecological distribution is indicated after species name (am, North America; as, Asia; eu, Europe; medit, Mediterranean; and af, Africa), and the infrageneric group for each Quercus species (C, Cerris; I, Ilex; L, Lobatae; and Q, Quercus).
ITS PCR–RFLP data

For a preliminary assessment of ITS polymorphism, nine restriction enzymes were tested in *Q. petraea*: AluI, BamHI, DpnI, EcoRI, HaeIII, HpaII, RsaI, Sau96I and TaqI. The four enzymes that provided the most clearly visible and polymorphic restriction fragments were selected: HaeIII (5′–GG∥CC-3′), HpaII (C∥CGG), Sau96I (G∥GNCC) and TaqI (T∥CGA). The molecular sizes of the restriction fragments are indicated in Table 2. The four selected restriction enzymes revealed polymorphisms among the 30 species. No specific pattern was observed according to *Quercus* infrageneric group, but all enzymes produced genus- and species-specific ITS PCR–RFLP patterns. The four restriction enzymes produced a total of 50 ITS PCR–RFLP patterns (Table 2), and several bands were common among patterns (Table 3). Each restriction enzyme gave rise to 12–13 patterns that occurred in a particular genus, group and/or species. Some patterns were shared among different taxa. Despite two patterns being present in all Lobatae species (Sau96I and TaqI), they could not be considered group-specific because they were shared with *Q. pyrenaica*. With the exception of TaqI, the other patterns of *Q. coccifera* were shared with oaks from several groups. In *Q. robur*, species-specific patterns were revealed for HaeIII and HpaII; for Sau96I and TaqI, shared patterns were observed in *Q. ilex* and *Q. ilex* subsp. rotundifolia. TaqI also produced a pattern specific to the genus Fagus. Again with the exception of TaqI in the Cerris, Ilex and Quercus groups, the other three enzymes produced monomorphic bands in the three genera and in all *Quercus* infrageneric groups (Table 3). An ITS PCR–RFLP independent analysis of the six taxonomic levels per restriction enzyme revealed levels of polymorphism that ranged from 0 to 100 %, and unique bands were found only for Fagus (Table 3).

Whenever the RFLP assay returns bands whose summed lengths exceed the uncut product, the presence of dissimilar loci in a genome is implied, reflecting the existence of sequence variants. In fact, Redecker et al. (1997) suggested that when the restriction patterns indicate the presence of two sets of sequences, because the overall length of the fragments is much larger than the original, uncut PCR product, this must be attributed to sequence heterogeneity. When considering the ITS PCR–RFLP data observed among all Fagaceae individuals and all restriction enzymes, 100 % polymorphism was observed (Table 4, first row). The rates of polymorphism ranged from 33.3 % in *Fagus* taxa to 100 % in Fagaceae taxa. A high number of phenotypes (12 or 13) were identified per restriction enzyme, which corroborates the medium levels of polymorphism observed.

The expected heterozygosity ($H_e$) was 0.2031 in the family Fagaceae (Table 4), with averages of 0.1512 among the three genera and 0.2013 among the four *Quercus* infrageneric groups (calculations from Table 4 data). The total diversity of the patterns, measured using Shannon’s diversity index ($I$), was 0.3297. Despite the low values exhibited for all taxa, Shannon’s diversity index varied according to the $H_e$. In the *Quercus* infrageneric groups, this index was similar in Cerris, Ilex and Quercus (~0.34). The much lower value in Lobatae (0.2008) was probably due to the geographic isolation of the American oaks. Overall, such relationships were reflected in the dendrogram (Fig. 1). These genetic diversity results are also consistent with those presented by Quijada et al. (1998) and Carvalho et al. (2009) using the same molecular marker technique with some of the restriction enzymes used here. Such low levels of heterozygosity might be due to the co-dominant nature of these markers.

The pool of the ITS PCR–RFLP data was used to construct an UPGMA dendrogram of genetic similarity among the 30 Fagaceae species under study in order to estimate their phylogenetic relationships based on these alternative co-dominant markers (Fig. 1). The high correlation coefficient (r = 0.89) ensured that the UPGMA clustering of the 30 Fagaceae species is an accurate representation of the data matrix, with 36 % total genetic similarity. Considering a cut-off value of 0.76, the dendrogram may be divided into six major clusters that correspond to each one of the four *Quercus* infrageneric groups (Cerris [Cluster I], Ilex [Cluster II], Quercus [Cluster III] and Lobatae [Cluster IV]) and to Fagus (Cluster V) and Castanea (Cluster VI) genera (Fig. 1). The genetic similarity among the 24 *Quercus* species was 0.57, confirming the moderate ITS PCR–RFLP polymorphisms visualized on the agarose gels (data not shown). The bootstrap analysis also corroborated several clusters at different taxonomic levels (Fig. 1). Although correctly clustered in the *Quercus* genus along with the other 19 oaks, five oaks (*Q. coccifera, Q. phillyraeoides, Q. pyrenaica, Q. robur,* and *Q. suber*) were not clustered according to the taxonomic infrageneric group. All of these oaks amplified a unique ITS1-5.8-ITS2 band and clustered among others with similar distribution areas, suggesting that such ITS PCR–RFLP resemblances could be due to the sharing of earlier ancestors.

Cluster I included oaks from the Cerris group, with only *Q. suber* excluded. Hybrids (particularly those occurring in the overlapping limits of the species distribution areas) tend to coexist with the parental species if these new intermediate genotypes have characters more suitable for the environmental circumstances. In fact, a genetic similarity of 96 % was found between *Q. crenata* and
Table 2. ITS PCR–RFLP patterns achieved with each restriction enzyme (RE) among the studied Fagaceae species (Quercus infrageneric groups in parentheses).

| RE pattern | Bands (bp) | Species with the same ITS PCR–RFLP pattern |
|------------|------------|------------------------------------------|
| **HaeIII** |            |                                          |
| a          | 180, 225, 415, 480 | Q. rubra (L); Q. mongolica (Q); Q. pyrenaica (Q) |
| b          | 180, 225     | Q. acutissima (C); Q. castaneifolia (C); Q. libani (C); Q. coccifera (I); Q. ilex (I); Q. phillyreaeoides (I); Q. kelloggii (L); Q. velutina (L); Q. serrata (Q) |
| c          | 155, 480, 620 | C. crenata                                  |
| d          | 100, 120, 180, 210, 290 | F. japonica                               |
| e          | 180, 225, 260, 415, 480 | Q. coccinea (L)                           |
| f          | 155, 180, 225, 260, 480 | Q. ilex subsp. rot. (I)                    |
| g          | 180, 225, 260, 415, 460 | Q. palustris (L)                           |
| h          | 180, 225, 380, 460 | Q. phellos (L)                             |
| i          | 180, 225, 315, 430, 480 | Q. robur (Q)                              |
| j          | 155, 180, 225, 480 | C. mollissima, C. sativa                   |
| k          | 100, 120, 180, 210, 460 | F. sylvatica, F. sylvatica Atropurpurea    |
| l          | 180, 225, 480 | Q. ceras (C); Q. crenata (C); Q. suber (C)  |
| m          | 180, 225, 460 | Q. faginea (Q); Q. fraeneto (Q); Q petraea (Q); Q. pubescens (Q) |
| **HpaII** |            |                                          |
| a          | 185, 250     | Q. acutissima (C); Q. kelloggii (L); Q. rubra (L) |
| b          | 185, 220, 250, 470 | Q. libani (C); Q. coccifera (I); Q. petraea (Q); Q. serrata (Q) |
| c          | 185, 220, 250 | Q. ceras (C); Q. crenata (C); Q. suber (C); Q. ilex (I); Q. fraeneto (Q); Q. mongolica (Q) |
| d          | 185, 250, 470 | Q. pubescens (Q); Q. pyrenaica (Q); Q. coccinea (L); Q. palustris (L); Q. phellos (L); Q. velutina (L) |
| e          | 140, 185, 270, 470 | C. mollissima                             |
| f          | 185, 250, 430, 470 | Q. castaneifolia (C)                      |
| g          | 185, 220, 250, 430 | Q. faginea (Q)                            |
| h          | 185, 220, 250, 270 | Q. ilex subsp. rot. (I)                   |
| i          | 140, 185, 220, 250, 400 | Q. phillyreaeoides (I)                   |
| j          | 185, 220, 250, 330, 470 | Q. robur (Q)                              |
| k          | 140, 185, 270 | C. crenata, C. sativa                     |
| l          | 220, 470     | Genus Fagus (all species)                 |
| **Sau96I** |            |                                          |
| a          | 100, 150, 350, 490 | Q. ilex (I); Q. ilex subsp. rot. (I); Q. robur (Q) |
| b          | 100, 150, 490 | Q. phillyreaeoides (I); Q. petraea (Q); Q. serrata (Q) |
| c          | 150, 490     | Q. acutissima (C); Q. libani (C); Q. suber (C); Q. coccifera (I); Q. faginea (Q); Q. mongolica (Q); Q. pubescens (Q) |
| d          | 215, 490     | Group Lobatae (all six species); Q. pyrenaica (Q) |
| e          | 150, 170, 490 | C. mollissima                             |
| f          | 100, 150, 450, 560, 690 | F. japonica                             |
| g          | 100, 150, 560, 650 | F. sylvatica                             |
| h          | 100, 150, 450, 490, 560 | F. sylvatica Atropurpurea               |

Continued
the parent Q. cerris, with only 66% similarity between Q. suber and the former two. Bellarosa et al. (2005) reported a contradictory result, with higher proximity between Q. crenata and Q. suber. Such a circumstance may be attributed to the molecular markers’ potential for the fingerprinting of natural hybrids. With respect to the uniparental inherited cpDNA, Muir and Schlötterer (2006) suggested that some areas of the hybrid genome could escape from selection towards species integrity. Despite the biparental inheritance of rDNA, homogenization of the entire rDNA arrays towards one parental type could take place in hybrids. Our results were similar to those of Conte et al. (2007), who emphasized that despite being auto-allogamous, ‘asymmetrical backcrossing’ of Q. crenata in a mixed population could lead the hybrid progeny towards the predominant parental species. Although in a different context, the hypothesis of ‘directed’ introgression was also noted by Casas et al. (2007) for the difficulties encountered in finding ‘morphologically identifiable hybrids’ between Q. coccifera and other Quercus species (except for Q. ilex). In our work, Q. coccifera (group Ilex) was included in Cluster I, separately from the other oaks of the same group. Taxonomically, this was a surprising clustering. It was also surprising in the molecular sense, since the large number of cpDNA haplotypes shared between Q. ilex and Q. coccifera led Jiménez et al. (2004) to suggest that both species might experience incomplete lineage sorting or hybridization. Denk and Grimm (2010) found ‘zero distance’ based on complete ITS sequencing, and Coutinho et al. (2014) found significant genetic similarity between Q. ilex and Q. coccifera using inter simple sequence repeats (ISSRs). If Q. coccifera clustered near Q. libani (Group Cerris), and since their distribution overlaps in the Mediterranean region, it is possible that both rDNAs experienced the same evolutionary pressures during speciation. In short, even if not in total agreement with the adopted taxonomy, both Clusters I and II are supported by the sharing of morphological (not homoplastic) and ecological features between the Cerris and Ilex groups.

Cluster II included two species from the Ilex group, Q. ilex and Q. ilex subsp. rotundifolia, and Q. robur from the Quercus group, with the three species exhibiting a Mediterranean-based distribution. The proximity between Q. ilex and Q. ilex subsp. rotundifolia may be due to rDNA array homogenization that preceded the speciation event. Nonetheless, Q. ilex and Q. robur share the natural hybrid Q. turneri Willd. A slow ‘molecular drive’ (Dover 1982, 1986) could explain such proximity; being Mediterranean species, both might have experienced similar adaptive conditions. Mutations, insertions or deletions are the changes in the recognition sites needed to
explain the presence in this cluster of Q. robur (Quercus group), the oak-type of the 'roburoid oaks'.

Constituting a single branch in Cluster III, the Q. phillyraeoides (Ilex group) only shares a Southwest Asia distribution with the Quercus group oaks. In a previous study using ISSRs, this species was clustered in the Ilex group (Coutinho et al. 2014). Quercus phillyraeoides exhibited two specific patterns revealed by the HpaII and TaqI enzymes, and shared patterns (produced by HaeIII) with two oaks from the Quercus group that dictated its inclusion in this cluster. The remaining species included in Cluster III are oaks from the Quercus group, with the exception of Q. suber (Cerris group). Similar clustering was recently found based on ISSRs (Coutinho et al. 2014). Quercus suber could have originated in the Middle East (Bellarosa et al. 2005 and references therein) and expanded westward (Lumaret et al. 2005). This feature could somehow explain its clustering along with oaks nowadays distributed throughout Eurasia. Regarding the inter-fertile Q. robur and Q. petraea (the two predominant white oaks in deciduous European forests), Muir et al. (2001) could not differentiate these based on ITS sequencing, and suggested that the split between the two may be too recent for rDNA to have diverged. Nevertheless, despite being in different clusters, the existing differences in the recognition sites allowed the ITS-RFLP markers to distinguish the two species.

Quercus serrata was not included in the studies of Denk and Grimm (2009, 2010) on the infrageneric classification of the Quercus genus. In our work, it clustered along with oaks from the Quercus group. This broad-leaved deciduous oak is widespread in the forests of eastern Asia, and was classified as belonging to the Lepidobalanus section (Camus 1938–39) along with the following: Q. faginea (subsection Gallifera);

**Table 3.** An individual analysis was performed in Castanea and Fagus genera, and each Quercus infrageneric group (Cerris, Ilex, Lobatae and Quercus) for the size (bp) of the monomorphic (M) and polymorphic (P) bands, and the polymorphism percentage (%P) detected per RE. The unique (U) bands were evaluated among the six studied taxa.

| RE      | Taxa     | M (bp)  | P (bp)   | %P | U (bp) |
|---------|----------|---------|----------|-----|--------|
| HaeIII  | Castanea | 155, 480| 180, 225, 620 | 60.0 | None   |
|         | Fagus    | 100, 120, 180, 210 | 290, 460 | 33.3 | 100, 120, 210 |
|         | Cerris   | 180, 225 | 480 | 33.3 | None   |
|         | Ilex     | 180, 225 | 155, 260, 480 | 60.0 | None   |
|         | Lobatae  | 180, 225 | 260, 380, 415, 460, 480 | 71.4 | None   |
|         | Quercus  | 180, 225 | 315, 415, 430, 460, 480 | 71.4 | None   |
| HpaII   | Castanea | 140, 185, 270 | 470 | 25.0 | None   |
|         | Fagus    | 220, 470 | None | 00.0 | None   |
|         | Cerris   | 185, 250 | 220, 430, 470 | 60.0 | None   |
|         | Ilex     | 185, 220, 250 | 140, 270, 400, 470 | 57.1 | None   |
|         | Lobatae  | 185, 250 | 470 | 33.3 | None   |
|         | Quercus  | 185, 250 | 220, 330, 430, 470 | 66.7 | None   |
| Sau96I  | Castanea | 150, 490 | 170 | 33.3 | None   |
|         | Fagus    | 100, 150, 560 | 450, 490, 650, 690 | 57.1 | 560 |
|         | Cerris   | 150, 490 | 100, 390, 650 | 60.0 | None   |
|         | Ilex     | 150, 490 | 100, 350 | 50.0 | None   |
|         | Lobatae  | 215, 490 | None | 00.0 | None   |
|         | Quercus  | 490 | 100, 150, 170, 215, 350 | 83.3 | None   |
| TaqI    | Castanea | 145, 260 | 200 | 33.3 | None   |
|         | Fagus    | 145, 260, 375 | None | 00.0 | 375 |
|         | Cerris   | None | 115, 145, 200, 265, 350 | 100 | None   |
|         | Ilex     | None | 115, 145, 200, 225, 265, 350 | 100 | None   |
|         | Lobatae  | 115, 145, 250 | None | 00.0 | None   |
|         | Quercus  | None | 115, 145, 200, 250, 265, 350 | 00.0 | None   |
The inclusion of *Castanea* clustered all the American oaks in a single cluster. Pending only on short recognition sites, the ITS PCR–RFLP clustered all the American oaks in a single cluster. The optimal number (K = 6 clusters; Fig. 2) agreed with the UPGMA clustering (Fig. 1).

The genetic structure analysis provided fixation index (*F_{ST}*) values that suggested high levels of differentiation (>0.50) for five clusters, corresponding to the *Castanea* genus (0.59), *Fagus* genus (0.61), *Cerris* group (0.51), *Lobatae* group (0.76) and *Quercus* group (0.5862). The sixth cluster, corresponding to the infrageneric *Ilex* group, revealed a reduced genetic differentiation value (*F_{ST} = 0.26*), sustaining the dispersion of these oaks through the dendrogram. The bar plot showed that four oaks of the *Ilex* group and one oak (*Q. robur*) of the *Quercus* group seemed to share some ancient genetic material with the *Castanea* genus. It is assumed that both *Quercus* and *Castanea* genera share an ancient origin, and the fact that these oaks have a narrow distribution area might have derived from different taxa.

**Table 4.** Genetic diversity analyses based on the pool of the RFLP data produced with the four REs, performed in *Castanea* and *Fagus* genera, and each *Quercus* infrageneric group (*Cerris*, *Ilex*, *Lobatae* and *Quercus*): total number of RFLP bands (*T*); number of polymorphic bands (%P); percentage of polymorphism (%P); Nei’s expected heterozygosity (*H_e*, Nei 1973); and Shannon’s information index (*I*).
have contributed to the conservation of such similarities and the lack of higher genetic fixation (as observed for the other species/groups). In the bar plot, it is apparent that three out of four Ilex individuals share material with the Cerris group, confirming the 0.74 genetic similarity between these groups found in the dendrogram (Fig. 1). The high F_	ext{ST} value for Fagus species suggested a defined ITS genetic structure and confirmed the ITS divergence from the other taxa. In the dendrogram, Q. phillyraeoides and Q. suber were clustered along with oaks of the Quercus group, and the bar plot suggested their high similarity. The same occurred between Q. pyrenaica (Quercus group) and oaks from the Lobatae group. Also, the Lobatae group was found to be the most genetically fixed, which could be explained by their ancient isolation in North America.

The genetic identities and genetic distances (calculated using POPGENE and the ITS PCR–RFLP pool data, data not shown) of the three genera and four Quercus infrageneric groups supported most of these results. Quercus and Castanea genera were evaluated to be more intimately related to each other (0.82) than to Fagus (0.69 and 0.64, respectively). The genetic relations in the infrageneric groups seem to resemble their geographical distributions: Lobatae exhibited the highest genetic distance relative to Cerris, Ilex and Quercus (0.153, 0.224 and 0.171, respectively); Cerris and Ilex were the most related (0.962); and Quercus revealed similar proximities to both Cerris (0.947) and Ilex (0.960).

**Conclusions**

This study of ITS genotaxonomy intended to evaluate the potential of the ITS-PCR–RFLP markers for inferences about the Fagaceae family taxonomy, particularly in the Quercus genus. In phylogenetics, given the stability of the sequence of the 5.8S rRNA gene, the main use of the ITS1-5.8S-ITS2 region has been for comparing the sequence of the two spacers. When using restriction enzymes, in which accuracy resides in short recognition sites defined by four or five nucleotides, even a single-nucleotide mutational event may constrain the final result. Nonetheless, the conservation of those enzyme cutting sites in closely related species may allow the use of RFLP patterns to make inferences about phylogenies. Indeed, according to the adopted taxonomy, correct clustering was observed for (i) the 30 individuals per genus, (ii) 19 of the 24 oaks per Quercus infrageneric group (almost 80 % accuracy) and (iii) 22 oaks per ecological area of distribution (>91 % accuracy). One must assume that site variability may distort the phylogenetic analysis, but our results and the advantages of cost-effectiveness, time consumption and higher discriminative power (especially between species with morphological similarities) suggest the effectiveness and utility of these markers for taxonomic discrimination in Fagaceae.

**Sources of Funding**

This work was supported by the PhD grant SFRH/BD/42837/2008 awarded by the Portuguese Foundation for Science and Technology (FCT) and, partially, by the Institute of Biotechnology and Bioengineering—Centre of Genomics and Biotechnology of the University of Tras-os-Montes and Alto Douro IBB-CGB/UTAD.
Contributions by the Authors

J.P.C. performed the practical work at the laboratory and analysed the results; J.P.C., A.C. and J.L.-B. contributed to the analysis and discussion of the results and to the writing of the manuscript.

Conflicts of Interest Statement

None declared.

Acknowledgements

The authors are very grateful to the botanical gardens (Table 1) for providing the seeds used in our work.

Literature Cited

Baldwin BG, Sanderson MJ, Porter JM, Wojciechowski MF, Capbell CS, Donoghie MJ. 1995. The ITS region of nuclear ribosomal DNA: a valuable source of evidence on angiosperm phylogeny. Annals of the Missouri Botanical Garden 82:247–277.

Belabbib N, Pemonge MH, Ouassou A, Sbey H, Cremer A, Petit RJ. 2001. Frequent cytoplasmic exchanges between oak species that are not closely related: Quercus suber and Q. ilex in Morocco. Molecular Ecology 10:2003–2012.

Bellarosa R, Simeone MC, Papini A, Schirone B. 2005. Utility of ITS and trnL-F sequence data for phylogenetic reconstruction of Italian Quercus spp. Molecular Phylogenetics and Evolution 34:355–370.

Camus A. 1938–39. Les chênes: monographie du genre Quercus, tome 2. Genre Quercus, sous-genre Euquerus (sections Lepido-balanus et Macrobalanus). Texte. Paris: Lechevalier.

Carvalho A, Guedes-Pinto H, Lima-Brito J. 2009. Genetic diversity among old Portuguese bread wheat cultivars and botanical varieties evaluated by ITS rDNA PCR–RFLP markers. Journal of Genetics 88:363–367.

Carvalho A, Polanco C, Lima-Brito J, Guedes-Pinto H. 2010. Differential rRNA genes expression in hexaploid wheat related to NOR methylation. Plant Molecular Biology Reporter 28:403–412.

Carvalho A, Guedes-Pinto H, Lima-Brito J. 2011a. Intergeneric spacer length variants in old Portuguese bread wheat cultivars. Journal of Genetics 90:203–208.

Carvalho A, Polanco C, Lima-Brito J, Guedes-Pinto H. 2011b. Physical localization of NORs and ITS length variants in old Portuguese durum wheat cultivars. Journal of Genetics 90:95–101.

Carvalho A, Polanco C, Lima-Brito J, Guedes-Pinto H. 2013. Differential rRNA genes expression in bread wheat and its inheritance. Genetica 141:319–328.

Casas RR, Cano E, Balaguer L, Pérez-Corona E, Manrique E, García-Verdugo C, Vargas P. 2007. Taxonomic identity of Quercus cocifera L. in the Iberian Peninsula is maintained in spite of widespread hybridisation, as revealed by morphological, ISSR and ITS sequence data. Flora 202:488–499.

Conte L, Cotti C, Cristofolini G. 2007. Molecular evidence for hybrid origin of Quercus crenata Lam. (Fagaceae) from Q. cerris L. and Q. suber L. Plant Biosystems 141:181–193.

Cottrell JE, Munro RC, Tabbener HE, Gillies ACM, Forrest GI, Deans JD, Lowe AJ. 2002. Distribution of chloroplast DNA variation in British oaks (Quercus robur and Q. petraea): the influence of postglacial colonisation and human management. Forest Ecology and Management 156:181–195.

Coutinho JP, Carvalho A, Lima-Brito J. 2014. Genetic diversity assessment and estimation of phylogenetic relationships among 26 Fagaceae species using ISSRs. Biochemical Systematics and Ecology 54:247–256.

Csaikl UM, Burg K, Fineschi S, König AO, Mátys G, Petit RJ. 2002. Chloroplast DNA variation of white oaks in the alpine region. Forest Ecology and Management 156:131–145.

Denk T, Grimm GW. 2009. Significance of pollen characteristics for infrageneric classification and phylogeny in Quercus (Fagaceae). International Journal of Plant Sciences 170:926–940.

Denk T, Grimm GW. 2010. The oaks of western Eurasia: traditional classifications and evidence from two nuclear markers. Taxon 59:351–366.

Denk T, Grimm G, Stögerer K, Langer M, Hemleben V. 2002. The evolutionary history of Fagus in western Eurasia: evidence from genes, morphology and the fossil record. Plant Systematics and Evolution 232:213–236.

Denk T, Grimsson F, Zetter R. 2010. Episodic migration of oaks to Iceland: evidence for a North Atlantic ‘Land Bridge’ in the latest Miocene. American Journal of Botany 97:276–287.

Denk T, Grimsson F, Zetter R. 2012. Fagaceae from the early Oligocene of Central Europe: persisting new world and emerging old world biogeographic links. Review of Palaeobotany and Palynology 169:7–20.

Dover GA. 1982. Molecular drive: a cohesive mode of species evolution. Nature 299:111–117.

Dover GA. 1986. Molecular drive in multigene families: how biological novelties arise, spread and are assimilated. Trends in Genetics 168:159–165.

Doyle JJ, Doyle JL. 1987. A rapid DNA isolation procedure for small quantities of fresh leaf tissue. Phytochemical Bulletin 19:11–15.

Felsenstein J. 1985. Confidence limits on phylogenies: an approach using the bootstrap. Evolution 39:783–791.

González-Rodríguez A, Bain JF, Golden JL, Oyama K. 2004. Chloroplast DNA variation in the Quercus affinis–Q. laurina complex in Mexico: geographical structure and associations with nuclear and morphological variation. Molecular Ecology 13:3467–3476.

Harpeke D, Peterson A. 2008. 5.8S motifs for the identification of pseudogenic ITS regions. Botany 86:300–305.

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

Lang P, Dane F, Kubisiak TL. 2006. Phylogeny of Castanea (Fagaceae) based on chloroplast trnT–L–F sequence data. Tree Genetics and Genomes 2:132–139.

Lang P, Dane F, Kubisiak TL, Huang H. 2007. Molecular evidence for an Asian origin and a unique westward migration of species in the genus Castanea via Europe to North America. Molecular Phylogenetics and Evolution 43:49–59.

Lewontin RC. 1972. Testing the theory of natural selection. Nature 236:181–182.

Lima-Brito J, Castro L, Coutinho J, Morais F, Gomes L, Guedes-Pinto H, Carvalho A. 2015. Genetic variability in Sambucus nigra L. clones: a preliminary molecular approach. Journal of Genetics Online Resources 90:e47–e52.
Lumaret R, Tryphon-Dionnet M, Michaud H, Sanu Y, Ipotesi E, Born C, Mir C. 2005. Phylogeographical variation of chloroplast DNA in cork oak (Quercus suber), *Annals of Botany* 96:853–861.

Manos PS, Doyle JJ, Nixon KC. 1999. Phylogeny, biogeography, and processes of molecular differentiation in Quercus subgenus Quercus (Fagaceae). *Molecular Phylogenetics and Evolution* 12:333–349.

Manos PS, Zhou ZK, Cannon CH. 2001. Systematics of Fagaceae: phylogenetic tests of reproductive trait evolution. *International Journal of Plant Sciences* 162:1361–1379.

Mayol M, Rossello JA. 2001. Why nuclear ribosomal DNA spacers (ITS) tell different stories in Quercus. *Molecular Phylogenetics and Evolution* 19:167–176.

McClintock B. 1934. The relationship of a particular chromosomal element to the development of the nucleoli in Zea mays. *Zeitschrift für Zellforschung und Mikroskopische Anatomie* 21:294–328.

Muir G, Schlötterer C. 2006. Moving beyond single-locus studies to characterize hybridization between oaks (Quercus spp.). *Molecular Ecology* 15:2301–2304.

Muir G, Fleming CC, Schlötterer C. 2001. Three divergent rDNA clusters predate the species divergence in Quercus petraea (Matt.) Liebl. and Quercus robur L. *Molecular Biology and Evolution* 18:112–119.

Nalin E, Bhagwat SG, Jawali N. 2007. Identification and characterization of some ITS variants from hexaploid wheat (*Triticum aestivum* L.). *Plant Science* 173:262–268.

Navashin M. 1934. Chromosomal alterations caused by hybridization and their bearing upon certain general genetic problems. *Cytophys* 5:169–203.

Nei M. 1973. Analysis of gene diversity in subdivided populations. *Proceedings of the National Academy of Sciences of the USA* 70:3321–3323.

Nei M. 1987. *Molecular evolutionary genetics*. New York: Columbia University Press.

Nixon KC. 2002. *The Oak (Quercus) Biodiversity of California and Adjacent Regions*. General Technical Reports PSW-GTR-184. USDA Forest Service, 3–20.

Nwakama DC, Pillay M, Okoli BE, Tenkouano A. 2003. PCR–RFLP of the ribosomal DNA internal transcribed spacers (ITS) provides markers for the A and B genomes in *Musa L*. *Theoretical and Applied Genetics* 108:154–159.

Page RDM. 1996. *TREEVIEW*: an application to display phylogenetic trees on personal computers. *Computer Applications in the Biosciences* 12:357–358.

Papini A, Simeone MC, Bellarosa R, Spada F, Schirone B. 2011. *Quercusmacranthera* Fisch. & Mey, ex Hohen. And *Quercus iberica* M. Bieb.: Taxonomic definition and systematic relationships with European oaks inferred from nuclear internal transcribed spacer (ITS) data. *Plant Biosystems* 145:37–49.

Pritchard JK, Stephens M, Donnelly P. 2000. Inference of population structure using multilocus genotype data. *Genetics* 155:945–959.

Pritchard JK, Wen X, Falush D. 2010. Documentation for structure software: version 2.3.38 pp. University of Chicago. http://pritchardlab.stanford.edu/structure_software/release_versions/v2.3.4/structure_doc.pdf (25 December 2014).

Quijada A, Liston A, Delgado P, Vázquez-Lobo A, Alvarez-Buylla ER. 1998. Variation in the nuclear ribosomal DNA internal transcribed spacer (ITS) region of *Pinus rzedowski* revealed by PCR–RFLP. *Theoretical and Applied Genetics* 96:539–544.

Redecker D, Thierfelder H, Walker C, Werner A. 1997. Restriction analysis of PCR-amplified internal transcribed spacers of ribosomal DNA as a tool for species identification in different genera of the order Glomales. *Applied and Environmental Microbiology* 63:1756–1761.

Rohlf FJ. 1998. *NTSYS-pc* ver. 2.02. *Numerical taxonomy and multivariate analysis system*. Setauket: Exeter Publishing.

Saini A, Reddy SK, Jawali N. 2008. Intra-individual and intra-species heterogeneity in nuclear rDNA ITS region of *Vigna* species from subgenus Ceratotropis. *Genetics Research* 90:299–316.

Saitou N, Nei M. 1987. The neighbour-joining method: a new method for reconstructing phylogenetic trees. *Molecular Biology and Evolution* 4:406–425.

Samuel R, Bachmair A, Jobst J, Ehrendorfer F. 1998. ITS sequences from nuclear rDNA suggest unexpected phylogenetic relationships between Euro-Mediterranean, East Asiatic and North American taxa of *Quercus* (Fagaceae). *Plant Systematics and Evolution* 211:129–139.

Thiers B. 2010. *Index Herbariorum*: a Global Directory of Public Herbaria and Associated Staff. *New York Botanical Garden’s Virtual Herbarium*. http://sweetgum.nybg.org/ih.

White TJ, Bruns T, Lee S, Taylor J. 1990. Amplification and direct sequencing of fungal ribosomal genes for phylogenetics. In: Innis MA, Gelfand DH, Sninsky JJ, White TJ, eds. PCR protocols: a guide to methods and applications. Orlando: Academic Press, 315–322.

Yap IV, Nelson RJ. 1996. *WinBoot*: a program for performing bootstrap analysis of binary data to determine the confidence limits of UPGMA-based dendrogram. *Discussion Paper Series* 14. Manila, Philippines: International Rice Research Institute.

Yeh FC, Yang RC, Boyle TBJ, Ye ZH, Mao JX. 1999. *Pappgene* version 1.32, the user-friendly shareware for population genetic analysis. Edmonton, Alberta: Molecular Biology and Biotechnology Centre, University of Alberta.