



Assessing molecular diversity among 87 species of the *Quercus* L. genus by RAPD markers

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Abstract Oaks (*Quercus* sp.) are among the most ecologically and economically important woody Angiosperms of the northern hemisphere. Nowadays, the reduction of *Quercus* biodiversity is becoming a matter of global concern, and several oak species have been included in the IUCN Red List of Threatened Species. Consequently, characterization and preservation strategies for the oak germplasm are largely promoted. Thus, in this work, the genetic diversity existing among 87 different *Quercus* species was assessed using the RAPD markers, in order to better typify these specimens, to show the amazing DNA variability of this plant genus, and to confirm or infer new putative molecular correlations. Our data were discussed taking into consideration the phylogenetic and phylogeographic relationships previously proposed by the literature. In general, the obtained results corroborated that the evolutionary pattern of *Quercus* genus has been

extremely intricate and continues to change rapidly, making it difficult to be fully resolved. The evidence collected in the present investigation would confirm the complex evolution of the oaks, due to their high migration capacity, divergence rate, and hybridization propensity. This research, performed on a so large series of species, represents a positive contribution for highlighting the genetic diversity within collections of *Quercus* germplasm and favouring *ex-situ* conservation programmes.

Keywords Oak · Fagaceae · Random amplification of polymorphic DNA · Phylogeny · Polymorphisms

Introduction

Population genetic studies performed on *Quercus* L. species (oaks), native to north America and Asia or present in the continuous distributions of the continental Europe, have suggested that the extraordinary biodiversity of this genus has been successfully subjected to several macro- and micro-evolutionary processes (Kremer and Hipp 2020).

Quercus genus includes both deciduous and evergreen plants and extends from cold latitudes to tropical areas, mainly in the northern hemisphere. About 450 species of oaks have been discovered all over the world, with a high percentage in the Americas (65%), where they represent about 20%

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and 29% of the US and Mexican forest biomass, respectively.

The literature has classified oaks repeatedly (Aldrich and Cavender-Bares 2011; Gailing and Curtu 2014; Cavender-Bares 2019). The last validated version has proposed the existence of two subgenus: *Cerris* and *Quercus* (Denk et al. 2017). The first one would include three monophyletic groups: (i) cycle cup oaks (section *Cyclobalanopsis*) occurring in Asia, (ii) cerris oaks (section *Cerris*) and (iii) ilex oaks (section *Ilex*), both located in Eurasia and north Africa. On the other hand, the second subgenus would be made up of five clusters: (i) live/evergreen oaks (section *Virentes*), (ii) species of the section *Ponticae* and (iii) white oaks (section *Quercus*) distributed across the Americas and Eurasia (the latter also in north Africa), (iv) red oaks (section *Lobatae*) and (v) golden cup oaks (section *Protobalanus*) restricted to the Americas.

Quercus genus shows a wide range of morphological and physiological traits. Nevertheless, in some cases, the difference among closely related specimens is often not clear-cut, making challenging their identification. For instance, white oaks usually exhibit similar characters, presenting overlapping systematic and ecological aspects (Gailing and Curtu 2014; McVay et al. 2017). This phenomenon is linked to the fact that oaks are prone to interspecific hybridization, introgression, and intricate speciation patterns, which involve incomplete lineage sorting (Pang et al. 2019). In addition, the sympatric occurrence of several *Quercus* species also promotes interbreeding and taxonomic confusion (Beatty et al. 2016). By contrast, the highest rates of species diversification have been detected in response to landscape changes and migration events into new territories (Kremer and Hipp 2020).

For centuries, oak classification has been based entirely on morphological traits, until the development of molecular approaches has changed remarkably the phylogeny of this plant genus (Denk et al. 2017). Among all, DNA analysis has been recognised as the most powerful tool for reconstructing *Quercus* evolutionary steps (Ardi et al. 2012). Dominant markers, such as AFLPs (Amplified fragment length polymorphisms), ISSRs (Inter Simple Sequence Repeats), and RAPDs (Random Amplification of Polymorphic

DNAs), have generally provided higher levels of discrimination than other techniques, even in presence of hybrid species (Cotti 2008).

AFLP has been one of the first method applied to *Quercus* genus, for instance, to: (i) highlight the similarity existing among four California red oak species (i.e., *Q. agrifolia*, *Q. parvula*, *Q. kelloggii*, and *Q. wislizeni*) (Dodd and Kashani 2003); (ii) assess the gene flow between *Q. suber* and *Q. ilex* samples from Portugal (Coelho et al. 2006); (iii) evaluate the degree of differentiation within indigenous populations of Flemish *Q. petraea* and *Q. robur* (Coart et al. 2002); (iv) investigate the hybridization rate between *Q. crispula* and *Q. dentata*, two dominant components of the cool-temperate forests across Japan (Ishida et al. 2003).

More recently, ISSR investigations have allowed to reconstruct the phylogenetic relationships among different Fagaceae genera (including *Quercus*) (Coutinho et al. 2014), to typify Russian varieties of *Q. robur* (Chokheli et al. 2016, 2018), and to select Moroccan ecotypes of *Q. suber* for conservation purposes (Laakili et al. 2018).

Although modern techniques, like the restriction-site associated DNA next-generation sequencing (RAD-seq), have been usefully applied for obtaining large-scale information about biogeography, niche modification, and diversification patterns of the American and European oaks (Hipp et al. 2018; 2020), RAPD method has been deemed as one of the best systems to investigate the evolution of the *Quercus* species, due to the high level of polymorphisms present in oak genomes and the easy applicability of this molecular analysis (Aykut 2016). In this regard, RAPDs have been employed for detecting genetic similarities among *Q. robur*, *Q. macranthera*, *Q. infectoria*, *Q. magnosquamata*, and *Q. libani* from forests of the north-west Iran (Ardi et al. 2012), for solving taxonomic issues relative to three Turkish evergreen oak species (i.e., *Q. coccifera*, *Q. ilex*, and *Q. aucheri*) (Aykut et al. 2013; Aykut 2016), or for distinguishing *Q. crassifolia* and *Q. crassipes* Mexican exemplars with partially overlapping distributions (Tovar-Sánchez and Oyama 2004; González-Rodríguez et al. 2004).

Nowadays, the degradation rate of oak biodiversity is becoming a matter of global concern. Uncorrected land exploitation, over-cutting, over-grazing, and climatic changes are the main factors that contribute

to the destruction of *Quercus* woods and trigger the threat of extinction to many species of this genus (Jawarneh et al. 2013). Therefore, characterization and preservation strategies of the oak genetics are largely favoured and promoted. In light of this, starting from 2010, a collection of about 430 oak exemplars, which represent 87 non-hybrid species deriving from different areas of the world, have been bedded out at the *Botanical Gardens of Rome Tor Vergata* (Rome, Italy). Thus, the aim of the current work consisted in the assessment of the genetic diversity of these 87 oak species by RAPDs, in order to: (i) better typify them at molecular level; (ii) highlight the amazing DNA variability existing in this plant genus; (iii) confirm or infer new putative genetic correlations among the studied specimens. Therefore, the obtained results represent the action of a project aimed at characterizing and promoting the *ex-situ* conservation of the *Quercus* germplasm.

Materials and methods

Plant material and ethical statements

The plant material was kindly provided by Prof. Antonella Canini (*Botanical Gardens of Rome Tor Vergata*, Rome, Italy; Lat: 41.84758, Long: 12.64385). The sampling occurred in Spring 2019 and consisted in the collection of leaves (without symptoms of pathology) from 87 oak species present in the living collection of the *Botanical Gardens of Rome Tor Vergata* (Supplementary Information 1). Each sampled specimen, today accessible and growing at the *Botanical Gardens of Rome Tor Vergata*, was tagged and included in a dedicated register; botanical vouchers (codes: Qsp1-430) are available at the *Botanical Gardens of Rome Tor Vergata*. For each species, from 3 to 5 individuals were sampled and analysed, to validate accuracy, repeatability, and validity of methods and results. After picking, plant tissues were carefully washed with sterile water, powdered by mortar and pestle in presence of liquid nitrogen, and stored at -80°C until the use. The experimental research performed in this study complies with relevant institutional, national, and international guidelines and legislation; in particular, the collection of the plant material was carried out in respect of the IUCN Policy

Statement. In addition, the current work promotes the conservation status of the investigated species not already available in public collection.

DNA extraction and quantitation

Total DNA was isolated from 100 mg of powdered plant material by using the NucleoSpin Plant II kit (Macherey–Nagel), according to manufacturers' instructions. However, this protocol was optimized and adapted for oak tissues, due to their high levels in secondary metabolites which negatively affect yield and purity of the nucleic acids. Thus, before the application of the kit, the sample was subjected to a preliminary purification step based on the CTAB method reported in Lefort and Douglas (1999) and Gismondi et al. (2012), with some modifications. In detail, the sample was resuspended in the extraction buffer (50 mM Tris–HCl pH 8.0; 20 mM EDTA; 0.7 M NaCl; 1% CTAB; 1% PVP; 2% SDS; 0.7% β -mercaptoethanol) for 15 min at 65°C , diluted with a quarter of volume by chloroform/isoamyl alcohol (24:1; v/v), vortexed, and centrifuged at 11.000 g for 10 min. Finally, the aqueous phase was recovered and treated as described in the guidelines of the extraction kit. DNA concentration and purity were determined by a NanoDrop spectrophotometer (Thermo Scientific), reading the absorbance of the samples at 260 nm and 280 nm.

RAPD-PCR

PCR reaction for RAPD analysis was carried out in a final volume of 50 μL which contained: 25 ng of template; 0.6 U of KAPA Taq DNA polymerase (Kapa Biosystems); 1X Taq DNA polymerase buffer; 0.4 μM for each primer; 0.2 mM of each dNTP; 3% DMSO. To optimize the amplification process, different combination of primers (Kit A, Operon Technologies, USA; OPA-1: 5'-CAGGCCCTTC-3'; OPA-2: 5'-TGCCGAGCTG-3'; OPA-4: 5'-AATCGG GCTG-3'; OPA-7: 5'-GAAACGGGTG-3'; OPA-8: 5'-GTGACGTAGG-3') were tested as reported here: (a) OPA-1, OPA-2, OPA-4, OPA-7, and OPA-8; (b) OPA-1, OPA-2, OPA-4, and OPA-7; (c) OPA-1, OPA-2, and OPA-4; (d) OPA-1, and OPA-2; (e) OPA-8 alone; (f) OPA-1 alone; (g) OPA-4 alone; (h) OPA-2 alone; (i) OPA-7 alone. In addition, two different concentrations of MgCl_2 (i.e., 0.5 mM and

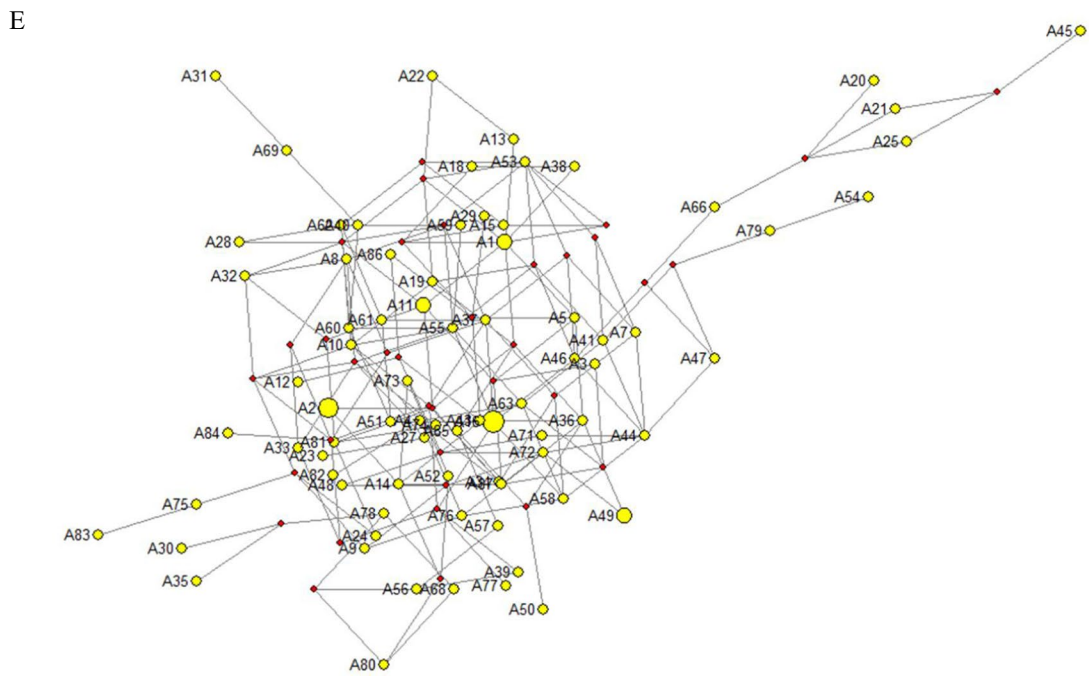
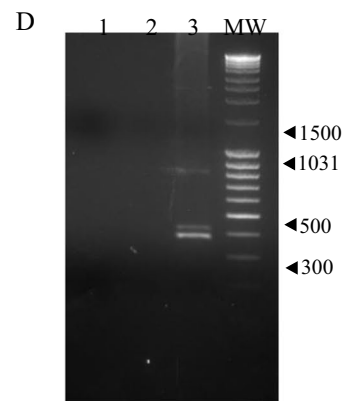
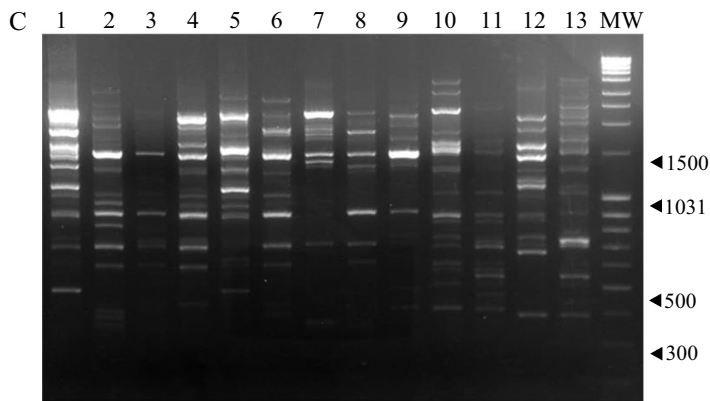
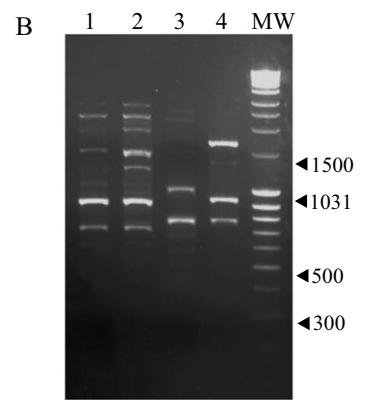
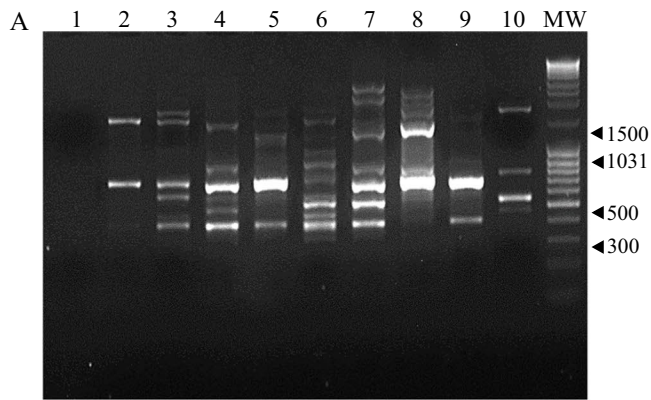


Fig. 1 **A** Screening of primers. Representative pattern of amplicons obtained by PCR, fractionated on 2% agarose gel, and visualized under UV-light. The combination of primers tested in the study were: no primer (lane 1); OPA-1, OPA-2, OPA-4, OPA-7, and OPA-8 (lane 2); OPA-1, OPA-2, OPA-4, and OPA-7 (lane 3); OPA-1, OPA-2, and OPA-4 (lane 4); OPA-1, and OPA-2 (lane 5); OPA-8 alone (lane 6); OPA-1 alone (lane 7); OPA-4 alone (lane 8); OPA-2 alone (lane 9); OPA-7 alone (lane 10). **B** Experimental setting-up. Agarose gel (2%) showing, under UV-light, a representative pattern of amplicons obtained by PCR (with OPA-1 primer alone) at different conditions: annealing temperature 52 °C+0.5 mM MgCl₂ (lane 1); 52 °C+0.75 mM MgCl₂ (lane 2); 54 °C+0.5 mM MgCl₂ (lane 3); 54 °C+0.75 mM MgCl₂ (lane 4). **C** RAPD profiles. Representative image of some genetic profiles obtained by RAPD analysis and visualized on agarose gel (2%), under UV-light. **D** PCR controls. Visualization on agarose gel (2%) of the representative PCR products obtained with: no Taq DNA polymerase (lane 1); no template (lane 2); 0.1 ng of template (lane 3). In all previous panels, MW lane shows the profile of the Mass Ruler DNA marker (Thermo Scientific); the arrows indicate specific fragments whose sizes are reported in base pairs. **E** RM-MJ Network. Network produced using RAPD data from the investigated *Quercus* species. The lab-codes identifying each sample refer to those reported in Supplementary Information 2. Node size is proportional to the number of species included in the hubs. In particular, A1 node included A1+A6 samples; A11 (A11+A17); A2 (A2+A26+A42); A16 (A16+A64+A65+A67); A49 (A49+A70)

0.75 mM) and two annealing temperatures (i.e., 52 °C and 54 °C) were evaluated. Amplifications were performed using a Bio-Rad IQ5 thermocycler whose conditions were set as follows: (i) 4 min of denaturation at 95 °C; (ii) 60 cycles, each one consisting in 20 s of further denaturation at 95 °C, 20 s of annealing at 52/54 °C, and 45 s of extension at 72 °C; (iii) final extension step at 72 °C for 10 min. PCR products were run immediately on agarose gel for the detection of the genetic profiles.

Agarose gel electrophoresis

After the amplification, PCR products were fractionated by electrophoresis on 2% agarose gel prepared using pH 8.5 TAE buffer (40 mM Tris-HCl; 1 mM EDTA; 20 mM acetic acid) and containing ethidium bromide (10 mg/mL). The run was carried out at 80 Volts for 120 min. DNA fragments were visualized under UV-light and photographed by a VersaDoc MP 4000 system (Bio-Rad). The molecular weight of the amplicons was estimated using the Mass Ruler DNA marker (Thermo Scientific) as reference.

Bioinformatics analysis

Polymorphic bands were detected in all species. Thus, RAPD profiles were defined using a binary method in which presence or absence of specific DNA fragments was coded as 1 or 0, respectively. Total number of bands, number of polymorphic bands, polymorphism in percentage, mean of the band frequencies, polymorphic information content value, marker index, and resolving power were calculated as reported in Guasmi et al. (2012), Ghosh et al. (2014), and Chesnokov and Artemyeva (2015). To simplify the analysis, each taxon was renamed using an internal lab-code (see Supplementary Information 2). The relationships among the genetic profiles were established using the reduced median–median joining (RM-MJ; $r=2$, $\epsilon=0$) approach (Bandelt et al. 1999) implemented by Network software (v. 10.0). Moreover, the matrix produced from the binomial data was employed to calculate the phylogenetic distance of the specimens, according to Nei and Li (1979) coefficient and UPGMA method, through the FreeTree program (Hapl et al. 2001). The relative dendrogram was drawn and viewed by TREEVIEW software (Page 1996).

Results and discussion

Oaks are among the most ecologically and economically important woody Angiosperms of the northern hemisphere. Therefore, the preservation of the *Quercus* genus appears fundamental for maintaining as balanced the environmental equilibria. Indeed, loss of biodiversity can cause severe consequences, including landscape fragmentation, phytosanitary problems, genetic erosion, and habitat degradation. To avoid such type of phenomena, conservation strategies and characterization studies of plant exemplars are crucial. For these reasons, in the present work, 87 different oak species were analysed by RAPD markers, to develop a specific identification system and to investigate the genetic diversity present in the oak germplasm collection present at the *Botanical Gardens of Rome Tor Vergata* for *ex-situ* conservation purposes.

To be more specific, the studied series included 19 oak species belonging to *Cerris* subgenus and 68 to *Quercus* one. The first group consisted

of specimens relative to 17 taxa from *Cerris* section and 2 from *Cyclobalanopsis*, while the second cluster presented individuals classified as follows: 28 species from *Lobatae* section, 38 from *Quercus*, 1 from *Protobalanus*, and 1 from *Virentes* (Supplementary Information 1).

The first step of the investigation included the setting up of the amplification protocol. Several combinations of primers were tested (see Materials and Methods section); among all, the most informative profiles, or rather those with the clearest and sharpest bands, were obtained using OPA-1 primer alone (Fig. 1A). This condition was further examined on varying two different annealing temperatures (i.e., 52 and 54 °C) and two MgCl₂ concentrations (i.e., 0.5 and 0.75 mM) (Fig. 1B). The best procedure, in terms of richness in bands, appeared to be that characterized by an annealing temperature of 52 °C in presence of 0.75 mM MgCl₂. Thus, this amplification system was selected to be applied on DNAs purified from the 87 oak species. The reproducibility of the banding patterns, generated using the standardized RAPD approach reported above, was checked by comparing the molecular profiles of the same oak DNA isolated in different extraction procedures and carrying out inter-laboratory replicates. The polymorphic nature of the *Quercus* DNAs can be observed in Fig. 1C, which shows OPA-1 genetic schemes for some representative specimens. To validate the experimental protocol, PCR controls were always carried out (Fig. 1D).

The selected RAPD primer produced 11 bands with sizes going from approximately 400 to 1100 bp. The fragments out of this range were not considered during the analysis. The binary profiles reflecting the RAPD results for all oak samples were reported in Supplementary Information 2. The molecular pattern showing the highest number of amplicons was relative to *Q. buckleyi* (i.e., 11/11), while *Q. grisea* and *Q. pubescens* were the species characterized by

the simplest profiles (i.e., 3/11). The most represented amplicons were those at 600 and 750 bp, which appeared in 74 and 73 species out of 87, respectively. By contrast, the band at 550 bp, detectable only in 31 taxa, was the less conserved among all.

RAPD profiles revealed that 92.8% of *Lobatae* species were characterized by the band at 400 bp, while 96.5% by the amplicon of 600 bp. On the other hand, the signal at 950 bp was absent in 78.5% of them. The bands at 600 bp and 900 bp typified 84.2% of the samples belonging to *Quercus* section. Surprisingly, the same percentage of specimens presented the absence of the fragment at 700 bp. About *Cerris* section, 88.2% of the oaks exhibited the longest amplicon (i.e., 1100 bp), while 94.1% the band at 750 bp. The amplicon at 580 bp was the less frequent for the same species, being detectable only in 11.8% of the molecular patterns. The reduction of the frequency (64.7%) of the band at 600 bp for the species included in the *Cerris* section, as well as other differences observed among distinct clusters, could be linked to migration and diversification events, considering the excellent adaptative capacity of oaks to several environments and climate conditions and the continuous gene flow existing within this genus (Cavender-Bares 2019).

The RAPD method employed in this study and based on the application of OPA-1 primer could be considered highly discriminant for oak species, according to the efficiency parameters calculated and reported in Table 1. In particular, OPA-1 was able to amplify a good number of bands, compared to other primers tested in RAPD analyses for *Quercus* species (Ardi et al. 2012), which were all polymorphic, allowing to reveal a great genetic variability in our samples. Indeed, the mean of the band frequencies (i.e., 0.576) suggested a general heterogeneity in the distribution of the amplicons among the species. In addition, the PIC value, often used as a measure for the informativeness of a molecular marker, was very high (0.488), taking into consideration that dominant

Table 1 Efficiency parameters of the applied method

Primer	Sequence (5'-3')	TNB	NPB	% Polym	MBF	PIC	MI	RP
OPA-1	CAGGCCCTTC	11	11	100	0.576	0.488	5.37	1.15

The sequence of the primer used for producing RAPD profiles and the relative efficiency parameters were reported: total number of bands (TNB); number of polymorphic bands (NPB); polymorphism in percentage (% Polym); mean of the band frequencies (MBF); polymorphic information content value (PIC); marker index (MI); resolving power (RP)

markers present a maximum PIC value of 0.5 (Serrote et al. 2020). Marker index and resolving power values, which show the capacity of the selected primer to detect genetic variability across the investigated plant material (Ghosh et al. 2014), revealed that OPA-1 had a discrete discriminatory potential, even with respect to other RAPD primers investigated in different species (Tonk et al. 2014).

To reduce the complexity of the obtained data, a network (Fig. 1E) was built by applying RM-MJ technique and giving equal scores to each character (locus/band). The outcome put in prominence the existence of 79 different genetic profiles out of 87 analysed species, confirming the high discriminating power of the selected RAPD method. Indeed, 90.1% of the samples appeared in the graph as single nodes (named using the lab-codes reported in Supplementary Information 2). In five cases (i.e., A1, A2, A11, A16, and A49), node size was bigger than the basal form, as proportional to the number of samples which included, representing taxa with identical molecular patterns. The largest node (tagged A16) contained 4 species (*Q. turbinella* A16, *Q. alentejana* A64, *Q. petraea* ssp. *iberica* A65, and *Q. hartwissiana* A67) which possess a very diversified area of origin but whose genetic proximity can be justified by the fact that they are all white oaks (*Quercus* section). The second node for dimension (tagged A2) consisted in the RAPD profile shared by 3 taxa of red oaks (*Q. gravesii* A2, *Q. imbricaria* A26, and *Q. shumardi* A42) belonging to *Lobatae* section and native of central and northern America. Finally, three nodes were made up of couples of species: node tagged A1 (*Q. seemanii* A1 and *Q. canbyi* A6); node tagged A11 (*Q. agrifolia* A11 and *Q. rysophylla* A17); node tagged A49 (*Q. glauca* A49 and *Q. coccifera* ssp. *rivas-martinezii* A70). The similarity showed by *Q. agrifolia* and *Q. rysophylla* might be expected, as they are both native to the same area (i.e., Mexico) and the only evergreen red oaks among those investigated in this study. The overlapping genotypes of *Q. seemanii* and *Q. canbyi* could suggest a common ancestor, also taking into account their membership to *Lobatae* section and their origin from Mexico. Surprisingly, *Q. glauca* and *Q. coccifera* ssp. *rivas-martinezii* exhibited the same RAPD fingerprint, although belonging to different sections, namely *Cyclobalanopsis* and *Cerris*, respectively.

The positioning of the species in the network did not allow to group the taxa according to their sections (e.g., *Quercus*, *Cerris*, *Lobatae*); however, the distribution pattern reflected the geographical localisation and/or the ecological role of the specimens, as suggested by other literature works. Indeed, such type of clustering has been already observed and explored by Kremer and Hipp (2020), looking for the ancestor of American white and red oaks. In the same context, the molecular distance registered between *Q. fabrei* (A83) and *Q. montana* (A45) is noteworthy. In fact, even if these two species belong to *Lobatae* section, they are native of different continents, in that order, Asia and America. Thus, events of evolutionary convergence, maybe dictated by specific environmental pressures, might have led the selection of peculiar genetic schemes closely correlated.

Other two nodes, that is A31 (*Q. marilandica*) and A69 (*Q. virgiliana*), distinguish themselves from the central core, spreading together toward an alternative direction. It is difficult to clearly understand the molecular and/or ecological mechanisms underlying this phenomenon, considering that A31 is a species of the *Lobatae* section native to central and eastern USA while A69 refers to *Quercus* section and originates from western Mediterranean.

RAPD data were also used to construct a similarity matrix (Supplementary Information 3), based on the coefficients of Nei and Li (1979), for assessing the genetic relationship existing among the 87 *Quercus* species considered in this work. The similarity indices ranged from a maximum of 1, when two RAPD profiles were identical, to 0, in case of couples of species with no band in common. Zero values were found for the following pairs: *Q. trojana* ssp. *euboica* (A68) and *Q. pubescens* (A75); *Q. pontica* (A84) and *Q. sinuata* var. *breviloba* (A12); *Q. pontica* (A84) and *Q. rotundifolia* (A54); *Q. trojana* ssp. *euboica* (A68) and *Q. pontica* (A84). These results cannot be explained as a consequence of membership to different sections, confirming the evidence inferred by the network analysis about the incapacity of the present RAPD approach (i.e., OPA-1 primer) to discriminate oak sub-genera and relative clusters.

Starting from the previous similarity matrix and using the unweighted pair-group method with arithmetic averages (UPGMA), an unrooted dendrogram (Fig. 2) showing the calculated genetic

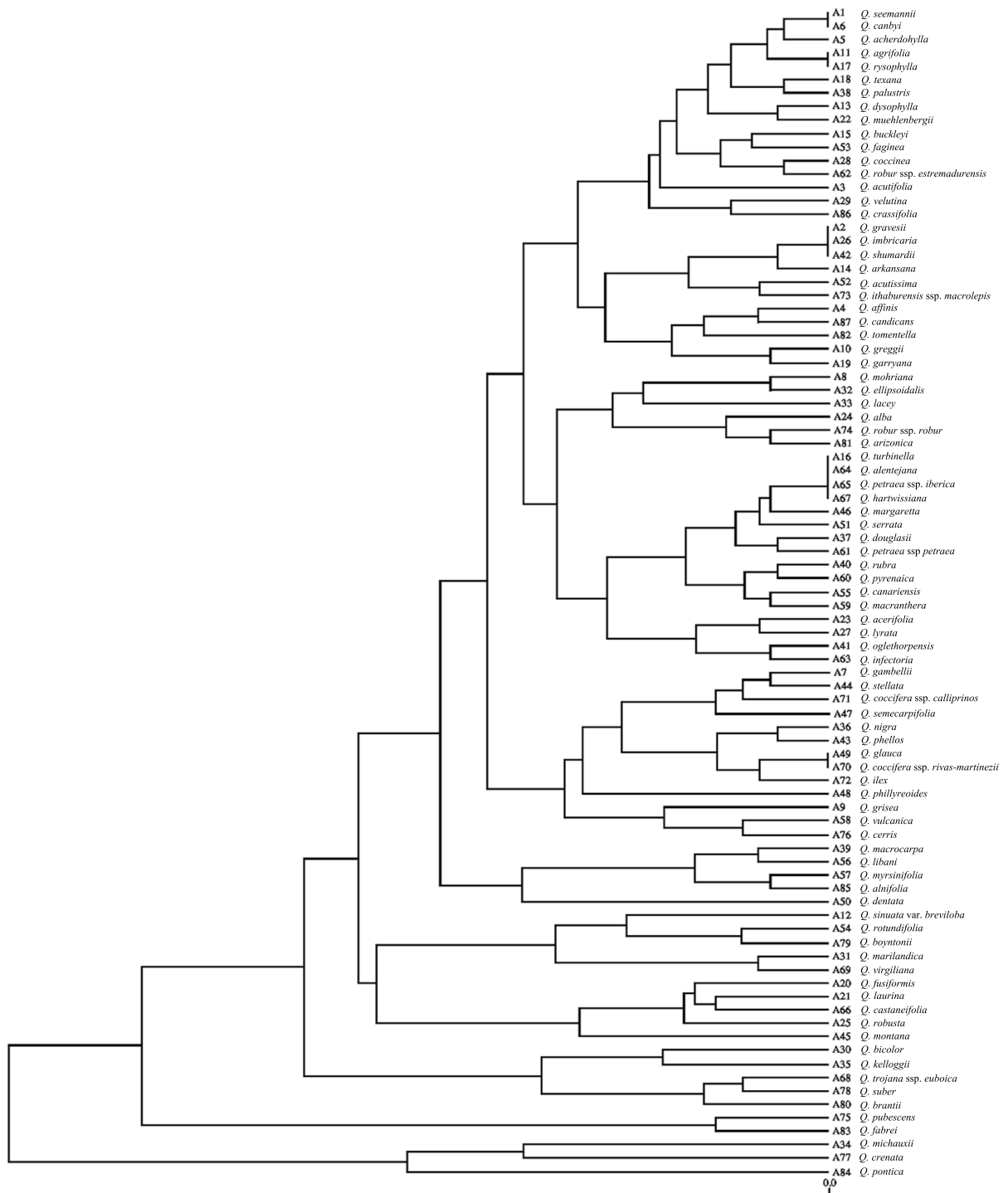


Fig. 2 Phylogenetic reconstruction. Dendrogram designed on RAPD data by UPGMA cluster analysis using Nei and Li coefficient

distances among the species was produced. Logically, the samples with identical genetic profiles clustered together (e.g., A1, and A6; A2, A26, and A42; etc.), as already observed and described in detail for the network (i.e., bigger nodes).

In the phylogenetic tree, *Q. pontica* (A84) appeared as the most genetically distinct species among all. This outcome could be expected, considering that *Q. pontica* has a very restricted geographical distribution, which confines itself on the Caucasus mountains of the western Asia (Denk et al. 2017). Indeed, this taxon has been considered as a Tertiary relict species markedly separated from the other white oaks, according to an analysis carried out on the nuclear ribosomal Internal Transcribed Spacer (ITS) barcode gene (Denk and Grimm 2010).

In the dendrogram, the major part of *Lobatae* characterized the upper region of the graph, while species from the *Quercus* section were mainly located in central branches. However, in general, oaks did not group distinctly based on their classification in sections, either in case of subspecies (i.e., *Q. petraea*, *Q. robur*, and *Q. coccifera*), but most probably because of a mixture of other factors, first of all the sharing of similar habitats. Paying attention to this aspect, it could be noticed that phylogeographic schemes were not fully respected; however, oaks are renowned for posing issues about the definition of species boundaries, which lead to taxonomic problems in classifying the specimens (Kashani and Dodd 2002). To justify this complex outcome, one should keep in mind that a lot of studies have described that the gene flow acts as a strong homogenizing agent able to reduce the genetic distance among *Quercus* species. Moreover, when species are not hybrids (like those investigated in this study), other elements, like shared ancestral polymorphisms and historical introgression events, could make the phylogeny more complicated and unclear (Tovar-Sánchez and Oyama 2004; McVay et al. 2017).

Particular attention should be paid to *Q. crenata*, a species strongly threatened with extinction. Indeed, nowadays, only about a thousand of exemplars have been recorded in Italy (where it is protected) and a few in France. Recent genetic studies have proposed this species as the remainder of the ancestral form from which *Q. suber* would have evolved (Simeone

et al. 2018), rejecting the theory of a stabilised hybridization between *Q. suber* and *Q. cerris* (Bellarosa et al. 2005). RAPD data reported in this research would be more in line with the first hypothesis, due to the higher proximity of *Q. crenata* (A77) to *Q. suber* accession, although this ambiguous species appeared quite isolated in the dendrogram from both its potential relatives.

Q. alentejana is another important taxon still under investigation. Some scholars consider it as a new independent species, others like a complex hybrid between *Q. pyrenaica* and *Q. marianica*. The scientific evidence here described did not reveal a high molecular similarity between *Q. alentejana* and *Q. pyrenaica*; however, no conclusion can be drawn because, unfortunately, *Q. marianica* was not among the studied samples.

The last peculiar aspect to be discussed in this work is relative to *Q. robusta*, whose presence is mainly limited to American stands of small size. The reduced distribution makes it as a species to be protected. According to Govaerts and Frodin (1998), *Q. robusta* would be the result of the hybridization between *Q. emoryi* and *Q. gravesii*. This assumption seems to be not corroborated by the phylogenetic reconstruction reported in the current study, which showed a considerable genetic distance between *Q. robusta* and *Q. gravesii*. Thus, it is possible that *Q. robusta* differentiated deeply from its ancestors or that the proposed hybridization model is not correct. In this regard, Muller (1952) had already confirmed this second hypothesis, explaining that *Q. emoryi* and *Q. gravesii* would not be able to fertilize each other, despite they share the same habitats and/or spatial regions.

In conclusion, we assessed the molecular diversity existing among 87 different *Quercus* species by applying a molecular method based on RAPD markers. With some exceptions, our data were generally coherent with the phylogenetic and phylogeographic relationships proposed by the previous literature. Our results confirmed that the evolutionary pattern of *Quercus* genus has been extremely complex and continues to change rapidly, making it difficult to be fully resolved. This phenomenon was surely due to several biological mechanisms typically linked to oaks, such as: (i) capacity to migrate rapidly, promoting ecological consequences on lineage differentiation; (ii) high

rates of evolutionary divergence; (iii) hybridization propensity, which contributes to adaptive introgression.

Some oak species included in this study are inserted in the IUCN Red List of Threatened Species, as vulnerable or endangered. Thus, the molecular characterization performed in this study, on a so large series of species, will surely represent a positive contribution for profiling the diagnostic genetic diversity of the *Quercus* species and for favouring preservation strategies of their genetics, with the final aim to promote *ex-situ* conservation programmes for oak germplasm.

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Availability of data and materials All data reported, used, and cited in this research are present in the main text or in the supplementary materials of the manuscript. The plant specimens sampled in this work are preserved in the *Botanical Garden of Rome Tor Vergata*.

Declarations

Ethical approval Not applicable.

Consent to participate Not applicable.

Consent for publication Not applicable.

Conflict of interest The authors declare no conflict of interest about the present work.

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References

- Aldrich PR, Cavender-Bares J (2011) *Quercus*. Wild crop relatives: genomic and breeding resources. Springer, Berlin Heidelberg, Berlin, Heidelberg, pp 89–129
- Ardi M (2012) Genetic variation among Iranian oaks (*Quercus* spp.) using random amplified polymorphic DNA (RAPD) markers. *Afr J Biotechnol*. <https://doi.org/10.5897/AJB12.325>
- Ardi M, Rahmani F, Siami A (2012) Genetic variation among Iranian oaks (*Quercus* spp.) using random amplified polymorphic DNA (RAPD) markers. *Afr J Biotechnol* 11:10291–10296. <https://doi.org/10.5897/AJB12.325>
- Bandelt HJ, Forster P, Röhl A (1999) Median-joining networks for inferring intraspecific phylogenies. *Mol Biol Evol* 16:37–48. <https://doi.org/10.1093/oxfordjournals.molbev.a026036>
- Beatty GE, Montgomery WI, Spaans F et al (2016) Pure species in a continuum of genetic and morphological variation: sympatric oaks at the edge of their range. *Ann Bot* 117:541–549. <https://doi.org/10.1093/aob/mcw002>
- Bellarosa R, Simeone MC, Papini A, Schirone B (2005) Utility of ITS sequence data for phylogenetic reconstruction of Italian *Quercus* spp. *Mol Phylogenet Evol* 34:355–370. <https://doi.org/10.1016/j.ympev.2004.10.014>
- Cavender-Bares J (2019) Diversification, adaptation, and community assembly of the American oaks (*Quercus*), a model clade for integrating ecology and evolution. *New Phytol* 221:669–692. <https://doi.org/10.1111/nph.15450>
- Chesnokov YV, Artemyeva AM (2015) Evaluation of the measure of polymorphism information of genetic diversity. *Agri Biol* 50:571–578. <https://doi.org/10.15389/agrobiology.2015.5.571eng>
- Chokheli V, Kozlovsky B, Sereda M et al (2016) Preliminary comparative analysis of phenological varieties of *Quercus robur* by ISSR-markers. *J Bot* 2016:1–7. <https://doi.org/10.1155/2016/7910451>
- Chokheli V, Kagan D, Rajput V et al (2018) Genetic Variability in cenopopulations of Pedunculate Oak (*Quercus robur*) in Rostov Region, Russia, with the Use of ISSR-Markers. *Int J Agric Biol* 20:2554–2548
- Coart E, Lamote V, De Loose M et al (2002) AFLP markers demonstrate local genetic differentiation between two indigenous oak species [*Quercus robur* L. and *Quercus petraea* (Matt.) Liebl.] in Flemish populations. *Theor Appl Genet* 105:431–439. <https://doi.org/10.1007/s00122-002-0920-6>
- Coelho AC, Lima MB, Neves D, Cravador A (2006) Genetic diversity of two evergreen Oaks [*Quercus suber* (L.) and *Quercus ilex* subsp. *rotundifolia* (Lam.)] in Portugal using AFLP Markers. *Silvae Genet* 55:105–118. <https://doi.org/10.1515/sg-2006-0016>
- Cotti C (2008) Molecular markers for the assessment of genetic variability in threatened plant species. *Mater Studiorum Università degli Studi di Bologna Ph.D. Thesis*. Chrome-extension://efaidnbmnnnibpcajpcglclefindmkaj/http://amsdottorato.unibo.it/660/1/Tesi_Cotti_Claudia.pdf.
- Coutinho JP, Carvalho A, Lima-Brito J (2014) Genetic diversity assessment and estimation of phylogenetic relationships among 26 Fagaceae species using ISSRs.

- Biochem Syst Ecol 54:247–256. <https://doi.org/10.1016/j.bse.2014.02.012>
- Denk T, Grimm GW (2010) The oaks of western Eurasia: Traditional classifications and evidence from two nuclear markers. *Taxon* 59:351–366. <https://doi.org/10.1002/tax.592002>
- Denk T, Grimm GW, Manos PS et al (2017) An updated infrageneric classification of the Oaks: review of previous taxonomic schemes and synthesis of evolutionary patterns. In: Gil-Pelegrín E, Peguero-Pina JJ, Sancho-Knapik D (eds) Oaks physiological ecology. Exploring the functional diversity of genus *Quercus* L. Springer International Publishing
- Dodd RS, Kashani N (2003) Molecular differentiation and diversity among the California red oaks (Fagaceae; *Quercus* section *Lobatae*). *Theor Appl Genet* 107:884–892. <https://doi.org/10.1007/s00122-003-1290-4>
- Gailing O, Curtu AL (2014) Interspecific gene flow and maintenance of species integrity in oaks. *Ann for Res* 57:5–18. <https://doi.org/10.15287/afr.2014.171>
- Ghosh BK, Mandal A, Datta AK, Das D (2014) RAPD analysis in *Andrographis paniculata* (Burm. F.) ness plant types. *Int J Res Ayurveda Pharm* 5:84–88. <https://doi.org/10.7897/2277-4343.05117>
- Gismondi A, Rolfo MF, Leonardi D et al (2012) Identification of ancient *Olea europaea* L. and *Cornus mas* L. seeds by DNA barcoding. *C R Biol* 335:472–479. <https://doi.org/10.1016/j.crvi.2012.05.004>
- González-Rodríguez A, Arias DM, Valencia S, Oyama K (2004) Morphological and RAPD analysis of hybridization between *Quercus affinis* and *Q. laurina* (Fagaceae), two Mexican red oaks. *Am J Bot* 91:401–409. <https://doi.org/10.3732/ajb.91.3.401>
- Govaerts R, Frodin DG (1998) World checklist and bibliography of Fagales. Royal Botanic Gardens, Kew
- Guasmi F, Elfalleh W, Hannachi H, Feres K, Touil L, Marzougui N et al (2012) The use of ISSR and RAPD markers for genetic diversity among south tunisian barley. *ISRN Agron* 952196:1–10. <https://doi.org/10.5402/2012/952196>
- Hapl V, Pavlíček A, Flegr J (2001) Construction and bootstrap analysis of DNA fingerprinting-based phylogenetic trees with the freeware program FreeTree: application to trichomonad parasites. *Int J Syst Evol Microbiol* 51:731–735. <https://doi.org/10.1099/00207713-51-3-731>
- Hipp AL, Manos PS, González-Rodríguez A et al (2018) Sympatric parallel diversification of major oak clades in the Americas and the origins of Mexican species diversity. *New Phytol* 217:439–452. <https://doi.org/10.1111/nph.14773>
- Hipp AL, Manos PS, Hahn M et al (2020) Genomic landscape of the global oak phylogeny. *New Phytol* 226:1198–1212. <https://doi.org/10.1111/nph.16162>
- Ishida TA, Hattori K, Sato H, Kimura MT (2003) Differentiation and hybridization between *Quercus crispula* and *Q. dentata* (Fagaceae): insights from morphological traits, amplified fragment length polymorphism markers, and leafminer composition. *Am J Bot* 90:769–776. <https://doi.org/10.3732/ajb.90.5.769>
- Jawarneh MS, Brake MH, Muhaidat R, et al (2013) Characterization of *Quercus* species distributed in Jordan using morphological and molecular markers. *Afr J Biotechnol* 12
- Kremer A, Hipp AL (2020) Oaks: an evolutionary success story. *New Phytol* 226:987–1011. <https://doi.org/10.1111/nph.16274>
- Laakili A, Belkadi B, Medraoui L et al (2018) Diversity and spatial genetic structure of natural Moroccan *Quercus suber* L. assessed by ISSR markers for conservation. *Physiol Mol Biol Plants* 24:643–654. <https://doi.org/10.1007/s12298-018-0538-z>
- Lefort F, Douglas GC (1999) An efficient micro-method of DNA isolation from mature leaves of four hardwood tree species *Acer*, *Fraxinus*, *Prunus* and *Quercus*. *Ann for Sci* 56:259–263. <https://doi.org/10.1051/forest:19990308>
- McVay JD, Hipp AL, Manos PS (2017) A genetic legacy of introgression confounds phylogeny and biogeography in oaks. *Proc R Soc B Biol Sci* 284:20170300. <https://doi.org/10.1098/rspb.2017.0300>
- Muller CH (1952) Ecological control of hybridization in *Quercus*: a factor in the mechanism of evolution. *Evolution* (n y) 6:147. <https://doi.org/10.2307/2405620>
- Nei M, Li WH (1979) Mathematical model for studying genetic variation in terms of restriction endonucleases. *Proc Natl Acad Sci* 76:5269–5273. <https://doi.org/10.1073/pnas.76.10.5269>
- Page RDM (1996) Tree view: an application to display phylogenetic trees on personal computers. *Bioinformatics* 12:357–358
- Pang X, Liu H, Wu S et al (2019) Species identification of Oaks (*Quercus* L., Fagaceae) from gene to genome. *Int J Mol Sci* 20:5940. <https://doi.org/10.3390/ijms20235940>
- Serrote CML, Reiniger LRS, Silva KB, dos Santos Rabaiolli SM, Stefanel CM (2020) Determining the polymorphism information content of a molecular marker. *Gene* 726:144175. <https://doi.org/10.1016/j.gene.2019.144175>
- Simeone MC, Cardoni S, Piredda R et al (2018) Comparative systematics and phylogeography of *Quercus* Section *Cerris* in western Eurasia: inferences from plastid and nuclear DNA variation. *PeerJ* 6:e5793. <https://doi.org/10.7717/peerj.5793>
- Tonk FA, Tosun M, Ilker E, Istipliler D, Tatar O (2014) Evaluation and comparison of ISSR and RAPD markers for assessment of genetic diversity in triticale genotypes. *Bulg J Agric Sci* 20:1413–1420
- Tovar-Sanchez E, Oyama K (2004) Natural hybridization and hybrid zones between *Quercus crassifolia* and *Quercus crassipes* (Fagaceae) in Mexico: morphological and molecular evidence. *Am J Bot* 91:1352–1363. <https://doi.org/10.3732/ajb.91.9.1352>
- Aykut Y (2016) Phylogenetic relationships of the genus *Quercus* L. (Fagaceae) from three different sections. *Afr J Biotechnol* 15:2265–2271. <https://doi.org/10.5897/ajb2016.15598>
- Aykut Y, Emel U, Tekin BM (2013) Molecular diversity among Turkish oaks (*Quercus*) using random amplified polymorphic DNA (RAPD) analysis. *Afr J Biotechnol* 12:6358–6365. <https://doi.org/10.5897/ajb2013.12299>
- Kashani N, Dodd RS (2002) Genetic differentiation of two California red oak species, *Quercus parvula* var. *shreveii*

and Q. wislizeni, based on AFLP genetic markers. US For Serv Gen Tech Rep PSW-GTR-184:417–426.

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