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Jana Ekhvaia, Marco Cosimo Simeone, Nana Silakadze & Otar Abdaladze

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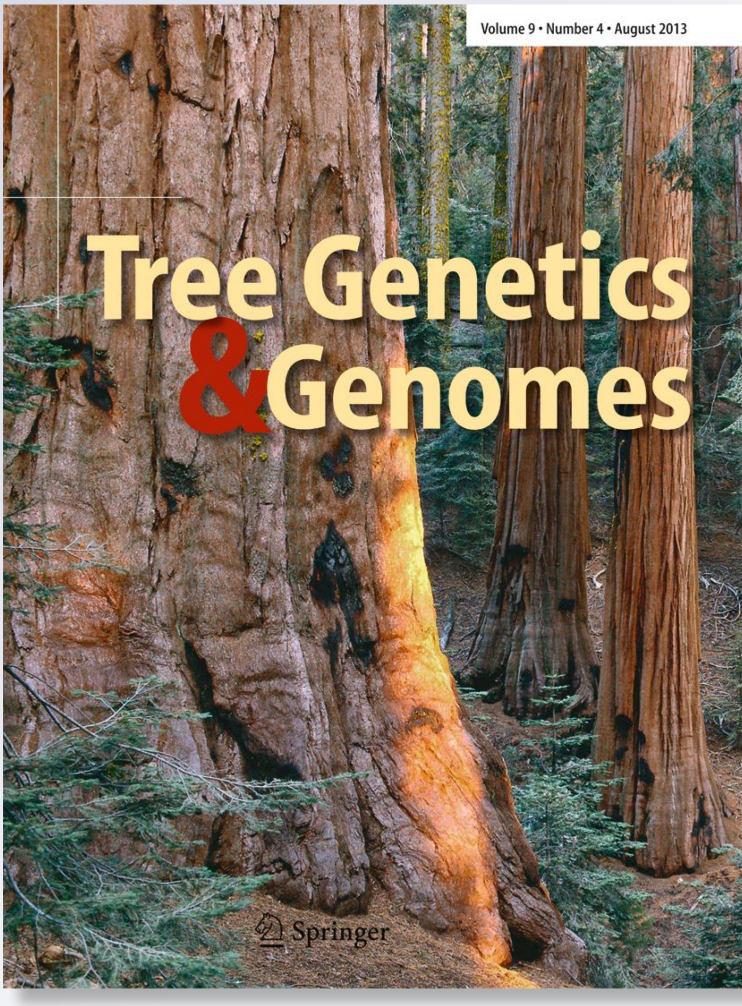

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ORIGINAL ARTICLE

Morphological diversity and phylogeography of the Georgian durmast oak (*Q. petraea* subsp. *iberica*) and related Caucasian oak species in Georgia (South Caucasus)

Jana Ekhvaia[1] · **Marco Cosimo Simeone**[2] · **Nana Silakadze**[1] · **Otar Abdaladze**[1]

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Abstract

The Caucasus region is one of the 25 global biodiversity hotspots and constitutes a shelter area for Neogene relict species as well as a center of ongoing radiation. In order to elucidate the taxonomic identity, divergence patterns, and evolutionary history of the largely widespread Georgian durmast oak (*Quercus petraea* subsp. *iberica*), we examined leaf morphology and chloroplast DNA (cpDNA) (*trn*H-*psb*A, *trn*K-*mat*K) sequence variation across its South Caucasian range. Six other oak taxa distributed throughout Georgia were included in the dataset and used for comparison. Evidence for differentiation in both sets of traits was found. Populations represented by different taxa from ecologically equivalent areas showed common morphological features and genetic structures. Molecular analysis clearly indicated the presence of two major haplotype lineages (West Caucasian vs. East Caucasian zonation type) and suggested a maternal lineage diversification of *Q. petraea* subsp. *iberica* in the Late Miocene, as a likely result of complex patterns associated with major orogenic and climatic changes. The Quaternary glacial oscillations resulted in a number of less common, derived haplotypes. Based on mismatch distribution analysis and neutrality tests, we found no evidence of demographic expansion for the populations from the West and East Caucasian zonation types. The two Caucasian provinces therefore acted as important shelter/diversification areas and as a lineage crossroad for the Georgian oaks. Close intra- and interspecific cpDNA relationships shared with other oaks from bordering countries support the relevant role played by the Colchis region as a primary refugium for the European temperate forest species.

Keywords *Quercus* · South Caucasus · Leaf morphology · cpDNA variation · Phylogeography · Population structure

Introduction

Georgia is located between latitude 41° 05′ N and longitude 44° 15′ E and occupies the central and western parts of the South Caucasus in the Southwestern Asian continent. Despite a small overall area (only 6970 km^2), Georgian forest vegetation is characterized by a huge diversity (Myers et al. 2000), mainly promoted by the land peculiar geographical position and diverse ecological features. The terrain of the country is mostly rugged and mountainous. Geologically, it belongs to the Alpine system of Eurasia (Milanovsky 1968) and is divided into the following landforms: the Greater Caucasus range in the north, the mountain system of the Lesser Caucasus in the south, and the Transcaucasian intermontane depression in the middle, divided by the Likhi range into the western and eastern parts (Fig.S1).

According to fossil records, forests were widespread in Georgia in the second half of Neogene and Pleistocene (Shatilova et al. 2011). Subtropical evergreen forests belonging to the Poltavian (e.g., *Sterculiaceae*, *Araliaceae*, *Lauraceae*, *Fagaceae*) and Turgai flora (e.g., *Salix*, *Pyrus*, *Carpinus*, *Juglans*, *Ulmus*) covered the region at the early

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✉ Jana Ekhvaia
ekhvaia@yahoo.com

[1] Alpine Ecosystems Research Program, Institute of Ecology, School of Life Sciences and Engineering, Ilia State University, 0162 Tbilisi, Georgia

[2] Department of Agriculture and Forestry Sciences (DAFNE), Università degli Studi della Tuscia, 01100 Viterbo, Italy

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stage of land formation in the middle Neogene. At the end of the Tertiary period, they were gradually replaced by temporary thermophilous forests mostly dominated by *Populus*, *Zelkova*, *Ulmus*, *Castanea*, *Carpinus*, *Quercus*, and *Fagus*, mainly widespread on the foothills.

Miocene is considered as a key period for the divergence of modern Angiosperms and a series of extinctions of ancient lineages (Tiffney and Manchester 2001). In the Caucasus region, huge orogenic movements in the Late Miocene (Messinian, 7.246–5.333 million years ago (Ma)) divided the territory of Georgia into two great western and eastern regions, separated by the Dzirula massif or Likhi range (Adamia et al. 2011). These two regions correspond to the Euxinian and Caucasian floristic provinces (Takhtajan 1978), respectively. The Late Miocene global cooling was the second main factor that shaped the current vegetation patterns in the Caucasus region, particularly for the Tertiary flora (Grossheim 1948; Gegechkori 2011; Nakhutsrishvili 2013; Tarkhnishvili 2014).

The climate changes of the Quaternary (i.e., over the past 2.6 million years) have also exerted a profound influence on the patterns of plant modern distribution and evolution at the global scale (Hewitt et al. 1996; Comes and Kadreit 1998). Typical responses of plants to such climate changes were adaptive evolution and migration, resulting in the alteration of geographical distributions (Etterson and Shaw 2001) and survival in a restricted number of small populations in glacial refugia. In Europe, present vegetation is thought to be the result of expansion from primary refugia, mostly located in the three main Mediterranean peninsulas (Iberian, Italian, and Balkan; Petit et al. 2002) and around the Black and Caspian seas (Hewitt 1999; Leroy and Arpe 2007). However, the role of key paleobiogeographic areas for temperate tree species, especially in the southeastern parts of the European continent, is still far from being completely understood (Médail and Diadema 2009; Ohlemüller et al. 2012). Recently, species distribution modeling (SDM) together with multilocus phylogeography and statistical analysis has improved our knowledge of the evolutionary patterns of dominant forest tree species worldwide (Gugger et al. 2013; Gavin et al. 2014; Ortego et al. 2015). Based on SDM, six major Quaternary refugia throughout Southeastern Europe and Western Asia were inferred by Tarkhnishvili et al. (2012): western Anatolia, western Taurus, the upper reaches of the Tigris River, Levant, Colchis, and Hyrcan. Among these, Colchis (at the eastern coast of the Black Sea) was suggested of major role in the preservation of relict mesophytic trees and shrubs, and as a lineage crossroad between Asia and Europe. Clearly, more phylogeographic evidence is needed to elucidate the evolutionary relevance of this region and provide a better understanding of its biodiversity.

Today, forests cover 36.7% of the total area of Georgia. The major climax formations are the broad-leaved deciduous forests dominated by *Fagus*, *Quercus*, *Carpinus*, *Castanea*, *Alnus*, and *Betula*, including many Caucasian endemic and still poorly known entities (e.g., *Betula medwedewii*, *Zelkova carpinifolia*, *Pterocarya pterocarpa*, among others). Oak forests are mostly represented by seven closely related taxa belonging to the infrageneric group *Quercus* (Denk and Grimm 2010), namely *Q. hartwissiana* Steven, *Q. macranthera* Fisch. & C.A.Mey. ex Hohen., *Q. petraea* subsp. *dshorochensis* (K.Koch) Menitsky, *Q. petraea* subsp. *iberica* (Steven ex M.Bieb.) Krassiln., *Q. robur* subsp. *imeretina* (Steven ex Woronow) Menitsky, *Q. robur* subsp. *pedunculiflora* (K.Koch) Menitsky, and *Q. pontica* K.Koch. As for numerous other *Quercus* taxa, the taxonomy of these oaks is rather controversial (Camus 1936–1954; Schwarz 1936–1939; Menitsky 2005). Their reliable and effective recognition is hampered by the relatively small sets of descriptors available, possibly exacerbated by a limited distribution and by the extended morphological overlaps due to ecological adaptation and incomplete reproductive isolation that characterize oaks in general (Burger 1975; Van Valen 1976; Petit et al. 2002).

The Georgian durmast oak (*Q. petraea* subsp. *iberica*) has the widest distribution and occurs from the western to the eastern part of the country (Fig.S1). It mostly grows in the lower mountain zone (500–1100 m above the sea level (a.s.l.)), on steep mountain slopes of dry southern exposures. Participation to the beech belt (1500 m a.s.l. and above) is not rare. For instance, in the inner river valleys of the Inguri basin (Svanetia, western Georgia), shielded from the humid sea winds by high mountain ranges, it can ascend to 1700–1800 m. a.s.l. (Menitsky 2005). Similarly, dry inner slopes of the Ajaristskali River (Ajara, western Georgia) and Turkish part of the Chorokh River to 800–1000 m a.s.l. are dominated by oak forests of *Q. petraea* subsp. *dshorochensis* (a possible synonym of subsp. *iberica*; Menitsky 2005) in an admixture with the less frequent *Q. hartwissiana*, an Eastern European oak finding its range limits on the Caucasus. Many regions on the drained river terraces of the Colchis lowlands are conservation areas of oak and oak-*Zelkova* forests with dominance of the Georgian endemic *Q. robur* subsp. *imeretina*. The upper mountain and subalpine belts of Colchis and Lazistan (Turkey), from deep gorges and slopes to high crests (1200–2300 m a.s.l.), are occupied by dense thickets of *Q. pontica*, another endemic, Tertiary relict tree species. This is the sole western representative of an ancient oak series (subsection Ponticae; Menitsky 2005), showing primeval leaf traits and striking genetic links with another narrow endemic oak in North America (*Quercus sadleriana* R.Br.ter; Denk and Grimm 2010). In the more humid regions of the Greater and Lesser Caucasus, forests of *Q. macranthera* spread and broaden gradually, above 1700 m a.s.l. Initially present only in mixed forests, dominated by coniferous species, this oak forms pure forests in the more xerophytic habitats of the upper mountains. Finally, the floodplain forests of

Q. robur subsp. *pedunculiflora*, an element of the Turgai flora, occur along the valleys of the Alasani and Iori river basins (Kakheti, eastern Georgia) and do not ascend onto the mountains.

Besides their socioeconomic importance, the ecological significance of these oaks is obvious for preserving Georgian territory and biodiversity. Clarifying their taxonomic issues and assessing the distribution of biological lineages would be fundamental steps to address appropriate conservation practices and effective management, for appropriate divulgation of biological research and to compare different studies. At the same time, a dissection of their evolutionary and diversity patterns could deepen our knowledge on the biodiversity richness of the whole Caucasus region and its role as both a shelter area and a diversification center for relict taxa and gene pools (Petit et al. 2002; Tarkhnishvili et al. 2008, 2012; Pokryszko 2011; Neiber and Hausdorf 2015; Maharramova 2015).

In angiosperms, chloroplast DNA (cpDNA) is appropriate to study seed dispersal and colonization routes, because the inheritance is predominantly maternal and it lacks recombination. Oaks were among the first plant taxa for which cpDNA variation was used to examine geographic variation within and among populations through Europe (Ferris et al. 1998; Petit et al. 2002), Asia (Liu et al. 2013), and America (Romero-Severson et al. 2003). More recently, Simeone et al. (2013, 2016) showed that some marker sequences of the oak cpDNA (*trn*H-*psb*A, *trn*K-*mat*K) can bear the imprints of very ancient and highly structured phylogeographic signals. However, no or extremely limited information is today available on the genetic diversity and structure of the Caucasian oaks (*Q. macranthera*: two populations investigated with PCR-RFLPs; Petit et al. 2002; *Q. macranthera* and *Q. petraea* subsp. *iberica*: phylogenetic analysis of the nuclear ribosomal DNA; Papini et al. 2011). In this work, we sought to fill this gap and performed detailed morphological and cpDNA sequence investigations on seven Caucasian oak taxa throughout their Georgian range. Our objectives were as follows: (1) to describe their phenotypic and genetic variation in relation to the reported taxonomy, (2) to investigate the diversity and evolutionary history of the most widespread oak in Georgia (*Q. petraea* subsp. *iberica*), and (3) to derive valuable insights on the role of key areas and events in shaping such diversity in the Caucasus region.

Materials and methods

Sample collection and morphological and molecular analyses

Our dataset included 37 total populations of seven oak taxa from two main zonation types (West and East Caucasian; Zazanashvili 2010). For the morphological analysis, five to seven individual trees were randomly sampled in 24 populations (Table 1); *Q. hartwissiana* could not be investigated due to the low number of collected samples (four individuals). Twenty fully expanded, healthy leaves were harvested from every oak tree. The morphological assessment was carried out according to Bruschi et al. (2003). In total, 24 macromorphological leaf characters (Table S2) were scored. Individual tree scores for each character were the mean value of 20 measurements, whereas population scores were the mean value of the individual tree scores from each population.

Total genomic DNA was extracted from silica gel-preserved leaf tissues of 93 individual trees with the DNeasy Plant Mini Kit (Qiagen), following the manufacturer's instructions (Table 1). Two plastid intergenic spacers (*trn*K-*mat*K, *trn*H-*psb*A) were amplified according to Simeone et al. (2013). Purified PCR products (with Illustra DNA Purification Kit, GE Healthcare) were sequenced at Macrogen (http://www.macrogen.com). Electropherograms were edited with Chromas 2.3 (http://www.technelysium.com.au) and checked visually. Multiple sequence alignments were obtained with MEGA 6.0 (Tamura et al. 2013). The *trn*K-*mat*K and *trn*H-*psb*A sequences of other European and Asian *Quercus* ($N = 17$), *Castanea* ($N = 2$), and *Lithocarpus* ($N = 2$) species were downloaded from NCBI to assess the genetic relationships shared with our dataset (Table S3).

Data analyses

Assumptions of normality and homogeneity were checked for all macromorphological characters with the Shapiro-Wilk test. Leaf characteristics like the number of lobes (NLR, NLL) and intercalary veins (NVR, NVL) on both sides showed a high right asymmetry. Normality for these traits was obtained after square root transformation (Sokal and Rohlf 1995). Analysis of variance (ANOVA) together with Tukey's HSD test ($P < 0.001$) was used to analyze leaf macromorphological differences in the investigated oak taxa. Initially, a principal component analysis (PCA) was used to remove highly correlated variables and replace the entire data file with a smaller number of uncorrelated variables (all extraction coefficients greater than 0.6). PCA was then carried out with the selected variables, and these were reduced to three principal components representing most of the information in the original dataset. A principal component (PC) solution was determined based on scree plot and Kaiser criterion (all eigenvalues greater than 1). All statistical analyses were performed using SPSS ver. 21.0 (https://www-01.ibm.com/).

DnaSP ver. 5.10 (Librado and Rozas 2009) was used to calculate the main diversity parameters of the two single and joined cpDNA regions across the whole dataset: nucleotide diversity (π), scaled mutation rate (θ), and haplotype diversity (H_d). Haplotypes were generated with the joined markers, and

Table 1 Sample list, origin, and haplotype distribution of the investigated oak dataset

Population ID	Species	Location	Latitude	Longitude	Floristic province/ zonation type	N^a	N^b	Haplotypes
DR01	*Q. petraea* subsp. *dshorochensis*	Batumi Botanical Garden, Ajara	41.69959	41.71970	Euxinus/West Caucasian	7	3*	H2 (1), H5 (1), H10 (1)*
DR02	"	Kintrishi Protected Area, Ajara	41.60945	41.75443	"	7	2	H5 (2)
HR03	*Q. hartwissiana*	Keda, Ajara	41.53770	42.03170	"	–	2	H8 (2)
HR04	"	Etseri, Samegrelo	42.06329	42.66443	"	–	2	H5 (2)
IB05	*Q. petraea* subsp. *iberica*	Tsageri, KvemoSvaneti	42.64047	42.76519	"	5	4	H2 (4)
IB06	"	Chrebalo, Racha-Lechkhumi	42.80450	42.51848	"	5	4	H2 (4)
IB07	"	Gali, Afkhazeti	42.68106	41.86958	"	5	4	H2 (4)
IB08	"	Kharagauli, Imereti	42.11213	43.30671	"	7	3	H5 (3)
IB09	"	Borjomi, Meskheti	41.91942	43.49325	"	7	3	H5 (3)
IB10	"	Akhaltsikhe, Meskheti	41.71949	42.98650	"	7	3	H2 (1), H5 (2)
IB11	"	Ajamethi Protected Area, Imereti	42.31241	42.63702	"	–	3	H5 (3)
IB19	"	Saguramo, Mtskheta-Mtianeti	41.86418	44.73266	Caucasus/East Caucasian	7	2	H5 (2)
IB20	"	Ananuri, Mtskheta-Mtianeti	42.15862	44.70415	"	7	3	H1 (2), H3 (1)
IB21	"	Manglisi, Trialeti	41.66995	44.33535	"	5	2	H1 (1), H3 (1)
IB22	"	Ateni, Trialeti	41.88861	43.99544	"	7	4	H1 (4)
IB23	"	Tskhvarichamia, Mtskheta-Mtianeti	41.88742	44.92234	"	–	2	H1 (2)
IB24	"	Kazbegi, Mtskheta-Mtianeti	42.43135	44.50056	"	–	2	H1 (1), H4 (1)
IB25	"	Mamkoda, Mtskheta-Mtianeti	41.85409	44.90428	"	–	2	H1 (1), H4 (1)
IB26	"	Kojori, Kartli	41.66297	44.70462	"	–	2	H1 (2)
IB27	"	Tskneti, Kartli	41.68416	44.69915	"	–	2	H3 (2)
IB28	"	Bodbe, Kakheti	41.59313	45.91830	"	7	3	H1 (1), H4 (2)
IB29	"	Sighnaghi, Kakheti	41.61348	45.87970	"	4	3	H4 (3)
IB30	"	Gombori, Kakheti	41.89313	45.37913	"	7	3	H4 (3)
IB31	"	Artsivi Gorge, Kakheti	41.49966	46.09797	"	0	3	H1 (3)
IB32	"	Dedoplistskaro, Kakheti	41.48669	46.13644	"	0	2	H1 (2)
IM12	*Q. robur* subsp. *imeretina*	Ajamethi Protected Area, Imereti	42.31241	42.63702	Euxinus/West Caucasian	7	2	H1 (1), H5 (1)
IM13	"	Etseri, Samegrelo	42.06328	42.66443	"	7	2	H2 (1), H5 (1)
PN14	*Q. pontica*	Keda, Ajara	41.53770	42.03170	"	7	4	H6 (2), H7 (2)
PN15	"	Bakhmaro, Guria	41.85167	42.33472	"	–	2	H5 (2)
MC16	*Q. macranthera*	Ateni, Trialeti	41.91048	44.00685	Caucasus/East Caucasian	7	2	H5 (2)
MC17	"	Manglisi, Trialeti	41.66995	44.33535	"	7	2	H1 (2)
MC18	"	Tetritskaro, Trialeti	41.51447	44.34425	"	7	2	H5 (2)
PD33	*Q. robur* subsp. *pedunculiflora*	Kvareli, Kakheti	41.94885	45.83988	"	7	2	H1 (2)
PD34	"	Korughi, Kakheti	41.65047	45.45032	"	7	2	H4 (2)
PD35	"	Sighnaghi, Kakheti	41.61348	45.87970	"	7	2	H2 (1), H4 (1)
PD36	"	Gardabani, KvemoKartli	41.37491	45.07426	"	7	2	H4 (2)
PD37	"	Vashlovani, Kiziki	41.27803	46.66564	"	–	2	H4 (2)

Brackets indicate the number of individuals

[a] The number of individuals investigated in the morphological analysis

[b] The number of individuals investigated in the molecular analysis

*Sample retrieved from GenBank

those with frequencies < 5% were defined as rare (Aoki et al. 2004). To visualize the relationships among cpDNA haplotypes, an unrooted haplotype network was constructed by coalescent simulations using the median-joining model implemented in NETWORK ver. 4.6 (Bandelt et al. 1999).

PERMUT ver. 1.2.1 (available at http://www.pierroton.inra.fr/genetics/labo/Software) was used to analyze population cpDNA diversity based on unordered and ordered alleles, as described in Pons and Petit (1995, 1996). Estimates of the total gene diversity among populations (H_T), the mean gene diversity within populations (H_S), and the level of population subdivision of diversity (G_{ST}) were calculated based on haplotype frequencies, as well as population differentiation based on haplotype frequencies and genetic divergence (N_{ST}) and V_T and V_S (analogues of H_T and H_S). The existence of a phylogeographic structure was tested by checking whether N_{ST} was greater than G_{ST}, indicating that closely related haplotypes occur more frequently in the same populations than less closely related haplotypes (Pons and Petit 1996); a 1000-random permutation test was performed using the same program (Burban et al. 1999).

To examine the effect of geographic distance and the relative contribution of migration and drift on the genetic structure (Hutchinson and Templeton 1999), the isolation-by-distance (IBD) analysis was performed with the Mantel test using the software GENALEX ver. 6.5 (Peakall and Smouse 2006). Analysis of molecular variance (AMOVA) was performed using ARLEQUIN ver. 3.5 (Excoffier and Lischer 2010) to calculate variance components and their significance levels for variation among and within populations for the whole dataset and separately for each floristic province. Pairwise genetic distances within and among floristic provinces were also calculated with the AMOVA. The significance of the covariance components was tested using a 1000-permutation test, and only P values lower than 0.05 were considered significant. Signals of demographic dynamics in the Georgian durmast oak were investigated with the mismatch distribution analysis from ARLEQUIN ver. 3.5. The goodness of fit under a sudden and spatial expansion model was tested with the sum of the squared deviations (SSD) and Harpending's raggedness index (H_{Rag}) (Harpending 1994) using 1000 parametric bootstrap replicates. Tajima's D (Tajima 1989) and Fu's Fs (Fu 1997) tests were also performed in DnaSP to discriminate mutation/drift equilibrium and to evaluate the hypothesis of population expansion through the significant excess of low-frequency haplotypes.

A Bayesian phylogenetic analysis was performed using the software BEAST ver. 1.8.2 (Drummond and Rambaut 2007) to estimate the timings of node divergence. The GTR substitution model with five-category discrete gamma distribution was selected by MEGA 6.0 using the Akaike information criterion (AIC; Kelchner and Thomas 2007). An uncorrelated lognormal relaxed clock and a Bayesian Skyline coalescent tree prior were applied for analysis. Posterior distributions of parameters were approximated using the Markov chain Monte Carlo (MCMC) method. As recommended by Sauquet et al. (2012), more accurate age estimates can be obtained by combining both ingroup and outgroup calibrations. Therefore, five (three outgroup and two ingroup) fossil dates were used to assign minimum age constrains on five internal stem nodes: *Castanea* (fossilized pistillate flowers from *Castanopsoidea* sp., 47.8–59.2 Ma; Crepet and Nixon 1989), *Lithocarpus* (fossilized leaves with cuticle from *L. saxonicus* H. Walther and Kvacek, 33.9–23.0 Ma; Sauquet et al. 2012; Kvacek and Teodoridis 2007), *Quercus* group Cerris (fossil fruits of *Q. cerricaecarpa*, 17.5–15 Ma; Song et al. 2000), and group *Quercus* (macrofossils from *Q. pontica miocenica* Kubat, 11.62–3.6 Ma; Palamerov and Tsenov 2004; macrofossils from *Q. iberica* Steven ex M.Bieb., 7.1–5.3 Ma; Shatilova et al. 2011). Assuming a normal distribution, priors for the calibration points were set on five internal stem nodes. MCMC was run for 1×10^8 generations, with sampling every 2000th generation. BEAST log files were inspected in Tracer ver. 1.6 (http://tree.bio.ed.ac.uk/software/tracer) to confirm sampling adequacy and convergence of the chains to a stationary distribution. The phylogenetic results during the burn-in period were removed, and the combined tree files were used to generate a lineage maximum credibility tree with median heights in TreeAnnotator ver. 1.8.2. Resulting chronograms were visualized in FigTree ver. 1.4.2 (http://tree.bio.ed.ac.uk/software/figtree).

Results

Morphological analysis

Eighteen statistically significant variables (extraction coefficients greater than 0.6; Table S2) out of the original 24 macromorphological leaf characters were identified by PCA. The ANOVA F-statistics showed significant differences among the six studied taxa (Table 2). Tukey's post hoc range tests indicated that leaves of *Q. pontica* were clearly differentiated from the other species, as they were significantly larger, had more lobes, and lacked intercalary veins. In addition, the two *Q. robur* subspecies could be differentiated from the remaining two *Q. petraea* subspecies and from *Q. macranthera* by their wider lobes, shorter length of the leaf petiole, distance of the principal vein to the sinus, smaller number of lobes, and larger number of intercalary veins. The investigated characters allowed no further discrimination between the two *Q. robur* subspecies, and neither between *Q. macranthera* and the two *Q. petraea* subspecies.

The PCA gave congruent results and revealed that the first three principal components account for 84.52% of the total variation in the dataset (40.32, 22.61, and 21.59%,

Table 2 Means, standard deviation, and F values for 18 leaf characters in six Caucasian oak taxa

Character	MR[a]	IB[b]	DR[c]	IM[d]	PD[e]	PN[f]	F
LL	10.58 ± 1.24^{abcde}	10.02 ± 1.10^{abcde}	10.95 ± 1.08^{abcde}	10.18 ± 0.46^{abcde}	10.38 ± 0.97^{abcde}	17.77 ± 1.12	67.05***
LP	1.17 ± 0.23^{acde}	1.65 ± 0.34^{abe}	1.46 ± 0.17^{abcde}	0.34 ± 0.03	1.02 ± 0.24^{acde}	1.28 ± 0.22^{abcde}	60.95***
MWL	7.06 ± 0.90^{ade}	6.03 ± 0.82^{bcde}	5.53 ± 0.35^{bcde}	6.35 ± 0.65^{abcde}	6.59 ± 0.77^{abcde}	8.54 ± 0.76	18.51***
HMW	4.61 ± 0.59^{abcde}	4.53 ± 0.55^{abcde}	3.85 ± 0.42^{abcde}	4.30 ± 0.38^{abcde}	4.17 ± 0.47^{abcde}	8.75 ± 1.62	71.48***
MDS	1.90 ± 0.34^{ade}	1.34 ± 0.33^{bc}	1.00 ± 0.09^{bcf}	2.07 ± 0.31^{ade}	2.16 ± 0.39^{ade}	0.62 ± 0.21^{f}	52.71***
WHL	1.65 ± 0.22^{ab}	1.48 ± 0.22^{ab}	1.10 ± 0.09	1.96 ± 0.21^{cd}	2.01 ± 0.24^{cd}	0.81 ± 0.11	61.19***
DVL	3.64 ± 0.47^{ade}	3.12 ± 0.43^{bcde}	2.87 ± 0.20^{bcde}	3.29 ± 0.35^{abcde}	3.43 ± 0.41^{abcde}	4.31 ± 0.26^{a}	16.45***
DS	1.98 ± 0.42^{abc}	1.99 ± 0.32^{abc}	2.29 ± 0.19^{abc}	1.42 ± 0.34^{de}	1.45 ± 0.41^{de}	3.96 ± 0.28	66.44***
NLR	2.86 ± 0.12^{abc}	2.82 ± 0.21^{abc}	3.10 ± 0.19^{abc}	2.55 ± 0.20^{de}	2.52 ± 0.14^{de}	4.56 ± 0.24	144.19***
NLL	2.87 ± 0.11^{abc}	2.82 ± 0.21^{abc}	3.05 ± 0.17^{abc}	2.57 ± 0.19^{de}	2.53 ± 0.14^{de}	4.47 ± 0.30	125.81***
NVR	0.31 ± 0.06^{abcf}	0.27 ± 0.02^{abcf}	0.00 ± 0.00^{abcf}	1.37 ± 0.02^{de}	1.25 ± 0.05^{de}	0.00 ± 0.00^{abcf}	121.25***
NVL	0.36 ± 0.06^{abcf}	0.33 ± 0.02^{abcf}	0.24 ± 0.16^{abcf}	1.48 ± 0.06^{de}	1.26 ± 0.06^{de}	0.00 ± 0.00^{abcf}	107.17***
TLL	11.78 ± 1.34^{abcde}	11.67 ± 1.24^{abcde}	11.64 ± 0.4^{abcde}	11.34 ± 1.09^{abcde}	11.43 ± 0.96^{abcde}	19.06 ± 1.24	54.13***
LLW	5.96 ± 0.78^{abcde}	5.47 ± 0.74^{abc}	6.34 ± 0.37^{abcde}	6.66 ± 0.8^{acde}	6.20 ± 0.64^{acde}	9.08 ± 0.71	36.97***
P%	9.92 ± 1.66^{acef}	14.13 ± 2.53^{bc}	12.57 ± 1.58^{abce}	2.65 ± 1.52^{df}	9.02 ± 2.24^{acef}	6.69 ± 1.05^{adef}	82.77***
DW%	60.10 ± 4.03^{ade}	51.78 ± 5.00^{bcdf}	47.58 ± 1.87^{bcdf}	55.87 ± 4.83^{abcde}	57.74 ± 4.70^{ade}	44.92 ± 4.77^{bcf}	22.38***
MWL/MDS	4.30 ± 0.74^{abcde}	4.72 ± 0.78^{abc}	5.66 ± 0.73^{abc}	3.15 ± 0.35^{ade}	3.57 ± 0.80^{ade}	14.81 ± 4.25	127.18***
LL/MWL	1.52 ± 0.11^{abcde}	1.62 ± 0.17^{abcde}	1.84 ± 0.06^{abcdef}	1.63 ± 0.56^{abcde}	1.59 ± 0.14^{abcde}	2.09 ± 0.23^{f}	10.62***

Letters in the same row indicate no significant differences at $P < 0.001$ according to Tukey's test; MR[a] = *Q. macranthera*; IB[b] = *Q. petraea* subsp. *iberica*; DR[c] = *Q. petraea* subsp. *dshorochensis*; IM[d] = *Q. robur* subsp. *imeretina*; PD[e] = *Q. robur* subsp. *pedunculiflora*; PN[f] = *Q. pontica*

***$P < 0.001$, significant

respectively; Table S2). The first PCA is mainly influenced by variables expressing leaf size and lobe number and clearly differentiates *Q. pontica* (Fig. 1). The second PCA is influenced by the number of intercalary veins and the dimensions related to the petiole leaf and identifies the two main groups constituted by most individuals of *Q. robur* s.l. and all individuals of *Q. macranthera* and *Q. petraea* s.l. The third PCA is related to maximal depth of sinus, width of the most handing lobe, and ratio between the length and width of lamina (Table S2) but provided no further subdivision.

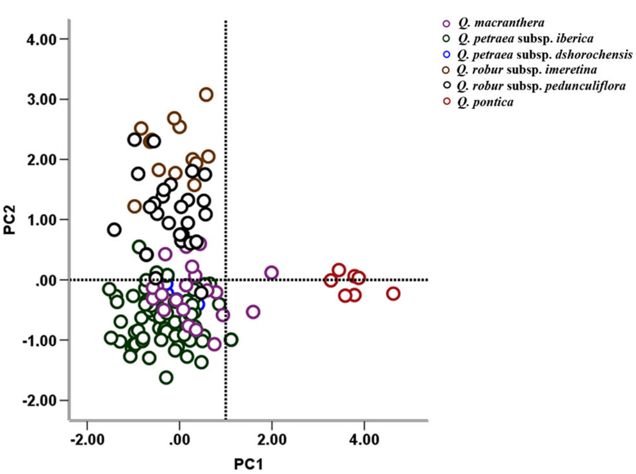

Fig. 1 Plot of the first two axes of the PCA from 18 macromorphological leaf data in six Caucasian oak taxa

cpDNA variation in Georgian oaks

The final dataset included *trn*K-*mat*K and *trn*H-*psb*A DNA sequences for 94 Georgian oak individuals (59 samples of *Q. petraea* subsp. *iberica*, ten samples of *Q. robur* subsp. *pedunculiflora*, six samples each of *Q. macranthera* and *Q. pontica*, four samples of *Q. petraea* subsp. *dshorochensis* plus one downloaded from GenBank, and four each of *Q. hartwissiana* and *Q. robur* subsp. *imeretina*; GenBank accession numbers listed in the "Data archiving statement" section). The multiple alignment of the two concatenated markers resulted in a matrix with 1223 characters (Table 3, Table S4). Site variation (π, θ) was moderate; with gaps considered, a total of nine haplotypes was generated in the Georgian dataset, with relatively high haplotype diversity (H_d). With the GenBank-retrieved sequences, the total haplotypes scaled up to 13, and five parsimony-informative characters (PICs) were scored. Haplotypes H1, H2, H4, and H5 were identified as common; all others were rare (Table S4). The Georgian samples of *Q. petraea* subsp. *iberica* displayed five haplotypes (H1–H5); two additional variants (H11, H12) were scored by the samples from Armenia and Iran. All other taxa showed two to three haplotypes. With the GenBank sequences included, seven haplotypes (H1–H5, H9, H10) were shared among different species or taxa and six were scored by single accessions (H6–H8, H11–H13).

Table 3 Summary of nucleotide variation of the two cpDNA marker regions in the investigated dataset

Marker region	Length (bp)	*S*	PICs	π	θ	H_d
Georgian oak individuals ($N = 94$)						
*trn*K-*mat*K	693	2	1	0.001 ± 0.000	0.001 ± 0.001	0.506 ± 0.018
*trn*H-*psb*A	530	3	3	0.002 ± 0.000	0.001 ± 0.001	0.599 ± 0.025
Total	1223	5	4	0.001 ± 0.000	0.001 ± 0.000	0.805 ± 0.000
Georgian oak individuals combined with GenBank-retrieved sequences ($N = 108$)						
*trn*K-*mat*K	693	3	2	0.001 ± 0.000	0.001 ± 0.001	0.545 ± 0.021
*trn*H-*psb*A	530	3	3	0.003 ± 0.000	0.001 ± 0.001	0.594 ± 0.024
Total	1223	6	5	0.001 ± 0.000	0.001 ± 0.000	0.818 ± 0.014

S = variable sites; π = nucleotide diversity; θ = scaled mutation rate; H_d = haplotype diversity; *N* = number of individuals

PICs parsimony-informative sites

Haplotype distribution and phylogenetic analysis

The geographical distribution of the eight haplotypes found in seven Caucasian oak taxa occurring in Georgia is reported in Fig. 2a (haplotype H10 was detected in a GenBank accession

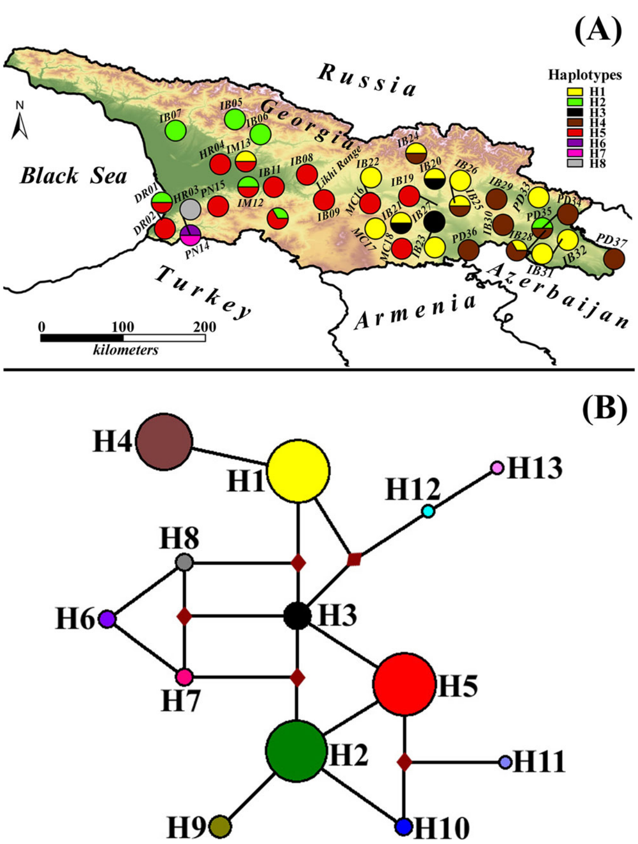

Fig. 2 **a** Distribution of 37 populations and 8 haplotypes of seven Caucasian oak taxa occurring in Georgia (abbreviations and regional affiliations are listed in Table 1). **b** Phylogenetic network of 13 cpDNA haplotypes of seven Caucasian oak taxa combined with East European-West Asian oak samples. Circle size is proportional to the frequency of a haplotypes across all populations. Solid dark red diamonds indicate unsampled or extinct haplotypes. Each line between two haplotypes represents one mutational step

for which no spatial annotations were provided), and details for each population are summarized in Table 1. Haplotypes of the Georgian populations of *Q. petraea* subsp. *iberica* are divided into two distinct geographic groups, corresponding to the Euxinian (West Caucasian zonation type) and Caucasian (East Caucasian zonation type) provinces. In particular, haplotypes H1, H3, and H4 were mainly found in samples from central-eastern and eastern Georgia (Caucasian province), while haplotypes H2 and H5 were predominant in southwestern Georgia (Euxinian province). These five haplotypes were also found in other oak taxa occurring in ecologically equivalent areas (e.g., *Q. petraea* subsp. *iberica* and *Q. robur* subsp. *pedunculiflora* or *Q. macranthera* in the Caucasian province; *Q. hartwissiana*, *Q. petraea* subsp. *dshorochensis*, *Q. petraea* subsp. *iberica*, and *Q. robur* subsp. *imeretina* in the Euxinian region; Fig. 2a, Table 1). Haplotypes H1–H5 were also identified in Eastern European-Western Asian samples of *Q. petraea* (Italy), *Q. petraea* subsp. *iberica* (Turkey), *Q. robur* subsp. *pedunculiflora* and *Q. hartwissiana* (Ukraine, Bulgaria), and *Quercus aliena* (Korea) (Table S3). The remaining haplotypes had a limited distribution and were restricted to *Q. pontica* (H6, H7), *Q. hartwissiana* (H8), and *Q. petraea* subsp. *dshorochensis* (H10). Haplotype H10 was also scored in a GenBank sample of *Quercus polycarpa* (*Q. petraea* subsp. *iberica*; Menitsky 2005) from Romania. Finally, haplotype H9 was found exclusively in the GenBank sequences of *Q. petraea* and *Q. robur* from Greece and Italy, whereas H11, H12, and H13 were exhibited by samples of *Q. petraea* subsp. *iberica* and *Q. macranthera* from other Caucasian bordering regions (Armenia, Iran, and Turkey).

The phylogenetic network of the 13 total haplotypes is shown in Fig. 2b. The main backbone includes the most common haplotypes arranged on a west (H2, H5) to east (H3, H1, H4) gradient, with only one mutational event separating the eastern (sub-) lineage from haplotype H3. This latter, located at the core of the network, could be an ancestral haplotype (Posada and Crandall 2001). Haplotypes H9–H13 are directly linked to the western and eastern sublineages. Haplotypes H6–H8 make a "star-like" relational cluster restricted to *Q. pontica* and *Q. hartwissiana*, separated from the main backbone by few unsampled or extinct haplotypes (Swofford 2003).

Genetic differentiation of *Q. petraea* subsp. *iberica* populations

Genetic differentiation estimates of the durmast oak populations are shown in Table 4. Diversity analysis based on haplotype frequencies and DNA divergence showed that the gene diversity among *Q. petraea* subsp. *iberica* populations (H_T = 0.87, V_T = 0.88) was higher than that within populations (H_S = 0.54; V_S = 0.44). N_{ST} was significantly higher than G_{ST} (0.51 and 0.38, respectively; $P < 0.05$), suggesting that the relative distribution of phylogenetically related haplotypes contributes to the overall geographical structure of the species (Pons and Petit 1996). However, the phylogeographic pattern of *Q. petraea* subsp. *iberica* failed to match the isolation-by-distance model as the Mantel test found no significant correlation between genetic and geographic distances ($r^2 = 0.054$, $P \geq 0.546$).

The general AMOVA revealed that most of the gene diversity could be attributed to differences among populations (66.82% of variance, $F_{ST} = 0.67$, $P \geq 0.000$), consistent with the PERMUT analysis (Table 5). Additionally, the highest pairwise F_{CT} values (65.94% of variance, $F_{CT} = 0.66$, $P \geq 0.000$) were scored between the two floristic provinces, suggesting a clear population differentiation between Euxinian and Caucasian populations. The AMOVA performed separately for the samples in the two provinces showed most of the variation residing among populations (87.17% of variance, $F_{ST} = 0.87$, $P \geq 0.000$) in the Western Caucasus and within populations (21.92% of variance, $F_{ST} = 0.22$, $P \geq 0.01$) in the Eastern Caucasus.

The mismatch distribution analysis conducted separately for all populations in the Euxinian and Caucasian provinces was both unimodal (data not shown). However, the neutrality tests of Tajima (*D*) and Fu (*Fs*) were non-significant in both the West and the East Caucasian zonation type (Table 6). In agreement, the non-significant SSD between observed and expected mismatch, and the raggedness index (H_{Rag}) in both provinces indicated no evidence of demographic expansion in the Georgian durmast oak populations.

Molecular divergence time estimates

The BEAST-derived cpDNA chronogram (Fig. 3, goodness-of-fit tests, and additional information provided as File S5A and File S5B) are consistent with the phylogenetic network reconstructed under coalescent simulations and revealed a subdivision of the Georgian *Q. petraea* subsp. *iberica* haplotypes (H1–H5) into two main lineages corresponding to the Euxinian province/West Caucasian zonation type and Caucasian province/East Caucasian zonation type, respectively. Haplotypes H10 (*Q. dshorochensis*) and H9 and H11–H13 (belonging to *Q. petraea* s.l., *Q. robur* s.l., *Q. petraea* subsp. *iberica*, and *Q. macranthera* samples collected outside Georgia and/or the Caucasus) derived more recently. Conversely, haplotypes H6 and H7 (*Q. pontica*) and H8 (*Q. hartwissiana*) were sensibly older, with time diversification of the former two at 8.36 Ma (95% HPD = 9.36–7.39 Ma). The divergence time of the most recent common ancestor (TMRCA) of the Georgian durmast oak was 5.44 Ma (95% HPD = 6.45–4.50 Ma). Based on this chronogram, the haplotypes of the East Caucasian zonation type began to diversify around 3.54 Ma (95% HPD = 5.80–0.72 Ma) and the haplotypes of the West Caucasian zonation type around 2.69 Ma (95%HPD = 5.27–0.42 Ma), respectively. The nucleotide substitution rate at the node of divergence between West and East Caucasian zonation types is equal to 1.0×10^{-9} s/s/y. This ratio is in agreement with the comparative low evolution ratio of some chloroplast intergenic spacers (including *trn*H-*psb*A) recently reported in the only other Eurasian member of group *Quercus* investigated so far (Xu et al. 2015).

Table 4 Genetic diversity and differentiation statistics for Georgian *Q. petraea* subsp. *iberica* populations combined with Caucasian (Armenia, Iran, and Turkey) samples ($N = 62$)

Statistical test	Estimated value	Standard error
Genetic diversity within populations (H_S)[a]	0.54	0.11
Total genetic diversity (H_T)[a]	0.87	0.04
Coefficient of genetic differentiation among populations (G_{ST})[a]	0.38	0.12
Genetic diversity within populations (V_S)[b]	0.44	0.11
Total genetic diversity (V_T)[a]	0.88	0.27
Coefficient of genetic differentiation among populations (N_{ST})[a]	0.51*	0.14

*$P \geq 0.05$

[a] Parameters of diversity and differentiation based on haplotype frequencies

[b] Parameters of diversity and differentiation based on haplotype frequencies and genetic divergence

Table 5 Summary analysis of molecular variance (AMOVA) for Georgian *Q. petraea* subsp. *iberica* populations combined with Caucasian (Armenia, Iran, and Turkey) samples ($N = 62$)

Source of variation	*df*	Sum of squares	Variance components	Percentage of variation	Fixation indices
Whole dataset					
Among populations	7	48.14	0.76***	66.82	$F_{ST} = 0.67$***
Within populations	55	21.57	0.38	33.18	
Total	62	69.70	1.14		
Among two floristic provinces (zonation types)					
Among the regions (provinces)	1	32.42	1.06***	65.94	$F_{CT} = 0.66$***
Among the populations within the regions (provinces)	20	22.77	0.33***	20.56	$F_{SC} = 0.60$***
Within the populations	41	8.67	0.22***	13.49	$F_{ST} = 0.86$***
Total	62	63.86	1.60		
Euxinian province/West Caucasian zonation type					
Among populations	3	5.13	0.28***	87.17	$F_{ST} = 0.87$***
Within populations	21	0.83	0.04	12.83	
Total	24	5.96	0.32		
Caucasian province/East Caucasian zonation type					
Among populations	4	6.74	0.16**	21.92	$F_{ST} = 0.22$**
Within populations	33	18.73	0.57	78.08	
Total	37	25.47	0.73		

***$P \geq 0.001$; **$P \geq 0.01$

Discussion

Leaf morphological variation and high rates of cpDNA sharing in Georgian oaks

Our univariate (ANOVA) and multivariate (PCA) leaf morphology analyses only allowed the recognition of *Q. pontica* as a well-defined species, sharply differentiated from all other oak taxa distributed in Georgia, and two main groups corresponding to *Q. robur* s.l. vs. *Q. macranthera*/*Q. petraea* s.l. complex. Accordingly, *Q. macranthera* and *Q. petraea* s.l. share a more pronounced leaf morphological similarity compared to *Q. robur* s.l. (Menitsky 2005). In addition, both *Q. macranthera* and *Q. petraea* subsp. *iberica* are highly drought- and cold-resistant mountain oaks and occur in the same geographical regions in the Caucasus, with the durmast oak occupying lower altitudes and being gradually replaced by the large-anthered oak at higher altitudes. Although the two species belong to different phylogenetic series (Menitsky 2005; Papini et al. 2011), sharing/convergence of many leaf characters is therefore plausible. As in the case of other sympatric and closely related oak species, this could be explained with the incomplete reproductive isolation that characterize oaks in general (Petit et al. 2004; Lepais et al. 2009) and overlapping morphological variation due to ecological adaptation (Burger 1975; Van Valen 1976). Conversely, *Q. petraea* s.l. and *Q. robur* s.l. generally grow in different ecological niches and areas in the Caucasus, with the second species occurring exclusively in lowland, mesophylous forests. The marked morphological differences recorded between the two species complexes might therefore reflect isolation and strong ecological adaptation to different environments (c.f. Kremer et al. 2002). No further separation at the subspecific level could be reliably done on the basis of the used morphological traits, pointing towards the dubious occurrence of "true" diagnostic characters other than specificity of occurrence in a geographical location (e.g., *Q. robur* subsp. *imeretina*) and adaptation to specific (micro-)habitats (e.g., the marked xerophyly of *Q. petraea* subsp. *dshorochensis*; cf. Menitsky 2005). Besides further morphological descriptors (e.g., flower organs, cupule scales, pubescence, trichome shape), the self-ecology of each taxon should be therefore

Table 6 Neutrality tests and mismatch distribution analysis for *Q. petraea* subsp. *iberica* populations combined with Caucasian (Armenia, Iran, and Turkey) samples ($N = 62$)

Region	Tajima's *D*	*P*	Fu's *Fs*	*P*	SSD	*P*	H_{Rag}	*P*
Euxinus/West Caucasian	0.535	0.751	0.557	0.587	0.032	0.440	0.241	0.061
Caucasus/East Caucasian	−0.302	0.929	−0.363	0.437	0.001	0.980	0.030	0.777

H_{Rag} = Harpending's raggedness index; P = no significant at $\alpha = 0.05$ level

SSD sum of the squared deviations (between observed and expected mismatch)

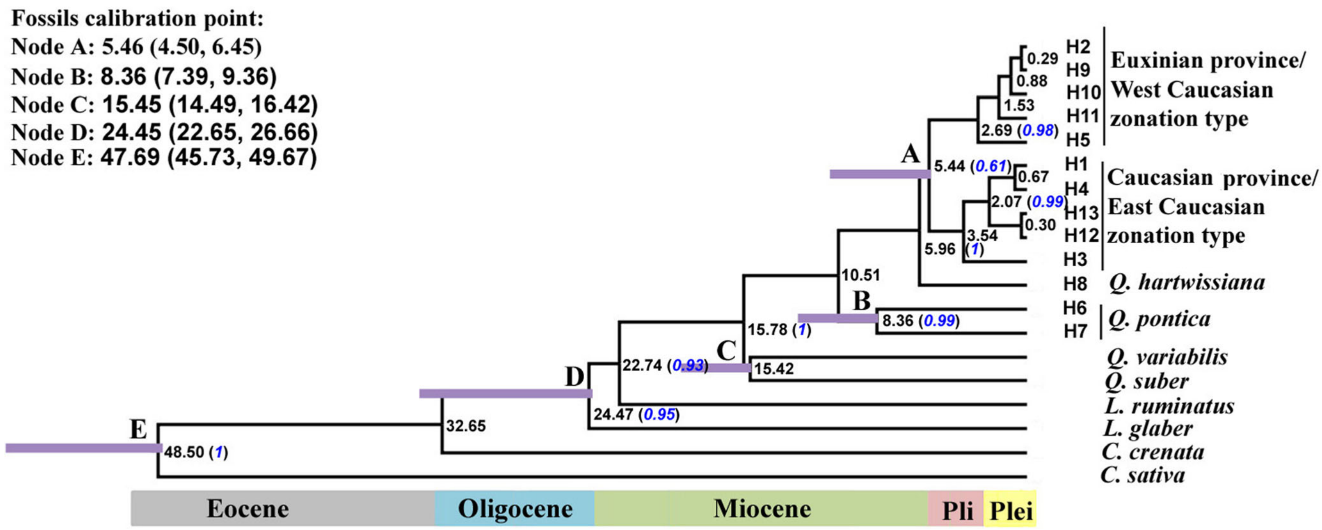

Fig. 3 BEAST chronogram of the 13 cpDNA oak haplotypes detected in this study with outgroup sequences from *Castanea*, *Lithocarpus*, and *Quercus*. Calibration points are marked with letters (A–E) and listed in the upper left with PP support > 95%. Mean divergence time (Ma, million years ago) estimates (black) and posterior probability (only PP > 0.6; blue) are shown below the nodes; bar represents the 95% HPD (Highest Posterior Density) of species divergence times; *Pli* Pliocene; *Ple* Pleistocene

extensively investigated in order to clearly assess the true taxonomic status of these oaks.

On the other hand, the large haplotype sharing between the Georgian populations of *Q. petraea* subsp. *iberica* and the other Caucasian oak taxa (with the exception of *Q. pontica*) indicates that haplotype variation is not correlated with the species identity. This is not an occasional phenomenon, but rather a common feature among *Quercus* species. Besides the limited variation of the used markers (discussed in Simeone et al. 2016), such species unspecificity of the cpDNA has been repeatedly interpreted as chloroplast capture, e.g., an active evolutionary mechanism proceeding over generations and leading to substantial exchanges of genetic variation between different taxonomical units (Petit et al. 2004; Dodd and Afzal-Rafii 2004; Excoffier et al. 2009). Among the white oaks, the *Q. robur*/*petraea* species pair has probably been the most studied interbreeding species system in Europe (e.g., Olalde et al. 2002; Curtu et al. 2007, 2009; Lepais et al. 2009; Lagache et al. 2013); however, no data are available for the Caucasian oaks, although Menitsky (2005) reports successful controlled crosses between *Q. robur* s.l. and *Q. petraea* s.l. in the Caucasus region. The common plastid signatures between interspecific population pairs from ecologically equivalent areas (e.g., population pairs HR04/IM13, IB11/IM12, IB21/MC17, IB28/PD35 in Fig. 2a) and the observed morphological overlapping of most taxa would make the hypothesis of adaptive introgression (mostly exerted by the nuclear genome) indeed plausible. On the other hand, the detected clear morphological definition of *Q. petraea* s.l. and *Q. robur* s.l. and the overall large extent of haplotype sharing make the possibility of reticulation and incomplete lineage sorting also possible (Simeone et al. 2016). This would be eventually supported by the finding of common genetic signatures in the Caucasian oaks and the West Asian *Q. aliena* (e.g., haplotype H3) and *Q. petraea*/*Q. robur* from Italy and the Balkans (haplotypes H2 and H5) (cf. Petit et al. 2002). Our data do not allow support of one hypothesis over the other, especially in consideration of the limited number of plastid loci investigated (c.f. Olalde et al. 2002). At the same time, a complex interplay between recent and ancient hybridization/introgression phenomena followed by strong ecological adaptation might explain the morphological and plastid scenario observed in our dataset (c.f. Vitelli et al. 2017). Clearly, extended comparative investigations at both the nuclear and plastid genomes are required to definitely assess species identity and relationships in this oak group. Conversely, both leaf morphology and cpDNA variation support the significance of *Q. pontica* as a phenotypically well-defined and genetically isolated oak species, as set forth by Denk and Grimm (2010) based on nuclear ribosomal data.

Genetic structure of *Q. petraea* subsp. *iberica* in Georgia

The detected parameters of DNA polymorphism appeared in line with previous studies (Simeone et al. 2016), and the geographic distribution of the haplotypes allowed the recognition of a clear genetic structure and some inferences on the evolutionary history of the Georgian durmast oak.

The missing correlation between genetic and physical distance matrices and the high cpDNA-based genetic differentiation among populations may be attributed to a limited cytoplasmatic gene flow via seed dispersal in *Q. petraea* subsp. *iberica*, which is constrained by a limited migratory capability and the complex topography of the Caucasus. In fact, natural populations of Georgian durmast oak occur in disjunct mountain areas including valleys and plateaus with high degrees of geographical isolation. Additionally, physical obstacles including the Likhi range and variable climatic conditions throughout the region (humid in the western and continental in the eastern part of the country) could have negatively affected seed dispersal between populations, resulting in a clear separation of the West and East Caucasian genetic lineages (Fig. 2a, b). This would be further supported by the detected significant value of N_{ST} over G_{ST} ($P < 0.05$; Table 4), pointing at a strong correlation between the phylogeny of haplotypes and their geographic locations. The presence of two main different eastern and western genetic lineages is consistent with the geographic structures detected in other woody (e.g., *Castanea sativa*, Mattioni et al. 2013; *Zelkova carpinifolia*, Kozlowski and Gratzfeld 2013; *Pterocarya fraxinifolia*, Mostajeran et al. 2016) and animal (e.g., the Miocene relict salamander *Mertensiella caucasica*; Tarkhnishvili et al. 2008) species in the Caucasus region, which supports the strong barrier to gene flow/migration of the Likhi range.

No significant expansion signal was detected for both the West and East Caucasian zonation types. However, the East Caucasian populations showed a substantial and significant reduction of the genetic variation (Table 5), compared with their western counterparts. This could be explained with strong population reduction and drift effects and, consistently, with the fossil evidence suggesting a significantly expanding distribution of oak-*Zelkova* forests in eastern Georgia during the Holocene (Shatilova et al. 2011), with founder events during postglacial re-colonization (Hewitt 1999, 2000). Likely, the Last Glacial Maximum (LGM) had much more impact on the forests of this province because of a longer distance from the mitigating effect of the Black Sea.

In their pioneering work on cpDNA variation of West Eurasian oaks, Petit et al. (2002) identified three main regions of Europe (the Iberian, Italian, and Balkan peninsulas) as primary refugia during the LGM and argued for the existence of additional shelter areas located further north (the Southern Carpathians) and east (the Black Sea coast). The presence of unique haplotypes in the Caucasian oak taxa and those shared with European oaks from bordering regions (e.g., Bulgaria, Turkey, Ukraine; Petit et al. 2002; this study) indeed supports this assumption. It is therefore possible that niches with a favorable microclimate have existed in marginal positions of mountainous areas, where small pockets of trees (Willis et al. 2000), shrubs (Daneck et al. 2011), herbaceous plants (Tyler 2002a; Stachurska-Swakoń 2012), and animals (Kotlík et al. 2006; Neiber and Hausdorf 2015) survived during the Quaternary climatic oscillations. Among the climate refugia recently postulated in several regions of Western Asia (Tarkhnishvili et al. 2012), Colchis (the eastern coast of the Black Sea) is considered of major role in the preservation of relict mesophytic trees and shrubs in the Caucasus as well as in triggering the adaptive radiation that shaped the modern flora (Kikvidze and Ohsawa 2001). This was the likely result of the long persisting warm and humid climate on this territory. Accordingly, a number of species typical of the Colchis flora died out many millions of years ago in Western Eurasia and, at present, their relatives have mainly survived in the mountains exposed to the summer monsoon in the Eastern and Southeastern Asia (Nakhutsrishvili 2013).

Finally, available literature data indicate that haplotypic richness is usually greater in the refugia than in re-colonized regions (e.g., Mediterranean peninsulas vs. Northern Europe), but gene diversity can be lower due to mixing of lineages at northern latitudes (Comps et al. 2001; Petit et al. 2003). Our results allow no inferences at this regard, due to the current lack of extensive data for comparisons with bordering regions. However, several findings combine with the rugged topography and high credibility of West Georgia as a primary refugium (Mèdail and Diadema 2009) to indicate the persistence of pre-Quaternary variation and postglacial admixture following a colonization that was mostly, but possibly not exclusively, limited in situ. Among the others, we may cite the overall moderate gene diversity detected, the relatively high number of haplotypes (five) observed in a single taxon (the Georgian durmast oak) and across a set of additional six taxa (nine, extensively shared, haplotypes) co-occurring on a small overall area, and the common genetic signatures displayed by some populations from close-by Central-Eastern Europe.

Evolutionary inferences

Based on the observed position in the network (Fig. 2b), the East Caucasian haplotype H3 may represent the ancestral haplotype (Posada and Crandall 2001). This would be supported by its occurrence also in *Q. aliena*, which has a wide range of distribution in the regions of warm temperate and subtropical climate of East Asia. As Menitsky (2005) pointed out: "The least specialized subspecies of *Q. petraea* is subsp. *iberica*, occurring in the Caucasus and Elburz mountains, very close to *Q. aliena*, which differs from the latter by having only somewhat fewer lateral veins." However, the most frequent haplotype H5 could also be considered very old because of its numerous connections to other haplotypes and high frequency in the western region. Therefore, the whole Caucasus region (eastern and western sides) would be confirmed as one of the most stable shelter areas in Western Eurasia, as well as an increasingly recognized center of species radiation (Pokryszko et al. 2011; Tarkhnishvili et al. 2012, 2014; Neiber and Hausdorf 2015). The restricted occurrence of *Q. pontica* in the Colchis region, a relict member of an ancient oak clade, possibly witnessing the primeval, uninterrupted distribution of *Quercus* in the Northern Hemisphere during the Tertiary period (Denk and Grimm 2010), is illustrative of this assumption. At the same time, the west Caucasian lineage (Fig. 3), including haplotype H9, found in Italian and Balkan peninsulas, and haplotypes H1, H2, H5, and H10 shared among the European and the Georgian oaks, supports the existence of strong phylogeographic links between these regions (Petit et al. 2002).

In agreement, the BEAST chronogram showed that the divergence among West Caucasian and East Caucasian maternal lineages likely occurred during the Late Miocene (Fig. 3). The use of fossils as calibration points involves both taxonomic and geological uncertainties (Sauquet et al. 2012). These fundamental issues have not always been adequately addressed in molecular dating studies dealing with *Quercus*, where the reliable assignation of fossils to extant species is still a matter of considerable debate (see, for instance, Denk et al. 2017). In addition, age correlation to the geologic timescale can be inaccurate, especially in the older literature. Such uncertainties can be limited by properly estimating the 95% confidence (or credibility interval) and combining more outgroup and ingroup age constraints (Sauquet et al. 2012). Nevertheless, age estimates should be necessarily cautious and supported by correlation with paleoecological data (e.g., Xu et al. 2015; Du et al. 2017). Since the Early Miocene, oaks of group *Quercus* were widespread across the entire Northern Hemisphere (Grímsson et al. 2016) and in this region as well (Shatilova et al. 2011). It is therefore highly likely that the genetic signatures we found to reflect pre-Quaternary speciation and differentiation processes, in agreement with numerous studies reporting older temporal scales than the Quaternary for the maternal lineage divergence in other Eurasian oaks (Late Miocene: Xu et al. 2015; Jiang et al. 2016; Du et al. 2017). In the close-by Southeast Mediterranean regions, modern types of oaks belonging to group *Quercus* were already present during Pliocene (Velitzelos et al. 2014).

The Miocene was a time of major climatic and vegetative changes worldwide, especially in the Northern Hemisphere, including major tectonic activity, orogeny, divergence, and extinctions of numerous plant lineages (Ramstein et al. 1997; Zachos et al. 2001; Tiffney and Manchester 2001). As Grossheim (1948) pointed out: "The radical turn in the Caucasus flora occurred considerably earlier than the Last Glacial Maximum (LGM) and was related with the Late Miocene global cooling." The significant uplift of the Dzirula massif during the Late Miocene (Adamia et al. 2011) divided the territory of Georgia into two large regions with diverse climatic features—humid conditions were predominant at the west, and continental climatic features were predominant over most of the eastern area (Zazanashvili et al. 2010). We may therefore infer that warm-temperate members of group *Quercus* apparently withstood the transformations that interested the region during Miocene and could be the phylogenetic sources of the Georgian oaks. Isolation and divergence of eastern vs. western haplotypes likely were the result of complex patterns associated with the Late Miocene orogeny events and major climatic changes. The complex topography of the Caucasus and the marked climatic differences between western and eastern Georgia allowed the preservation of relict taxa (e.g., *Q. pontica*) and lineages (e.g., haplotypes H5, H3) and, at the same time, promoted new habitat formation, with isolation and/or reiterated contacts of different populations, adaptation to local environments, and further maternal lineage diversification. Relationships established with Central-Eastern Europe populations during the Quaternary are probable but yet awaiting further confirmation.

Conclusion

This work reports the first comprehensive study on the biological diversity of Caucasian oak taxa and the genetic structure of *Q. petraea* subsp. *iberica* populations occurring in Georgia, using leaf morphometry and cpDNA sequence variation. Except for *Q. pontica*, no clear correlation between phenotype, genetic variation, and the proposed taxonomy was found. The Pontian oak represents an extremely ancient and isolated species, deserving further deeper investigations in force of its suggested status of New World-Old World disjunct. Of the remaining taxa, only two major morphotypes ("*petraea*-like" and "*robur*-like") could be reliably identified. Genetically, the durmast oak seems to bear the ancestral cpDNA variants and cover most of the variation found across the country. All gathered data reflect the well-known taxonomical controversies on oaks at the subspecific level, with habitat specificity and geographic circumscription apparently being the only reliable indicators for a prompt recognition of these (sub-)taxa to date. The biogeographic relevance of the Caucasus region is remarked, thanks to its geologic history, climatic features, and topography. Likely, since the Late Miocene, two main regions corresponding to the Euxinian (Western Caucasus) and Caucasian (Eastern Caucasus) provinces played major roles as a lineage crossroad and in both preserving and triggering diversity. More extensive investigations with additional markers (e.g., from the nuclear genome), bio-ecological descriptors, further populations, and species from border regions are needed to fully understand the true extent of the oak biodiversity in the Caucasus and assist conservation of this important biome.

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