Analysis of nuclear microsatellites reveals limited differentiation between Colchic and Hyrcanian populations of the wind-pollinated relict tree *Zelkova carpinifolia* (Ulmaceae)[1]

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- *Premise of the study:* The Caucasus represents one of the world's biodiversity hotspots and includes the climatic refugia Hyrcan on the southern coast of the Caspian Sea and Colchis on the eastern coast of the Black Sea, where different species survived during the Quaternary climatic oscillations. We evaluated the genetic diversity of the relict tree *Zelkova carpinifolia* shared between the two refugia and distributed throughout the Caucasus and adjacent areas.
- *Methods:* Specimens were collected from 30 geographical sites in Azerbaijan, Georgia, Iran, and Turkey and screened for variability at eight nuclear microsatellite loci. The genetic diversity among and within populations was assessed using a set of statistical measures.
- *Key results:* We detected 379 different genotypes from a total of 495 individuals with varying degrees of clonal reproduction at the different sites. Low to intermediate levels of genetic diversity were observed at all sites, and strong differentiation between sampling sites was absent. In addition, we observed no clear genetic differentiation between the Colchis and Hyrcan. Bayesian clustering of the genotypes revealed three populations with high levels of admixture between the sampling sites.
- *Conclusions:* The lack of strong genetic structure of studied populations of *Z. carpinifolia* contrasts with a previous study based on chloroplast markers and suggests that long-distance pollen dispersal is an important factor of gene flow among populations of *Z. carpinifolia*. The present study does not reveal any particular site with particularly isolated genotypes that would deserve more attention for conservation purposes than others, although some sites should be considered for further investigation.

Key words: Caucasus biodiversity hotspot; Colchis; gene flow; Hyrcan; relict tree; Ulmaceae; *Zelkova carpinifolia*.

Conservation of relict plants that were able to survive through the Quaternary climatic oscillations and persist today in isolated refugial areas is crucial for maintaining the global diversity of plants, especially considering the current climate trend of increasing temperatures and water stress (Petit et al., 2005; Kozlowski et al., 2012). The Arcto-Tertiary relict flora, that covered large parts of the northern hemisphere during the Eocene but since the Miocene has moved southward due to a shift toward a dryer and cooler climate (Chaney, 1947; Milne and Abbott, 2002; Milne, 2006), is represented today by many temperate tree genera, among which *Zelkova* gained a particular interest in the recent years (Fineschi et al., 2002; Denk and Grimm, 2005; Kvavadze and Connor, 2005; Søndergaard and Egli, 2006; Garfi et al., 2011; Kozlowski et al., 2012; Christe et al., 2014). The fossil record supports the past wide distribution of the genus (Wang et al., 2001; Denk and Grimm, 2005) and its extinction in North America, North Africa, and Europe as a result of climatic shifts in the Quaternary period (Chaney, 1947; Kvavadze and Connor, 2005). The last appearances of *Zelkova* in mainland Europe were in Rome (Italy) and date back to 31 thousand years ago (ka) (Follieri et al., 1986).

Zelkova carpinifolia (Pall.) C. Koch. is the only species of the genus *Zelkova* that occurs in western Asia. The remaining *Zelkova* species are restricted to Mediterranean islands or have a wider distribution in East Asia. It has been shown that six regions in western Asia could have served as climate refugia for forest vegetation during the Last Glacial Maximum (LGM): Colchis, western Anatolia, western Taurus, upper reaches of the Tigris River, Levant, and the southern Caspian basin (Tarkhnishvili et al., 2012). There are two relict forest regions in the Caucasus and adjacent areas: the Hyrcanian forest on the southern coast of the Caspian Sea, bounded by the Talysh and Alborz mountain ranges, and the Colchic forest on the eastern coast of the Black Sea, bounded by the western Greater and Lesser Caucasus mountains and the Likhi Ridge (Nakhutsrishvili et al., 2011). *Zelkova carpinifolia* occurs naturally in the Hyrcan region of Azerbaijan and Iran and the Colchis of western

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Georgia, but also grows in the south Lesser Caucasus (Garabagh, Azerbaijan), eastern Georgia (Babaneuri Strict Nature Reserve), Zagros Mountains (Iran), and the eastern part of Turkey (Gulisashvili, 1961; Davis, 1982; Kvavadze and Connor, 2005; Akhani et al., 2010). The upheaval of the Greater Caucasus and the Lesser Caucasus mountain ranges began, respectively, at the end of the Miocene-Pliocene era (Avdeev and Niemi, 2011) and the Pliocene (Sosson et al., 2010), whereas Colchic and Hyrcanian forests are believed to have emerged during the Upper Pliocene (Kolakovsky, 1961). These forests are now completely separated from each other and consist of distinctive plant communities, with several common relict plant species (Nakhutsrishvili et al., 2011). Climatic oscillations during the Holocene influenced the distribution of *Z. carpinifolia* as it was shown for Georgia (Kvavadze and Connor, 2005) and Iran (Djamali et al., 2008; Ramezani et al., 2008).

The phylogenetic relationships in the genus *Zelkova* were studied by Denk and Grimm (2005) using morphological characters and ribosomal internal transcribed spacer (ITS). Fineschi et al. (2002) addressed the genetic variation in natural populations of two Mediterranean species (*Z. sicula* and *Z. abelicea*) and their comparison to *Z. carpinifolia* using different molecular techniques, but no variation within the Mediterranean species was found. Christe et al. (2014) addressed the phylogeographical patterns in three western Eurasian species: *Z. sicula*, *Z. abelicea*, and *Z. carpinifolia* using two chloroplast (*trnH-psbA* and *trnL*) and nuclear (ITS1 and ITS2) regions and detected high levels of genetic variation and strong phylogeographical structure using chloroplast markers.

Currently, *Z. carpinifolia* mainly occurs in lowlands and middle mountain ranges, where, more recently, tree populations have become fragmented due to anthropogenic activities such as selective logging for timber, silvopasture, tree lopping and cutting for fuel, agricultural developments, construction of roads, tourism developments, fires, and extensive urbanization and industrialization (Gulisashvili, 1961; Scharnweber et al., 2007; Ramezani et al., 2008; Akhani et al., 2010; Kozlowski and Gratzfeld, 2013). As a consequence, *Z. carpinifolia* is listed as "Near threatened" (Güner and Zielinski, 1998) according to the criteria of the International Union for Conservation of Nature (IUCN).

The main aims of the current study were to (1) assess the nuclear genetic diversity across natural populations of *Z. carpinifolia*, (2) evaluate the differentiation between Hyrcanian and Colchic populations at nuclear loci, and (3) identify possible refugial areas as areas of high conservation value.

MATERIALS AND METHODS

Study species and sampling sites—*Zelkova carpinifolia* is a mesophytic deciduous tree species that occurs mainly in mixed lowland, riverside, and ravine forests with trees such as *Quercus* sp., *Carpinus* sp., *Acer* sp., *Ulmus* sp., *Parrotia persica*, *Fraxinus excelsior*, *Gleditsia caspia*, *Albizia julibrissin*, *Diospyros lotus*, and *Pterocarya fraxinifolia.* Some individuals grow to 40 m tall and 2–3 m in diameter and have an expected lifespan of more than 300 yr. At high elevations, up to 1500 m a.s.l., the species occurs in the form of small shrubs. *Zelkova carpinifolia* is andromonoecious with male flowers clustering together at the basis of the current year's twigs, while bisexual flowers are sitting alone in the leaf axils. Flowering takes place in March and April, and pollination is by wind. The fruit is a greenish, angular, rugous drupe, that is usually dispersed with part of the twig by wind.

Between 2010 and 2012, leaf samples were collected from 495 individuals of *Z. carpinifolia* among 30 geographical sites (10–20 individuals per site): 19 sites from Azerbaijan, 6 sites from Georgia, 4 sites from Iran, and 1 site from Turkey (Fig. 1, Appendix S1, see Supplemental Data with the online version of this article). Specimens were collected from both protected and unprotected areas, more or less untouched forests, forests degraded by human activities in

Fig. 1. Geographical distribution of *Zelkova carpinifolia* in the Caucasus and Iran. Gray circles represent the distribution according to Browicz and Zielinski (1982); black circles represent sites sampled in this study. Red dotted lines enclose Caucasus ecoregion as designated by the World Wildlife Fund (WWF). Map was created in ArcGIS Desktop version 10.2.2 (ESRI, 2014). Country borders, streams, water bodies and terrain shapefiles were taken from ArcGIS Online (ESRI, 2014).

the past and naturally regenerating forests, and park-like forest stands and remnant tree stands in villages. The distances among sites varied from 1 to 1000 km. The minimum distance between sampled trees was approximately 10 m, except for small sites where minimum distance was lower, but sampling of neighboring trees was prevented. Per site, one voucher specimen was collected, and voucher specimens were deposited in the herbaria of the Botanical Garden and Botanical Museum Berlin-Dahlem (Germany) and the Natural History Museum in Fribourg (Switzerland). Collection numbers were not assigned for specimens from Iran, Georgia, and Turkey, and herbarium vouchers were not collected for sites ZE053_IR and ZE069_AZ. Collected leaves were dried and stored in silica gel until DNA extraction.

***DNA extraction and microsatellite genotyping*—**Total genomic DNA was extracted from silica-gel-dried leaves using the NucleoSpin Plant II kit (Macherey Nagel, Düren, Germany) following the manufacturer's protocol and individuals were initially genotyped at 10 microsatellite loci described previously for *Z. carpinifolia* (Maharramova et al., 2014). Except for loci ZMS_4 and ZMS_9, microsatellite markers were amplified by multiplex polymerase chain reaction (PCR) using three mixes of oligonucleotide primers: mix 1 amplified microsatellite loci ZMS_12 and ZMS_13; mix 2 amplified loci ZMS_1, ZMS_2, ZMS_5 and ZMS_7; and mix 3 amplified loci ZMS_3 and ZMS_8. PCRs were performed in 25-μL reaction volumes containing 20–40 ng template DNA, 0.4 μM of each forward and reverse primer (Eurofins MWG Operon, Ebersberg, Germany), 1× TaqBuffer S (PeqLab, Erlangen, Germany), 1.5 mM $MgCl_2$, 250 μM of each dNTP, 0.2 mg/μL bovine serum albumin (BSA), and 0.75 U HotTaq polymerase (PeqLab). Either the forward or reverse primer of each primer combination was labeled with a fluorescent dye (6FAM, VIC, NED or PET; Applied Biosystems, Warrington, UK; Appendix S2, see online Supplemental Data). The PCRs were carried out using the following temperature profile: initial denaturation at 96°C for 2 min, annealing at 57°C (mix 1), 60°C (mixes 2 and 3), 62°C (for ZMS_9) or 52°C (for ZMS_4) for 1 min, and primer extension at 72°C for 1 min; 30 cycles of denaturation at 95°C for 30 s, annealing at the aforementioned temperatures for 30 s, and primer extension at 72°C for 30 s; final extension at 72°C for 15 min. Proper PCR amplification was checked by agarose gel electrophoresis and PCR products were cleaned up using the Gel/PCR DNA Fragment Extraction Kit (Avegene Life Sciences, Taipeh, Taiwan). Fragment analysis was performed by Macrogen (Seoul, Korea) using GeneScan 500 LIZ as internal size standard.

***Statistical analysis*—**Genotypes were scored using GeneMarker version 1.95 (SoftGenetics, State College, Pennsylvania, USA) and manually adjusted where necessary. Because *Z. carpinifolia* is actively reproducing by sprouting, identity analysis as implemented in the program CERVUS version 3.0.3 (Kalinowski et al., 2007) was performed to identify clones. Multilocus genotype diversity was estimated as a modification of the Simpson index (Pielou, 1969; Berg and Hamrick, 1994): $D_G = 1 - \Sigma n_i(n_i - 1)/N(N - 1)$, where n_i is the number of individuals of genotype i and N is the total number of individuals. The clone size was calculated as the ratio N_r/N_g and the frequency of clones per site as $1 - N_g/N_r$, where N_r is the total number of individuals sampled per site (ramets) and N_g is the number of different genotypes per site (genets) (McClintock and Waterway, 1993; Chung and Epperson, 2000). Genetic diversity parameters are usually measured with an exclusion of clonal individuals (Setsuko et al., 2004; Wei et al., 2013). However, it was shown that clonal individuals do not affect the levels of genetic diversity significantly (Chung et al., 2005), but rather affect the spatial genetic structure of populations (Berg and Hamrick, 1994; Setsuko et al., 2004; Chung et al., 2005; Schueler et al., 2006). Except for the analysis of null alleles, all further analyses were performed using both the data set with all ramets and the data set with genets only to assess whether the measured parameters are affected by the inclusion of clonal individuals.

Failed PCR amplifications that could be caused by the presence of null alleles or other technical issues were treated as missing data, and the percentage of missing data were calculated manually. However, null alleles can also occur at heterozygous loci and cause a homozygote excess. The program MicroChecker version 2.2.3 (Van Oosterhout et al., 2004) was used to identify null alleles and to calculate null allele frequencies for all loci.

Genetic diversity was estimated in the program Arlequin version 3.5 (Excoffier and Lischer, 2010) as allelic richness (A), observed heterozygosity (H_O) and expected heterozygosity (H_E) under the assumption of Hardy–Weinberg (HW) genotypic proportions. Polymorphism information content (PIC), commonly used in linkage analysis as a measure of polymorphism for a marker locus (Botstein et al., 1980), was calculated in CERVUS. The occurrence of alleles private to a single site was assessed. To correct for differences in sample size between the geographical sites, we calculated allelic richness and private allelic richness using rarefied subsamples of 10 individuals (excluding ZE061_TUR with only 8 individuals) with HP-rare version 1.1 (Kalinowski, 2005).

Arlequin was used to assess deviations from HW equilibrium using a locus-by-locus exact test (1 000 000 Markov chain steps and 100 000 dememorization steps), to calculate Wright's fixation index F_{IS} (Weir and Cockerham, 1984) and to assess pairwise linkage disequilibrium (LD) between loci using a likelihood ratio test with 10 000 permutations to calculate the significance of the observed likelihood ratios.

An exact test of population differentiation based on genotype frequencies was performed with Arlequin using 100 000 Markov chain steps and 10 000 dememorization steps. For visualizing the genetic structure of sampled sites, multidimensional scaling was performed using the cmdscale routine in the program R version 3.0.2 (R Core Team, 2013) with a matrix of pairwise R_{ST} distances between geographic sites calculated in Arlequin.

Population genetic structure was investigated using the program STRUCTURE version 2.3.4 (Pritchard et al., 2000) with a model that allows mixed ancestry of individuals and assumes correlated allele frequencies within populations. Five independent runs were performed with the number of clusters (K) varying from 2 to 25, a burn-in period of 100 000 iterations and a data collection period of 1 000 000 iterations. Bayesian clustering is based on the assignment of individuals to K clusters (populations) by estimating the membership coefficients for each individual in each cluster and indicating the maximal number of clusters using the posterior probability of the data for a given K [Ln $P(D)$]. However, Evanno et al. (2005) showed that the maximum value of Ln $P(D)$ does not always indicate the "true" number of clusters, and instead ΔK, the rate of change in the log probability of data between successive K values, should be used. The program STRUCTURE HARVESTER web version 0.6.93 (Earl and vonHoldt, 2012) was used to analyze the clustering results, to calculate ΔK and to produce input files for the program Clumpp version 1.1.2 (Jakobsson and Rosenberg, 2007), which permutes the results of different clustering runs and produces a single table with individual membership coefficients. The results were visualized using the program Distruct version 1.1 (Rosenberg, 2004).

For partitioning the total genetic variation among groups, among sites within groups, and within sampling sites, analysis of molecular variance (AMOVA) across all loci was performed in Arlequin using pairwise genetic distances defined as R_{ST} and 10 000 permutations to assess the significance of the variance components. For this purpose, the sampling sites were initially grouped together based on their geographical distribution into a Colchic group with samples from West Georgia, Turkey, and East Georgia (ZE054_GEO is situated outside the Colchis, but placed here due to its close proximity) and a Hyrcanian group with the samples from Azerbaijan and Iran. A second AMOVA was performed using only non-admixed populations as suggested by the STRUCTURE results. Pairwise genetic distances between sampling sites, estimated as R_{ST} (Slatkin, 1995) and F_{ST} (Wright, 1949), were obtained in Arlequin, and significances were assessed using a permutation test with 10 000 permutations. The adjustment of P values for multiple comparisons were implemented in R (R Core Team, 2013) using the method controlling the false discovery rate (FDR; Benjamini and Hochberg, 1995). Correlation between R_{ST} and F_{ST} was assessed in R (R Core Team, 2013) using a Mantel test (Mantel, 1967) implemented in the "vegan" package (Legendre and Legendre, 1998). To evaluate the relationship between genetic and geographic distances, we performed a Mantel test in Arlequin (significance was assessed using 100 000 permutations) using pairwise R_{ST} as genetic distances and pairwise geographic distances obtained using the Geographic Distance Matrix Generator 1.2.3 (Ersts, 2012).

RESULTS

***Genetic diversity in* Zelkova carpinifolia—**Identical multilocus genotypes were found in 28 sampling sites and the multilocus genotypic diversity (D_G) ranged between 0 (all multilocus genotypes identical) for ZE061_TUR and 1 (no identical multilocus genotypes) for ZE072_AZ and ZE054_GEO (average value of D_G = 0.922; Table 1). Excluding ZE061_TUR, the lowest value of D_G (0.767) and the largest clone size (3.2) were observed for site ZE031_AZ. The individuals (ramets) with identical multilocus genotypes were considered to belong to the same genet. From the 495 ramets that were screened, 379 genets were detected. The frequency of clones

TABLE 1. Genetic diversity parameters calculated for 30 geographical sites of *Zelkova carpinifolia*.

No.	Sampling site	Region	N_r	N_g	A	A_R	P_{AR}	$H_{O(r)}$	$H_{E(r)}$	$H_{O(g)}$	$H_{E(g)}$	$F_{IS(r)}$	$F_{IS(g)}$	D_G	N_r/N_g	$Freq_c$
1	ZE001_AZ	H	20	14	3.63	3.22	—	0.719	0.555	0.714	0.567	–0.455	–0.359	0.963	1.4	0.300
2	ZE007_AZ	H	20	12	3.88	3.54	—	0.769	0.567	0.750	0.580	–0.255	–0.175	0.947	1.7	0.400
3	ZE013_AZ	H	18	13	3.13	2.81	—	0.575	0.490	0.567	0.520	0.070	0.045	0.948	1.4	0.278
4	ZE017_AZ	H	20	16	3.38	3.16	—	0.560	0.534	0.558	0.531	0.012	–0.024	0.974	1.3	0.200
5	ZE019_AZ	H	20	18	4.00	3.49	0.02	0.469	0.474	0.479	0.487	0.027	0.028	0.984	1.1	0.100
6	ZE021_AZ	H	20	16	4.13	3.42	0.14	0.431	0.453	0.452	0.473	0.170	0.189	0.974	1.3	0.200
7	ZE027_AZ	H	20	14	3.38	2.89	—	0.493	0.446	0.486	0.452	–0.195	–0.227	0.953	1.4	0.300
8	ZE031_AZ	H	17	5	2.50	2.35	—	0.518	0.455	0.600	0.559	–0.257	–0.139	0.767	3.2	0.688
9	ZE034_AZ	H	18	14	3.75	3.42	0.05	0.569	0.527	0.545	0.514	0.030	0.033	0.967	1.3	0.222
10	ZE036_AZ	H	15	9	3.50	3.29	—	0.629	0.552	0.603	0.566	0.071	0.157	0.924	1.7	0.400
11	ZE043_AZ	H	18	16	3.25	2.94	0.03	0.556	0.493	0.563	0.508	–0.049	–0.033	0.987	1.1	0.111
12	ZE044_AZ	H	20	14	3.63	3.24	0.01	0.530	0.527	0.534	0.547	–0.030	–0.013	0.968	1.4	0.300
13	ZE051_AZ	H	10	8	3.63	3.63	—	0.600	0.560	0.589	0.574	–0.146	–0.068	0.956	1.3	0.200
14	ZE062_AZ	H	10	8	2.75	2.75	—	0.514	0.468	0.505	0.450	–0.257	–0.191	0.956	1.3	0.200
15	ZE067_AZ	H	20	17	3.75	3.29	0.02	0.619	0.559	0.610	0.562	–0.127	–0.034	0.979	1.2	0.150
16	ZE069_AZ	H	13	12	3.63	3.46	0.10	0.505	0.457	0.505	0.464	–0.122	–0.110	0.987	1.1	0.077
17	ZE072_AZ	H	13	13	3.75	3.59	0.12	0.602	0.604	0.602	0.604	–0.120	–0.120	1.000	—	—
18	ZE074_AZ	H	20	16	3.50	3.05	—	0.436	0.438	0.455	0.450	–0.036	–0.086	0.974	1.3	0.200
19	ZE075_AZ	H	20	18	3.50	3.10	0.01	0.524	0.518	0.512	0.525	–0.011	0.077	0.984	1.1	0.100
20	ZE053_IR	H	21	14	3.50	3.05	—	0.524	0.485	0.503	0.475	–0.140	0.055	0.942	1.3	0.250
21	ZE076_IR	H	12	10	3.75	3.57	—	0.413	0.518	0.410	0.542	–0.081	–0.111	0.955	1.2	0.167
22	ZE077_IR	H	13	7	3.50	3.33	0.06	0.549	0.476	0.551	0.502	0.135	0.182	0.872	1.9	0.462
23	ZE079_IR	H	11	5	3.00	2.94	—	0.511	0.417	0.525	0.467	0.198	0.386	0.818	2.2	0.545
24	ZE054_GEO	EG	20	20	3.00	2.78	—	0.478	0.425	0.478	0.425	–0.030	–0.030	1.000	—	—
25	ZE055_GEO	C	13	11	3.25	3.17	—	0.567	0.544	0.545	0.537	–0.023	–0.016	0.974	1.2	0.154
26	ZE057_GEO	C	18	14	3.50	3.18	—	0.590	0.506	0.583	0.538	–0.122	–0.074	0.947	1.4	0.300
27	ZE058_GEO	C	10	9	3.25	3.25	—	0.588	0.567	0.583	0.577	–0.277	–0.210	0.978	1.1	0.100
28	ZE059_GEO	C	20	18	3.75	3.30	0.06	0.459	0.494	0.470	0.505	0.055	0.087	0.989	1.1	0.100
29	ZE060_GEO	C	20	17	3.75	3.38	—	0.446	0.522	0.462	0.537	0.196	0.153	0.988	1.1	0.105
30	ZE061_TUR	C	8	1	1.25	—	—	—	—	—	—	–1.000	0.000	0.000	8.0	0.875

Notes: Region corresponds to Hyrcan (H), Colchis (C), or East Georgia (EG). N = number of individuals, A = allelic richness, A_R = rarefied allelic richness, P_{AR} = private allelic richness, H_O = observed heterozygosity, H_E = expected heterozygosity, F_{IS} = fixation index ($P > 0.05$, non-significant), D_G = multilocus genotypic diversity, N_r/N_g = clone size, $Freq_c$ = frequency of clones per site, r = ramets, g = genets. The mean values are presented.

varied from 0 (ZE072_AZ and ZE054_GEO) over 0.688 (ZE031_AZ) to 0.875 (ZE061_TUR).

Disregarding clonal individuals, the highest estimated null allele frequencies (NAF) were detected for loci ZMS_5 (0.037), ZMS_7 (0.054), ZMS_4 (0.184), and ZMS_9 (0.235). Loci ZMS_4 and ZMS_9 were not amplified in, respectively, 1.6% and 15% of the individuals, whereas ZMS_5 and ZMS_7 were amplified in almost all individuals. Locus ZMS_2, which showed 5% of missing data, did not show any evidence for the presence of null alleles, indicating that missing data are not caused by the presence of null alleles alone. General homozygote excess caused by the presence of null alleles was observed in nine sites at locus ZMS_9 and in 13 sites at locus ZMS_4. Loci ZMS_4 and ZMS_9 were excluded from all subsequent analyses.

In total, 51 alleles were observed for eight microsatellite loci. The number of alleles per locus (A) ranged from three alleles at locus ZMS_3 to 10 alleles at loci ZMS_2 and ZMS_8 (overall mean = 6.38 alleles). The total number of di- and trinucleotide repeats (R) ranged between 2 and 11 repeats per locus with an average of 6.25 repeats across all loci. The highest gene diversity (H_E) and/or polymorphism information content (PIC) were observed for loci ZMS_2 (respectively, 0.693 and 0.726) and ZMS_8 (respectively, 0.677 and 0.731), and the lowest for locus ZMS_5 (respectively, 0.114 and 0.123). Average gene diversity across all loci was 0.460 (see Table 2).

Genetic diversity parameters per sampling site are shown in Table 1. The average number of alleles across loci per site (A) ranged between 1.25 (ZE061_TUR) and 4.13 (ZE021_AZ), and the rarefied allelic richness (A_R) per site (calculated excluding ZE061_TUR) between 2.35 (ZE031_AZ) and 3.63 (ZE051_AZ). Average private allelic richness was also low (0.01–0.14), and private alleles were detected in 11 sampled sites (for up to three loci). The mean observed heterozygosity (H_O) per site varied between 0.410 (ZE076_IR) and 0.750 (ZE007_AZ), whereas the mean expected heterozygosity (H_E) varied between 0.425 (ZE054_GEO) and 0.604 (ZE072_AZ). ZE061_TUR was not considered due to its clonal structure. Twelve sampling sites significantly deviated from HWE for one to three loci. The mean fixation index F_{IS} over all sites was slightly negative (−0.092), with F_{IS} ranging between −0.455 (ZE001_AZ) and 0.198 (ZE079_IR), but deviations of F_{IS} from zero were not significant ($P > 0.05$). Among a total of 840 tests for pairwise linkage disequilibrium among the eight loci, only 55 were significant ($P < 0.05$). Significant linkage disequilibria were detected in 21 sites for at least one locus pair, with two pairs of loci (ZMS_2 and ZMS_8; ZMS_2 and ZMS_7) linked in five different sites. Therefore, we assumed no physical linkage between loci. As shown in Table 1, the inclusion of clonal individuals did not change the estimates of genetic diversity much, although it did affect locus by locus significance of HWE and LD (data not shown).

***Genetic structure of* Zelkova carpinifolia *populations*—** Among 435 tests for pairwise differentiation among 30 geographical sites with clonal individuals, only 73 were non-significant ($P > 0.05$). However, all pairwise population (site) comparisons were non-significant when clonal individuals were excluded from the analysis. No significant difference was found between matrices of F_{ST} and R_{ST} (Mantel test, 1000 permutations, $r = 0.519$, $P < 0.05$) showing the independence of our results from the applied microsatellite mutation model. Among a total of 435 R_{ST} pairwise comparisons, 146 were not significantly different from zero when clonal individuals were included in the analysis, and 251 were not significantly different from zero when clones were excluded (online Appendix S3). Including clonal individuals, the lowest significant pairwise R_{ST} was between ZE001_AZ and ZE043_AZ (0.027, $P < 0.05$), and the highest was between ZE027_AZ and ZE053_IR (0.410, $P < 0.05$). When clones were excluded, the lowest pairwise R_{ST} was observed between ZE043_AZ and ZE067_AZ (0.054, $P < 0.05$), and the highest was between ZE027_AZ and ZE053_IR (0.489, $P < 0.05$). In general, ZE027_AZ and ZE053_IR were not only more distant from each other, but also from all other sampled sites. It can also be seen in Fig. 2, which presents the results of a multidimensional scaling. When clones were removed, the R_{ST} distances for ZE067_AZ, ZE062_AZ and ZE079_IR were not significantly different from zero (Appendix S4). Additionally, Mantel test revealed no significant correlation between genetic and geographic distances in both datasets ($r = 0.093$, $P > 0.05$).

Hierarchical AMOVA revealed low differentiation between the Colchic and Hyrcanian groups and among the sampled sites within the groups (respectively, 0.71% and 10.55%), with a non-significant fixation index for among group variation. The differences among individuals within sampling sites exhibited the major part of the total variation (88.74%; Table 3). Exclusion of the clonal individuals changed the results only slightly, preserving the same tendency for higher within-site differentiation.

Analysis of the results of the Bayesian clustering as implemented in STRUCTURE revealed that the posterior probability of the data for a given K [Ln P(D)] increases with an increasing number of clusters (K) and that it reaches a maximum for $K = 5$.

TABLE 2. Characteristics of eight microsatellite loci for *Zelkova carpinifolia*.

Locus	A (total)	A (mean ± SD)	R (total)	R (mean ± SD)	H_E (total)	H_E (mean ± SD)	PIC
ZMS_1	7	4.03 ± 1.098	7	5.03 ± 1.450	0.684	0.606 ± 0.151	0.634
ZMS_2	10	5.50 ± 1.526	11	7.10 ± 2.578	0.752	0.693 ± 0.104	0.726
ZMS_3	3	2.07 ± 0.365	2	1.07 ± 0.365	0.477	0.425 ± 0.118	0.366
ZMS_5	5	1.53 ± 0.629	4	0.60 ± 0.770	0.127	0.114 ± 0.156	0.123
ZMS_7	6	3.77 ± 0.971	5	3.57 ± 0.971	0.660	0.568 ± 0.159	0.621
ZMS_8	10	5.03 ± 1.189	10	6.43 ± 0.935	0.765	0.677 ± 0.095	0.731
ZMS_12	4	2.60 ± 0.724	3	2.23 ± 0.935	0.258	0.247 ± 0.161	0.247
ZMS_13	6	2.70 ± 0.794	8	2.27 ± 1.617	0.397	0.352 ± 0.154	0.355
mean	6.38	3.40 ± 0.539	6.25	3.87 ± 0.596	0.515	0.460 ± 0.080	0.475

Notes: A = number of alleles, R = allelic range (difference between minimum and maximum number of repeats), H_E = expected heterozygosity, PIC = mean polymorphism information content.

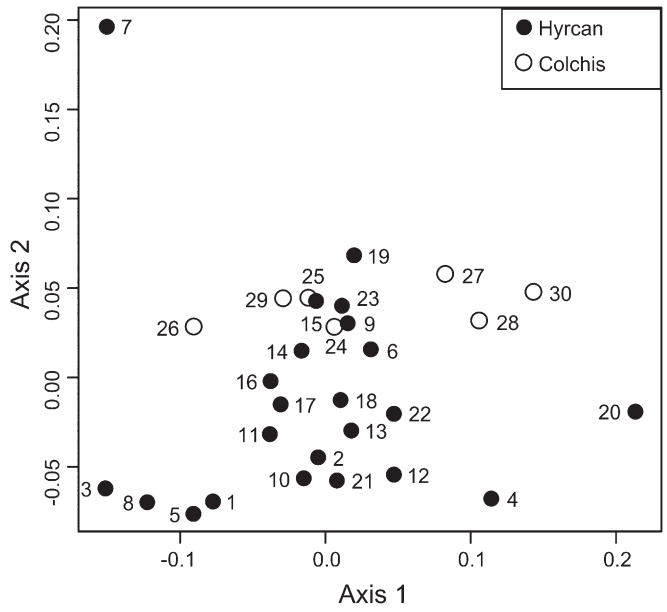

Fig. 2. Multidimensional scaling of 30 sites of *Zelkova carpinifolia* using pairwise R_{ST} as genetic distance (including clonal individuals). Circles represent sampling sites. Numbers correspond to the sites as shown in Table 1.

However, the optimal number of clusters suggested by the ΔK method corresponded to $K = 3$ (Fig. 3). A visual representation of individuals' membership coefficients also shows that three clusters capture the major structure in the data (Fig. 4). The STRUCTURE analysis separated Colchic (blue cluster) and Hyrcanian (yellow cluster) groups with the third cluster (pink) inside the Hyrcanian group. Nevertheless, many sites are admixed with individuals sharing their memberships in two or all three clusters. The second AMOVA, using only non-admixed populations as suggested by the STRUCTURE results, showed 25.9% differentiation among sites ZE001_AZ, ZE027_AZ, and ZE058_GEO.

DISCUSSION

Genetic diversity at nuclear microsatellite loci and frequency of clonal reproduction—The present study reports low to intermediate levels of genetic diversity in 30 sites sampled throughout the range of the relict tree *Zelkova carpinifolia* based on eight nuclear microsatellite loci. The lack of similar comprehensive studies of other trees from the Caucasus region prevents the recognition of general patterns of genetic diversity in its relict forests. Higher values of genetic diversity parameters (H_E = 0.6–0.7, A = 4–4.6) and high among population differentiation were shown for *Juglans regia* (Ibrahimov et al., 2010; Karimi et al., 2010), albeit that population sampling in both studies was biased toward low-distance scales. Usually long-lived woody species are likely to possess higher levels of genetic diversity than other life forms as they possess a higher proportion of polymorphic loci and more alleles per locus within their populations (Hamrick et al., 1992). However, similar levels of genetic diversity at neutral loci (H_E = 0.48, A = 3.4) were described for *Ulmus leavis*, a close relative of *Zelkova,* from glacial refugia of the Iberian Peninsula (Spain) (Venturas et al., 2013).

Vegetative reproduction could delay the time among generations in disturbed environments and, therefore, buffer against the effect of fragmentation on the genetic diversity (Wei and Jiang, 2012). Root sprouting and stump shooting are also characteristic for *Zelkova* species (Gulisashvili, 1961; Nakagawa et al., 1998; Søndergaard and Egli, 2006), and almost all studied sites of *Z. carpinifolia* contained clonal individuals with the large clone size for ZE031_AZ severely affected by past logging and lopping. The site ZE061_TUR from Trabzon (Turkey) described previously as *Z. carpinifolia* subsp. *yomraensis* (Anşin and Gercek, 1991) seems to be established by only one individual, since all eight individuals represent the same multilocus genotype.

Gene flow between Hyrcanian and Colchic populations as a result of wind pollination—The current distribution of common shared relicts of Colchic and Hyrcanian forests such as *Zelkova carpinifolia* and *Pterocarya fraxinifolia* outside the climatic refugia could be a result of expansion processes during interglacials. However, the occurrence of their fossils in different parts of Georgia since the Miocene (Stuchlik and Kvavadze, 1998; Kvavadze and Connor, 2005) suggests that they also covered the region before the uplift of topographical barriers and formation of Colchic and Hyrcanian forests in the Upper Pliocene (Kolakovsky, 1961). Climatic oscillations during the Holocene and recent anthropogenic disturbance influenced the current distribution of *Z. carpinifolia* in the Caucasus. Isolation as well as human-induced fragmentation is expected to cause a reduction of the gene flow among populations and an increase of inbreeding and random genetic drift in populations, resulting in strong genetic structure among regions and isolated populations (Young et al., 1996; Lowe et al., 2005). Such effects could also occur in *Zelkova* populations in the Caucasus. However, the obtained results indicate the same levels of genetic diversity, absence of inbreeding, and low differentiation between the two regions, with some private alleles detected for Hyrcan. Many temperate tree species are characterized by high diversity within populations and low differentiation among populations (Hamrick and Godt, 1996; Young et al., 1996; Sun et al., 2011; Lesser et al., 2013; Wei et al., 2013) due to the long lifespan, woody life form, outcrossing mating system, and wind pollination (Loveless and Hamrick, 1984; Heuertz et al., 2004). Wind

TABLE 3. Results of hierarchical AMOVA for relict tree *Zelkova carpinifolia*.

Source of variation	df	Sum of squares	Variance components	Percentage of variation, %	Fixation indices
Among groups	1	111.424	0.11488	0.71	F_{CT} = 0.00708
Among sites within groups	28	1979.74	1.71169	10.55	F_{SC} = 0.10625*
Within sites	960	13822.982	14.39894	88.74	F_{ST} = 0.11257*
Total	989	15914.145	16.2255		

Notes: *P < 0.05.

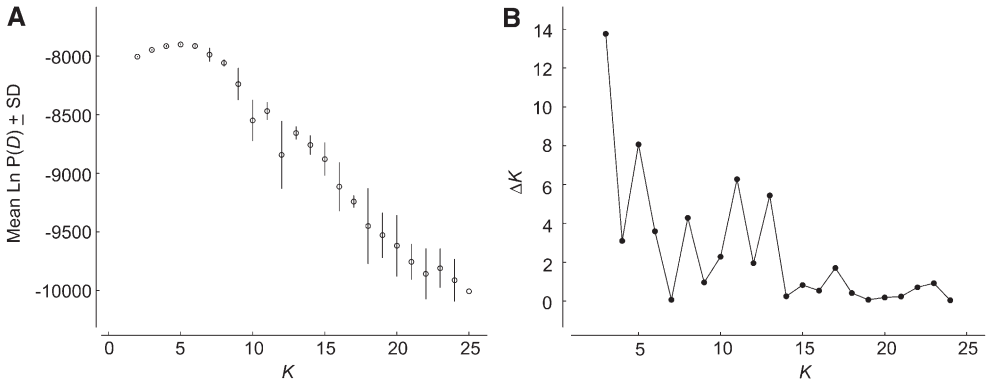

Fig. 3. Bayesian inference of the number of clusters (*K*) over five replicates for each *K*, using software STRUCTURE, based on 495 individuals of *Zelkova carpinifolia* collected from 30 geographical sites in the Caucasus. (A) Mean posterior probability of data for a given *K* [Ln P(*D*)]. (B) Rate of change in the log probability of data between successive *K* values (Δ*K*).

pollination allows gene flow among populations that could override inbreeding and genetic drift and cause low among population differentiation and elimination of geographical structure (Lesser et al., 2013).

However, we found about 26% of variation among three non-admixed populations each representing one of the three clusters revealed by STRUCTURE analysis: ZE001_AZ of shrubs found at a high altitude (1205 m a.s.l.) near Zuvand highland (Lerik, Azerbaijan); ZE027_AZ, a small but dense tree stand surrounding the cemetery in Tengerud (Astara, Azerbaijan); and ZE058_GEO, a very small tree stand in the village of Rokiti (Baghdati, West Georgia) close to the Ajameti Nature Reserve. The observed admixture of the rest of the sampling sites could also be explained by pollen-mediated gene flow among populations within and between Colchis and Hyrcan. Even if *Z. carpinifolia* is a long-lived woody species and our sampling included overlapping generations of trees that could lead to an underestimation of the effects of the recent human-induced fragmentation as shown for black walnut (Victory et al., 2006), fragmentation caused by climatic changes and long-lasting isolation of the two regions would have been obvious from the detected patterns of genetic differentiation. Pollen of *Z. carpinifolia* was shown to be scarcely represented in sediments of Georgia (Stuchlik and Kvavadze, 1993), which was explained by the low yield of pollen production or poor pollen preservation in sediments. The limited pollen content (5–10%) was also described from fossil spectra of the Middle Miocene and Pliocene-Pleistocene from Georgia (Stuchlik and Kvavadze, 1993). Long-distance dispersal of pollen of *Z. carpinifolia* up to 100 km was shown by Kvavadze and Connor (2005). However, our findings indicate the possibility for pollen dispersal over larger distances (200 km or more). So far, no other cases of such long-distance pollen dispersal were described in the literature (Petit and Hampe, 2006; Heslewood et al., 2014). However, we do not exclude that more stepping-stone populations of *Zelkova carpinifolia*, which connected the regions in the past have already been eliminated due to climate change or anthropogenic influence, and so our results can also illuminate the past gene flow in a long-lived tree.

Incongruent patterns of diversification at nuclear and chloroplast loci—High haplotype diversity was detected in natural populations of *Z. carpinifolia* using chloroplast markers in the recent study of Christe et al. (2014). Fifteen haplotypes clustered in two groups, separating western Colchic from eastern Hyrcanian populations. The groups were separated by 19 mutations, and among population differentiation was significant, and constituted about 87% of the total variation. A discrepancy between chloroplast and nuclear markers was shown previously for other tree species (Pakkad et al., 2008; Sun et al., 2011) and likely corresponds to a difference in seed and pollen dispersal. Chloroplast markers tracking uniparental inheritance revealed significant genetic structuring and differentiation among populations, providing evidence for limited seed dispersal in this species. Fruiting twigs separating from the mother trees can only disperse over very short distances in *Zelkova* species

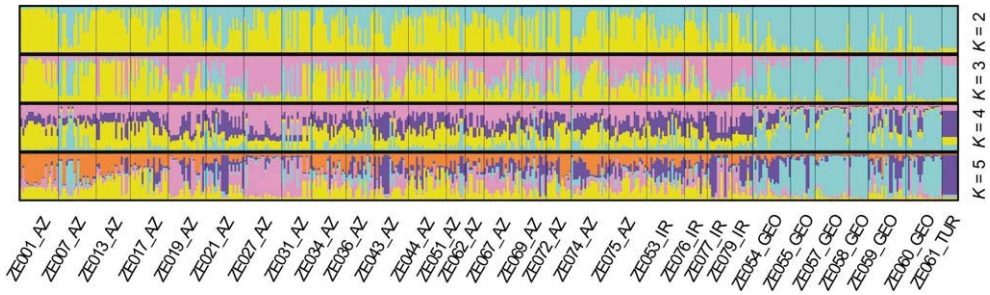

Fig. 4. Results of Bayesian clustering of 495 specimens of relict tree *Zelkova carpinifolia* for *K* = 2–5, where the most probable number of clusters is *K* = 3 with blue cluster representing Colchic group of populations and yellow and pink clusters representing the Hyrcanian group.

(Hoshino, 1990). However, both pollen and seed production was shown to be irregular in *Zelkova* (Nakagawa et al., 1998; Søndergaard and Egli, 2006; Garfi et al., 2011). The intensity of fruiting in *Z. carpinifolia* differs from year to year and among individuals, and 30–80% of fruits are empty due to unfavorable environmental conditions during flowering (Gulisashvili, 1961). Nuclear markers are biparentally inherited and track both pollen and seed dispersal. The analysis of ITS variability in populations of *Z. carpinifolia* (Christe et al., 2014) did not reveal a clear differentiation between Hyrcan and Colchis regions, which could also be interpreted in the light of pollen-mediated gene flow due to wind pollination. Moreover, the greater proportion of pollen flow over the seed flow was indicated in other studies of temperate and tropical tree species (Ennos, 1994; Petit and Hampe, 2006).

Since our results obtained at neutral loci differed from chloroplast data, it should also be taken into account that microsatellites are prone to size homoplasy (Estoup et al., 2002) and that evaluation of the genetic diversity could depend on their location in the genome (genic vs. nongenic) (DeFaveri et al., 2013), so they would not reflect genome-wide diversity (Vali et al., 2008).

***Implications for conservation*—**Despite of in situ protection of *Z. carpinifolia* in Nature Reserves, National Parks and Protected areas in Georgia, Iran, and Azerbaijan, the species is still under the threat of human influence and ongoing climate change. It was also shown that very limited ex situ collections of this relict species are found in the countries of its origin (Kozlowski et al., 2012). So, ex situ and in situ conservation planning is still a challenge. Furthermore, abandoned former plantations and clear-cuttings in Talysh lowlands (Azerbaijan) could be used for reforestation purposes (Scharnweber et al., 2007).

In the planning of conservation strategies, both plastid and nuclear data should be used (Moritz, 1994), and priority should be given to the measure of allelic richness compared with allelic frequencies (Petit et al., 1998). Our findings based on nuclear multilocus genotypic data show low values of allelic richness in all studied geographical sites. We identified some populations with non-admixed ancestry based on the distribution of allele frequencies, all of them residing outside the protected areas. None of these populations contained any private alleles or were characterized by higher genetic diversity. Although some other sites exhibit low frequencies of private alleles, no individual site could be indicated as more valuable for conservation. However, they could be the areas close to the source (refugium) populations and could be considered for future investigations. The results suggest that spatially isolated populations are not isolated genetically as a result of gene flow, which prevents inbreeding in and differentiation among the populations; however, high gene flow could be hazardous in terms of outbreeding depression when fitness of the progeny is reduced (Ellstrand, 1992). The reductions in a seed set, production of empty seeds, and irregular flowering in *Zelkova* are usually attributed to unfavorable environmental conditions, but they could be argued to represent the consequences of outbreeding depression as a result of intraspecific gene flow. In conservation genetics, if populations experience outbreeding depression, then management should be directed to reduce the gene flow (Ellstrand, 1992). However, more investigations are required to support the evidence of long-distance gene flow by pollen and the effect of outbreeding depression in *Zelkova* to understand how conservation management should be planned.

LITERATURE CITED

Akhani, H., M. Djamali, A. Ghorbanalizadeh, and E. Ramezani. 2010. Plant biodiversity of Hyrcanian relict forests, N Iran: An overview of the flora, vegetation, palaeoecology and conservation. *Pakistan Journal of Botany* 42: 231–258.

Anşin, R., and Z. Gercek. 1991. A new *Zelkova* taxon for the flora of Turkey: *Zelkova carpinifolia* (Pall.) C. Koch subsp. *yomraensis* Anşin & Gercek, subsp. *nova. Turkish Journal of Agriculture and Forestry* 15: 564–575.

Avdeev, B., and N. A. Niemi. 2011. Rapid Pliocene exhumation of the central Greater Caucasus constrained by low-temperature thermochronometry. *Tectonics* 30: TC2009 10.1029/2010TC002808.

Benjamini, Y., and Y. Hochberg. 1995. Controlling the false discovery rate—A practical and powerful approach to multiple testing. *Journal of the Royal Statistical Society,B. Methodological* 57: 289–300.

Berg, E. E., and J. L. Hamrick. 1994. Spatial and genetic-structure of 2 sandhills oaks — *Quercus laevis* and *Quercus margaretta* (Fagaceae). *American Journal of Botany* 81: 7–14.

Botstein, D., R. L. White, M. H. Skalnick, and R. W. Davies. 1980. Construction of a genetic linkage map in man using restriction fragment length polymorphism. *American Journal of Human Genetics* 32: 314–331.

Browicz. K., and J. Zielinski. 1982. Chronology of trees and shrubs in south-west Asia and adjacent regions. Polish Scientific Publishers, Warsaw, Poland.

Chaney, R. W. 1947. Tertiary centers and migration routes. *Ecological Monographs* 17: 139–148.

Christe, C., G. Kozlowski, D. Frey, S. Bétrisey, E. Maharramova, G. Garfì, S. Pirintsos, and Y. Naciri. 2014. Footprints of past intensive diversification and structuring in the genus *Zelkova* (Ulmaceae) in south-western Eurasia. *Journal of Biogeography* 41: 1081–1093.

Chung, M. G., and B. K. Epperson. 2000. Clonal and spatial genetic structure in *Eurya emarginata* (Theaceae). *Heredity* 84: 170–177.

Chung, M. Y., Y. Suh, J. López-Pujol, J. D. Nason, and M. G. Chung. 2005. Clonal and fine-scale genetic structure in populations of a restricted Korean endemic, *Hosta jonesii* (Liliaceae) and the implications for conservation. *Annals of Botany* 96: 279–288.

Davis, P. H. 1982. Flora of Turkey and the East Aegean islands. Edinburgh University Press, Edinburgh, UK.

DeFaveri, J., H. Viitaniemi, E. Leder, and J. Merila. 2013. Characterizing genic and nongenic molecular markers: Comparison of microsatellites and SNPs. *Molecular Ecology Resources* 13: 377–392.

Denk, T., and G. W. Grimm. 2005. Phylogeny and biogeography of *Zelkova* (Ulmaceae sensu stricto) as inferred from leaf morphology, ITS sequence data and the fossil record. *Botanical Journal of the Linnean Society* 147: 129–157.

Djamali, M., J. L. de Beaulieu, M. Shah-Hosseini, V. Andrieu-Ponel, P. Ponel, A. Amini, H. Akhani, et al. 2008. A late Pleistocene long pollen record from Lake Urmia, NW Iran. *Quaternary Research* 69: 413–420.

Earl, D., and B. vonHoldt. 2012. STRUCTURE HARVESTER: A website and program for visualizing STRUCTURE output and implementing the Evanno method. *Conservation Genetics Resources* 4: 359–361.

Ellstrand, N. C. 1992. Gene flow by pollen—implications for plant conservation genetics. *Oikos* 63: 77–86.

Ennos, R. A. 1994. Estimating the relative rates of pollen and seed migration among plant populations. *Heredity* 72: 250–259.

Ersts, P. J. 2012. Geographic distance matrix generator, version 1.2.3. Website http://biodiversityinformatics.amnh.org/open_source/gdmg [accessed 27 November 2013].

ESRI. (2014) ArcGIS Desktop, release 10.2.2. Redlands, CA: Environmental Systems Research Institute. Available from http://esri.com.

Estoup, A., P. Jarne, and J. M. Cornuet. 2002. Homoplasy and mutation model at microsatellite loci and their consequences for population genetics analysis. *Molecular Ecology* 11: 1591–1604.

Evanno, G., S. Regnaut, and J. Goudet. 2005. Detecting the number of clusters of individuals using the software STRUCTURE: A simulation study. *Molecular Ecology* 14: 2611–2620.

Excoffier, L., and H. E. L. Lischer. 2010. Arlequin suite ver 3.5: A new series of programs to perform population genetics analyses under Linux and Windows. *Molecular Ecology Resources* 10: 564–567.

Fineschi, S., M. Anzidei, D. Cafasso, S. Cozzolino, G. Garfi, R. Pastorelli, D. Salvini, et al. 2002. Molecular markers reveal a strong genetic differentiation between two European relic tree species: *Zelkova abelicea* (Lam.) Boissier and *Z. sicula* Di Pasquale, Garfi & Quezel (Ulmaceae). *Conservation Genetics* 3: 145–153.

Follieri, M., D. Magri, and L. Sadori. 1986. Late Pleistocene *Zelkova* extinction in Central Italy. *New Phytologist* 103: 269–273.

Garfi, G., F. Carimi, S. Pasta, J. Ruhl, and S. Trigila. 2011. Additional insights on the ecology of the relic tree *Zelkova sicula* di Pasquale, Garfi et Quezel (Ulmaceae) after the finding of a new population. *Flora* 206: 407–417.

Gulisashvili, V. Z. 1961. Dendroflora of the Caucasus. Academy of Sciences of Georgian SSR, Tbilisi, Georgia [in Russian].

Güner, A., and J. Zielinski. 1998. *Zelkova carpinifolia*, IUCN 2013. IUCN Red List of Threatened Species, version 2013.1. Website http://www.iucnredlist.org [accessed 22 August 2013].

Hamrick, J. L., and M. J. W. Godt. 1996. Effects of life history traits on genetic diversity in plant species. *Philosophical Transactions of the Royal Society of London, B, Biological Sciences* 351: 1291–1298.

Hamrick, J. L., M. Godt, and S. Sherman-Broyles. 1992. Factors influencing levels of genetic diversity in woody plant species. *New Forests* 6: 95–124.

Heslewood, M. M., A. J. Lowe, D. M. Crayn, and M. Rossetto. 2014. Contrasting levels of connectivity and localised persistence characterise the latitudinal distribution of a wind-dispersed rainforest canopy tree. *Genetica* 142: 251–264.

Heuertz, M., J. F. Hausman, O. J. Hardy, G. G. Vendramin, N. Frascaria-Lacoste, and X. Vekemans. 2004. Nuclear microsatellites reveal contrasting patterns of genetic structure between western and southeastern European populations of the common ash (*Fraxinus excelsior* L.). *Evolution* 58: 976–988.

Hoshino, Y. 1990. Fruiting shoot as a wind-dispersed diaspore assisting the fruit dispersal of *Zelkova serrata* (Thunb.) Makino. *Japanese Journal of Ecology* 40: 35–41.

Ibrahimov, Z., G. H. McGranahan, C. A. Leslie, and M. Aradhya. 2010. Genetic diversity in walnut (*Juglans regea*) from the Caucasus nation of Azerbaijan. *In* D. L. McNeil [ed.], Proceedings of the Sixth International Walnut Symposium in Melbourne, Australia, 2009, *ISHS Acta Horticulture* 1: 163–170.

Jakobsson, M., and N. A. Rosenberg. 2007. CLUMPP: A cluster matching and permutation program for dealing with label switching and multimodality in analysis of population structure. *Bioinformatics* 23: 1801–1806.

Kalinowski, S. T. 2005. HP-RARE 1.0: A computer program for performing rarefaction on measures of allelic richness. *Molecular Ecology Notes* 5: 187–189.

Kalinowski, S. T., M. L. Taper, and T. C. Marshall. 2007. Revising how the computer program CERVUS accommodates genotyping error increases success in paternity assignment. *Molecular Ecology* 16: 1099–1106.

Karimi, R., A. Ershadi, K. Vahdati, and K. Woeste. 2010. Molecular characterization of persian walnut populations in Iran with microsatellite markers. *HortScience* 45: 1403–1406.

Kolakovsky, A. A. 1961. The plant world of Colchis. Publishing House of Moscow University, Moscow, Russia [in Russian].

Kozlowski, G., D. Gibbs, F. Huan, D. Frey, and J. Gratzfeld. 2012. Conservation of threatened relict trees through living ex situ collections: Lessons from the global survey of the genus *Zelkova* (Ulmaceae). *Biodiversity and Conservation* 21: 671–685.

Kozlowski, G., and J. Gratzfeld. 2013. *Zelkova*—An ancient tree. Global status and conservation action. Natural History Museum, Fribourg, Switzerland.

Kvavadze, E. V., and S. E. Connor. 2005. *Zelkova carpinifolia* (Pallas) K. Koch in Holocene sediments of Georgia—An indicator of climatic optima. *Review of Palaeobotany and Palynology* 133: 69–89.

Legendre, P., and L. Legendre. 1998. Numerical ecology, 2nd English ed. Elsevier Science BV, Amsterdam, Netherlands.

Lesser, M. R., T. L. Parchman, and S. T. Jackson. 2013. Development of genetic diversity, differentiation and structure over 500 years in four ponderosa pine populations. *Molecular Ecology* 22: 2640–2652.

Loveless, M. D., and J. L. Hamrick. 1984. Ecological determinants of genetic structure in plant populations. *Annual Review of Ecology and Systematics* 15: 65–95.

Lowe, A. J., D. Boshier, M. Ward, C. F. E. Bacles, and C. Navarro. 2005. Genetic resource impacts of habitat loss and degradation; reconciling empirical evidence and predicted theory for neotropical trees. *Heredity* 95: 255–273.

Maharramova, E. H., L. A. Muller, N. Korotkova, and T. Borsch. 2014. Development of nuclear microsatellites for the Arcto-Tertiary tree *Zelkova carpinifolia* (Ulmaceae) using 454 pyrosequencing. *Applications in Plant Sciences* 2: 1300072.

Mantel, N. 1967. The detection of disease clustering and a generalized regression approach. *Cancer Research* 27: 209–220.

McClintock, K. A., and M. J. Waterway. 1993. Patterns of allozyme variation and clonal diversity in *Carex lasiocarpa* and *C. pellita* (Cyperaceae). *American Journal of Botany* 80: 1251–1263.

Milne, R. I. 2006. Northern hemisphere plant disjunctions: A window on tertiary land bridges and climate change? *Annals of Botany* 98: 465–472.

Milne, R. I., and R. J. Abbott. 2002. The origin and evolution of tertiary relict floras. *Advances in Botanical Research* 38: 281–314.

Moritz, C. 1994. Defining 'evolutionarily significant units' for conservation. *Trends in Ecology & Evolution* 9: 373–375.

Nakagawa, T., G. Garfi, M. Reille, and R. Verlaque. 1998. Pollen morphology of *Zelkova sicula* (Ulmaceae), a recently discovered relic species of the European Tertiary flora: description, chromosomal relevance, and palaeobotanical significance. *Review of Palaeobotany and Palynology* 100: 27–37.

Nakhutsrishvili, G., N. Zazanashvili, and K. Batsatsashvili. 2011. Regional profile: Colchic and Hyrcanic temperate rainforests of the Western Eurasian Caucasus. *In* D. A. Della Sala [ed.], Temperate and boreal rainforests of the world: Ecology and conservation, 214–221. Island Press/Center for Resource Economics, Washington, D.C., USA.

Pakkad, G., S. Ueno, and H. Yoshimaru. 2008. Genetic diversity and differentiation of *Quercus semiserrata* Roxb. in northern Thailand revealed by nuclear and chloroplast microsatellite markers. *Forest Ecology and Management* 255: 1067–1077.

Petit, R. J., A. El Mousadik, and O. Pons. 1998. Identifying populations for conservation on the basis of genetic markers. *Conservation Biology* 12: 844–855.

Petit, R. J., and A. Hampe. 2006. Some evolutionary consequences of being a tree. *Annual Review of Ecology, Evolution and Systematics* 37: 187–214.

Petit, R. J., A. Hampe, and R. Cheddadi. 2005. Climate changes and tree phylogeography in the Mediterranean. *Taxon* 54: 877–885.

Pielou, E. C. 1969. An introduction to mathematical ecology. John Wiley, New York, New York, USA.

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

Ramezani, E., M. R. M. Mohadjer, H. D. Knapp, H. Ahmadi, and H. Joosten. 2008. The late-Holocene vegetation history of the Central Caspian (Hyrcanian) forests of northern Iran. *Holocene* 18: 307–321.

R Development Core Team. 2013. R: A language and environment for statistical computing. Website http://www.R-project.org/.

Rosenberg, N. A. 2004. distruct: A program for the graphical display of population structure. *Molecular Ecology Notes* 4: 137–138.

Scharnweber, T., M. Rietschel, and M. Manthey. 2007. Degradation stages of the Hyrcanian forests in southern Azerbaijan. *Archiv für Naturschutz und Landschaftsforschung* 46: 133–156.

Schueler, S., A. Tusch, and F. Scholz. 2006. Comparative analysis of the within-population genetic structure in wild cherry (*Prunus avium* L.) at the self-incompatibility locus and nuclear microsatellites. *Molecular Ecology* 15: 3231–3243.

Setsuko, S., K. Ishida, and N. Tomaru. 2004. Size distribution and genetic structure in relation to clonal growth within a population of *Magnolia tomentosa* Thunb. (Magnoliaceae). *Molecular Ecology* 13: 2645–2653.

Slatkin, M. 1995. A measure of population subdivision based on microsatellite allele frequencies. *Genetics* 139: 457–462.

Søndergaard, P., and B. R. Egli. 2006. *Zelkova abelicea* (Ulmaceae) in Crete: Floristics, ecology, propagation and threats. *Willdenowia* 36: 317–322.

Sosson, M., Y. Rolland, C. Müller, T. Danelian, R. Melkonyan, S. Kekelia, S. Adamia, et al. 2010. Subductions, obduction and collision in the Lesser Caucasus (Armenia, Azerbaijan, Georgia), new insights. *Geological Society of London, Special Publications* 340: 329–352.

Stuchlik, L., and E. Kvavadze. 1993. Spore–pollen spectra of surface samples from *Zelkova* forest in the Babaneuri Reservation. *Acta Palaeobotanica* 33: 357–364.

Stuchlik, L., and E. Kvavadze. 1998. Subfossil pollen spectra of floodplain forest of *Pterocarya pterocarpa* in the Alazani valley (East Georgia). *Acta Palaeobotanica* 38: 217–222.

Sun, Y., X. Y. Wen, and H. W. Huang. 2011. Genetic diversity and differentiation of *Michelia maudiae* (Magnoliaceae) revealed by nuclear and chloroplast microsatellite markers. *Genetica* 139: 1439–1447.

Tarkhnishvili, D., A. Gavashelishvili, and L. Mumladze. 2012. Palaeoclimatic models help to understand current distribution of Caucasian forest species. *Biological Journal of the Linnean Society* 105: 231–248.

Vali, U., A. Einarsson, L. Waits, and H. Ellegren. 2008. To what extent do microsatellite markers reflect genome-wide genetic diversity in natural populations? *Molecular Ecology* 17: 3808–3817.

Van Oosterhout, C., W. F. Hutchinson, D. P. M. Wills, and P. Shipley. 2004. MICRO-CHECKER: Software for identifying and correcting genotyping errors in microsatellite data. *Molecular Ecology Notes* 4: 535–538.

Venturas, M., P. Fuentes-Utrilla, R. Ennos, C. Collada, and L. Gil. 2013. Human-induced changes on fine-scale genetic structure in *Ulmus laevis* Pallas wetland forests at its SW distribution limit. *Plant Ecology* 214: 317–327.

Victory, E. R., J. C. Glaubitz, O. E. Rhodes, and K. E. Woeste. 2006. Genetic homogeneity in *Juglans nigra* (Juglandaceae) at nuclear microsatellites. *American Journal of Botany* 93: 118–126.

Wang, Y. F., D. K. Ferguson, R. Zetter, T. Denk, and G. Garfi. 2001. Leaf architecture and epidermal characters in *Zelkova*, Ulmaceae. *Botanical Journal of the Linnean Society* 136: 255–265.

Wei, X. Z., and M. X. Jiang. 2012. Limited genetic impacts of habitat fragmentation in an "old rare" relict tree, *Euptelea pleiospermum* (Eupteleaceae). *Plant Ecology* 213: 909–917.

Wei, Z. Z., Q. Z. Du, J. F. Zhang, B. L. Li, and D. Q. Zhang. 2013. Genetic diversity and population structure in Chinese indigenous poplar (*Populus simonii*) populations using microsatellite markers. *Plant Molecular Biology Reporter* 31: 620–632.

Weir, B. S., and C. C. Cockerham. 1984. Estimating *F*-statistics for the analysis of population structure. *Evolution* 38: 1358–1370.

Wright, S. 1949. The genetical structure of populations. *Annals of Eugenics* 15: 323–354.

Young, A., T. Boyle, and T. Brown. 1996. The population genetic consequences of habitat fragmentation for plants. *Trends in Ecology & Evolution* 11: 413–418.