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ORIGINAL ARTICLE

Footprints of past intensive diversification and structuring in the genus *Zelkova* (Ulmaceae) in south-western Eurasia

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ABSTRACT

Aim To elucidate the phylogeographical patterns in three Cenozoic relict species: *Zelkova sicula*, *Z. abelicea* and *Z. carpinifolia* (Ulmaceae).

Location Sicily, Crete and Transcaucasia.

Methods Two chloroplast loci (*trn*H–*psb*A and *trn*L) and the nuclear ribosomal markers ITS1 and ITS2 were sequenced for 154 samples collected from 14 populations of *Z. abelicea*, 16 populations of *Z. carpinifolia*, and the two known populations of *Z. sicula*. We obtained georeferenced data, calculated median joining networks and carried out diversity analyses. A few *ex situ* samples collected from botanical gardens, of the East Asian species *Zelkova serrata*, *Z. schneideriana* and *Z. sinica*, were also analysed for comparative purposes.

Results High levels of variability were found in the chloroplast markers within *Z. carpinifolia* (15 haplotypes) and *Z. abelicea* (33 haplotypes), in association with strong phylogeographical structure. Conversely, *Z. sicula* was characterized by low diversity, with each population exhibiting a single haplotype. Lower variability was found for ITS1 and ITS2 within *Z. carpinifolia* and *Z. abelicea* (13 and 7 ribotypes per species, respectively), with *Z. carpinifolia* showing a high proportion of populations with no intragenomic polymorphism. In the triploid and clonal *Z. sicula*, all individuals displayed intragenomic polymorphism and seven ribotypes were identified.

Main conclusions The chloroplast diversity of *Z. abelicea* and *Z. carpinifolia* suggests a very ancient history of diversification and structuring, with footprints of past expansions and more recent bottlenecks. *Zelkova sicula* has had a history of severe isolation and is likely to be of hybrid origin.

Keywords
Chloroplast markers, Crete, internal transcribed spacer, phylogeography, Sicily, Transcaucasia, *Zelkova abelicea*, *Zelkova carpinifolia*, *Zelkova sicula*.

INTRODUCTION

Climatic oscillations during the Quaternary had dramatic effects on the distributions of species worldwide (Hewitt, 2000). South-western Eurasia, and especially Europe, was greatly affected by the glaciations (Connor, 2009), because both the Mediterranean Sea and east–west-orientated mountains prevented many species from migrating southwards. Several genera of the prominent Cenozoic tree flora vanished from the European continent or only survived in isolated and disjunct refugia of the Transcaucasian region, in the Balkan Peninsula and on Mediterranean islands (Milne & Abbott, 2002; Quézel & Médail, 2003).

Species of *Zelkova* Spach (Ulmaceae) were important elements of the vast forests that prevailed throughout the Northern Hemisphere during much of the Cenozoic Period (Mai, 1995; Fineschi *et al.*, 2004; Walker *et al.*, 2012). Today, the genus comprises six species with disjunct distribution

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Journal of Biogeography

patterns (Denk & Grimm, 2005): three in eastern Asia [*Zelkova serrata* (Thunb.) Makino; *Zelkova schneideriana* Hand.-Mazz.; and *Zelkova sinica* C. K. Schneid.], one in south-western Asia [*Zelkova carpinifolia* (Pall.) C. Koch] and two on the Mediterranean islands of Sicily (*Zelkova sicula* Di Pasq., Garfì & Quézel) and Crete [*Zelkova abelicea* (Lam.) Boiss.]. The oldest fossils attributed to *Zelkova* date from the early Eocene (55 Ma) in western North America, where the genus is extinct today (Burnham, 1986).

Phylogeography, using chloroplast and mitochondrial markers, has mostly focused on the Quaternary and the influence of the cycles of glaciation on species distribution and structure (Schönswetter *et al.*, 2005; Terrab *et al.*, 2008). Phylogeography has, however, also been used to document more ancient patterns (Petit *et al.*, 2005a; Rodríguez-Sánchez *et al.*, 2009), with some of them presumably dating as far back as the early Miocene (Magri *et al.*, 2007). The retrieval of ancient patterns may be specific to tree species, which are assumed to evolve more slowly than herbaceous plants and shrubs (Petit & Hampe, 2006). *Zelkova* trees live for centuries (Fazan *et al.*, 2012), which is a good indication that ancient patterns might be recovered using molecular markers.

A few phylogenetic and biogeographical studies have already been carried out on *Zelkova* (Fineschi *et al.*, 2002, 2004; Denk & Grimm, 2005; Fukatsu *et al.*, 2012), but these studies had small sample sizes or weak representation of wild populations. Our phylogeographical analysis, based on *trn*H–*psb*A, *trn*L and internal transcribed spacer regions 1 and 2 (ITS1 and ITS2), is the first to use a comprehensive sampling of natural populations from nearly all the disjunct regions where *Z. abelicea*, *Z. carpinifolia* and *Z. sicula* presently grow. We aimed to assess the diversity within and among species using DNA from two cellular compartments that have different modes of inheritance and trace different histories (Petit *et al.*, 2005b). For comparative purposes, we added specimens of the three East Asian *Zelkova* species, using *ex situ* collections.

The following questions were addressed: (1) What are the relationships of the three south-western Eurasian species, and how are they related to the East Asian species? (2) What are the phylogeographical patterns of the south-west Eurasian species, and what do they convey about the colonization and diversification patterns within species? (3) Do the chloroplast and nuclear loci give congruent information?

MATERIALS AND METHODS

Study group

Zelkova abelicea is endemic to Crete (Greece); it has a fragmentary distribution in the four main mountain regions of Crete (Lefka Ori, Psiloritis, Dikti and Thrypti), between 900 and 1800 m a.s.l., which corresponds to the upper timberline (Kozlowski *et al.*, 2014). It grows mainly on north-facing slopes or in and around rocky river-beds and gullies which remain moist during dry summers (Egli, 1997; Søndergaard & Egli, 2006). The species is highly endangered through habitat fragmentation and destruction, overgrazing, fire and water stress (Phitos *et al.*, 1995; Søndergaard & Egli, 2006; Kozlowski *et al.*, 2014).

Zelkova carpinifolia grows in the Transcaucasian countries of western Asia: Georgia, Armenia, Azerbaijan, Iran and Turkey (Güner & Zielinski, 1998). This area is considered to be one of the most important refugial zones of the Cenozoic relict flora in south-western Eurasia (Mai, 1995; Milne & Abbott, 2002). Despite having a relatively wide distribution range, *Z. carpinifolia* is rare and/or threatened in many regions (Davis, 1982; Kozlowski *et al.*, 2012). Two additional taxa have been recognized: *Z. carpinifolia* subsp. *yamraensis* Anşin & Gercek was described from Trabzon in north-eastern Turkey, and some populations from southern Azerbaijan have been distinguished as *Z. hyrcana* Grossh. & Jarm. None of these putative taxa have been investigated using molecular methods.

Zelkova sicula, discovered in 1991, is endemic to south-eastern Sicily (Di Pasquale *et al.*, 1992). The species has been identified as the most threatened in the genus, with only two known populations and a total of 1800 individuals (Garfì, 2006). *Zelkova sicula* is triploid and produces sterile seeds (Garfì *et al.*, 2011; G. Garfì, Institute of Biosciences and Bioresources, Palermo). Although several studies have been carried out on one population of this taxon (Nakagawa *et al.*, 1998; Fineschi *et al.*, 2002; Garfì *et al.*, 2002), the phylogenetic position of *Z. sicula* has not yet been elucidated.

Sampling

Leaf material of *Z. abelicea*, *Z. carpinifolia* and *Z. sicula* was retrieved from up to five individuals per population for a total of 154 trees. The sampling included 14, 16 and 2 populations, respectively; 19 *ex situ* samples of *Z. serrata*, *Z. schneideriana* and *Z. sinica* collected from botanical gardens were also used (see Appendix S1 in Supporting Information). All voucher specimens (one individual per population) were deposited in the herbarium of the Natural History Museum in Fribourg, Switzerland.

DNA extraction, amplification and sequencing

Total genomic DNA was extracted using the NucleoSpin Plant II kit (Macherey-Nagel, Düren, Germany). One cpDNA spacer (*trn*H–*psb*A; Shaw *et al.*, 2005), one cpDNA intron (*trn*L; Taberlet *et al.*, 1991) and two nrDNA markers (ITS1: Denk *et al.*, 2002; ITS2: White *et al.*, 1990) were used. Amplifications were carried out in a Biometra thermocycler (Göttingen, Germany) as detailed in Appendix S2. The sequencing reactions were performed using BigDye Terminator v3.1 Cycle Sequencing Kit and run on an ABI 377 automated sequencer (Applied Biosystems, Foster City, CA, USA). For ITS1, sequencing reactions were performed using the GenomeLab DTCS Quick Start Kit and run on a

GenomeLab GeXP automated sequencer (Beckman Coulter, Fullerton, CA, USA).

Some samples of *Z. sicula* produced illegible ITS2 sequences, and required cloning. The PCR products were ligated into the pGEM-T plasmid of the Promega pGEM-T Vector System I kit (Promega, Fitchburg, WI, USA). The ligated products were used to transform JM109 competent cells (Promega, Fitchburg, WI, USA) and five clones per PCR product were subsequently sequenced using the same protocol as before. No legible sequences were obtained for ITS1 in population A2 of *Z. sicula* despite several attempts.

Data analyses

All sequences were assembled with SEQUENCHER (GeneCodes Corporation, Ann Arbor, MI, USA) and manually aligned in BIOEDIT 7.0.3.5 (Hall, 1999). The cpDNA sequences were clustered into haplotypes on the basis of the aligned sequences. Indels and inversions were coded manually following Simmons & Ochoterena (2000) then used in all analyses, as they provide relevant phylogeographical information (Ingvarsson *et al.*, 2003). For ITS1 and ITS2, ambiguous positions were sometimes found; in order to determine the sequence phase in single individuals, we used the software PHASE 2.1 (Stephens *et al.*, 2001). The inferred haplotypes were named ribotypes and used in further analyses. In order to exclude putative pseudogenes, the GC content of each ribotype was computed using BIOEDIT, and ITS2 ribotypes were checked for the presence of the two conserved motifs M2 (5′-GAATTGCAGAATCC-3′) and M3 (5′-TTTGAACGCA-3′; Hřibová *et al.*, 2011). Haplotype richness and ribotype richness (*Rs*) were computed for each species using the rarefaction method (Petit *et al.*, 1998) in order to account for differences in sampling size, using $n = 10$ for the chloroplast data and $n = 8$ for the nuclear data. The chloroplast haplotypes and ITS genotypes sequences were deposited in GenBank (accession numbers JX399087–JX399147, JX110824–JX110837 and KF561728–KF561819).

Linkage disequilibrium between the two chloroplast loci was checked using Fisher's exact test, prior to their combination as chloroplast haplotypes. The haplotype diversity (h) and nucleotide diversity (π; Nei, 1987) were estimated for *Z. abelicea*, *Z. carpinifolia* and *Z. sicula*, and tested for deviation from neutral evolution using Tajima's D (Tajima, 1983) and Fu's F_S (Fu, 1997) statistics. Analyses of molecular variance (AMOVAs; Excoffier *et al.*, 1992) were conducted to assess genetic differentiation among populations (F_{ST}) or groups of populations (F_{CT}) using pairwise differences as the measure of genetic distance between haplotypes. Mantel tests were computed for *Z. abelicea* and *Z. carpinifolia* to check for the significance of isolation by distance between populations, using one matrix of $F_{ST} / (1 - F_{ST})$ values and one of either geographical distances between populations or their natural logarithms (Rousset, 1997). Mismatch distributions were computed within species and within regions, and the observed pattern was compared to those obtained under a sudden demographic expansion or a spatial expansion scenario. Goodness of fit, based on the sum of the squared differences (SSD) between the observed and the simulated data, was used as a statistical test to accept or reject a given scenario (Excoffier *et al.*, 2006). Each time a scenario could not be rejected at the 5% level, an estimate of the parameter of the spatial or demographic expansion was obtained. For a demographic expansion, a stationary haploid population at equilibrium is assumed to have suddenly passed from N_0 to N_1 individuals T generations ago. For a spatial expansion, it is assumed that a population of N_0 individuals expanded in space T generations ago as an infinite number of demes of size N that exchange migrants with other demes (Ray *et al.*, 2003; Excoffier, 2004). The estimated parameters are $\theta_0 = 2\mu N_0$, $\theta_1 = 2\mu N_1$ and $\tau = 2\mu T$. Because the chloroplast mutation rate is unknown for *Zelkova*, only τ is given to allow comparisons among species, assuming that the mutation rate is the same among species. All former analyses were performed using ARLEQUIN 3.1.5.2 (Excoffier *et al.*, 2006), and significance tests were conducted using 10,000 permutations.

The median joining network (MJN) of combined chloroplast haplotypes was drawn using the software NETWORK (Bandelt *et al.*, 1999). Site mutations, indels and inversions were equally weighted. As no Asian species could be sequenced for ITS1, GenBank accessions were used instead for *Z. serrata* and *Z. schneideriana*. Consequently, ITS1 and ITS2 sequences were not combined for these species and two different MJNs were built using the same rules as before. The geographical distribution of chloroplast haplotypes was visualized using ARCMAP GIS.

RESULTS

Chloroplast haplotype diversity and population differentiation

From a 1530-bp alignment, 91 polymorphic sites and 58 combined haplotypes were detected for the two cpDNA loci in the 173 individuals analysed (Appendix S3). Only two haplotypes were shared between species, both of them among East Asian species (Appendix S3).

Two of the three East Asian species display equivalent diversities, with four haplotypes each (*Z. serrata* and *Z. schneideriana*), followed by *Z. sinica*, with two haplotypes. *Zelkova abelicea*, *Z. carpinifolia* and *Z. sicula* exhibit 33, 15 and 2 haplotypes, respectively, all of which are species-specific (Appendix S3). The lowest overall gene diversity (h) is found in *Z. sicula*, and the highest in *Z. abelicea* and *Z. carpinifolia* (Table 1). *Zelkova sicula* also displays the lowest nucleotide diversity, while *Z. carpinifolia* has the highest (Table 1). Haplotype richness was equivalent in *Z. abelicea* and *Z. carpinifolia* (*Rs* = 7.8 and *Rs* = 7.3, respectively), but higher than the two haplotypes found in *Z. sicula*.

Of the 32 populations of *Z. abelicea*, *Z. carpinifolia* and *Z. sicula*, 22 are polymorphic: 13 in *Z. abelicea* and 9 in

Table 1 Populations of *Zelkova abelicea*, *Z. carpinifolia* and *Z. sicula* used in this study. For each population, the country, region, coordinates, collector, number of analysed individuals (*n*), number of combined haplotypes, and genotypes are given together with gene (π) and nucleotide (*h*) diversity for both genomes.

Population	Country	Region	Longitude	Latitude	Coll*	*n*	Chloroplast haplotypes	*h* (±SD)	π (±SD)	*n*	ITS1 genotypes	*n*	ITS2 genotypes	ITS1 + ITS2 *h* (±SD)	ITS1 + ITS2 π (±SD)
Zelkova abelicea															
OMA1	GR, Crete	Lefka Ori	23.912	35.316	DF	5	$WB^{(1)}$, $WH^{(3)}$, $WJ^{(1)}$	0.70 ± 0.22	0.0007 ± 0.0007	5	$N/P^{(3)}$, $F/P^{(2)}$	5	$D/D^{(5)}$	0.69 ± 0.10	0.0011 ± 0.0010
OMA2	GR, Crete	Lefka Ori	23.903	35.309	DF	5	$LJ^{(1)}$, $TJ^{(1)}$, $XJ^{(1)}$, $WH^{(2)}$	0.90 ± 0.16	0.0033 ± 0.0023	4	$F/P^{(1)}$, $P/P^{(3)}$	5	$B/B^{(2)}$, $D/D^{(3)}$	0.61 ± 0.16	0.0017 ± 0.0013
OMA3	GR, Crete	Lefka Ori	23.913	35.348	DF	5	$WH^{(3)}$, $WB^{(2)}$	0.60 ± 0.18	0.0005 ± 0.0005	3	$P/Q^{(3)}$	3	$D/D^{(3)}$	0.60 ± 0.13	0.0015 ± 0.0013
OMA4	GR, Crete	Lefka Ori	23.911	35.345	DF	5	$WG^{(1)}$, $WF^{(1)}$, $WL^{(1)}$, $WO^{(1)}$, $WH^{(1)}$	1.00 ± 0.13	0.0013 ± 0.0011	0	—	5	$D/D^{(5)}$	—	—
AMB1	GR, Crete	Lefka Ori	23.986	35.367	DF	5	$XO^{(2)}$, $WC^{(2)}$, $XC^{(1)}$	0.80 ± 0.16	0.0020 ± 0.0015	1	$F/F^{(1)}$	4	$D/D^{(4)}$	—	—
AMB2	GR, Crete	Lefka Ori	23.981	35.355	DF	5	$WH^{(3)}$, $WJ^{(2)}$	0.60 ± 0.18	0.0005 ± 0.0005	4	$F/Q^{(2)}$, $N/Q^{(2)}$	5	$D/D^{(5)}$	0.71 ± 0.13	0.0013 ± 0.0010
NIA	GR, Crete	Lefka Ori	24.155	35.288	DF	5	$WH^{(4)}$, $WJ^{(1)}$	0.40 ± 0.24	0.0003 ± 0.0004	5	$F/Q^{(1)}$, $P/Q^{(4)}$,	4	$D/D^{(4)}$	0.57 ± 0.09	0.0014 ± 0.0012
ELI	GR, Crete	Lefka Ori	24.015	35.260	DF	5	$WH^{(1)}$, $VB^{(1)}$, $VH^{(1)}$, $VJ^{(2)}$	0.90 ± 0.16	0.0018 ± 0.0014	4	$P/Q^{(3)}$, $F/R^{(1)}$	5	$D/D^{(5)}$	0.79 ± 0.11	0.0017 ± 0.0013
KED	GR, Crete	Kedros	24.627	35.188	DF	5	$RA^{(1)}$, $TA^{(1)}$, $TF^{(2)}$, $TO^{(1)}$	0.90 ± 0.16	0.0011 ± 0.0010	2	$F/Q^{(2)}$	5	$D/D^{(5)}$	0.67 ± 0.20	0.0016 ± 0.0015
PSI	GR, Crete	Psiloritis	24.929	35.179	DF	5	$LL^{(1)}$, $MF^{(1)}$, $LF^{(3)}$	0.70 ± 0.22	0.0010 ± 0.0009	3	$N/Q^{(1)}$, $P/Q^{(1)}$, $N/P^{(1)}$	5	$D/D^{(5)}$	0.80 ± 0.12	0.0013 ± 0.0012
LAS1	GR, Crete	Dikti	25.538	35.170	DF	2	$UC^{(2)}$	—	—	5	$Q/S^{(4)}$, $R/N^{(1)}$	2	$D/D^{(2)}$	0.67 ± 0.20	0.0025 ± 0.0021
LAS2	GR, Crete	Dikti	25.513	35.137	DF	5	$OA^{(2)}$, $OF^{(2)}$, $SF^{(1)}$	0.80 ± 0.16	0.0008 ± 0.0008	2	$P/Q^{(2)}$	5	$D/D^{(5)}$	0.67 ± 0.20	0.0016 ± 0.0015
LAS3	GR, Crete	Dikti	25.513	35.138	DF	5	$OF^{(4)}$, $QF^{(1)}$	0.40 ± 0.24	0.0007 ± 0.0007	4	$P/P^{(2)}$, $P/R^{(2)}$	5	$D/D^{(5)}$	0.43 ± 0.17	0.0005 ± 0.0006
STA	GR, Crete	Thrypti	25.888	35.081	DF	5	$KJ^{(1)}$, $KA^{(1)}$, $DB^{(1)}$, $KF^{(1)}$, $KB^{(1)}$	1.00 ± 0.13	0.0024 ± 0.0018	3	$N/N^{(1)}$, $N/Q^{(2)}$	5	$D/D^{(5)}$	0.53 ± 0.17	0.0006 ± 0.0007
Total						67	33	0.92 ± 0.02	0.0043 ± 0.0024	45	12	63	2	0.75 ± 0.03	0.0016 ± 0.0011
Zelkova carpinifolia															
BAB	Georgia	Barbaneuri	45.371	42.081	DF	5	$JS^{(5)}$	—	—	5	$A/A^{(5)}$	5	$E/E^{(5)}$	—	—
NER	Georgia	Nergeeti	42.826	42.046	DF	4	$BX^{(4)}$	—	—	1	$A/A^{(1)}$	4	$F/F^{(1)}$, $E/E^{(3)}$	—	—

Table 1 Continued

Population	Country	Region	Longitude	Latitude	Coll*	*n*	Chloroplast haplotypes	*h* (±SD)	π (±SD)	*n*	ITS1 genotypes	*n*	ITS2 genotypes	ITS1 + ITS2 *h* (±SD)	ITS1 + ITS2 π (±SD)
IAN	Georgia	Ianeti	42.419	42.172	DF	4	$EY^{(2)}$, $BY^{(2)}$	0.67 ± 0.20	0.0017 ± 0.0014	4	$A/A^{(1)}$, $A/B^{(1)}$, $A/J^{(1)}$, $A/L^{(1)}$	4	$E/E^{(4)}$	0.64 ± 0.18	0.0039 ± 0.0025
ANR	Georgia	Ajametis	42.763	42.143	DF	5	$AZ^{(2)}$, $EZ^{(3)}$	0.60 ± 0.18	0.0010 ± 0.0009	3	$A/A^{(1)}$, $A/E^{(1)}$, $A/L^{(1)}$	5	$E/E^{(5)}$	0.60 ± 0.22	0.0034 ± 0.0024
MAR	Georgia	Martvili	42.378	42.405	DF	5	$EY^{(1)}$, $AY^{(3)}$, $BY^{(1)}$	0.70 ± 0.22	0.0010 ± 0.0009	5	$A/A^{(5)}$	5	$E/E^{(5)}$	—	—
ROK	Georgia	Rokiti	42.793	42.114	DF	5	$BZ^{(5)}$	—	—	3	$A/A^{(3)}$	5	$E/E^{(5)}$	—	—
VAN	Georgia	Vani	42.565	42.089	DF	5	$BX^{(1)}$, $BZ^{(4)}$	0.40 ± 0.24	0.0003 ± 0.0004	1	$A/A^{(1)}$	5	$E/E^{(5)}$	—	—
KUT	Georgia	Kutaisi	42.700	42.250	GK	5	$EZ^{(1)}$, $AZ^{(4)}$	0.40 ± 0.24	0.0007 ± 0.0007	0	—	3	$E/E^{(3)}$	—	—
TBI	Georgia	Tbilissi	44.783	41.717	GK	4	$JS^{(4)}$	—	—	4	$A/A^{(3)}$, $A/B^{(1)}$	2	$E/E^{(2)}$	—	—
TRA	Turkey	Trabzon	39.866	40.950	IK	5	$EY^{(2)}$, $EU^{(3)}$	0.60 ± 0.18	0.0010 ± 0.0009	4	$A/A^{(4)}$	5	$E/E^{(5)}$	—	—
XAN	Azerbaijan	Xanbulan	48.800	38.661	EG	5	$IT^{(5)}$	—	—	3	$A/E^{(1)}$, $A/L^{(1)}$, $D/L^{(1)}$	5	$E/E^{(5)}$	0.87 ± 0.13	0.0046 ± 0.0031
PAR	Azerbaijan	Parakand	48.805	38.659	EG	5	$HR^{(5)}$	—	—	1	$A/J^{(1)}$	5	$E/E^{(5)}$	1.00 ± 0.50	0.0074 ± 0.0080
GUN	Azerbaijan	Güneshli	48.469	38.805	EG	5	$JT^{(1)}$, $IT^{(4)}$	0.40 ± 0.24	0.0007 ± 0.0007	3	$F/M^{(1)}$, $K/M^{(1)}$, $G/H^{(1)}$	3	$E/E^{(2)}$, $E/F^{(1)}$	1.00 ± 0.18	0.0059 ± 0.0044
TA1	Azerbaijan	Talysh	48.773	38.452	GK	5	$JT^{(4)}$, $J3T^{(1)}$	0.40 ± 0.24	0.0007 ± 0.0007	4	$C/L^{(1)}$, $A/L^{(3)}$	5	$E/E^{(5)}$	0.78 ± 0.11	0.0051 ± 0.0032
TA2	Azerbaijan	Talysh	48.433	38.729	GK	5	$HR^{(5)}$	—	—	1	$A/D^{(1)}$	5	$E/E^{(5)}$	1.00 ± 0.50	0.0025 ± 0.0030
GO	Iran	Golestan	54.580	36.726	MJ	5	$CP^{(3)}$, $CK^{(2)}$	0.60 ± 0.18	0.0005 ± 0.0005	1	$A/I^{(1)}$	5	$E/E^{(5)}$	1.00 ± 0.50	0.0061 ± 0.0067
Total						77	15	0.93 ± 0.01	0.0115 ± 0.0058	43	12	71	3	0.45 ± 0.07	0.0027 ± 0.0017
Zelkova sicula															
A1	I, Sicily	Buccheri	37.17	14.86	GK	5	$FV^{(5)}$	—	—	4	$F/U^{(3)}$, $T/W^{(1)}$	4	$B/I^{(1)}$, $B/K^{(1)}$, $B/H^{(1)}$, $B/C^{(1)}$	0.93 ± 0.12	0.0044 ± 0.0030**
A2	I, Sicily	Ciranna	37.21	15.04	GK	5	$GW^{(5)}$	—	—	0	—	5	$E\text{-}E^{(5)}$	—	—
Total						10	2	0.56 ± 0.07	0.0029 ± 0.0018	4	2	9	5	0.93 ± 0.12	0.0044 ± 0.0030

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**Depending on the phase between ITS1 and ITS2 ribotypes, five or six combined ribotypes were found and π and *h* changed accordingly. All combinations were tested. Figures are given for five combined ribotypes. For six ribotypes, π = 1.00 ± 0.10 and *h* = 0.0041 ± 0.0029.

Z. carpinifolia (Table 1). For *Z. carpinifolia*, both neutrality tests were non-significant or nearly so (D = 1.196, P = 0.090; F_S = 7.279, P = 0.049). For *Z. abelicea*, the neutrality tests gave contrasting results, with a non-significant Tajima's D (D = 0.558, P = 0.251) and a highly significant F_S (F_S = −17.759, P = 0.000), indicating that more haplotypes were recorded than would have been expected under neutrality.

The haplotype MJN is clustered into groups separated by many mutations (Fig. 1a). The haplotypes belonging to *Z. abelicea* show reticulate evolution and are grouped corresponding to the major Cretan mountains (Fig. 1c). Haplotypes of *Z. carpinifolia* are clustered into two groups, separated by a minimum of 19 mutations. The haplotypes of the first group (AY, AZ, BX, BY, BZ, EU, EY and EZ; Fig. 1d) are found only in the western populations. The second group of haplotypes (JS, JT, J3T, IT and HR) is found in the five eastern and the two central populations, whereas haplotypes CP and CK are seen in the population from Golestan (north-eastern Iran). The only two known populations of *Z. sicula* display one haplotype each (FV and GW; Fig. 1b), located between those of *Z. carpinifolia* and *Z. abelicea*. The eight haplotypes found in the three East Asian species (*Z. sinica*, *Z. serrata* and *Z. schneideriana*; SH4E, YD, YE, ZD, ZE, Z2E, Z3E and Z5E) are grouped together in a different part of the network which is joined to the first cluster of *Z. carpinifolia*.

F_{ST} is very high within species, and is significant for *Z. abelicea* and *Z. carpinifolia* (Table 2). For these species, the populations were further clustered into four groups according to geography (Fig. 1c,d). For both species, the variation among geographical groups was also significantly large (Table 2). Mismatch distributions were computed for the three species, although the numbers of populations and individuals were low for *Z. sicula*. For the latter, the tests rejected both scenarios. Conversely, none of the demographic or spatial expansion scenarios could be rejected at the 5% level for *Z. abelicea* or *Z. carpinifolia* (Table 3). Taking into account the SSD values, a demographic scenario provided a better fit than a spatial expansion scenario for *Z. abelicea*,

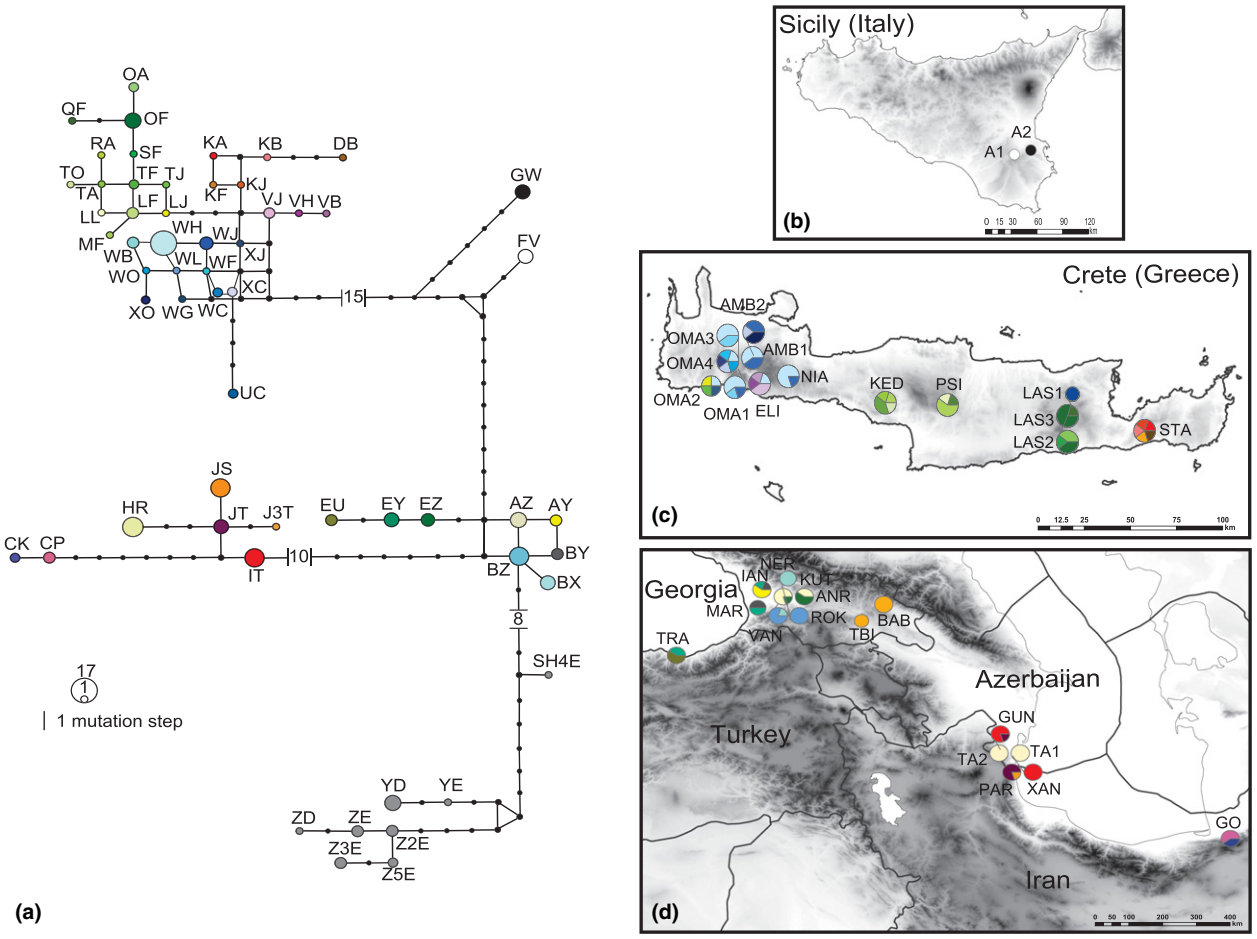

Figure 1 (a) Haplotype network based on the two chloroplast markers *trn*H–*psb*A and *trn*L in three Eurasian *Zelkova* species. The name of each haplotype is constituted from the identifier of the *trn*H–*psb*A sequence followed by the identifier of the *trn*L sequence, as given in Appendix S3. The size of each haplotype is proportional to the number of individuals that share it. The black dots without names on the lines are missing or non-sampled haplotypes. (b) Geographical distribution of *Z. sicula* haplotypes. (c) Geographical distribution of *Z. abelicea* haplotypes. (d) Geographical distribution of *Z. carpinifolia* haplotypes.

Table 2 F_{ST} and F_{CT} values for *Zelkova abelicea*, *Z. carpinifolia* and *Z. sicula* on the combined chloroplast data set (*trn*H–*psb*A and *trn*L).

Species	Source of variation	d.f.	Sum of squares	Variance component	Percentage of variation	*F*-indices
Zelkova abelicea	Among groups	3	94.112	$V_a = 2.093$	59.26%	$F_{CT} = 0.593$**
	Among populations	10	40.067	$V_b = 0.685$	19.38%	$F_{ST} = 0.786$**
	Within populations	53	40.000	$V_c = 0.755$	21.36%	
	Total	66	174.179			
Zelkova carpinifolia	Among groups	3	427.405	$V_a = 8.456$	86.99%	$F_{CT} = 0.870$**
	Among populations	12	60.166	$V_b = 0.986$	10.14%	$F_{ST} = 0.971$**
	Within populations	61	17.000	$V_c = 0.279$	2.87%	
	Total	76	504.571			
Zelkova sicula	Among populations	1	15.000	$V_a = 0.300$	1.00%	$F_{ST} = 1.000$*
	Within populations	8	0.000	$V_b = 0.000$	0.00%	
	Total	9	15.000			

*$P < 0.01$; **$P < 0.001$.

Table 3 Probabilities for a demographic or a spatial expansion that occurred T generations ago (T in million generations ago, MGA) computed on the combined chloroplast data set (*trn*H–*psb*A and *trn*L) from three species of *Zelkova*.

	Spatial expansion			Demographic expansion		
Scenario	*P*-values	SSD	$\tau = 2\mu T$	*P*-values	SSD	$\tau = 2\mu T$
Zelkova abelicea	0.475	0.0110	3.2	0.784	0.0056	8.0
West (Lefka Ori)**	0.553	0.0094	1.7	0.662	0.0073	3.1
Central West (Psiloritis)	0.484	0.0127	2.0	0.526	0.0127	2.0
Central East (Dikti)**	0.813	0.0012	1.0	0.844	0.0013	1.0
East (Thrypti)	0.566	0.0471	1.9	0.600	0.0471	1.9
Zelkova carpinifolia	0.633	0.0146	19.2	0.336	0.0268	0.0
West (Turkey & Georgia)	0.808	0.0011	1.2	0.012	0.0689	—
Central West (Georgia)	n/a*	n/a	n/a	n/a	n/a	n/a
Central East (Azerbaijan)	0.175	0.0663	4.5	0.077	0.1038	5.3
East (Iran)	0.310	0.0543	0.9	0.505	0.0543	0.9
Zelkova sicula	0.008	0.2284	—	0.046	0.3675	—

*n/a: not available due to the lack of polymorphism within the region. **Without the two populations that showed signs of long-distance dispersal (OMA2 and LAS1). SSD, sum of the squared differences.

whereas the reverse was true for *Z. carpinifolia* (Table 3). When computed on the different regions within species, the results were found to be in agreement (Table 3), except for the western part of the distribution range of *Z. carpinifolia*.

For *Z. abelicea*, the correlation between geographical and genetic distances was highly significant and positive whatever geographical distance was used, whereas Mantel tests were non-significant for *Z. carpinifolia* at the 5% level (Table 4).

Nuclear genotype diversity and population differentiation

ITS1 and ITS2 had consensus alignments of 381 bp and 434 bp, respectively (Appendix S3). The two matrices display 12 polymorphic sites for ITS1 and 6 for ITS2. For ITS1, 24 genotypes were recorded and PHASE identified 21 different ribotypes, whose GC contents ranged between 60.6% and 61.7%, with no significant differences among species. Only one ribotype is shared among the three species (ITS1_F; Table 1, Fig. 2a). For ITS2, a total of nine genotypes were found, consisting of eight ribotypes (Table 1). The GC contents were more variable and ranged between 54.2% and 64.3%, but did not differ significantly among species. The conserved motifs M2 and M3 were found in all ribotypes. Three ribotypes were shared: ITS1_F (*Z. sicula*,

Table 4 Mantel tests for *Zelkova abelicea* and *Z. carpinifolia* using chloroplast pairwise F_{ST} as the genetic distance.

Units of distance	Measurement	*Z. abelicea*	*Z. carpinifolia*
km	Correlation coefficient	0.404	−0.027
	P(random ≥ observed)	**0.003**	0.614
log(km)	Correlation coefficient	0.399	0.016
	P(random ≥ observed)	**0.001**	0.480

P(random ≥ observed) is the percentage of correlations calculated on randomized data that are higher than the observed correlation. Values in bold are significant at least at the 1% level.

Z. abelicea and *Z. carpinifolia*), ITS2_B (*Z. sicula* and *Z. abelicea*) and ITS2_E (*Z. sicula* and *Z. carpinifolia*) (Appendix S3).

ITS ribotype richness was similar in *Z. abelicea* and *Z. carpinifolia* (*Rs* = 3.9 and *Rs* = 3.0, respectively), but still lower than the six ribotypes found in population A1 of *Z. sicula* (Table 1). Contrasting results were obtained for ITS1 and ITS2: whereas most populations of the three species are monomorphic for ITS2, higher diversity is recorded for ITS1. This is particularly true for *Z. abelicea* (12 polymorphic populations for ITS1 versus one for ITS2) and for *Z. carpinifolia* (nine polymorphic populations for ITS1 versus two for ITS2; Table 1). For *Z. sicula*, four ribotypes were found in ITS1, and six in ITS2. Gene diversity was higher in *Z. sicula* than in *Z. abelicea* and *Z. carpinifolia* (Table 1), and nucleotide diversities were higher in *Z. carpinifolia* than in *Z. abelicea*, with *Z. sicula* again showing the highest value.

The ribotype MJNs display different patterns for ITS1 and ITS2 (Fig. 2a,b). For ITS1, all the ribotypes for the three studied species are derived from two sequences from *Zelkova serrata* (Zse3 and Zse4). Ribotype F, which is shared among the three species, is in an internal position and gives rise to most of the other ribotypes (except A, D, H and G). The ribotypes seen in *Z. sicula* (population A1) are close to those of *Z. abelicea*. The ribotypes seen in *Z. carpinifolia* are clustered into two groups, one branching directly from ribotype F and the other branching off from the *Z. serrata*

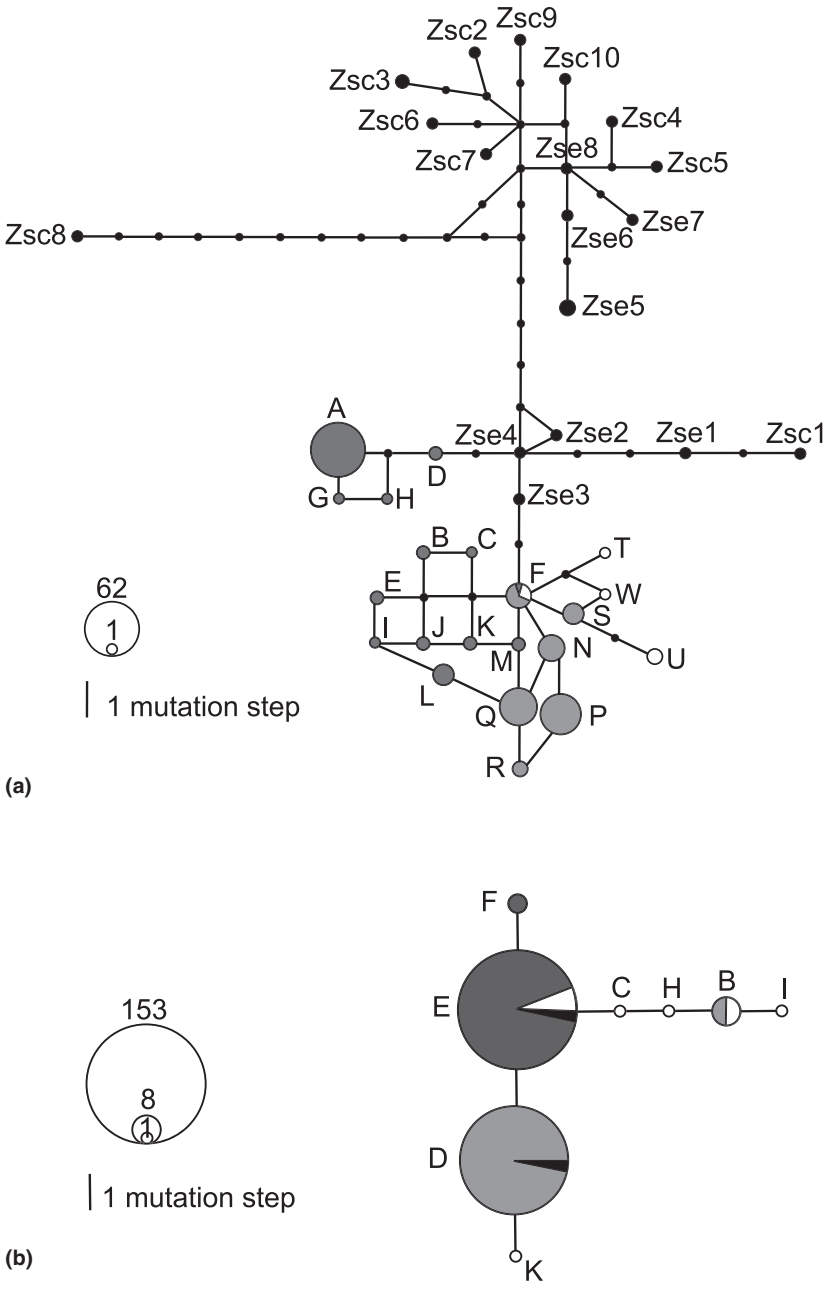

Figure 2 Median joining network for ribotypes of the nuclear ITS1 (a) and ITS2 (b) in six Eurasian *Zelkova* species. The size of each ribotoype is proportional to the number of individuals that share it and ribotypes are coloured according to the species they belong to (light grey, *Z. abelicea*; dark grey, *Z. carpinifolia*; white, *Z. sicula*; black, *Z. schneideriana*, *Z. serrata* and *Z. sinica*). For *Z. serrata*, Zse1–8 refer to GenBank accessions AJ622874, AJ622875, AJ622876, AJ622877, AJ622870, AJ622873, AJ622868 and AJ622869, respectively. For *Z. schneideriana*, Zsc1–10 refer to GenBank accessions AJ622867, AJ622860, AJ622858, AJ622861, AJ622862, AJ622857, AJ622864, AJ622856, AJ622865 and AJ622866, respectively. The black dots without names on the lines are missing or non-sampled ribotypes.

ribotype Zse4. The ribotypes seen in *Z. serrata* are found in the middle of the network (Zse1 to Zse4), or (Zse5 to Zse8) close to those in *Z. schneideriana*. For ITS2, two ribotypes are predominant (D and E), with only one mutation separating them. Ribotype K of *Z. sicula* is derived from ribotype D, which is predominantly found in *Z. abelicea*, whereas ribotypes C, H, B and I, also from *Z. sicula*, are serially derived from ribotype E, which is found predominantly in *Z. carpinifolia* but also in *Z. sinica* and *Z. scheideriana*. Differentiation indices within species were significant for *Z. abelicea* and *Z. carpinifolia* (*Z. abelicea*: $F_{ST} = 0.29$, $P < 0.001$; *Z. carpinifolia*: $F_{ST} = 0.26$, $P < 0.05$).

DISCUSSION

An overall high molecular diversity

A very large number of chloroplast haplotypes was found in each of *Z. abelicea* and *Z. carpinifolia*, coupled with significant genetic structure. Such high numbers of haplotypes are seldom found in tree species (but see Fukatsu *et al.*, 2012, for *Z. serrata*), even in species that are thought to have diversified long ago (Magri *et al.*, 2007; Rodríguez-Sánchez *et al.*, 2009; Caetano & Naciri, 2011). Tree species are usually characterized by long generation times and low mutation rates (Petit & Hampe, 2006). Such high diversity suggests an ancient origin of the species and limited genetic drift through time. In contrast, the history of *Z. sicula* is clearly marked by one or more severe bottlenecks.

Structured diversity was found for ITS, and especially for ITS1. In contrast with the chloroplast haplotypes however, some ribotypes are shared among species. ITS is known to have a multicopy structure, and hundreds or thousands of copies are generally found in a typical plant genome. The amplified sequence is therefore the consensus of many targets in the cell (Poczai & Hyvönen, 2010). Intragenomic polymorphism due either to heterozygosity or to paralogy was recorded in all three species for ITS1 but only in *Z. sicula* for ITS2. For ITS1, a particularly stable GC content was recorded both among and within species, indicating that no pseudogenes were amplified. More variation among species in GC content was recorded for ITS2, but the variation within species was very low, except for *Z. sicula*, which had one ribotype (ITS2_C) with a higher GC content than average. Nonetheless, no pseudogenes could be clearly identified, as all the sequences contained the two highly conserved motifs M2 and M3. For ITS1 and ITS2, the mean GC contents (61% and 59%, respectively) were found to be at the lower end of the range of those recorded in other genera (Grimm & Denk, 2008; Hřibová *et al.*, 2011). The discrepancy between ITS1 and ITS2 variability, as observed in *Zelkova*, is probably due to ITS2 experiencing stronger constraints than ITS1 due to its secondary structure (Coleman, 2007; Hřibová *et al.*, 2011).

A number of authors have highlighted the fact that ITS can be used to trace events of polyploidization or hybridization (Mayol & Rosselló, 2001; Nieto Feliner & Rosselló, 2007; Grimm & Denk, 2008; Hřibová *et al.*, 2011). Buckler *et al.* (1997) indicated that heterogeneity in the ITS sequence is more frequently found in organisms that are either hybrids or polyploids. We will therefore use the chloroplast data to infer species phylogeography, because chloroplasts are exclusively inherited through seeds, the vectors of colonization. ITS sequences will be used to better understand the role of past hybridization and polyploidization in the genus *Zelkova*.

Zelkova abelicea: diversification and structuring

Zelkova abelicea has a high level of genetic variation, as reported by Fineschi *et al.* (2004), and a high proportion of polymorphic populations (93%), indicating that the colonization of Crete is ancient. Most populations (57%) are found in the Lefka Ori massif and host 48% of the haplotypes. The genetic diversity of *Z. abelicea* is structured according to the four mountain chains of the island (Fig. 1c), with signs of isolation by distance, as indicated by Mantel tests; 59% of the total genetic variance is explained by variation among the four massifs, which indicates that gene flow mediated by seeds must have been negligible for a long time. This is corroborated by the fact that seed dispersal in *Zelkova* occurs only over short distances (Wang *et al.*, 2001). Only two populations have haplotypes that are not consistently clustered with geography (LAS1 and OMA2; Fig. 1c): this might be the result of long-distance dispersal events (LDD), mediated by animals or humans. A demographic expansion scenario is more likely for *Z. abelicea* than a spatial expansion scenario. The F_S statistic further supports this demographic hypothesis, as Fu (1997) noticed that this statistic is very sensitive to demographic expansion, which usually leads to large negative F_S values, as recorded here.

By the early Miocene (12–23 Ma), Crete had already emerged and was separated by a sea, at its northern edge, from the lowland that occupied almost all of the current Aegean Sea (Triantis & Mylonas, 2009). At the end of the Miocene and during the Pliocene (2–12 Ma), most of the Aegean region was flooded, and Crete was isolated and split into three islands, corresponding to the Lefka Ori mountains, the Psiloritis massif and the Dikti/Thrypti region. Whereas the colonization of Crete by *Z. abelicea* probably took place before the early Miocene, the latter period might be the time at which population structuring began. When calculated for the four regions separately, the demographic scenario was also supported. When excluding populations that may have been founded by LDD events, Lefka Ori may have been the first massif where *Z. abelicea* experienced a demographic expansion, according to estimates of τ (Table 3).

Zelkova carpinifolia: diversification and massive extinctions

This species is the second most diverse of the three, displaying 15 haplotypes clustered into two equal groups separated

by 19 mutations. This diversity translates into the highest nucleotide diversity recorded for the three species, although the gene diversity and haplotype richness are similar to those of *Z. abelicea*. Because 56% of the populations are monomorphic, F_{ST} is also high. This lack of within-population diversity might be a sign of past spatial expansions with haplotype surfing (Excoffier & Ray, 2008). Accordingly, the spatial scenario is preferred to the demographic one; the non-significant Mantel test also supports this hypothesis.

One of the peculiarities of *Z. carpinifolia* is the large gap between the two haplotype clusters: the first comprises all eastern populations from the Hyrcanian region (Talysh and Alborz mountains), and the second comprises all the western populations from the Colchic region (western Georgia and north-eastern Turkey). These correspond to two known biogeographical regions, separated by a geographical barrier (Browicz, 1982). The large mutational gap between the two clusters suggests a very ancient history of diversification followed by extinctions that erased all the intermediate haplotypes. This can be interpreted as the footprint of a more wide-ranging ancestral species that occupied nearly all of the Northern Hemisphere during the Miocene and then became fragmented following climate changes. Alternative explanations could be LDD or chloroplast capture. LDD would introduce some incongruence between geographical distribution and haplotype genetic distance, which is clearly not the case, and chloroplast capture usually involves one haplotype rather than a whole cluster of haplotypes. The hypothesis that is most strongly supported is therefore that of an initial intraspecific polymorphism previously far more important than that recorded now. This corresponds to a clear genetic characteristic of a relict species.

Our molecular results fail to support the taxonomic distinctiveness of populations from the Talysh mountains (GUN), described as *Z. hyrcana*, or that of the population from Trabzon in Turkey (TRA), described as *Z. carpinifolia* subsp. *yamraensis*.

Zelkova sicula: a result of ancient hybridization?

Zelkova sicula has very characteristic patterns for both types of markers. The two known populations are both characterized by a unique chloroplast haplotype. The two haplotypes are separated by several mutations and are located between those of *Z. abelicea* and *Z. carpinifolia*. As this species is triploid and clonal, the two populations may have developed from a very small number of individuals.

Zelkova sicula might be of hybrid origin with parents close to the ancestral species of *Z. abelicea* and *Z. carpinifolia*. Accordingly, incomplete concerted evolution was found for ITS2, which is otherwise highly conserved, and this concurs with other studies that demonstrate higher ITS polymorphism in species of hybrid origin (Poczai & Hyvönen, 2010). Fossil leaves dating from 300,000 BP in Italy (Riano Romano) and from Miocene–Pliocene deposits in France (Lac Chambon) were attributed to *Z. carpinifolia* and then later interpreted as being from *Z. sicula* once this species had been described (Nakagawa *et al.*, 1998). The leaf morphology of the two populations supports the hybrid origin, with population A1 being closer to *Z. abelicea* and population A2 closer to *Z. carpinifolia* (Rucinska, 2012). Accordingly, we found that some ITS1 and ITS2 ribotypes in population A1 are identical to those of *Z. abelicea* whereas the ITS2 ribotype of population A2 is similar to that of *Z. carpinifolia*.

According to pollen records (Follieri *et al.*, 1998; Magri *et al.*, 2010), *Zelkova* expanded in the Italian Peninsula during the last interglacial period. It is thought to have disappeared from this region 31,000 BP, having been abundant between 128,000 and 75,000 BP (Follieri *et al.*, 1986, 1988). Many populations from different ancestors could have persisted at times in refugia in Sicily, where *Zelkova* persisted in relative abundance until *c.* 20,000 BP (Sadori *et al.*, 2008). Habitat disturbance is suspected to have increased the frequency of hybridization events, as reported for some alpine plants (Choler *et al.*, 2004), and hybridization events might have occurred during one of the glacial cycles when the vegetation in Europe was forced to migrate southwards. This process must have been accompanied by strict bottlenecks, because only two haplotypes escaped extinction. *Zelkova sicula* is therefore an extremely important taxon and merits immediate conservation action.

CONCLUSIONS

Our results have addressed the three main questions we identified. First, the genus can be divided into two main groups: the south-western Eurasian group, comprising *Z. sicula*, *Z. abelicea* and *Z. carpinifolia*; and the East Asian group, comprising *Z. sinica*, *Z. serrata* and *Z. schneideriana*. *Zelkova carpinifolia* is genetically closer the East Asian taxa according to chloroplast markers. The three south-western Eurasian species have haplotypes that are distinctly clustered according to species assignment.

Second, although *Z. abelicea* covers a relatively small geographical area, it is highly genetically diverse within each of the four mountain massifs where it occurs, representing separate genetic units. For *Z. carpinifolia*, two main clades were identified, an eastern clade in Azerbaijan and Iran, including the highly isolated population in eastern Georgia, and a western clade in western Georgia and north-eastern Turkey. The large gap between the two clusters suggests a very ancient history of diversification followed by widespread extinctions. *Zelkova sicula* was confirmed to be genetically impoverished and is suspected to have a hybrid origin with parents that were close to the widespread ancestral species of *Z. abelicea* and *Z. carpinifolia*. This assumption needs further investigation, and additional molecular markers are required to better test this hypothesis.

Third, haplotype and ribotype diversities showed similar trends for *Z. abelicea* and *Z. carpinifolia*. Discrepancies between the chloroplast and nuclear data sets exist, such as

ITS2 showing reduced diversity, probably due to strict evolutionary constraints, or the south-western Eurasian group being closer to *Z. serrata* for ITS1, in agreement with Denk & Grimm (2005). Moreover, the ITS data set suggests a putative hybrid origin for *Z. sicula*, a conclusion that would have not been retrieved from the chloroplast markers alone.

Our results provide a unique tool for both *in situ* and *ex situ* approaches aimed at conserving the genetic diversity in *Zelkova*, which represent a significant evolutionary heritage (Petit *et al.*, 2005a).

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Appendix S1 The institutions and number of *ex situ* individuals of *Zelkova schneideriana*, *Z. serrata* and *Z. sinica* used in this study.

Appendix S2 Sequence names and source of primers.

Appendix S3 The substitutions and indels for the chloroplast haplotypes at the locus *trn*H–*psb*A and *trn*L, and for the nuclear ribotypes at ITS1 and ITS2. The number of individuals and the species identity are given for each haplotype and each ribotype.

BIOSKETCH

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Author contributions: Y.N. and G.K. developed the experimental ideas; D.F., E.M., G.G., S.P. and S.B. conducted the field work; C.C. analysed the samples under the supervision of Y.N.; and C.C, Y.N. and G.K. led the writing of the manuscript.

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