ORIGINAL ARTICLE

Extending glacial refugia for a European tree: genetic markers show that Iberian populations of white elm are native relicts and not introductions

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Conservation policies usually focus on *in situ* protection of native populations, a priority that requires accurate assessment of population status. Distinction between native and introduced status can be particularly difficult (and at the same time, is most important) for species whose natural habitat has become both rare and highly fragmented. Here, we address the status of the white elm (*Ulmus laevis* Pallas), a European riparian tree species whose populations have been fragmented by human activity and is protected wherever it is considered native. Small populations of this species are located in Iberia, where they are unprotected because they are considered introductions due to their rarity. However, Iberia and neighbouring regions in southwestern France have been shown to support discrete glacial refuge populations of many European trees, and the possibility remains that Iberian white elms are native relicts. We used chloroplast RFLPs and nuclear microsatellites to establish the relationship between populations in Iberia and the Central European core distribution. Bayesian approaches revealed significant spatial structure across populations. Those in Iberia and southwestern France shared alleles absent from Central Europe, and showed spatial population structure within Iberia common in recognized native taxa. Iberian populations show a demographic signature of ancient population bottlenecks, while those in Central European show a signature of recent population bottlenecks. These patterns are not consistent with historical introduction of white elm to Iberia, and instead strongly support native status, arguing for immediate implementation of conservation measures for white elm populations in Spain and contiguous areas of southern France.
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INTRODUCTION

Populations at the periphery of a species' range are important in the conservation of endangered species, and of biological diversity in general (Channell and Lomolino, 2000). For many widespread tree species, it is precisely the populations at the edge of the distribution range that accumulate most of the intraspecific genetic variation, either because they correspond to glacial (micro-) refugia (for example, Kelly *et al.*, 2010; Anderson *et al.*, 2011), or because they represent contact zones between migrants from such refugia (Hewitt, 2004; Magri *et al.*, 2006). Conservation of natural marginal populations of species has therefore long been considered crucial for the preservation of genetic diversity and evolutionary potential (Newton *et al.*, 1999; Moritz, 2002). Usually, only populations thought to be native are prioritized for conservation (for example, in the Red List of the International Union for Conservation of Nature (IUCN, 2001)), and populations thought to be introduced or of dubious status are typically not protected (Decocq *et al.*, 2004). However, it can be difficult to apply the full range of ecological, physiological and biogeographical criteria considered desirable in assessment of native status at the margins of a species' range, raising the importance of assessing population status using genetic information.

The European white elm (*Ulmus laevis* Pallas) is a broadleaved, deciduous tree characteristic of river margins and damp lowland forests. It is one of the few trees tolerant to prolonged flooding (Collin, 2003), and constitutes an important foundation species in suitable habitats. Suitable riparian habitats have been widely and extensively disturbed by transformation into agricultural and pasture land, flow regulation of rivers and mining (Hooke, 2006). A combination of habitat destruction and Dutch elm disease have compromised the long-term survival of *U. laevis* through most of its distribution range, leaving many small and isolated populations vulnerable to genetic drift (Collin *et al.*, 2004). The European Forest Genetic Resources programme therefore identifies *U. laevis* as a species requiring conservation (Collin, 2003).

The natural distribution of white elm is currently regarded as extending across central and eastern Europe, from the Ural Mountains to eastern France, and from southern Finland to the Caucasus and Bosnia (Jalas and Suominen, 1999; Collin, 2003). Populations have also long been recorded in Spain, including the Pyrenees (Lapeyrouse, 1813) and Asturias (Pastor, 1853), and were included in the first Iberian floras (Willkomm and Lange, 1861). However, on the basis of only its rarity and in the absence of any historical evidence of

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introduction, this species is currently considered introduced in Spain (Navarro and Castroviejo, 1993; Collin, 2003) and it has been excluded from subsequent Spanish floras (Ceballos, 1966; Ruiz de la Torre, 1971). As a result, populations of *U. laevis* in the Iberian Peninsula and neighbouring regions of southern France (Timbal and Collin, 1999) are unprotected. The alien status of white elm in Iberia was challenged by Segura (1973) following discovery of a previously unknown population in Central Iberia, and over the last 10 years 53 Spanish locations for this species have been found during field surveys by the Spanish Elm Conservation and Breeding Programme (SECBP). The key question is whether these geographically widespread but very local populations represent native relict fragments, or the dispersed introductions inferred for some other species (Decocq *et al.*, 2004; Gil *et al.*, 2004).

Fossil records, particularly pollen, can provide conclusive evidence of native status for some plants (Webb, 1985; Postigo-Mijarra *et al.*, 2008). However, pollen is only identifiable at the genus level for European elms (Stafford, 1995). In the absence of fossil data, neutral genetic markers can offer evidence as to the native status of populations of uncertain origin. Native Iberian populations of widespread European trees are commonly divergent genetically from populations in central and eastern Europe, reflecting confinement to, and limited gene flow between, discrete glacial refugia through the Pleistocene (King and Ferris, 1998; Petit *et al.*, 2002; Hampe *et al.*, 2003; Rendell and Ennos, 2003; Heuertz *et al.*, 2004; Magri *et al.*, 2006; Maliouchenko *et al.*, 2007). Such separation is also commonly associated with region-specific genetic variation in Iberia and immediately contiguous regions of southern France that is absent from Central Europe and regions further east.

In contrast, introduced populations are characterized by possessing a subset of genetic diversity found in source populations (Stone and Sunnucks, 1993). If introduced from a single alternative refuge population, introductions are often genetically depauperate (Webb, 1985; Stone and Sunnucks, 1993; Bossdorf *et al.*, 2005), though introductions or colonizations from multiple populations can, together, show higher genetic diversity than any single source (for a natural example in *Fagus*, see Comps *et al.*, 2000).

Under the 'Relict hypothesis', we expect Iberian populations to possess regionally private genetic diversity absent from Central Europe. Under the 'Introduction hypothesis' we expect Iberian populations to contain a subset of genetic diversity associated with the Central European core distribution of white elm, and to lack regionally private genetic variants. Here, we test these hypotheses using chloroplast (cpDNA) RFLP and nuclear (nDNA) microsatellite markers for European populations of *U. laevis* sampled from Spain, neighbouring regions of southern France and Central Europe. We build on a previous analysis using only samples from Europe outside Iberia (Whiteley, 2004) that identified three cpDNA haplotypes in *U. laevis*: a common haplotype A, extending from France to NW Russia, and two rare haplotypes—haplotype B confined to southern France and haplotype C confined to the Balkans and SW Russia. Whiteley (2004) argued that the presence of both haplotypes A and C in Russia is compatible with a core Russian glacial refuge, from which current white elm populations would have originated by range expansion. This idea was first proposed by Huntley and Birks (1983). However, Whiteley (2004) also recognizes that the southern distributions of haplotypes B and C could indicate additional refugia.

In order to further discriminate between the Relict and Introduction hypotheses, we also use a combination of analyses (Williamson-Natesan, 2005) that permit detecting population bottlenecks over recent and longer timescales. If Iberian white elms are descendants of

repeated isolated introductions of small numbers of individuals from larger source populations, we expect them to show signatures of recent population bottlenecks (Garza and Williamson, 2001).

MATERIALS AND METHODS
Sampling
Our analysis incorporates data for 242 elms from 20 populations, 186 individuals from 11 populations in Spain and 56 individuals in 9 populations from the rest of Europe. Sampling in Iberia spanned the known distribution (Figure 1, Supplementary Table S1). Four Spanish populations (ATA, ERR, ORO and TDU) correspond to the populations considered 'naturalized' (but of no specified origin) in the Flora Iberica (Navarro and Castroviejo, 1993). The remainder comprises locations discovered during SECBP field surveys. Whenever possible we sampled trees separated by >50 m in order to avoid collecting leaves from closely related individuals, as this could result in a bias in allele frequency and diversity parameters estimates (Ruiz-Guajardo *et al.*, 2010).

The 56 European *U. laevis* DNA samples from France, Belgium and Germany were obtained from the Royal Botanic Garden of Edinburgh (RBGE) (see Figure 1). These are a subset of samples from the EU project 'Coordination for conservation, characterization, collection and utilization of genetic resources of European Elms' (RESGEN CT96-78) (Collin *et al.*, 2004) and were included to represent genetic diversity in the core distribution for this species (shaded grey in Figure 1).

DNA extraction and scoring of molecular markers
We extracted DNA from fresh leaves using a modified protocol from the study by Dumolin *et al.* (1995), in which ∼1 cm^2 of ground leaf material and 20–30 mg of insoluble polyvinylpirrolidone were combined with 1 ml of 1× ATMAB buffer, plus 0.1% *w/v* 1,4-Diothiothreitol.

Chloroplast DNA (cpDNA) was analysed by PCR–RFLP using regions *trn*S–*trn*fM, *trn*H–*trn*K and *trn*D–*trn*T (Demesure *et al.*, 1995), previously
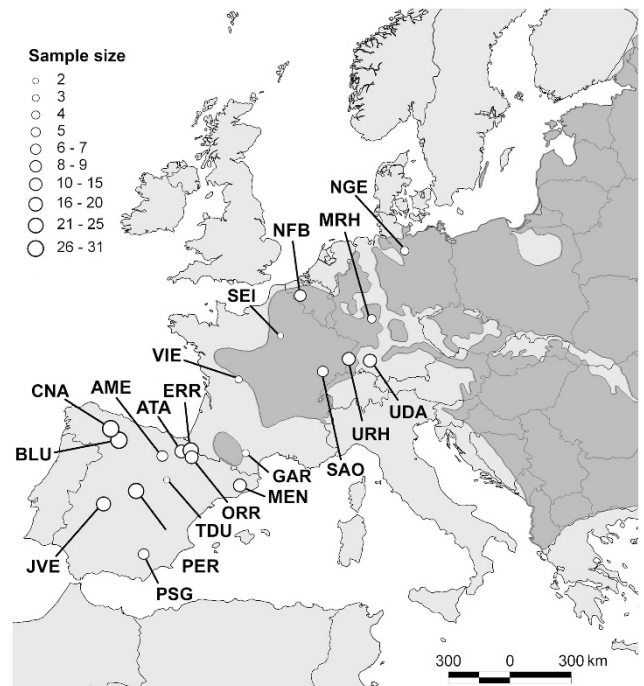

Figure 1 Location of the 20 populations of *U. laevis* included in this study (white circles). Population codes correspond to Table 1. Spanish populations were chosen to cover the known species range. The shaded area shows the most recent distribution map of *U. laevis* published by the European Forest Genetic Resources Programme (EUFORGEN), including the populations of South France (Collin, 2003). (Map created using Lambert's equal-area projection).

shown to vary in European elms (Gil *et al.*, 2004; Whiteley, 2004). Amplicons were digested with restriction enzymes (fragment-enzyme combinations: SfM-*Mva*I, HK-*Hae*III, DT-*Taq*I). Restriction fragments were separated by electrophoresis in 8% non-denaturing polyacrylamide gels and visualized using silver staining. Chloroplast haplotypes were then assigned by combining the observed polymorphisms of each PCR fragment-restriction enzyme combination. Correspondence of observed haplotypes to those previously described (Whiteley, 2004) was established with reference to 39 common samples stored at the RBGE (Whiteley, pers. comm.).

To verify sequence conservation of the PCR–RFLP haplotypes across their distribution ranges, the two observed haplotypes for the SfM digest region (the only variable region) were sequenced for eight samples (four from Spain, two from France, one from Belgium and one from Germany, specified in Supplementary Table S1), spanning the distribution of both haplotypes. SfM products were sequenced on a CEQ 8000 Beckman-Coulter automated sequencer (Beckman-Coulter, Brea, CA, USA), and edited and aligned using Geneious Pro 5.1.7 (Biomatters, Auckland, New Zealand).

Five nuclear microsatellite loci (*ulm2*, *ulm3*, *ulm6*, *ulm9* and *ulm19*) developed for *U. laevis* were screened following PCR conditions given in Whiteley *et al.* (2003). PCR products were denatured and separated in 6% (*w/v*) acrylamide:bisacrylamide (19:1) gels using a Li-Cor 4300 automatic sequencer (Li-Cor Biosciences, Lincoln, NE, USA), and alleles were scored using Gene ImagIR 3.56 (Scanalytics, Milwaukee, WI, USA). A molecular size standard (Sequamark, Invitrogen, Carlsbad, CA, USA) was included in each gel for the identification of allele sizes. All 242 samples were screened for all loci.

Bayesian identification of genotype pools in *U. laevis*

Two Bayesian clustering programs were used to assess population structure in the microsatellite data set: STRUCTURE v.2.1 (Pritchard *et al.*, 2000) and BAPS v.4.0 (Corander *et al.*, 2008). First, we used STRUCTURE to reveal individual-based patterns in genetic variation without prior information on sample geographic origin. We performed three independent runs for each value of K from 1–21 (number of sampling locations plus one). The no admixture ancestry model was assumed, as it is more successful at detecting subtle differentiation (Pritchard and Wen, 2003). We used a MCMC length of 1 200 000 with burnin length of 200 000 (enough for the summary statistics to have converged). For values of $K \geqslant 2$ the proportion of each individual's genotype assigned to each genetic cluster was roughly symmetric, indicating no population structure (Pritchard and Wen, 2003). To detect subdivision within the two clusters observed, the analysis was repeated independently for individuals included within them (Pritchard and Wen, 2003). Second, we used BAPS to incorporate sample geographic information. The 56 samples from the RBGE represent individuals sampled over larger geographical areas rather than from discrete populations. To allow incorporation of geographic location information for these samples, we divided them into nine groups based on geographic proximity and shared watershed (Supplementary Table S1). Geographic coordinates of each group were approximated using the geographic centroid of the individual sample origins. These nine groups were added to the 11 Iberian populations to give 20 geographic groupings in the BAPS analysis using the 'clustering of groups of individuals' option. There was not enough information in the data set to get conclusive results using individual clustering. We carried out 10 independent simulations for each value of K from 1–21.

Measures of genetic diversity and population structure

We compared the diversity and distribution of genetic variation for both cpDNA and microsatellite markers for each of the 11 Spanish populations, and for the genotype groupings supported by BAPS. We did not consider the nine *post hoc* Central European sample sets as populations in this context. Using the distance between observed haplotypes, cpDNA genetic diversity parameters corrected for variation in population sizes (Pons and Petit, 1995) were calculated for each population (h_k), and region (h_S), and overall populations (h_T), together with the coefficient of genetic differentiation among subpopulations (G_{ST}) within Iberia and among BAPS clusters using HAPSTEP (http://www.pierroton.inra.fr/genetics/labo/Software/; Pons and Petit, 1996). An analysis of molecular variance (AMOVA) was run using GENALEX (Peakall

and Smouse, 2006) to test significance of genetic diversity partitioning among BAPS clusters, among groups of individuals within BAPS clusters, and within populations, based on 999 permutations.

From the microsatellite data, we estimated mean number of alleles (A), mean allelic richness per sample (Rs), the number of regionally private alleles (Ap), percentage of polymorphic loci (P), mean expected (H_E) and observed (H_O) heterozygosities and inbreeding coefficients (F_{IS}) using FSTAT (Goudet, 2001). We calculated these parameters for the Spanish populations and, at the regional level, for the BAPS groups. The latter regional analysis is also intended to reduce potential biases introduced by small sample sizes in the Central European populations. We tested the statistical significance of differences in diversity parameters between regions in FSTAT using either a Mann–Whitney U-test (A) or permutation tests (Rs, H_E and F_{IS}). We tested departures from Hardy-Weinberg equilibrium (HWE) within populations, within regions and overall using permutation tests in FSTAT. We estimated the population differentiation parameter F_{ST} (Weir and Cockerham, 1984) for populations within Iberia, and between BAPS groups using FSTAT; significant deviations from zero population differentiation were assessed with permutation tests. We carried out an AMOVA analysis of microsatellite diversity using GENALEX at the same hierarchical levels used in analysis of the cpDNA data.

Finally, we used BayesAss (Wilson and Rannala, 2003) to estimate rates of recent migration (over the last several generations, which correspond to the time scale of putative human-mediated movement of *U. laevis*) among BAPS groups. We performed the analysis with 30 000 000 iterations, with a burnin period of 999 999, and a sampling frequency of 2000. Delta values for allele's frequencies, migration rate and inbreeding were set to 0.15 (default value).

Demographic analysis

We used M-value (Garza and Williamson, 2001) and BOTTLENECK v.1.2.02 (Cornuet and Luikart, 1996) analysis of microsatellite data to assess the evidence for population bottlenecks in all Iberian populations containing >11 individuals, and, across western Europe, in the genetic clusters supported by BAPS. M-value is the mean ratio of the number of alleles to the range in allele size. This ratio decreases when a population is reduced in size, and M-values of <0.68 are indicative of a genetic bottleneck (Garza and Williamson, 2001). For the BOTTLENECK analysis, we ran a Wilcoxon sign rank test with 10 000 iterations under the infinite alleles (IAM), stepwise mutation (SMM) and the two-phase (TPM) models. The SMM and IAM represent two extreme mutation models, with the SMM being the most conservative model to test for heterozygosity excess (Cornuet and Luikart, 1996). As the TPM may more closely capture the mutation behaviour of microsatellites (Di Rienzo *et al.*, 1994; Williamson-Natesan, 2005), here we emphasize results with this approach. Simulation studies (Williamson-Natesan, 2005) have shown that tests based on the range in allele size conditioned on the number of alleles (M-value) are better able to detect a bottleneck lasting several generations followed by demographic recovery (see also Garza and Williamson, 2001). In contrast, tests based on heterozygosity given the number of alleles (BOTTLENECK) are better able to identify recent, less-severe bottlenecks. Our premise is that because Iberian populations have been rare throughout recorded history, a bottleneck signature in allele-size distributions is more likely to be associated with remnant native populations than a recent introduction.

RESULTS

Bayesian analysis of population structure

STRUCTURE analysis supported division of Europe-wide samples into two clusters (named *I* and *II*) that correspond to two geographic regions (Table 1). Cluster *I* comprised six populations in southwestern Europe: five in western and southern Spain (CNA, AME, BLU, JVE and PSG), all with a high (>0.750) proportion of membership assigned to cluster *I*, and the southern-most French group of individuals (GAR), with an assignment probability of 0.649. The remaining Iberian populations and European groups were assigned to Cluster *II* with probabilities ranging from 0.537–0.995. When each cluster was analysed independently, only cluster *II* showed evidence of further substructure, and was divided into two subclusters

Table 1 Classification of *U. laevis* populations into genetically homogeneous clusters and subclusters inferred from the STRUCTURE analysis (Figure 1)

Population	Country	N	Cluster-subcl.	Prob cl (subcl)
JVE	Spain	20	I	0.935
PSG	Spain	6	I	0.753
BLU	Spain	31	I	0.907
CNA	Spain	30	I	0.854
AME	Spain	7	I	0.983
GAR	France	3	I	0.649
PER	Spain	25	II (a)	0.662 (0.631)
ATA	Spain	13	II (a)	0.588 (0.633)
ORO	Spain	15	II (a)	0.779 (0.756)
ERR	Spain	23	II (a)	0.829 (0.857)
MEN	Spain	14	II (a)	0.866 (0.912)
VIE	France	3	II (a)	0.537 (0.926)
TDU	Spain	2	II (b)	0.973 (0.885)
SEI	France	2	II (b)	0.940 (0.657)
SAO	France	6	II (b)	0.769 (0.608)
URH	France–Germany	12	II (b)	0.864 (0.571)
UDA	Germany	13	II (b)	0.825 (0.706)
NFB	Belgium–France	9	II (b)	0.821 (0.743)
MRH	Germany	4	II (b)	0.988 (0.777)
NGE	Germany	4	II (b)	0.995 (0.799)

The proportion of individuals from each population assigned to each group is indicated.
N = number of samples per population included in the analysis.

Figure 2 Distribution of cpDNA haplotypes and clusters of populations based on five nuclear microsatellites for *U. laevis*. The two cpDNA haplotypes are shown in blue (**A**) and red (**B**). Pie charts represent the proportion of individuals observed for each haplotype in each population (Table 1), with sizes proportional to the number of individuals analysed. For the nuclear SSR markers, the map shows the Voronoi tessellation and assignment to the five clusters (**A**–**E**) obtained with BAPS (map created using Lambert's equal-area projection).

(*IIa* and *IIb*, Table 1). Subcluster *IIa* is formed by five populations from central and northern Spain (PER, ATA, ERR, ORO and MEN) plus a group of individuals from southern France (VIE). Subcluster *IIb* comprises the groups from the core distribution of the species in central Europe (SAO, SEI, NFB, URH, UDA, MRH and NGE), plus one Spanish population (TDU, with only two individuals). STRUCTURE analysis is a conservative grouping approach agnostic of prior geographic origin. Grouping of TDU in subcluster *IIb* is the only geographic inconsistency observed, suggesting that it is valid to use BAPS to incorporate further geographic information.

BAPS results provided strong posterior probability (88%) support for allocation of individuals into five genetic groups showing clear geographical structure (Figure 2). Cluster *A* includes the core Central European populations from northern and eastern France, Belgium and Germany, whereas clusters *B*–*E* comprise populations in Spain, western and southern France. Cluster *B* comprises 5 populations in central and northern Spain and the two southern-most French populations. This BAPS cluster contains most of the same individuals in STRUCTURE's subcluster *IIa*, with the addition of the individuals from site TDU in central Spain (the outlier in STRUCTURE subcluster *IIb*) and GAR (a population assigned with relatively low probability to in STRUCTURE cluster *I*). Clusters *C* and *E* each consisted of one Spanish population (MEN and CNA respectively). Cluster *D* comprises four Spanish populations located in separate watersheds (AME, BLU, JVE and PSG), also grouped together in STRUCTURE cluster *I*. It is worth noticing how the incorporation of geographical information in the BAPS analysis seems to help detecting more detailed genetic grouping than STRUCTURE.

Genetic diversity
Chloroplast DNA. Of the three cpDNA regions analysed by PCR–RFLP, only fragment SfM was polymorphic, with two size variants in one of the two fragments ob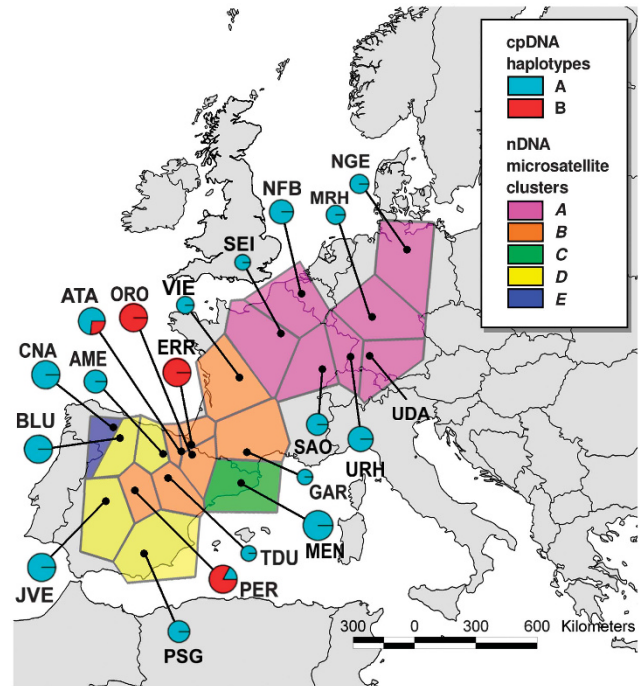tained after digestion with *Mva*I. This polymorphism distinguished two cpDNA haplotypes—A (1241 base pairs, bp) and B (1248 bp) differing by a 7-bp sequence (TAATAAA) present in haplotype B. Haplotype A was the commonest haplotype (75.3% of 150 samples analysed) (Table 2, Figure 2). It was the only variant detected in Central European samples, and was also found in nine Spanish populations (of which it was fixed in seven; Table 2). Haplotype B (24.7% of individuals) was restricted to Iberia, being found in four Spanish populations, and fixed in two (ERR, ORO) (Table 2). Iberian populations showed low within-population diversity ($h_S = 0.067$). However, the presence of haplotype B in these populations resulted in moderate total diversity ($h_T = 0.440$) and high population differentiation ($G_{ST} = 0.847$). When BAPS groupings were considered, all cpDNA variation was restricted to cluster B in central-north Iberia plus southern France ($h_k = 0.440$), with all other clusters fixed for haplotype A, resulting in lower total genetic diversity ($h_T = 0.274$). Genetic differentiation among clusters was relatively high ($G_{ST} = 0.679$), although lower than among Iberian populations alone. AMOVA for Iberian populations showed that the majority (52%) of the genetic variation was found among BAPS clusters, with ~35% of variation observed among populations within clusters and only 13% within clusters (Supplementary Table S2). These percentages represent significant (<0.001) spatial structure at each level.

Nuclear microsatellites. Most loci were polymorphic in all 11 Iberian populations, except for 3 of them (AME, JVE and PSG) that were monomorphic at one locus (Table 2). Overall allelic diversity of Iberian populations was relatively low ($A = 2.62$, $Rs = 1.91$). In the

Table 2 Genetic variability parameters of twenty populations of *Ulmus laevis* based on cpDNA and nuclear SSR markers at the population, regional and global level

Population	Chloroplast DNA				Nuclear SSR								
	N	*h.A*	*h.B*	h_k	*N*	*A*	*Rs*	*Ap*	*P*	H_O	H_E	F_{IS}	
Iberia													
AME	7	7	0	0.000	7	2.2 (1–4)	1.59 (0.19)	0	80	0.314 (0.123)	0.312 (0.104)	−0.008	ns
ATA	11	8	3	0.436	13	2.4 (2–4)	1.84 (0.18)	0	100	0.462 (0.081)	0.433 (0.072)	−0.067	ns
BLU	12	12	0	0.000	31	3 (2–6)	1.77 (0.15)	1	100	0.465 (0.092)	0.395 (0.068)	−0.174	*
CNA	12	12	0	0.000	30	2.8 (2–5)	2.01 (0.23)	0	100	0.553 (0.113)	0.496 (0.089)	−0.116	ns
ERR	12	0	12	0.000	23	2.6 (2–4)	1.92 (0.24)	0	100	0.435 (0.103)	0.470 (0.113)	0.074	ns
JVE	12	12	0	0.000	20	2.4 (1–4)	1.73 (0.19)	0	80	0.450 (0.125)	0.387 (0.100)	−0.163	ns
MEN	14	14	0	0.000	14	2.6 (2–4)	1.95 (0.22)	0	100	0.510 (0.102)	0.477 (0.101)	−0.069	ns
ORO	12	0	12	0.000	15	3 (2–4)	1.96 (0.17)	1	100	0.587 (0.100)	0.481 (0.077)	−0.220	*
PER	12	2	10	0.303	25	3.2 (2–6)	2.04 (0.25)	0	100	0.584 (0.090)	0.501 (0.090)	−0.166	**
PSG	6	6	0	0.000	6	2.2 (1–4)	1.81 (0.33)	0	80	0.433 (0.180)	0.397 (0.139)	−0.092	ns
TDU	2	2	0	0.000	2	2.4 (2–3)	2.40 (0.24)	0	100	0.600 (0.100)	0.700 (0.122)	0.143	ns
Overall/mean	10.2				16.9	2.62	1.91	0.18	95	0.490	0.459	−0.078	ns
BAPS groups													
Cluster A	33	33	0	0.000	50	5 (2–7)	3.87 (0.66)	8	100	0.568 (0.135)	0.513 (0.077)	0.047	ns
Cluster B	54	17	37	0.440	83	3.4 (2–6)	2.98 (0.59)	1	100	0.518 (0.146)	0.477 (0.101)	−0.010	ns
Cluster C	14	14	0	0.000	14	2.6 (2–4)	2.59 (0.39)	0	100	0.510 (0.229)	0.496 (0.089)	−0.069	ns
Cluster D	37	37	0	0.000	64	3.4 (2–6)	2.67 (0.47)	1	100	0.441 (0.204)	0.404 (0.077)	−0.092	ns
Cluster E	12	12	0	0.000	30	2.8 (2–5)	2.73 (0.51)	0	100	0.553 (0.253)	0.596 (0.071)	−0.116	ns
Overall/mean	30.0				48.2	3.44	2.96	2.00	100	0.518	0.497	−0.029	ns
Global overall	150				242	5.4 (2–8)	2.16 (0.21)		100	0.526 (0.047)	0.511 (0.062)	−0.072	ns

Abbreviations: *N*, number of samples; *h.A*, *h.B*, number of samples corresponding to haplotypes A and B, respectively; h_k, intrapopulation genetic diversity; *A*, mean number of alleles per locus (range); *Rs*, mean estimated allelic richness per sample (s.d., standard deviation); *Ap*, number of private alleles per population (frequency; only in individual populations); *P*, percentage of polymorphic loci; H_O, observed heterozygosity (s.d.); H_E, expected gene diversity (s.d.); F_{IS}, inbreeding coefficient; ns, not significant; *, $P < 0.05$; **, $P < 0.01$.

Spanish populations the lowest values for most genetic diversity parameters were observed in AME ($A = 2.2$, $Rs = 1.59$), and the highest in PER ($A = 3.2$) and TDU ($Rs = 2.40$) (Table 2). Allele frequencies for all groupings were in Hardy–Weinberg equilibrium.

Microsatellite data revealed significant population structure among Iberian populations ($F_{ST} = 0.155$; $P < 0.001$) and across all groups of individuals (global $F_{ST} = 0.143$; $P < 0.001$). At the regional level, the test for population differentiation among BAPS clusters was highly significant ($F_{ST} = 0.126$; $P < 0.001$; Supplementary Table S3). Cluster A (Central Europe) showed the highest values in genetic diversity parameters, in terms of mean number of alleles per locus (5.0), mean allelic richness per sample (3.87) and gene diversity ($H_E = 0.513$). Three BAPS clusters showed private alleles: cluster A (with eight private alleles) in Central Europe, and clusters B and D in SW Europe (with one private allele each). The AMOVA among BAPS clusters also showed significant ($P < 0.001$) differentiation at all levels of partitioning (Supplementary Table S2): most (71%) of the variation was found within populations, with 19% among BAPS clusters and only 10% among populations within clusters.

Migration rates among BAPS clusters. Only in two pair-wise comparisons between BAPS clusters were BayesAss migration rates greater than 10%. These were migration from cluster B into cluster A ($m = 0.21$, s.e. $= 0.04$) and migration from cluster D into cluster E ($m = 0.12$, s.e. $= 0.10$) (Supplementary Table S4). The observed migration rate (and 95% confidence interval) in simulated data sets of five populations for when there is no information in the data is 0.0417 (1.79×10^{-5}, 0.185). Assuming normality in the distribution of migration rates, only the first migration rate (from cluster B into cluster A) resulted to be significantly different from the no-information migration rate (*t*-test $P = 0.001$).

Demographic analysis

The results of the bottleneck tests varied with the method used (Table 3). Analysis of allele size distributions (*M*-values) identified bottlenecks in six out of the eight Iberian populations tested (*M*-value < 0.68), and did not detect a bottleneck signal in the two European ones (URH and MRH; Table 3). All BAPS clusters from SW Europe (B, C, D and E) showed a signal of bottlenecking with *M*-value.

Analyses of heterozygosity (BOTTLENECK) showed significant heterozygosity excess ($P < 0.05$, Wilcoxon test), indicative of population bottlenecking for five populations under the two-phase model (Table 3), comprising four Spanish populations (ATA, CNA, ERR and PER). Only BAPS clusters *B* and *E* showed evidence of bottlenecking by this test. No population or genetic cluster showed a heterozygosity deficit indicative of population expansion.

DISCUSSION

Is *U. laevis* native to the Iberian Peninsula?

Our analysis substantially extends the range sampled in previous work on *U. laevis* (Whiteley, 2004; Nielsen and Kjær, 2010). We show that though populations in Iberia have low absolute genetic diversity, they contain regionally private genetic variation in both chloroplast sequence (haplotype B) and nuclear microsatellites. Differentiation between Iberia/SW France and Central Europe, and spatial structure among Iberian populations are further supported by regional differentiation of BAPS genotype clusters.

Table 3 Summary of population size reduction tests

Population	N	M	# H exc.	Bottleneck test			Expansion test		
				SMM	TGM (40%)	IAM	SMM	TGM (40%)	IAM
ATA	13	0.717	4/5	**0.0469**	**0.0313**	**0.0313**	0.9688	0.9844	0.9844
BLU	31	**0.558**	3/5	0.5000	0.4063	0.1094	0.5938	0.8906	0.9219
CNA	30	**0.549**	5/5	**0.0156**	**0.0156**	**0.0156**	1.0000	1.0000	1.0000
ERR	23	0.925	4/5	0.1094	**0.0469**	**0.0313**	0.9219	0.9688	0.9844
JVE	20	**0.604**	4/5	0.1563	0.0625	**0.0313**	0.9063	0.9688	1.0000
MEN	14	**0.525**	4/5	0.0781	0.0781	**0.0313**	0.9531	0.9531	0.9844
ORO	15	**0.590**	4/5	0.5938	0.0781	**0.0469**	0.5000	0.9531	0.9688
PER	25	**0.583**	5/5	**0.0313**	**0.0156**	**0.0156**	0.9844	1.0000	1.0000
URH	12	0.736	5/5	**0.0469**	**0.0313**	**0.0156**	0.9688	0.9844	1.0000
MRH	13	0.730	5/5	0.4063	0.0781	**0.0156**	0.8906	0.9688	1.0000
CLUSTER A	50	0.702	5/5	0.8906	0.0781	**0.0156**	0.3125	0.9843	1.0000
CLUSTER B	84	**0.608**	5/5	**0.0156**	**0.0156**	**0.0156**	0.9844	1.0000	1.0000
CLUSTER C	14	**0.525**	4/5	0.0781	0.0781	**0.0313**	0.9531	0.9687	0.9844
CLUSTER D	64	**0.608**	3/5	0.8906	0.5000	0.1094	0.3125	0.6875	0.8906
CLUSTER E	30	**0.549**	5/5	**0.0156**	**0.0156**	**0.0156**	1.0000	1.0000	1.0000

Abbreviations: IAM, infinite-alleles model; SMM, stepwise mutation model; TPM (40%), two-phase model with 40% mutations following SMM.
M-statistic (Garza and Williamson, 2001) averaged among microsatellite loci. Values <0.68 are highlighted in bold; #H exc., fractions of loci showing heterozygosity deficiency (He observed, He equilibrium) (Cornuet and Luikart, 1996); Wilcoxon test *P*-values for heterozygosity tests considering all microsatellite loci: test for heterozygosity excess (Bottleneck test) and deficit (Expansion test). Significant (*P*<0.05) values are highlighted in bold.

Two general explanations exist for this pattern. The first is that Iberian populations of *U. laevis* represent previously unrecognized native populations. Many widespread temperate trees in the Western Palaearctic show strong longitudinal population structure associated with discrete putative glacial refugia (for example, Petit *et al.*, 2002; Hampe *et al.*, 2003; Magri *et al.*, 2010). In all cases so far studied, each refuge is characterized by private genetic variation, accumulated during glacial advance. In many tree species, and as we find for *U. laevis*, genetic variants diagnostic of native Iberian populations also extend into southern France, particularly in the west (Petit *et al.*, 2002; Magri *et al.*, 2010). Iberian populations do not show a simple subset of genetic diversity present in Central Europe, a pattern expected if Iberian populations are recently derived by introduction from this source (Stone and Sunnucks, 1993). Furthermore, the only significant recent migration detected is from southwestern Europe into Central Europe, and not the other way around. Unless we posit that the Iberia-specific genetic variation evolved rapidly since an introduction (and this is unlikely due to the low mutation rate of cpDNA (Clegg *et al.*, 1994)), the genetic diversity observed in Iberian populations is not compatible with introduction from Central Europe.

A second possible explanation is that Iberian populations are the result of one or more introductions from alternative, genetically divergent, sources within the native range of *U. laevis* (Figure 1) that harbour genotypes differing from those in Central Europe. In contrast to many European trees, *U. laevis* is absent from Italy, which can thus be discounted as a possible source. White elm is native to the Balkans, which could thus represent a possible source. However, previous work by Whiteley (2004) indicates that this region is occupied by a different haplotype, C, also present in Russia. In the absence of an Italian refuge for *U. laevis*, the Balkans are also the most likely refugial origin of the Central European populations sampled in our study and fixed for chloroplast haplotype A. Both lines of reasoning argue against a Balkan origin for Iberian populations, but cannot absolutely exclude this possibility. Further sampling of Balkan populations is required to test this hypothesis.

Regardless of their origin, demographic analyses also argue against recent introduction of Iberian populations. Populations and BAPS clusters from Spain and southwestern France show a more consistent bottleneck signature in allele-size distributions than in heterozygosity excess. This in turn suggests that underlying demographic effects lasted for several generations, rather than being recent phenomena (Garza and Williamson, 2001; Williamson-Natesan, 2005). It is possible for introduced populations to carry with them demographic signatures from their source population(s)—but only where the introduction comprises relatively large numbers of individuals (for example, Stone *et al.*, 2007). There is no historical evidence for any large-scale transport of white elm (in contrast, for example, to Field elm *Ulmus minor*; Gil *et al.*, 2004). We suggest that the bottleneck signatures in Iberian populations reflect natural population fragmentation associated with Holocene expansion of more xeric habitats in Iberia, rather than impacts of recent introduction for gardening as suggested by Navarro and Castroviejo (1993), and for which no documentary evidence exists. Associated genetic drift could explain the lower allelic richness observed in southwestern Europe compared with Central Europe, where more suitable climatic conditions promote larger population sizes. In contrast, recent bottlenecks detected by heterozygosity excess in half of the Spanish populations, in Central Europe (URH) and in the Netherlands (Nielsen and Kjaer, 2010) may have resulted from intense and historically recent anthropogenic landscape transformation (Valbuena-Carabaña *et al.*, 2010).

Palaeobotanic and modelling evidence are also congruent with the native status hypothesis of *U. laevis*. Palaeobotanical records show the presence of *Ulmus* in the Iberian Peninsula since the Miocene and throughout the Pleistocene and Holocene (López, 2000), proving that Iberia was a glacial refuge for *Ulmus*. Unfortunately, *U. minor*, *U. glabra* and *U. laevis* wood (Schweingrüber, 1990) and pollen (Stafford, 1994) cannot be distinguished. Fossil macro-remains such as fruits or leaves discriminate between elm species, but unfortunately these are very rare (García-Amorena *et al.*, 2008). Iberia has also been determined to be a glacial refugia for the three elm species by climatic modelling (Svenning *et al.*, 2008). The combination of palaeoecology,

phylogeography and species distribution modelling have a great potential to inform each other, as they provide complementary information, and have helped interpret glacial refuge for several tree species in Iberia (Rodríguez-Sánchez *et al.*, 2010).

Assuming that Iberia acted as a glacial refugium, the phylogeographic pattern observed in *U. laevis* is similar to those observed in other widely distributed, non-cultivated, European tree taxa (that is, Petit *et al.*, 2002; Magri *et al.*, 2006). Before *U. laevis*, other tree taxa previously considered introduced in the Iberian peninsula have been proven to be native by the use of genetic markers, like chestnut (Fineschi *et al.*, 2000), olive (Breton *et al.*, 2006), stone pine (Vendramin *et al.*, 2008). The latter, like *U. laevis*, showed very little genetic diversity across its broad Mediterranean range. Although in these species ancient cultivation by humans may have justified some doubts about their native status, this has never been the case for *U. laevis*, which has been considered introduced in the absence of any historical evidence solely due to its rarity in Iberia. In contrast, the genetic data presented in this study provide evidence that *U. laevis* populations in SW Europe are more likely to be native.

Regional variation in population structure

Our results (*F*~ST~, AMOVA and BAPS clusters) show significant genetic differentiation within Iberia, and between Central and SW Europe, but genetic homogeneity within Central Europe. Similarly, Whiteley (2004) found no differentiation among five Central and North Eastern European populations of *U. laevis*, but significant differentiation between these populations and each of southwestern France and Finland. Genetic differentiation is frequently higher within natural than introduced populations (Bossdorf *et al.*, 2005), but reduced gene flow between recently established populations can also cause genetic subdivision where no differentiation is found in the centre of a species' range (Stone and Sunnucks, 1993). Population substructure alone is thus agnostic on the introduced versus native status of *U. laevis*. However, if Iberian populations are considered native, differentiation among them may be significant for conservation and management of regional diversity.

Whatever its cause, the small size of Iberian populations of *U. laevis* together with self-incompatibility make this species particularly susceptible to population bottlenecks and associated genetic drift (Nielsen and Kjaer, 2010). The greatest risk faced by *U. laevis* in Iberia is the on-going fragmentation and small size of its populations. In Spain, we only found *U. laevis* in 53 locations, 21 (40%) comprising just one or two isolated individuals. Similarly, in South France *U. laevis* appears in groups of trees or scattered individuals found in just nine sites (Timbal and Collin, 1999). Human disruption of riparian habitats, by agriculture, sand and gravel mining, hydrological control of rivers and urbanization, are the greatest threats to these *U. laevis* populations (Collin, 2003). An example of the latter is the population of Palazuelos de Eresma (PER). This stand has lost 22% of its mature elms (28 of 130) in the last 5 years while being transformed into a golf course and urban complex. Tardajos de Duero (TDU) has also experienced severe recent population size reduction, from several individuals (Segura, 1973) to just two trees when sampled in 2005.

In Spain and South France, white elm populations occupy a total area of <10 km². These severely fragmented populations also show a continuous decline in area, quality of habitat and number of mature individuals. Following the IUCN Red List criteria (IUCN, 2001), if *U. laevis* is considered native in Iberia it should be considered as critically endangered (B2ab(iii + v)). However, as a presumed alien species in Iberia, *U. laevis* currently remains excluded from the Red List of Spanish Vascular Flora (VVAA, 2000). Within the European Union, the Habitat and Species Directive (Council Directive 92/43/EEC) established the list of natural habitat types of community interest for which special areas of conservation (Natura 2000 network) have been designated since 1992. Although habitat 91F0 (Section 91) of the Directive set up the basic framework for the conservation of populations of *U. laevis* in Central and Eastern Europe, none of the prioritized habitats listed for Mediterranean deciduous forests (Section 92, Annex I) supports conservation of *U. laevis* in the Iberian Peninsula and southwestern France. Inclusion of a species in these lists is a *de facto* prerequisite for direct action towards population protection or restoration (Mace, 2004). We argue that the balance of evidence now supports inclusion of *U. laevis* in the Iberian Red List, and initiation of associated regional conservation measures. At the habitat level, native status for Iberian white elm would strengthen the argument for inclusion of Mediterranean riparian forests with *U. laevis* under Section 92 of the Directive. Unless effective action is taken immediately, the issue will become moot because there will be no Iberian populations left to conserve.

We propose the definition of evolutionary significant units, and of management units within these (Moritz, 2002), for *U. laevis* conservation (Supplementary Information). The wide range of *in situ* and *ex situ* conservation measures suggested for *U. laevis* by Collin *et al.* (2004) should be based on the proposed management units. Efforts should focus on augmentation of remnant populations and, where possible, introduction to suitable riparian environments to facilitate connectivity among existing populations. Populations with very low numbers of adult trees (that is, PSG and TDU) could be increased with seedlings from larger populations within the same management unit to minimize risks of inbreeding depression (Moritz, 1999) and maintaining their evolutionary potential.

Although susceptible to Dutch elm disease, *U. laevis* is far less attractive to elm bark beetles transmitting Dutch elm disease than *U. minor* (Webber, 2000), and in many Central European rivers has replaced the latter as the most common elm species following Dutch elm disease pandemics (although with much lower population sizes) (Mackenthum, 2004). As long as the insect vectors do not change their feeding preferences, the genetic resources of *U. laevis* are not really endangered by Dutch elm disease. After the great losses suffered by *U. minor* in the last decades, and with other riparian trees currently declining due to other fungal diseases (i.e., alder, *Alnus glutinosa*; Solla *et al.*, 2010), conservation and promotion of *U. laevis* can be very valuable for the restoration and preservation of SW European riparian ecosystems.

CONCLUSIONS

Our data shows that Iberian populations of *U. laevis*, traditionally considered non-native and of no conservation interest, are genetically distinct from populations in Central Europe and may represent relict populations of an Iberian glacial refuge. This phylogeographic pattern would be in accordance with those observed in other European tree taxa (Petit *et al.*, 2002; Magri *et al.*, 2006). Therefore, attending to our genetic data, and to historic and palaeobotanic evidence, we consider it is much more likely that Iberian populations of *U. laevis* are native rather than introduced. Should this native status be confirmed by a larger scale study, it would represent a rare addition of a new tree species to *Flora ibérica*, and it would also corroborate native status for *U. laevis* in South France, as proposed by Timbal and Collin (1999). Nevertheless, these populations require immediate and appropriate management measures for the protection of existing individuals and of the species genetic resources they represent.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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