

Article

# Genetic Diversity in Relict and Fragmented Populations of *Ulmus glabra* Hudson in the Central System of the Iberian Peninsula

María Martín del Puerto <sup>1</sup>, Felipe Martínez García <sup>2</sup>, Aparajita Mohanty <sup>3</sup> and Juan Pedro Martín <sup>1,\*</sup>

<sup>1</sup> Departamento de Biotecnología-Biología Vegetal, Escuela Técnica Superior de Ingeniería Agronómica, Alimentaria y de Biosistemas, Universidad Politécnica de Madrid, Avda. Puerta de Hierro 2-4, 28040 Madrid, Spain; maria.martin.delpuerto@upm.es

<sup>2</sup> Departamento de Sistemas y Recursos Naturales, Escuela Técnica Superior de Ingeniería de Montes, Forestal y del Medio Natural, Universidad Politécnica de Madrid, Paseo de las Moreras s/n, 28040 Madrid, Spain; felipe.martinez@upm.es

<sup>3</sup> Department of Botany, Gargi College, University of Delhi, Sirifort Road, New Delhi 110049, India; aparajita.gargi@gmail.com

\* Correspondence: juanpedro.martin@upm.es; Tel.: +34-913-365-662

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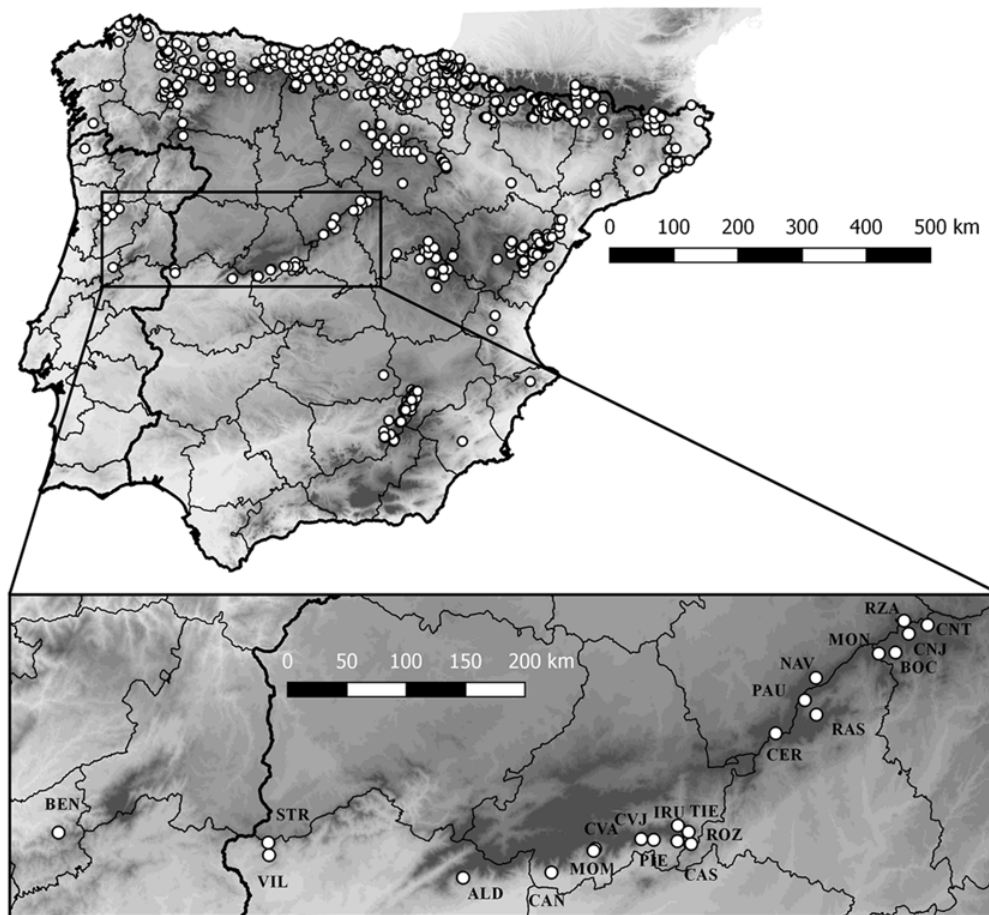
**Abstract:** *Ulmus glabra* Hudson, or Wych elm, occurs as fragmented and relict natural populations in the Central System, which acts as a refugium in the Iberian Peninsula. Considering the importance of the Central System populations of *U. glabra*, the main objective was to assess their genetic diversity using nuclear microsatellite markers. A total of 360 different genotypes were detected in the 427 *U. glabra* individuals analyzed. Wych elm populations showed a highly significant genetic differentiation (24%;  $p = 0.0001$ ). Of the 22 populations studied, population of Rozas de Puerto Real (ROZ) showed the highest values of effective number of alleles (2.803), mean Shannon's diversity (1.047) and expected heterozygosity (0.590). Populations of ROZ and Mombeltrán (MOM) showed the highest values of observed heterozygosity (0.838 and 0.709, respectively), and highly negative values for inbreeding coefficient ( $-0.412$  and  $-0.575$ , respectively). Also, most of putative hybrids (50 of 55) were observed in these two populations. Demographic analysis revealed signals for recent (four populations) and ancestral (fifteen populations) bottlenecks. Fragmented populations with diminishing number of individuals, along with anthropogenic intervention and Dutch elm disease (DED), are the main threats to *U. glabra* populations. From a future perspective, the information generated can be considered in the formulation of conservation strategies for *U. glabra* populations in the Central System.

**Keywords:** fragmented populations; genetic diversity; introgression; microsatellite markers; population bottleneck; *Ulmus glabra*; Wych elm

## 1. Introduction

Wych elm (*Ulmus glabra* Hudson) is a deciduous tree distributed mainly in the Eurosiberian region. *U. glabra* is spread over central and northern Europe, reaching up to the Ural Mountains and further extending towards eastern and southern Europe (i.e., Mediterranean region) [1]. The Iberian Peninsula is the southwestern limit for Wych elm [2]. The continuity and abundance of *U. glabra* populations in the peninsula is greater in the northern strip and Pyrenean-Cantabric axis. The most meridional populations are separated by hundreds of kilometers [2]. Some of these populations belong to the Central System (Figure 1). Due to its location, orientation and altitude, this Central System is

important in modulating the general climate and acting as an island. These climatic and geographical characteristics have enabled the persistence of some areas with Eurosiberian conditions surrounded by zones with Mediterranean climate [3]. The persistence of these areas acting as refugia with a favorable climate, has enabled the survival of the Eurosiberian species till the present [4,5].



**Figure 1.** Map depicts the locations of *Ulmus glabra* populations in the Iberian Peninsula. The details of distribution of the studied populations along the Central System are shown in the rectangle (see Table 1 for codes).

The presence of the genus *Ulmus* in the Iberian Peninsula dates back to the Miocene and also has been reported throughout the Pleistocene and Holocene [6]. Paleobotanic and paleoclimatic studies confirm a temperature increase over the Late Glacial and, also, in the Holocene [7]. Therefore, the Mediterranean climate has gradually expanded to the Iberian Peninsula. Consequently, Eurosiberian vegetation has been reduced both latitudinally and altitudinally [4,8,9]. In addition to the climatic factors, the development of human-related activities has drastically reduced the woody vegetation in these mountains [10,11], thus affecting the genus *Ulmus* [12].

Besides landscape, climatic and anthropogenic factors, natural interspecific hybridization and introgression among relatives of the genus *Ulmus* have had significant consequences on the genetic diversity, genetic structure and conservation of their natural populations [13–15]. In the Iberian Peninsula, three *Ulmus* species are considered natives: *U. glabra*, *U. minor* Mill. and *U. laevis* Pall. [16,17]. *U. minor* and *U. glabra* are genetically most close [18], so hybridization and introgression between the two species have been described in natural populations where Field elms and Wych elms coexist [15,19]. Both these *Ulmus* species are monoecious, self incompatible, wind pollinated and their seed dispersal is also by wind [1].

**Table 1.** Location, number of sampled individuals (*Ns*) and estimated total number of individuals (*Nt*) of *Ulmus glabra* and *U. minor* populations studied along the Central System of the Iberian Peninsula.

Populations									
No	Code	Locality	Province	Species	Latitude	Longitude	Altitude (m)	<i>Ns</i>	<i>Nt</i> <sup>1</sup>
1	CNT	Cantalojas	Guadalajara	<i>U. glabra</i>	41.24913	−3.23853	1393	1	1
2	CNJ	Cantalojas (Arroyo de la Zarza)	Guadalajara	<i>U. glabra</i>	41.20193	−3.34225	1557	38	194
3	BOC	El Cardoso de la Sierra-Bocígano	Guadalajara	<i>U. glabra</i>	41.13182	−3.41177	1314	4	8
4	RZA	Riaza-Becerril	Segovia	<i>U. glabra</i>	41.26585	−3.36955	1556	22	27
5	NAV	Navafría	Segovia	<i>U. glabra</i>	41.01932	−3.84353	1512	14	14
6	MON	Montejo de la Sierra-El Cardoso de la Sierra	Madrid	<i>U. glabra</i>	41.13687	−3.51304	1463	7	9
7	RAS	Rascafría-Morcuera	Madrid	<i>U. glabra</i>	40.86605	−3.83780	1451	33	37
8	PAU	El Paular (Pista de las Poyatas)	Madrid	<i>U. glabra</i>	40.92471	−3.90225	1670	1	1
9	CER	Cercedilla	Madrid	<i>U. glabra</i>	40.78363	−4.05626	1260	6	6
10	ROZ	Rozas de Puerto Real	Madrid	<i>U. glabra</i>	40.31348	−4.49671	801	46	46
11	TIE	El Tiemblo	Ávila	<i>U. glabra</i>	40.35743	−4.51245	1163	42	91
12	IRU	El Barraco (Valle de Iruelas)	Ávila	<i>U. glabra</i>	40.38169	−4.56854	1260	57	>6500
13	CAS	Casillas	Ávila	<i>U. glabra</i>	40.32280	−4.57195	955	21	78
14	PIE	Piedralaves	Ávila	<i>U. glabra</i>	40.33955	−4.70401	1042	7	14
15	CVJ	Casavieja	Ávila	<i>U. glabra</i>	40.32676	−4.76926	1170	20	60
16	CVA	Cuevas del Valle	Ávila	<i>U. glabra</i>	40.27778	−5.01679	797	11	17
17	MOM	Mombeltrán	Ávila	<i>U. glabra</i>	40.26935	−5.02413	757	10	18
18	CAN	Candeleda	Ávila	<i>U. glabra</i>	40.17660	−5.24816	654	8	11
19	ALD	Aldeanueva de la Vera	Cáceres	<i>U. glabra</i>	40.13357	−5.72310	696	10	11
20	VIL	Villamiel	Cáceres	<i>U. glabra</i>	40.18574	−6.77448	680	15	22
21	STR	San Martín de Trevejo-Eljas	Cáceres	<i>U. glabra</i>	40.23256	−6.78812	820	29	>5000
22	BEN	Benfeita	Arganil (Portugal)	<i>U. glabra</i>	40.21813	−7.91848	546	25	195
23	PAUm	El Paular	Madrid	<i>U. minor</i>	40.88794	−3.88643	1164	5	NA
24	VILm	Villamiel	Cáceres	<i>U. minor</i>	40.16955	−6.79228	573	5	NA
25	ESBm	Escalos de Baixo	Castelo Branco (Portugal)	<i>U. minor</i>	39.87995	−7.38771	374	5	NA

<sup>1</sup> NA = not available.

Thus, the available data about vegetation evolution together with the current distribution of *U. glabra* in the Iberian Peninsula emphasize the relict and peripheral distribution of Wych elm populations in the Central System. According to Rossignoli and Génova [2], these populations are not only relict but also have fragmented distribution. Collin et al. [20] have emphasized the need for special attention to marginal populations of *U. glabra* because of their rarity and adaptability to unique geographical conditions. These populations are also important for the natural heritage preservation, particularly in view of the fact that major threats to their survival were found [2]. The scattered distribution, small population size and impact of human activities, can increase the fragmentation of populations along with genetic erosion, mainly towards the southern limits of the species distribution, causing them to be more susceptible to stochastic, genetic, and demographic events [21].

Moreover, in the last decades, Dutch elm disease (DED) has reduced the number of trees in Wych elm populations [2,16]. Fifteen populations of *U. glabra* from the Central System have been previously described [2] as having low numbers of individuals in general and with a high contribution of young individuals combined with re-sprouting stumps. For the present study, these 15 populations and an additional seven populations have been included. Hence, 22 populations of *U. glabra*, located along a 600-kilometer stretch of the large mountainous region, which includes the Spanish provinces of Guadalajara, Segovia, Madrid, Ávila and Cáceres, and one population found in the Serra da Estrela in Portugal (Figure 1), have been analyzed in the present investigation.

For understanding and establishing adequate conservation management strategies in the natural populations of *U. glabra* in the Central System of Iberian Peninsula, characterization of genetic diversity is the first necessary step. To study genetic variability in populations of elm species, molecular markers such as Random Amplified Polymorphic DNAs (RAPDs) [22–24], Inter Simple Sequence Repeats (ISSRs) [23], and Amplified Fragment Length Polymorphisms (AFLPs) [15,16,25] have been used. AFLP markers have also been used to investigate interspecific hybridization between *U. minor* and *U. glabra* in Flanders [15].

In addition, numerous microsatellite markers have been developed for different *Ulmus* species, such as *U. laevis* [26], *U. minor* [27] and *U. rubra* Muhl. [28], and used for testing of cross-amplification in other elm species, including *U. glabra*. Many *U. laevis* populations located along Europe have been studied using these markers [17,29,30]. Genetic diversity of a few localized natural populations of *U. minor* (e.g., in Balearic Islands, [31]; in the Netherlands, [32]; in Croatia, [33]) and *U. glabra* (e.g., in Denmark, [34]) have also been studied using microsatellite markers. The most extensive analysis of European populations of these two species together, using restriction fragment-length polymorphisms in chloroplast DNA, AFLPs and nuclear microsatellites [16], included only five populations of Wych elm from Spain, of which one was from the Central System. Moreover, there are some studies using microsatellite markers focused on detecting hybridization and introgression between elm species, e.g., between invasive *U. pumila* L. and native *U. rubra* in USA [13,35] or native *U. minor* in Italy [14,36].

Keeping in view the importance of Central System populations of *U. glabra* in the Iberian Peninsula and simultaneous lack of studies on genetic diversity, interspecific hybridization and introgression, the major objectives of the present investigation were (i) to assess the level of the genetic diversity of *U. glabra* populations in the Central System; (ii) to analyze possible demographic processes (e.g., evidence of bottleneck events) they have undergone; and (iii) to detect possible introgression between *U. glabra* and *U. minor*, which can significantly influence the genetic diversity and genetic structure in Wych elm populations.

## 2. Materials and Methods

### 2.1. Sampling of Plant Material

Leaf material of 427 individuals from 22 populations of *U. glabra* in the Central System was sampled for the present study (Table 1, Figure 1). Whenever possible, leaf material was collected from

clearly-separated individuals to reduce the risk of sampling ramets. In addition, fifteen individuals of *U. minor* were sampled in three different Central System locations (Table 1). Also, since *U. glabra* populations have a fragmented distribution in the Central System and the number of estimated individuals/population ( $Nt$ ) was extremely varied (see Table 1), sampling was done so as to reflect the distribution of the species in the Central System. Hence, a representative number of individuals (approximately between 20 and 60) which were mostly at a uniform separation within the population, were collected. For populations that had less than 20 estimated individuals/population (see Table 1), collection of all possible individuals was done, after taking care to avoid the collection of ramets. In such cases the number of individuals per population was less (less than 20). Leaf material of all individuals was stored at  $-80\text{ }^{\circ}\text{C}$  until DNA extraction.

## 2.2. DNA Extraction and Microsatellite Genotyping

Total genomic DNA was extracted from frozen leaf material following the protocol supplied in the “NucleoSpin<sup>®</sup> Plant II Kit” (Macherey-Nagel GmbH & Co. KG, Düren, Germany). Extracted DNA was quantified by visual comparison with known concentrations of lambda DNA on 1.2% agarose gels, and a working solution of DNA (approx. 10 ng/ $\mu\text{L}$ ) was made.

Twenty two microsatellite loci comprising of seven developed in *U. laevis* (Ulm2, Ulm3, Ulm6, Ulm8, Ulm9, Ulm12 and Ulm19; [26]), four in *U. minor* (Ulmi1-21, Ulmi1-98, Ulmi1-165 and Ulmi2-16; [27]), and eleven in *U. rubra* (UR101, UR123, UR138, UR141, UR153, UR158, UR159, UR173a, UR173b, UR175 and UR188a; [28]) were screened using 32 samples of *U. glabra* (one from each population with  $\leq 20$  samples and two from populations with  $>20$  samples). One sample of each of the three locations of *U. minor* was also used for this preliminary screening. Eleven of 22 microsatellite markers (Ulm2, Ulm3, Ulm8, Ulmi1-21, Ulmi1-98, Ulmi1-165, UR123, UR138, UR153, UR173a and UR175), were shortlisted for use in the present study based on interpretable polymorphisms in both elm species. However, UR138 and UR173a showed multiband patterns in *U. minor*, and therefore, they were not used for analysis in this species.

One primer of each pair was labeled with a fluorescent dye (6-FAM<sup>™</sup>, VIC<sup>®</sup>, NED<sup>™</sup> or PET<sup>™</sup>; Applied Biosystems<sup>™</sup>, Foster City, CA, USA) at the 5' end. Polymerase chain reactions were performed in 20 $\mu\text{L}$  of final volume containing 2  $\mu\text{L}$  of genomic DNA (about 20 ng), 0.5  $\mu\text{M}$  of each of the forward and reverse primers, 0.15 mM of each deoxynucleotide triphosphate (dNTP), 2 mM of  $\text{MgCl}_2$  and 0.8 U of *Tth* DNA polymerase in 1X of the manufacturer's reaction buffer (BIOTOOLS, B&M Labs, Madrid, Spain). DNA amplifications were carried out in a PTC-100 thermalcycler (MJ Research, Inc., Waltham, MA, USA) with heated lid, using the same protocol for all microsatellite loci: an initial denaturalization step of 4 min at 94  $^{\circ}\text{C}$ , followed by 35 cycles of 45 s at 94  $^{\circ}\text{C}$ , 1 min at 50  $^{\circ}\text{C}$  and 1 min 30 s at 72  $^{\circ}\text{C}$ , with a final extension step of 72  $^{\circ}\text{C}$  for 10 min. Amplified products were separated by capillary electrophoresis using an ABI PRISM<sup>®</sup> 3730 DNA analyzer (Applied Biosystems, Foster City, CA, USA). GeneScan-500LIZ (Applied Biosystems, Foster City, CA, USA) was used as an internal size standard. Alleles were scored using Peak Scanner<sup>™</sup> software version 1.0 (Applied Biosystems, Foster City, CA, USA).

## 2.3. Data Analysis

### 2.3.1. Genetic Diversity

Genetic diversity parameters were calculated based on data from eleven microsatellite loci analyzed in 427 Wych elm samples from 22 populations. For each locus: allele frequencies, total number of alleles ( $N_A$ ) and effective number of alleles ( $N_E$ ), number of different genotypes observed ( $G_O$ ), observed heterozygosity ( $H_O$ ) and expected heterozygosity ( $H_E$ ), and Shannon's information index ( $I$ ) were calculated, using GenAlEx software version 6.5 [37,38]. Polymorphic information content per locus ( $PIC$ ) was also calculated according to Bostein et al. [39].

Furthermore, the discrimination power ( $D$ ; [40,41]) of microsatellite markers was estimated. This parameter is an estimate of the probability that two randomly-sampled trees could be distinguished by their microsatellite profiles, and was calculated for each locus as  $D = 1 - C$ , where  $C$  is the probability of coincidence, i.e., two samples match by chance at one locus ( $C = \sum P_i^2$ , and  $P_i$  is the frequency of different genotypes observed at that locus). The discrimination power for all loci combined ( $m = 11$ ) was calculated as  $D_T = 1 - C_T$ , where  $C_T = \Pi C_m$ , and represents the probability of coincidence cumulative for all loci.

Considering the overall microsatellite loci analyzed, for each population the percentage of polymorphic loci ( $P$ ) and  $G_O$  were calculated. Also, other parameters, such as the mean over all loci of  $N_E$ ,  $I$ ,  $H_O$ ,  $H_E$  and inbreeding coefficient ( $F$ ), were also calculated using the GenAlEx 6.5 software [37,38].

Relationships among 19 *U. glabra* populations (with at least six sampled individuals) were obtained using the UPGMA (unweighted pair group with arithmetic mean) method with NTSYS-pc software version 2.2 [42], based on Nei's standard genetic distances [43]. A co-phenetic matrix was derived from the genetic distance matrix to test the goodness-of-fit of the clusters by comparing the two matrices using the Mantel matrix correspondence test in the MxComp program of the NTSYS-pc package, using 10,000 random permutations. The robustness of the dendrogram was also tested using 10,000 bootstraps implemented with the Phylip software version 3.6 [44].

Additionally, in order to determine the significance of partitioning of genetic diversity among and within populations, an analysis of molecular variance (AMOVA; [45]) was conducted using the GenAlEx 6.5 software. Levels of significance of variance component estimates were computed by non-parametric permutational procedures using 10,000 random permutations. Genetic differentiation ( $F_{ST}$ ) was also calculated over all populations in a group and then among all pairs of populations, and their significance was tested by a permutation procedure using 10,000 permutations.

### 2.3.2. Demographic Analysis

The effective population size ( $N_e$ ) is a critical parameter to study the evolution and conservation in natural populations. As estimates of  $N_e$  can be affected by recent population size changes, the test for evidence of recent bottlenecks in *U. glabra* populations was performed using the  $T_2$  statistic implemented in the program BOTTLENECK version 1.2 [46,47]. This statistic represents an average over loci of the deviation of the actual  $H_E$  from the  $H_E$  expected from the number of alleles in the population assuming mutation–drift equilibrium in a constant size population. The program tests for excess of heterozygosity using several mutation models including the stepwise mutation model (SMM), infinite allele model (IAM), and a two-phase model (TPM). For microsatellite data, the TPM is suggested to be the most appropriate mutation model [47–49]. Thus, the TPM model was applied assuming 95% single-step mutations and 5% multiple-step mutations [47], and a variance among multiple steps of 26. Positive values of  $T_2$  indicate bottleneck, whereas negative values are consistent with recent population expansions. Also, considering the number of polymorphic loci used in the present study, the Wilcoxon's signed rank test was chosen to determine the  $T_2$  significance as recommended by Piry et al. [47]. The test was run with 10,000 iterations.

Additionally, the  $M$ -ratio values (calculated as a ratio of the number of alleles to the range in allele size at a microsatellite locus [50]) were calculated using Arlequin software version 3.5 [51].  $M$ -ratio value was used to detect reductions in effective population size. In comparison to the BOTTLENECK methods that are tailored to detect recent bottlenecks,  $M$ -ratio reflects a population size decline over a longer timescale and may be more powerful for detecting ancestral bottlenecks [48,50]. Based on empirical evidence, Garza and Williamson [50] indicated that in any population study where seven or more loci are analyzed and if the population shows  $M$ -ratio below 0.68, then it may be assumed that they have undergone a genetic bottleneck [17]. Both  $T_2$  bottleneck statistic and  $M$ -ratio method used to detect bottleneck events were carried out in only 15 *U. glabra* populations that were comprised of at least 10 sampled individuals.

Finally, the effective population size for each of the 10 *U. glabra* populations with at least 20 sampled individuals, was calculated using two different single-sample estimation methods: the linkage–disequilibrium information and approximate Bayesian computation. LDNe software version 1.31 [52] was used, which employs linkage–disequilibrium information among alleles at different loci caused by genetic drift in finite populations. This method corrects for biases associated with small sample sizes [52,53].  $N_e$  was estimated assuming random mating and excluding alleles with frequencies less than 0.05. This critical value has been shown to produce little bias from rare alleles while maintaining moderate precision [54]. The 95% confidence intervals for  $N_e$  were obtained using a Jackknife method as recommended in Waples and Do [52].

Approximate Bayesian computations to estimate variance  $N_e$  from summary statistics that are related to  $N_e$  were performed using ONE-SAMP software version 1.1 [55]. The user defines prior estimates of  $N_e$  (lower and upper bounds) and ONE-SAMP generates 50,000 simulated populations drawn randomly from the distribution of  $N_e$  priors. Summary statistics are calculated and compared to the actual data and similar summary statistics are retained for use in generating an estimate of  $N_e$  using weighted local regression [55]. Several lower and upper bounds were tested on the prior estimates for  $N_e$  per population. Finally, 2–7000 for two populations (STR and IRU; see Table 1 for population codes) and 2–200 for the other eight populations tested, were used to estimate their  $N_e$  along with 95% confidence intervals.

### 2.3.3. Introgression between *U. glabra* and *U. minor*

To identify genetic groups within *U. glabra* populations and the relationships with *U. minor* genotypes, STRUCTURE software version 2.3.4 [56] was used. This Bayesian approach uses no a priori classification and assigns samples to  $K$  genetic clusters based on the allele frequencies at each locus. A first analysis was carried out on 427 *U. glabra* individuals using data from the eleven microsatellite markers, and the range of possible groups ( $K$ ) tested was from 1 to 25, i.e., the total number of sampled populations plus three [57]. Then, to know the relationships between *U. glabra* and *U. minor* genotypes, a second analysis was performed by adding the 15 *U. minor* samples, using only data from the nine microsatellite loci that were amplified in both species, and wherein the number of groups tested ranged from 1 to 10 considering the results of previous analysis.

The estimate of the most likely number of genetic groups was performed following the procedure of Evanno et al. [57], which proposed the ad hoc statistic  $\Delta K$ . Program settings used were the admixture ancestry and correlated allele frequencies models. The degree of admixture alpha was inferred from the data, and lambda, the parameter of the distribution of allelic frequencies, was set to 1 [57,58]. The program was run 20 independent times for each  $K$  value. In each run, a burn-in period of 10,000 iterations, and 100,000 post-burning MCMC (Markov chain Monte Carlo) simulations, were carried out. Finally, among the 20 runs performed for the optimal  $K$  value estimated, the run with the least negative log–likelihood value [59] was used to obtain the membership coefficients ( $q$ ) for each individual in each of the  $K$  inferred groups.

In the second STRUCTURE analysis, admixed individuals could represent putative hybrids between *U. glabra* and *U. minor*, so an analytical tool more focused on hybrid detection was also applied. Bayesian statistical methods provided in the program NewHybrids version 1.1 beta [60] were used to estimate posterior probabilities of whether each analyzed individual belonged to parental classes (*U. glabra* and *U. minor*) or hybrid categories (i.e.,  $F_1$ ,  $F_2$  and backcrosses  $F_1$ ). This was performed using prior information about individual assignments corresponding to genetic clusters previously detected by STRUCTURE analysis. The program was carried out using the default parameters for the six genotype class frequencies and ten independent runs with a burn-in period of 100,000 iterations followed by 600,000 MCMC simulations.

### 3. Results

#### 3.1. Genetic Diversity

An analysis of 11 microsatellite loci in 442 elm individuals (427 *U. glabra* and 15 *U. minor*) revealed a total of 101 alleles. Of the 101 alleles, 92 were detected in *U. glabra* (see Tables S1 and S2). Frequencies of 50 out of 92 alleles were below 0.05, whereas five alleles showed frequencies above 0.5 (Table S2).

Results of the genetic diversity analysis in *U. glabra* per locus are presented in Table 2. The number of alleles per locus ranged from 3 in UR123 to 23 in Ulmi1-165, with an average of 8.4 alleles per locus. The average effective number of alleles per locus was 3.6, and the range varied between 10.4 for Ulmi1-165 and 1.2 for Ulm2. The average number of observed genotypes was 23.6, with a maximum (95) in Ulmi1-165 and a minimum (5) in UR123. The means of observed and expected heterozygosity were 0.477 and 0.566, respectively. The maximum values of  $H_E$  and  $H_O$  were obtained in Ulmi1-165 and minimum values were observed in Ulm2. The mean of Shannon's diversity index over all loci was 1.272 and varied between 2.639 (Ulmi1-165) and 0.356 (Ulm2). Similar trends were observed for polymorphic information content and discrimination power. For  $PIC$ , the maximum (0.899) and minimum (0.141) values were for Ulmi1-165 and Ulm2 respectively, and the mean value was 0.539 (Table 2). The maximum and minimum values obtained for  $D$  were also for Ulmi1-165 (0.974) and Ulm2 (0.269) respectively, with a cumulative probability for all loci close to 1 (Table 2). In general, microsatellite loci developed in *U. minor* (Ulmi1-SSR series) showed the highest values for  $N_A$ ,  $N_E$ ,  $G_O$ , heterozygosity,  $PIC$  and  $D$ , and the lowest values in Ulm-SSR series, developed in *U. laevis*.

**Table 2.** Genetic diversity parameters at eleven microsatellite loci analyzed in 427 *Ulmus glabra* individuals from the Central System of the Iberian Peninsula.

Loci	$N_A$	$N_E$	$G_O$	$H_O$	$H_E$	$I$	$PIC$	$C$	$D$
Ulm2	5	1.169	6	0.148	0.144	0.356	0.141	0.731	0.269
Ulm3	9	1.420	13	0.260	0.296	0.687	0.283	0.525	0.475
Ulm8	4	1.325	6	0.246	0.245	0.523	0.234	0.577	0.423
Ulmi1-21	9	5.757	34	0.520	0.826	1.979	0.809	0.068	0.932
Ulmi1-98	10	4.444	32	0.670	0.775	1.736	0.743	0.091	0.909
Ulmi1-165	23	10.365	95	0.806	0.904	2.639	0.899	0.026	0.974
UR123	3	1.373	5	0.234	0.272	0.527	0.252	0.599	0.401
UR138	7	3.928	18	0.712	0.745	1.570	0.706	0.109	0.891
UR153	6	3.287	15	0.604	0.696	1.327	0.646	0.137	0.863
UR173a	9	2.514	20	0.539	0.602	1.254	0.552	0.196	0.804
UR175	7	3.512	16	0.513	0.715	1.393	0.665	0.135	0.865
Mean	8.4	3.554	23.6	0.477	0.566	1.272	0.539	---	---
Cumulative	92	---	260	---	---	---	---	$8.4 \times 10^{-9}$	0.99999992

$N_A$ —number of alleles,  $N_E$ —effective number of alleles,  $G_O$ —number of observed genotypes,  $H_O$ —observed heterozygosity,  $H_E$ —expected heterozygosity,  $I$ —Shannon's information index,  $PIC$ —polymorphic information content,  $C$ —probability of coincidence, and  $D$ —discrimination power.

Microsatellite-based genotyping for the 11 combined SSR loci in 427 individuals of *U. glabra* revealed a high number (360) of distinct genotypes (Table S1). Only 25 of 360 genotypes were common to two or more individuals. The three Ulmi1-SSR loci alone showed a discrimination power of 0.99984. They could distinguish 301 different genotypes, which is about 84% of the total genotypes detected using the 11 SSR loci.

The results of genetic diversity analysis in populations of *U. glabra* are presented in Table 3. Percentages of polymorphic loci varied between 100% (ROZ, IRU and CVJ populations) and 46% (CNT). Three populations (BOC, PAU and CER) with a low number of individuals showed relatively low values of  $P$  (64%). The number of different genotypes observed across all loci in each population was similar to the number of sampled individuals, except in CER, ROZ and MOM populations where number of genotypes was <30% of the sampled individuals (Table 3). The highest values of effective number of alleles ( $N_E = 2.803$ ), mean Shannon's diversity ( $I = 1.047$ ) and expected heterozygosity ( $H_E = 0.590$ ) were observed in ROZ population while the lowest values ( $N_E = 1.711$ ,  $I = 0.547$  and



$H_E = 0.310$ ) were detected in RAS population (Table 3), when populations with sample size below 5 (CNT, BOC, and PAU) and population with one unique genotype (i.e., CER) were excluded from the analyses. Populations of ROZ and MOM showed the highest values of observed heterozygosity ( $H_O = 0.838$  and  $0.709$ , respectively), which corresponded to highly negative values for inbreeding coefficient ( $F = -0.412$  and  $-0.575$ , respectively; Table 3).

**Table 3.** Genetic diversity parameters calculated for *Ulmus glabra* populations from the Central System of the Iberian Peninsula using data from eleven microsatellite loci. See Table 1 for population codes.

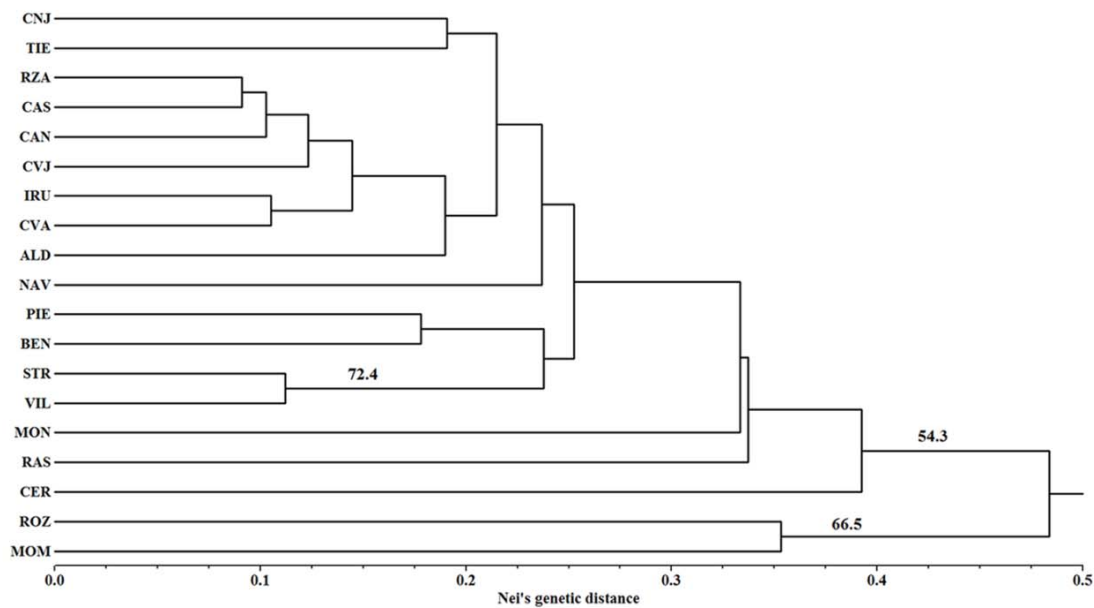
Population	P (%)	$G_O$ (Ns)	$N_E$	I	$H_O$	$H_E$	F
CNT	46	1 (1)	---	---	0.455	---	---
CNJ	82	38 (38)	1.959	0.678	0.428	0.393	-0.086
BOC	64	2 (4)	---	---	0.568	---	---
RZA	82	22 (22)	2.090	0.751	0.397	0.413	0.038
NAV	82	14 (14)	1.897	0.640	0.429	0.399	-0.090
MON	73	7 (7)	2.045	0.728	0.416	0.404	-0.028
RAS	82	29 (33)	1.711	0.547	0.309	0.310	0.017
PAU	64	1 (1)	---	---	0.636	---	---
CER	64	1 (6)	---	---	0.636	---	---
ROZ	100	13 (46)	2.803	1.047	0.838	0.590	-0.412
TIE	73	39 (42)	2.213	0.769	0.394	0.443	0.089
IRU	100	56 (57)	2.222	0.867	0.445	0.435	0.006
CAS	82	21 (21)	2.136	0.695	0.398	0.376	-0.024
PIE	73	5 (7)	2.048	0.666	0.442	0.371	-0.185
CVJ	100	17 (20)	2.078	0.848	0.482	0.435	-0.089
CVA	82	10 (11)	2.187	0.672	0.405	0.385	-0.052
MOM	82	2 (10)	2.083	0.717	0.709	0.450	-0.575
CAN	91	7 (8)	2.505	0.847	0.409	0.444	0.026
ALD	73	10 (10)	2.020	0.655	0.455	0.388	-0.132
VIL	73	12 (15)	1.890	0.637	0.394	0.355	-0.097
STR	82	28 (29)	2.161	0.807	0.464	0.434	-0.074
BEN	91	25 (25)	2.233	0.828	0.491	0.475	-0.061

P—percentage of polymorphic loci,  $G_O$ —number of observed genotypes across all loci. Mean values of  $N_E$ , I,  $H_O$ ,  $H_E$  and inbreeding coefficient (F) calculated over all loci per population.  $N_E$ , I,  $H_E$  and F parameters were not calculated for populations with a sample size less than 5 (CNT, BOC and PAU) or population with one unique genotype observed (CER).

In the present investigation, ten unique or private alleles were detected in seven of the 22 populations (NAV, MON, RAS, IRU, CAS, CVJ and MOM; see Table S1). The abundance of three of the unique alleles (Ulm2-111, Ulm3-164, UR173a-185 found in CAS, MON and RAS, respectively) was >50% (Table S1).

### 3.2. Population Relationships

The UPGMA dendrogram shows the relationships among 19 *U. glabra* populations with more than five sampled individuals (Figure 2). The Mantel test revealed a good and significant co-phenetic correlation ( $r = 0.87$ ;  $p = 0.0001$ ), which indicates a good fit to the cluster analysis [42]. The dendrogram shows the populations grouped in two main clusters, separated at a genetic distance level of 0.48. Populations of ROZ and MOM group together in a cluster with a support of 66.5%, and in the other cluster are the remaining 17 populations with a support of 54.3% (Figure 2). Within the second cluster, CER, RAS and MON populations are distanced from the remaining 14 populations (Figure 2). On the other hand, the western populations (VIL, STR and BEN) group together, and especially VIL and STR are located in the same subgroup and are separated from the rest with a support of 72.4% (Figure 2).



**Figure 2.** Unweighted pair group with arithmetic mean (UPGMA) clustering showing the relationships among the 19 *Ulmus glabra* populations from the Central System of the Iberian Peninsula with at least six sampled individuals (see Table 1 for codes), based on 11 microsatellite loci data using Nei's genetic distance. The numbers at nodes are support (>50%) by bootstrap analysis (10,000 replicates).

Results of AMOVA analysis revealed a highly significant genetic differentiation among the 19 *U. glabra* populations (24%;  $p = 0.0001$ ). A major portion of variation was harbored within populations (76%). Genetic differentiation values among all population pairs were also calculated (Table S3), and all of them were highly significant ( $p < 0.0005$ ). The highest  $F_{ST}$  value was observed between RAS and MOM populations (43.6%) and the lowest value was between CAS–CAN and RZA–CAN population pairs (8.5% in both cases).

### 3.3. Demographic Analysis

The results of demographic analysis are shown in Tables 4 and 5.  $T_2$  statistic using the TPM model implemented in BOTTLENECK program showed a significant ( $p < 0.05$ ) excess of heterozygosity (positive values of  $T_2$ ) in four (NAV, ROZ, TIE and MOM) of the 15 populations studied (Table 4), suggesting that these populations could have suffered a recent bottleneck. On the other hand, values of  $M$ -ratio (a recommended statistical parameter to detect a bottleneck lasting several generations followed by a demographic recovery) revealed bottleneck marks (based on  $M$ -ratio  $< 0.68$ ; [50]) in all the analyzed populations (Table 4).

The effective population size values for the ten Wych elm populations that were analyzed are shown in Table 5. The  $N_e$  was less than 20 in eight and seven of the ten analyzed populations using ONeSAMP and LDNe programs, respectively. The lowest  $N_e$  values were observed in ROZ population using both programs (Table 5).

**Table 4.** Detection of genetic bottlenecks in the 15 *Ulmus glabra* populations (see Table 1 for codes) with at least 10 sampled individuals, using the  $T_2$  statistic and  $p$ -values of the Wilcoxon’s signed rank test under the two-phase model (TPM) as implemented in Bottleneck v. 1.2, and mean values over loci for the  $M$ -ratio as calculated in Arlequin v. 3.5.

Population	$T_2$ Statistic	Wilcoxon’s Test ( $p$ values) <sup>1</sup>	$M$ -ratio
CNJ	0.2056	0.2480	0.2950
RZA	0.0192	0.5000	0.2725
NAV	0.6597	<i>0.0068</i>	0.3559
RAS	−0.1479	0.7148	0.2892
ROZ	0.6865	<i>0.0061</i>	0.3702
TIE	0.6194	<i>0.0195</i>	0.2643
IRU	−1.6151	0.9976	0.2780
CAS	0.0206	0.3672	0.2867
CVJ	−0.9445	0.9976	0.3193
CVA	0.3550	0.1250	0.3320
MOM	0.9936	<i>0.0010</i>	0.2902
ALD	0.5103	0.1563	0.2149
VIL	−0.0650	0.4727	0.2798
STR	−0.1423	0.5898	0.2749
BEN	0.3321	0.1377	0.3196

<sup>1</sup> Significant  $p$  values (<0.05) are in italics.

**Table 5.** Estimates for the effective population size ( $N_e$ ) in the 10 *Ulmus glabra* populations (see Table 1 for codes) with at least 20 sampled individuals, using approximate Bayesian computation (ONeSAMP v. 1.1) and linkage–disequilibrium information (LDNe v. 1.31). In parentheses are the lower and upper values at 95% confidence intervals (CI).

Population	$N_e$ (95% CI)	
	ONeSAMP	LDNe
CNJ	18.3 (14.7–24.6)	11.4 (4.7–23.1)
RZA	17.9 (14.0–26.2)	12.8 (7.3–25)
RAS	16.6 (12.4–24.5)	2.2 (1.4–3.3)
ROZ	9.3 (7.3–12.1)	0.9 (0.8–1.1)
TIE	15.9 (12.8–23.1)	3 (1.8–6.8)
IRU	668.0 (253.4–5277.2)	16.6 (10.9–25.2)
CAS	18.5 (15.3–24.2)	29.1 (11.6–1550.5)
CVJ	10.0 (8.1–12.8)	1.2 (1–1.4)
STR	36.7 (21.4–96.4)	51.3 (18.8–∞)
BEN	18.8 (15.6–26.9)	48.4 (19.3–∞)

### 3.4. Introgression between *U. glabra* and *U. minor*

In order to analyze the existence of a possible introgression between *U. glabra* and *U. minor*, genetic data from nine microsatellite loci that amplified in both species were used. Seventy six alleles in 427 *U. glabra* individuals from 22 populations, and 28 alleles in 15 *U. minor* individuals from three locations in the Central System, were detected (see Table S2). Nineteen of the 28 alleles detected in *U. minor* were common to *U. glabra* also. Two of these alleles (Ulm3-182 and UR123-252) showed high frequency ( $\geq 0.5$ ) in both species (Table S2). All *U. glabra* samples shared at least two alleles with individuals of *U. minor* (see Table S1).

STRUCTURE analysis carried out on 427 *U. glabra* individuals using data from the 11 microsatellite markers, showed the modal value of the distribution of  $\Delta K$ , i.e., the optimal number of groups estimated, for  $K = 2$ . Similar results were obtained when a second analysis was performed using data only from the nine microsatellite loci in 442 samples, 427 *U. glabra* and 15 *U. minor* (Figure S1).

With respect to 427 *U. glabra* individuals, STRUCTURE analysis revealed the existence of two clearly-defined genetic clusters (A and B), based on coefficients  $q \geq 0.9$  for one of the two groups [61]. Thus, 368 individuals were assigned to genetic group A, 47 to group B, and 12 were considered as admixture samples, because they showed admixture values below 0.9 (Table 6 and Table S4). The 15 *U. minor* individuals were clustered with the 47 *U. glabra* from group B (Table S4).

**Table 6.** Number and percentage of individuals assigned to clusters (A and B) or considered as admixture samples as defined by STRUCTURE analysis in each *Ulmus glabra* population studied. See Table 1 for population codes.

Population	Ns	Cluster A (%)	Cluster B (%)	Admixture Samples
CNT	1	1 (100)	---	---
CNJ	38	37 (97.4)	---	1 (2.6%)
BOC	4	4 (100)	---	---
RZA	22	22 (100)	---	---
NAV	14	14 (100)	---	---
MON	7	7 (100)	---	---
RAS	33	33 (100)	---	---
PAU	1	1 (100)	---	---
CER	6	6 (100)	---	---
ROZ	46	1 (2.2)	38 (82.6)	7 (15.2)
TIE	42	42 (100)	---	---
IRU	57	55 (96.5)	---	2 (3.5)
CAS	21	21 (100)	---	---
PIE	7	7 (100)	---	---
CVJ	20	18 (90.0)	2 (10.0)	---
CVA	11	11 (100)	---	---
MOM	10	3 (30.0)	7 (70.0)	---
CAN	8	7 (87.5)	---	1 (12.5)
ALD	10	10 (100)	---	---
VIL	15	15 (100)	---	---
STR	29	29 (100)	---	---
BEN	25	24 (96.0)	---	1 (4.0)

For performing NewHybrids analysis, the 368 *U. glabra* individuals which had clustered into group A (by STRUCTURE analysis) and the 15 *U. minor* individuals which had clustered into group B (by STRUCTURE analysis), were treated as reference samples for *U. glabra* and *U. minor* respectively. Similar to STRUCTURE analysis, all individuals with a posterior assignment probability  $p \geq 0.9$  [61], were placed in one of the six predefined genotypic classes (Table S4). Based on NewHybrids analysis, 372 individuals were assigned to parental class of *U. glabra*, 22 (all from ROZ population and with the same genotype; see Table S1) together with 15 *U. minor* samples were assigned to *U. minor* class, and 26 to F<sub>1</sub> hybrid class (16 from ROZ population, 7 from MOM, 2 from CVJ and 1 from IRU). The remaining seven samples (5 from ROZ population, 1 from IRU and 1 from CAN) showed values of posterior assignment probability lower than 0.9 and were not assigned to any parental or hybrid class (Table S4). The 55 individuals (22 of ROZ + 26 of F<sub>1</sub> hybrid class + 7 unassigned) which were not classified into *U. glabra* parental class following NewHybrids analysis, showed the highest number of heterozygotic loci (7 to 10; see Table S1) and these individuals also shared 5 to 10 alleles with *U. minor* (see Table S1).

## 4. Discussion

### 4.1. Genetic Diversity and Population Relationship

An analysis of microsatellite diversity values ( $N_s = 427$ ,  $H_O = 0.477$  and  $H_E = 0.566$ ; see Table 2) of populations of *U. glabra* from the Central System yielded lower values than a similar study in the

Suserup Forest of Denmark ( $N_s = 29$ ,  $H_O = 0.759$  and  $H_E = 0.774$ ; [34]). However, as each study analyses a different number of samples, one should exercise caution while comparing the results. In the present analysis as well as in the study by Nielsen and Kjær [34], Ulmi1-165 was the most polymorphic and informative locus, suggesting that Ulmi1-165 is an appropriate locus to study genetic diversity of *U. glabra*.

Populations with the highest number of individuals (IRU,  $N_t > 6500$ ; STR,  $N_t > 5000$ ) showed high diversity indexes (Table 3). However, this trend cannot be generalized (e.g., populations MOM and CAN which have small population size also show high diversity values). The location of IRU and STR in protected areas (Natural Reserve of Valley of Iruelas and ZEPA—Zona de Especial Protección para las Aves—of Sierra de Gata, respectively) has probably helped to maintain the high number of individuals. Also, most of the IRU and STR individuals are young with very limited rates of sexual reproduction [2]. According to Rossignoli and Génova [2], in the last few decades, DED has affected the populations of Wych elm from the Central System. As a consequence of this, many ramets are formed by sprouting from the stumps of the old trees, resulting in an increase of young individuals in a population. Thus, DED may be the reason behind the high number of young individuals, as the older elms are preferred by bark beetles and therefore perish due to disease [62].

Population CER had only six young individuals in nature, located in an area of approximately 20 m<sup>2</sup>, which showed the same genotype. This probably indicates that they may be sprouts of one adult tree, which does not exist anymore. In general, the presence of sprouting was detected in all *Ulmus* populations analyzed in this study. Wych elm does not show root sprouts but sprouts on trunks of young trees have been described by Cox et al. [15]. Vegetative reproduction by sprouting helps to diminish the effects of fragmentation and genetic drift, thus avoiding allelic loss [63] and contributing to clonal restoration of *U. glabra* populations on disturbed sites [34].

In general, signs of population isolation and low gene flow were detected throughout the Central System ( $F_{ST} = 0.239$ ) compared to that reported for some other wind-pollinated temperate forest species:  $F_{ST} = 0.049$  in *Quercus garryana* Douglas ex Hook [64],  $F_{ST} = 0.052$  in *Populus euphratica* Olivier [65],  $\Phi_{ST} = 0.013$  in *Fraxinus excelsior* L. [66], and  $F_{ST} = 0.074$  in *Castanea sativa* Mill. [67]. In the present study, high and significant  $F_{ST}$  values among population pairs indicated both high genetic differentiation and low average diversity within populations, probably due to small population size in most of them. Pollen flow up to 1000 m was estimated in *U. glabra* [34], so geographic distances between elm populations appear to have favored population differentiation observed in the Central System.

The UPGMA tree showed a main cluster of 17 populations and another of two populations. Populations MOM and ROZ group together and form a separate cluster, probably because both these populations are similar in that they contain a high number of putative hybrids (38/46 in ROZ and 7/10 in MOM) with *U. minor* and also low  $F_{ST}$  amongst them. Within the majority cluster, MON and RAS populations are separated from the rest, probably because they are geographically isolated populations. Also, these populations showed high frequency of private alleles. The presence of private alleles has been associated with low gene flow and, therefore, population isolation [68]. The clustering results are related to geographic isolation and suggest a decrease in gene flow because of larger geographical distances [69]. In addition, the presence of BEN in the majority cluster suggests its genetic relatedness with other Iberian population in the Central System. This indicates *U. glabra* in Portugal (BEN) is a part of natural populations, as proposed by Monteiro-Henriquez et al. [70].

#### 4.2. Demographic Analysis

Demographic analyses using Wilcoxon's test indicated bottlenecks in fewer populations (4 of 15; NAV, ROZ, TIE and MOM) compared to  $M$ -ratio values, where the 15 analyzed populations revealed bottlenecks. A similar trend of detection of bottlenecks by the two statistical methods was observed in *U. laevis* populations by Fuentes-Utrilla et al. [17]. Low  $M$ -values in the 15 populations indicated an ancestral and extended bottleneck [50]. Bottlenecks described in Spanish populations of *U. laevis* were attributed to Holocene expansion of more xeric habitats in the Iberian Peninsula [17] or glacial

periods in Pleistocene [30]. It is probable that the same factors have affected the Wych elm, contributing to an ancestral and progressive loss of trees in the Central System, reducing the interpopulation gene flow (shown by high genetic differentiation) and leading to fragmented populations.

Four of the studied populations (NAV, ROZ, TIE and MOM) showed recent bottleneck signals as detected by  $T_2$  statistic and Wilcoxon's test. In contrast to the  $M$ -value,  $T_2$  bottleneck statistic is more effective in detecting bottlenecks in populations that have experienced a recent and severe reduction in the number of individuals [47].

In most populations of *U. glabra* from the Central System, clear signs of anthropogenic activity were observed, which can be attributed to different forestry land-uses (i.e., wood removals, grazing, etc.). In the case of NAV (now a managed pinewood forest), there is a history of successive and high wood removals [71]. Thus, it is possible that the *Ulmus* stands from NAV population also have been affected. According to the National Plan of Aerial Orthophotography (PNOA, © Instituto Geográfico Nacional; <http://pnoa.ign.es/>), in TIE population also a similar process of wood removal was observed in the 1970's. Therefore, it is possible that forest land-uses could be the reason for the existence of a recent bottleneck in TIE and NAV populations. In such cases, bottlenecks can further increase the interpopulation differentiation that existed prior to the anthropogenic intervention [72].

In the MOM and ROZ populations, only two genotypes in 10 samples and 13 genotypes in 46 samples, respectively, were observed (i.e., <30% of the sampled individuals). This may be a consequence of high rates of sprouting in these populations. The recent bottlenecks observed in these populations may be due to samples being the ramets of the same clone which the software recognized as a bottleneck. Also, repeated genotypes due to vegetative reproduction cause a reduction in  $N_e$  of populations. Another effect of asexual reproduction is the absence of segregation, leading to maintenance of some of the allelic combinations in heterozygotes, which resulted in negative  $F$  values [73].

Furthermore, a very low  $N_e$  (except in IRU), was observed in populations of the Central System. A reduction of  $N_e$  of population is caused by factors leading to genetic drift, and such populations with low  $N_e$  have a greater probability of extinction [74]. Wych elm is a self-incompatible species [34], and therefore even more susceptible to habitat fragmentation and reduction in population sizes. Thus, population fragmentation in Wych elm in the Central System is evident, with a scarce number of populations and a limited number of trees.

#### 4.3. Introgression between *U. glabra* and *U. minor*

In the present study of populations of the Central System, introgression between *U. glabra* and *U. minor* was detected. Such introgression has also been observed in other European populations of Wych elm [15,75]. The genetic proximity between *U. glabra* and *U. minor* [18], and the lack of reproductive barriers between them [15], have favored natural hybridization. These two factors (i.e., genetic relatedness and absence of reproductive barriers) are probably responsible for the large number of alleles shared between both species, some of them in high frequency.

Putative hybrids constituted 13% (55 of 427 individuals) and were detected in five of 22 populations of the Central System (ROZ, IRU, CVJ, MOM and CAN). Although significant introgression was observed, it was less compared to other studies, e.g., in natural populations of Wych elm in northern Belgium (46%; [15]). The attributed reason is that in Belgium, *U. glabra* is more abundant and coexists with *U. minor* at the same altitude [15]. However, in the Central System the presence of *U. glabra* is limited and its populations can be found at an average altitude of 1150 m reaching <1800 m [76], while *U. minor* does not usually surpass 1200 m [77], thus introgression in the Central System is more restricted.

Besides co-existence of the two species at the same altitudes, introgression is influenced by anthropogenic activities as well [78–80]. Traditionally, *U. minor* is the more cultivated species and is frequently naturalized [76]. Populations of ROZ and MOM are located close to individuals of *U. minor* and are exposed to anthropogenic activities. These factors have probably resulted in

maximum hybridization (38/46 individuals in ROZ and 7/10 individuals in MOM). The presence of large numbers of putative hybrids in ROZ and MOM is responsible for its high observed heterozygosity and estimated diversity values. Also, the inbreeding coefficient of the MOM and ROZ populations was highly negative, indicating excess of heterozygosity. Similar results were obtained by Zalapa et al. [13], where the extent of hybridization in naturalized populations of *U. pumila* influenced the genetic diversity.

Throughout the study area, 26 individuals were identified as possible F<sub>1</sub> hybrids by NewHybrids analysis. These samples were heterozygous for the majority of loci (9 or 10 of 11) analyzed, as expected in hybrid individuals. Zalapa et al. [13] have shown a similar increase of genetic diversity due to hybridization between *U. pumila* and *U. rubra*. In addition, the putative hybrid individuals detected in the present study shared numerous alleles with *U. minor*, some of which were present in high frequency in *U. minor* and uncommon in Wych elm, which indicated asymmetric introgression of *U. minor* into *U. glabra*. This could happen because at < 1200 m of altitude in the Central System, *U. minor* is in abundance compared to *U. glabra*, thus facilitating asymmetric gene flow. Due to large differences in abundance of parental species, asymmetric gene flow has been observed in other studies [81,82].

The NewHybrids program also classified 22 individuals of *U. glabra* from ROZ population, which presented the same genotype, into *U. minor* parental class. The lack of sufficient numbers of species-specific exclusive alleles can complicate the classification of individuals when using the NewHybrids software [60]. In the present investigation, the 15 samples of *U. minor* analyzed (which presented six different genotypes and eight exclusive alleles) could be insufficient to assign correctly some hybrid individuals. Morphologically, the 22 samples did not differ much from the rest of *U. glabra* samples collected. The morphological characteristics have been shown to be questionable in the classification of the genus *Ulmus*, especially in hybrid individuals [13,14,23,32,36]. These samples showed a high number of alleles shared with *U. minor* (9 of 18 possible alleles), and were heterozygous for most loci (10 of 11) analyzed. Consequently, these 22 individuals probably have undergone a high level of introgression with *U. minor*.

As in *U. minor*, root suckering was also observed in introgressed individuals from ROZ and MOM populations, which increases the number of introgressed samples with the same genotype in these populations. Similar observations were made by [15].

In the present study, despite extensive sampling, no F<sub>2</sub> individuals were detected using the NewHybrids program. Cox et al. [15] also did not find F<sub>2</sub> offspring of F<sub>1</sub> hybrids among elms sampled in northern Belgium. One possible explanation for failing to detect F<sub>2</sub> hybrids could be that an increase of homozygous incompatibility in F<sub>2</sub> individuals would lead to loss of fitness [83]. The other possibilities are the existence of pollen–stigma incompatibilities in F<sub>1</sub> hybrid, which would contain S-alleles from the two parental sources [35], or because of outbreeding depression [84].

## 5. Conclusions and Future Prospects

Populations of ROZ and MOM showed lowest numbers of genotypes, high heterozygosity, lowest genetic differentiation between the two populations, and highly negative values of inbreeding coefficient. This points towards the existence of introgression and propagation through vegetative means (as indicated by high  $H_O$ , low  $G_O$  and highly negative  $F$ ) and that the populations are genetically not very different (low  $F_{ST}$ ). The estimated population size is much lower (ROZ = 46, MOM = 18) compared to IRU (>6500) and STR (>5000). This could probably be due to the location of the IRU and STR populations in protected and undisturbed areas. Populations IRU and STR not only maintained a high number of individuals but also represented a very high number of genotypes (>95% of the sampled individuals), indicating low frequency of clonal propagation. A distinct feature of ROZ and MOM populations was that maximum numbers of putative hybrids (with *U. minor*) were detected, which enhanced their genetic diversity indexes. Population of RAS on the other hand did not show any putative hybrids and revealed lowest diversity indexes. However, a high number of genotypes with private alleles was detected (>87% of the analyzed individuals) in RAS population compared to ROZ

and MOM (<30% of the sampled individuals), and high genetic differentiation between RAS–ROZ and RAS–MOM population pairs was observed. Thus, the RAS population appears to be more isolated with respect to interpopulation gene flow. Such information can play an important role in the formulation of appropriate conservation strategies for *Ulmus* populations of the Central System.

In general, populations of *U. glabra* analyzed in the Central System showed recent and/or ancestral bottlenecks and low effective population size. As *U. glabra* is a self-incompatible species, has bottlenecks and reduced  $N_e$ , the chances of population fragmentation and decrease in number of individuals, and probability of extinction increases. At this point, it is also important to note that existence of clonal propagation in *U. glabra* populations possibly contributes to reducing the effects of fragmentation and genetic drift and hence allelic loss. Also, existence of natural hybridization with *U. minor* influences genetic diversity and may elevate the number of alleles and effective alleles in *U. glabra* populations.

To summarize, the fragmented and relict natural populations of *U. glabra* of the Central System of the Iberian Peninsula are marked by moderate genetic diversity, high genetic differentiation and ancestral as well as extended bottlenecks. The small number of fragmented populations with diminishing number of individuals in most populations, along with anthropogenic intervention and the biotic stress of DED are the main threats to Wych elm in the Central System. Further, there is no continuity of *U. glabra* populations of the Central System with the populations of the rest of the peninsula and no strategies for conservation of these relict populations. This is especially true in the province of Avila, where most of the populations have aggregated. Therefore, the present information generated from this study (i.e., high genetic differentiation and lack of continuity of Central System populations with the rest of the peninsula) emphasizes the need for formulating regional conservation strategies to secure the future of the Central System populations.

**Supplementary Materials:** The following are available online at [www.mdpi.com/1999-4907/8/5/143/s1](http://www.mdpi.com/1999-4907/8/5/143/s1), Figure S1: Estimation of the optimal number of genetic groups ( $K$ ) based on the rate of change in the statistic  $\Delta K$  between successive  $K$  values [57], for  $K = 1$  to 25 (using data from 11 microsatellite markers analyzed in 427 *U. glabra* individuals; a) and  $K = 1$  to 10 (using data from the nine microsatellite loci that were amplified in both species, 427 *U. glabra* individuals and 15 *U. minor* samples; b)); Table S1: Allele sizes in base pairs at each of eleven microsatellite loci analyzed in 427 *U. glabra* individuals from 22 populations and 15 *U. minor* individuals from three locations sampled in the Central System; Table S2: Allele sizes (in base pairs) and frequencies at each of eleven microsatellite loci analyzed in 427 *U. glabra* and 15 *U. minor* individuals sampled from the Central System; Table S3: Pairwise  $F_{ST}$  (below the diagonal) and  $p$  (above the diagonal) values among 19 *U. glabra* populations (see Table 1 for codes) based on eleven microsatellite loci data using the AMOVA method. Significance was obtained on 10,000 random permutations; Table S4: Results of Structure and NewHybrids analysis obtained in 442 elm individuals (427 *U. glabra* and 15 *U. minor*) studied.

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