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Indications of Genetic Admixture in the Transition Zone between *Fagus sylvatica* L. and *Fagus sylvatica* ssp. *orientalis* Greut. & Burd

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Abstract: Two subspecies of European beech (*Fagus sylvatica* L.) can be found in southeast Europe: *Fagus sylvatica* ssp. *sylvatica* L. and *Fagus sylvatica* ssp. *orientalis* (Lipsky) Greut. & Burd. (*Fagus orientalis* Lipsky). In a previous study, based on genetic diversity patterns and morphological characters, indications of hybridization between both subspecies were found in northeastern Greece, a known contact zone of *F. sylvatica* and *F. orientalis*. Nevertheless, potential genetic admixture has not been investigated systematically before. Here, we investigated genetic diversity and genetic structure of 14 beech populations originating from Greece and Turkey as well as of two reference *F. sylvatica* populations from Germany based on nine expressed sequence tag-simple sequence repeat (EST-SSR) markers. Very low genetic differentiation was detected among *F. sylvatica* populations (mean G_{ST}: 0.005) as well as among *F. orientalis* populations (mean G_{ST}: 0.008), but substantial differentiation was detected between populations of the two subspecies (mean G_{ST}: 0.122). Indications for hybridization between both subspecies were revealed for one population in Greece. One of the genetic markers showed specific allele frequencies for *F. sylvatica* and *F. orientalis* and may be used as a diagnostic marker in future studies to discriminate both subspecies.

Keywords: hybridization; genetic diversity; microsatellites; Fagaceae; forest; beech; diagnostic marker

1. Introduction

In southeast Europe, two subspecies of *Fagus sylvatica* L. can be found: *Fagus sylvatica* ssp. *sylvatica* (hereafter *F. sylvatica*) and *Fagus sylvatica* ssp. *orientalis* (Lipsky) Greut & Burd. (hereafter *F. orientalis*) [1,2], whereby the status of these two taxa as subspecies or species and their phylogeny still needs to be clarified [3,4]. *F. sylvatica* is distributed over large areas in Europe, whereas *F. orientalis* ranges from the southeastern Balkan to northern Iran [5]. Several studies were conducted to investigate morphological and genetic variation patterns within the distribution area of the two subspecies. For instance, Denk et al. [6] conducted a morphological analysis of beech populations covering the range of species in western Eurasia. The authors detected a west-east gradient of morphological characteristics with overlapping variability in morphological types. Differences in morphological traits were also revealed on a more regional scale. Thus, morphological forms resembling *F. sylvatica* were found in western parts of Greece, whereas morphological types resembling *F. orientalis* were found in the eastern parts of the country [7]. Populations of *F. sylvatica* and *F. orientalis* were also investigated using different types of genetic markers such as amplified fragment-length polymorphisms (AFLPs), chloroplast microsatellites, internal transcribed

spacer (ITS) sequences, and isozymes [6,8–13]. Genetic analyses revealed clinal variation of increasing genetic diversity from west to east [9,11]. Much higher haplotype diversity was found for beech in southeastern Europe compared to central and western Europe [8,10,13], likely due to the migration history of the species during the Pleistocene. Based on AFLPs [10] and isozymes [12], it was possible to group beech populations into *F. sylvatica* and *F. orientalis*, albeit no clear species-specific alleles were identified.

Papageorgiou et al. [9] investigated both subspecies in their potential transition zone in the Rodopi Mountains in northeastern Greece. Morphological traits and genetic variation revealed characteristics resembling *F. sylvatica* mainly in the western parts of the mountains and at higher altitudes, whereas characteristics resembling *F. orientalis* were mainly found in the eastern parts of the mountains and at lower elevations. Intermediate phenotypes were also detected in the investigated populations [9]. The intermediate phenotypes and higher genetic diversity compared to other beech populations indicate introgression between *F. sylvatica* and *F. orientalis* in this area [7]. Here, we further analyzed the genetic structure of beech populations in the potential transition zone between *F. sylvatica* and *F. orientalis* in Greece and Turkey. Based on nine expressed sequence tag-simple sequence repeat (EST-SSR) markers, genetic diversity and differentiation of 16 beech populations were determined, and potential genetic admixture among populations was analyzed.

2. Materials and Methods

2.1. Plant Material

In total, 16 beech populations were investigated (Figure 1, Table 1). DNA samples of six Turkish *F. orientalis* populations (Covakici, Duezce, Catalca, Inegoel, Izmit, and Karabuek) were obtained (ten samples per population) from a previous study [10]; they originate from trees of a provenance trial in Germany that was established in 1986/1987 [14]. Furthermore, DNA samples from two North German *F. sylvatica* populations (Calvoerde and Goehrde, used as *F. sylvatica* reference populations in this study) were obtained (24 samples per population) from Seifert [15]. Four potential (based on morphological assessment) *F. sylvatica* populations were sampled in Northwest Greece (Alevitsa, Varnuntas, Aetomilitsa, and Tsepelovo), and two potential *F. sylvatica* populations were sampled in West Rodopi in Northeast Greece (Frakto, Lepida). Finally, one potential *F. orientalis* population was sampled in East Rodopi in Northeast Greece (Hilia), and one potential *F. orientalis* population was sampled in Northwest Turkey (Demirkoy). In each population, leaves from 24 randomly selected individuals, with a minimum distance of 100 m among each other, were sampled. For an easier identification of the populations in the manuscript, we will use the prefixes "Fs" for *F. sylvatica* and "Fo" for *F. orientalis*, and the country name will be used as a suffix to indicate the population origin (e.g., "Fo-Duezce-Turkey" for the *F. orientalis* population Duezce from Turkey).

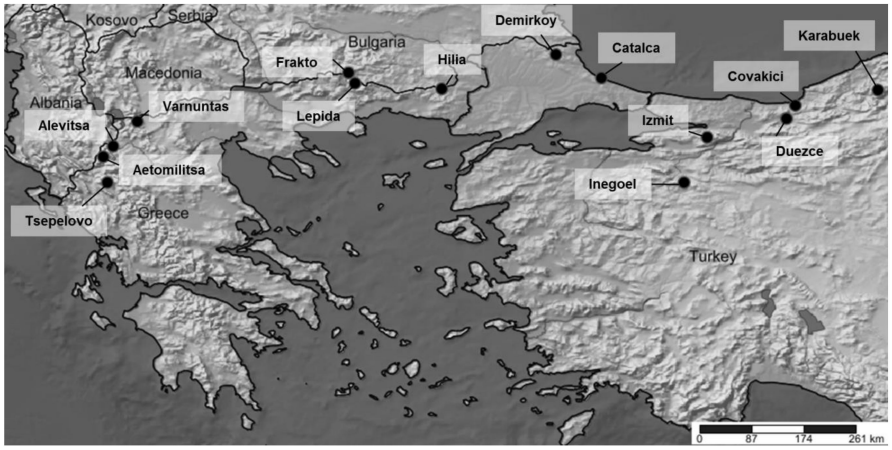

Figure 1. Locations of the sampled populations in Greece and Turkey. The German reference populations are not shown. The map was generated with SimpleMappr [16].

Table 1. Population characteristics.

Population Name	Origin	No. of Samples	Subspecies	Latitude	Longitude
Calvoerde	Germany	24	*F. sylvatica*	52.403967	11.261017
Goehrde	Germany	24	*F. sylvatica*	53.122983	10.820400
Aetomilitsa	Greece	24	*F. sylvatica* **	40.272958	20.813221
Tsepelovo	Greece	24	*F. sylvatica* **	39.882826	20.876105
Alevitsa	Greece	24	*F. sylvatica* **	40.432116	20.963033
Varnuntas	Greece	24	*F. sylvatica* **	40.806592	21.328064
Frakto	Greece	24	*F. sylvatica* **	41.545455	24.523565
Lepida	Greece	24	*F. sylvatica* **	41.386269	24.621609
Hilia	Greece	24	*F. orientalis* **	41.302195	25.934421
Demirkoy	Turkey	24	*F. orientalis* **	41.819996	27.662091
Catalca	Turkey *	10	*F. orientalis*	41.466670	28.350000
Inegoel	Turkey *	10	*F. orientalis*	39.883330	29.600000
Izmit	Turkey *	10	*F. orientalis*	40.566670	29.950000
Duezce	Turkey *	10	*F. orientalis*	40.850000	31.150000
Covakici	Turkey *	10	*F. orientalis*	41.050000	31.283330
Karabuek	Turkey *	10	*F. orientalis*	41.283330	32.533330

* Samples were obtained from a provenance trial in Germany (see Materials and Methods). ** Based on morphological assessment [9,17].

2.2. DNA Extraction and Genotyping

Total DNA was extracted from dried leaves of the newly sampled populations with the DNeasy 96 Plant Kit (Qiagen, Hilden, Germany). All samples were genotyped at 9 EST-SSR markers (Table S1) obtained from previous studies [18,19]. The primer FS_C4971 was analyzed in a separate PCR, while the other primers were compiled into multiplex reactions (multiplex 1: FgSI0006, FgSI0024, FS_C1968, FS_C2361; multiplex 2: FgSI0009, FS_C7377; multiplex 3: FS_C6785, FS_C7797). The following touchdown PCR program was used for all reactions: an initial denaturation of 95 °C for 15 min, followed by 10 touchdown cycles of 94 °C for 1 min, 60 °C (−1 °C per cycle) for 1 min, and 72 °C for 1 min, 25 cycles of 94 °C for 1 min, 50 °C for 1 min, and 72 °C for 1 min, followed by a final extension step of 72 °C for 20 min. The PCR mix consisted of 1 µL DNA (ca. 0.6 ng/µL), 1.5 µL 10× reaction buffer B (Solis BioDyne, Tartu, Estonia), 1.5 µL $MgCl_2$ (25 mM), 1 µL dNTPs (2.5 mM each dNTP), 0.2 µL (5 U/ µL) HOT FIREPol *Taq* DNA polymerase (Solis BioDyne, Tartu, Estonia), 0.2 µL (5 picomole/µL) tailed forward primer (a M13-specific sequence (5′-CACGACGTTGTAAACGAC-3′) was added to the 5′ end of the primer [20,21]), 0.5 µL (5 picomole/ µL) PIG-tailed (the sequence 5′- GTTTCTT-3′ was added to the 5′ end of the primer [22]) reverse primer, 1 µL (5 picomole/µL) dye labeled (6-FAM or 6-HEX) M13 primer, and H_2O (filled up to a volume of 12.4 µL). Fragments were separated on an ABI 3130xl Genetic Analyzer (Applied Biosystems, Foster City, USA) using GS 500 ROX (Applied Biosystems, Foster City, USA) as size standard. Allele scoring was conducted with the GeneMapper 4.0 software (Applied Biosystems, Foster City, CA, USA).

2.3. Data Analysis

The GenAlEx 6.5 software [23,24] was used to calculate the number of alleles (N_a), the observed heterozygosity (H_o), and the expected heterozygosity (H_e). Furthermore, the software was used to calculate pairwise G_{ST} values [25,26] between populations based on 999 permutations. The inbreeding coefficient (F_{IS}) and allelic richness (A_R) were calculated with the FSTAT 2.9.4 software [27]. F_{IS} values were corrected for multiple testing using a sequential Bonferroni correction [28] implemented in FSTAT. The software was further used to test for significant differences in A_R, H_o, and H_e between *F. sylvatica* populations and *F. orientalis* populations (excluding the Fo-Hilia-Greece population, since it was revealed to be a hybrid population between the two subspecies) based on 1000 permutations. The presence of null alleles was checked with the MICRO-CHECKER 2.2.3 software [29]. Outlier analyses were performed with the LOSITAN 1.0 software [30] and the BayeScan 2.1 software [31]. For the LOSITAN

analysis, the stepwise mutation model, 70,000 simulations, and a false discovery rate (*FDR*) of 0.05 were used. For the BayeScan analysis, default parameters were selected and loci with a *q*-value lower than 5% were considered to be outliers. The populations 1.2.32 software [32] was used to generate a neighbor-joining (NJ) dendrogram based on Nei's genetic distances [33]. Bootstrap values were calculated based on 1000 permutations over loci. The tree was visualized using Tree Explorer implemented in MEGA 7.0.26 [34]. The STRUCTURE 2.3.4 software [35] was used to infer population structure. The admixture model and correlated allele frequencies were selected. A burn-in period of 30,000 and Markov chain Monte Carlo (MCMC) replicates of 100,000 were used. Potential clusters (*K*) from 1 to 20 were tested using 10 iterations. The Δ *K* method [36] was used to determine the most likely number of *K* with the STRUCTURE HARVESTER 0.6.94 program [37]. The CLUMPAK software [38] was employed for summation and graphical representation of the STRUCTURE results.

3. Results

The mean number of alleles (N_a) over all populations ranged from 2.6 for marker FS_C6785 to 9.6 for marker FS_C1968 (Table 2). The observed heterozygosity (H_o) ranged from 0.284 for marker FS_C6785 to 0.769 for marker FS_C1968, and the expected heterozygosity (H_e) ranged from 0.278 for marker FS_C6785 to 0.761 for marker FS_C1968. For no marker, F_{IS} values significantly different from zero were detected (Table 2).

Table 2. Mean genetic diversity indices over all populations for each marker.

Marker	*N*	N_a	H_o	H_e	F_{IS}	G_{ST}
FgSI0006	18.6	3.0	0.345	0.349	0.029	0.244 *
FgSI0024	18.6	4.6	0.589	0.600	0.057	0.014
FS_C1968	18.4	9.6	0.769	0.761	0.019	0.032 *
FS_C2361	18.4	3.8	0.564	0.534	−0.032	0.118 *
FgSI0009	17.4	3.0	0.447	0.473	0.085	0.099 *
FS_C6785	18.7	2.6	0.284	0.278	0.012	0.469 *
FS_C7377	18.6	3.2	0.521	0.509	−0.006	0.158 *
FS_C7797	18.5	3.6	0.415	0.346	−0.145	0.016 *
FS_C4971	18.7	5.1	0.706	0.636	−0.078	0.095 *
Mean	18.4	4.3	0.515	0.498	−0.007	0.132 *

N—number of individuals, N_a—number of alleles, H_o—observed heterozygosity, H_e—expected heterozygosity, F_{IS}—inbreeding coefficient, G_{ST}—fixation index (* $p < 0.05$).

The mean number of alleles (N_a) ranged from 3.2 for the Fo-Covakici-Turkey population to 5.2 for the Fo-Demirkoy-Turkey population (Table 3). Mean allelic richness (A_R) ranged from 2.9 for the Fs-Goehrde-Germany population to 4.2 for the Fo-Inegoel-Turkey population. The observed heterozygosity (H_o) ranged from 0.421 for the Fs-Goehrde-Germany population and 0.599 for the Fo-Karabuek-Turkey population (mean H_o: 0.515), while the expected heterozygosity (H_e) ranged from 0.402 for the Fs-Goehrde-Germany population to 0.582 for the Fo-Hilia-Greece population (mean H_e: 0.498). The mean F_{IS} value was −0.002 over all populations, and F_{IS} was not significantly different from zero in any population (Table 3). Mean genetic diversity indices (A_R, H_o, and H_e) were higher for *F. orientalis* populations compared to *F. sylvatica* populations, but only A_R was significantly higher in *F. orientalis* (mean in *F. sylvatica*: 3.3, mean A_R in *F. orientalis*: 3.7).

Mean pairwise G_{ST} was 0.005 among *F. sylvatica* populations (excluding the two German reference populations), 0.008 among *F. orientalis* populations (without the potentially admixed Fo-Hilia-Greece population, see below), and 0.122 between *F. sylvatica* and *F. orientalis* populations (Table 4). Evidence for null alleles was only detected for markers FS_C1968 and FgSI0009 in population Fo-Hilia-Greece as well as for marker FS_C73377 in population Fo-Catalca-Turkey. Based on LOSITAN, four outlier loci (FgSI0006, FS_C2361, FS_C6785, and _FS_C7377) were detected, potentially under directional selection. With BayeScan, one outlier locus (FS_C1968) was found, which was potentially under balancing or

purifying selection. Locus FS_C6785, located in a sequence annotated as a putative *ribosomal protein* [18], showed a high genetic differentiation between *F. sylvatica* and *F. orientalis* (G_{ST}: 0.504). The allele 189 at this locus showed a frequency of 0.849 in *F. sylvatica*, whereas the allele frequency was 0.070 in *F. orientalis*. The allele 192 showed a much lower frequency in *F. sylvatica* (0.148) compared to *F. orientalis* (0.842) (Figure 2). See Figure S1 for an electropherogram showing an example of peaks 189 and 192 of marker Fs_C6785.

Table 3. Sample size and mean genetic diversity indices for all populations.

Population	*N*	*Na*	A_R	H_o	H_e	F_{IS}
Fs-Calvoerde-Germany	24	3.8	3.1	0.500	0.464	−0.056
Fs-Goehrde-Germany	24	3.4	2.9	0.421	0.402	−0.026
Fs-Aetomilitsa-Greece	24	5.0	3.5	0.551	0.528	−0.022
Fs-Tsepelovo-Greece	24	4.2	3.4	0.516	0.519	0.028
Fs-Alevitsa-Greece	24	4.4	3.3	0.506	0.499	0.009
Fs-Varnuntas-Greece	24	4.8	3.4	0.527	0.512	−0.007
Fs-Frakto-Greece	24	4.7	3.3	0.532	0.503	−0.034
Fs-Lepida-Greece	24	4.1	3.4	0.485	0.546	0.133
Fo-Hilia-Greece	24	4.8	3.8	0.563	0.582	0.053
Fo-Demirkoy-Turkey	24	5.2	3.7	0.569	0.500	−0.117
Fo-Catalca-Turkey	10	3.9	3.7	0.460	0.472	0.079
Fo-Inegoel-Turkey	10	4.4	4.2	0.468	0.512	0.143
Fo-Izmit-Turkey	10	4.2	3.9	0.543	0.490	−0.054
Fo-Duezce-Turkey	10	4.0	3.8	0.558	0.500	−0.062
Fo-Covakici-Turkey	10	3.2	3.1	0.447	0.421	−0.002
Fo-Karabuek-Turkey	10	4.0	3.8	0.599	0.522	−0.094
Mean	18.8	4.3	3.5	0.515	0.498	−0.002

N—number of individuals, N_a—number of alleles, A_R—allelic richness, H_o—observed heterozygosity, H_e—expected heterozygosity, F_{IS}—inbreeding coefficient.

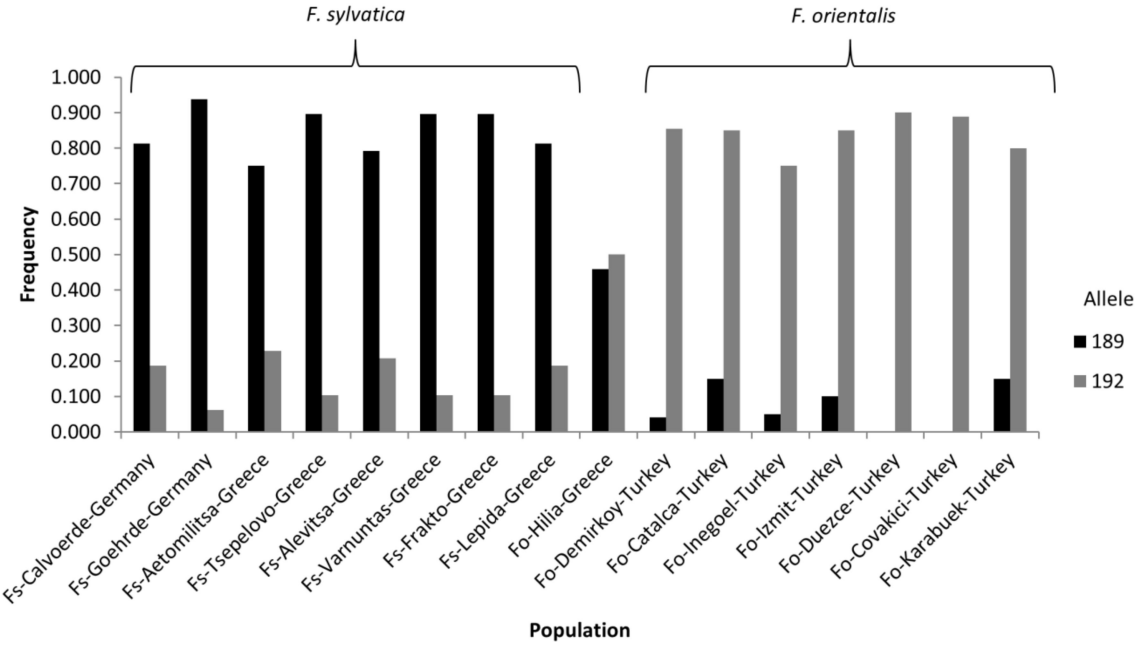

Figure 2. Frequencies of alleles 189 and 192 of locus Fs_C6785 for the different populations.

Table 4. Pairwise G_{ST} values between populations.

Populations	Fs-Calvoerde-Germany	Fs-Goehrde-Germany	Fs-Aetomilitsa-Greece	Fs-Tsepelovo-Greece	Fs-Alevitsa-Greece	Fs-Varnuntas-Greece	Fs-Frakto-Greece	Fs-Lepida-Greece	Fo-Hilia-Greece	Fo-Demirkoy-Turkey	Fo-Catalca-Turkey	Fo-Inegoel-Turkey	Fo-Izmit-Turkey	Fo-Duezce-Turkey	Fo-Covakici-Turkey	Fo-Karabuek-Turkey
Fs-Calvoerde-Germany	0.000															
Fs-Goehrde-Germany	0.016	0.000														
Fs-Aetomilitsa-Greece	0.014	0.039	0.000													
Fs-Tsepelovo-Greece	0.014	0.026	0.001 $^{+}$	0.000												
Fs-Alevitsa-Greece	0.015	0.046	0.000 $^{+}$	0.010	0.000											
Fs-Varnuntas-Greece	0.009	0.032	0.003 $^{+}$	0.001 $^{+}$	0.006	0.000										
Fs-Frakto-Greece	0.009	0.032	0.004 $^{+}$	0.002 $^{+}$	0.012	0.005 $^{+}$	0.000									
Fs-Lepida-Greece	0.020	0.043	0.005 $^{+}$	0.009	0.013	0.010	0.000 $^{+}$	0.000								
Fo-Hilia-Greece	0.073	0.104	0.054	0.047	0.069	0.062	0.039	0.035	0.000							
Fo-Demirkoy-Turkey	0.150	0.190	0.125	0.121	0.143	0.140	0.116	0.106	0.019	0.000						
Fo-Catalca-Turkey	0.159	0.205	0.119	0.120	0.139	0.141	0.114	0.099	0.024	0.013	0.000					
Fo-Inegoel-Turkey	0.124	0.158	0.111	0.103	0.127	0.121	0.101	0.097	0.018	0.006 $^{+}$	0.019	0.000				
Fo-Izmit-Turkey	0.139	0.183	0.115	0.112	0.136	0.131	0.102	0.092	0.012	0.004 $^{+}$	0.000 $^{+}$	0.000 $^{+}$	0.000			
Fo-Duezce-Turkey	0.137	0.172	0.117	0.115	0.136	0.136	0.106	0.092	0.015	0.002 $^{+}$	0.017 $^{+}$	0.000 $^{+}$	0.000 $^{+}$	0.000		
Fo-Covakici-Turkey	0.185	0.231	0.152	0.145	0.177	0.168	0.144	0.136	0.034	0.001 $^{+}$	0.010 $^{+}$	0.011 $^{+}$	0.005 $^{+}$	0.014 $^{+}$	0.000	
Fo-Karabuek-Turkey	0.136	0.173	0.114	0.103	0.129	0.126	0.109	0.106	0.024	0.015	0.017	0.009 $^{+}$	0.013 $^{+}$	0.014 $^{+}$	0.004 $^{+}$	0.000

$^{+}$ Not significant ($p \geq 0.05$); all other G_{ST} values are significant ($p < 0.05$).

The neighbor-joining dendrogram showed a separation between the Turkish populations and the German and Greek populations, with Fo-Hilia-Greece clustering with *F. orientalis* from Turkey in a basal position (Figure 3). Furthermore, the two West Rodopi populations Fs-Frakto-Greece and Fs-Lepida-Greece grouped together with *F. sylvatica* populations of North Western Greece.

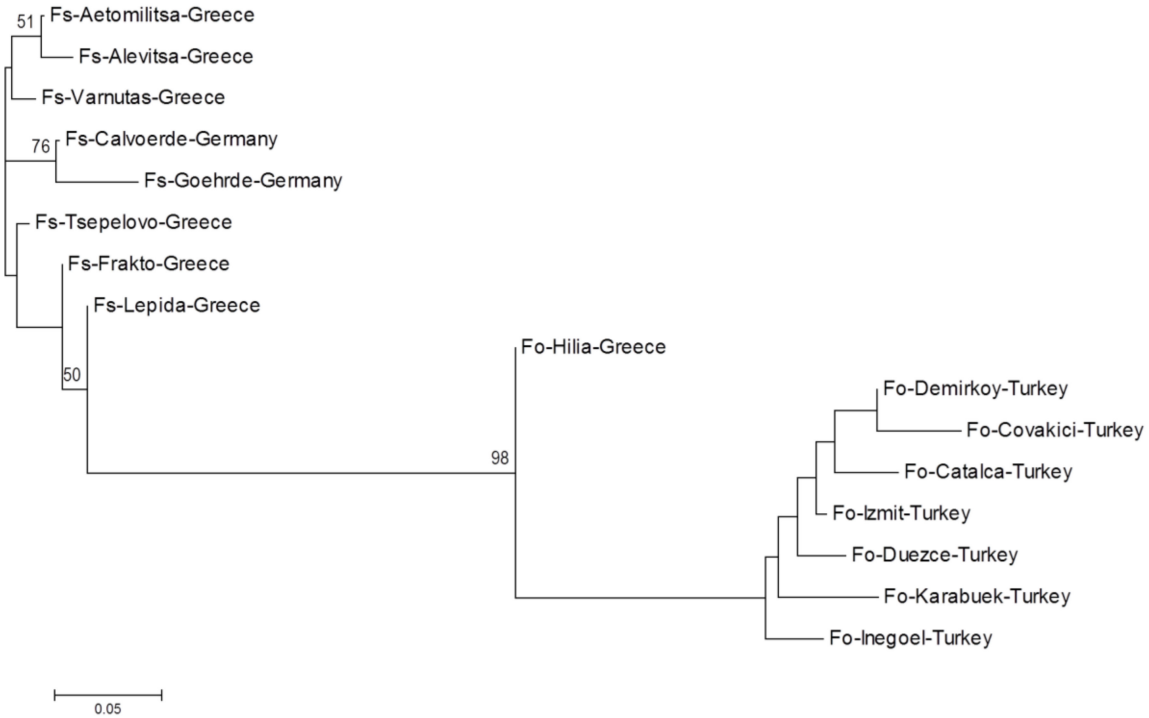

Figure 3. Neighbor-joining (NJ) dendrogram for all populations. Bootstrap values ≥ 50 are shown.

Similar results were obtained from the STRUCTURE analysis. The ΔK method revealed a most likely number of two clusters ($K = 2$) (Figure S2), whereby the German and Greek populations formed one cluster and the Turkish populations the second one. The Fo-Hilia-Greece population was not assigned to one of the two clusters and shows a high degree of admixture (Figure 4). The two West Rodopi populations Fs-Frakto-Greece and Fs-Lepida-Greece cluster together with *F. sylvatica* populations.

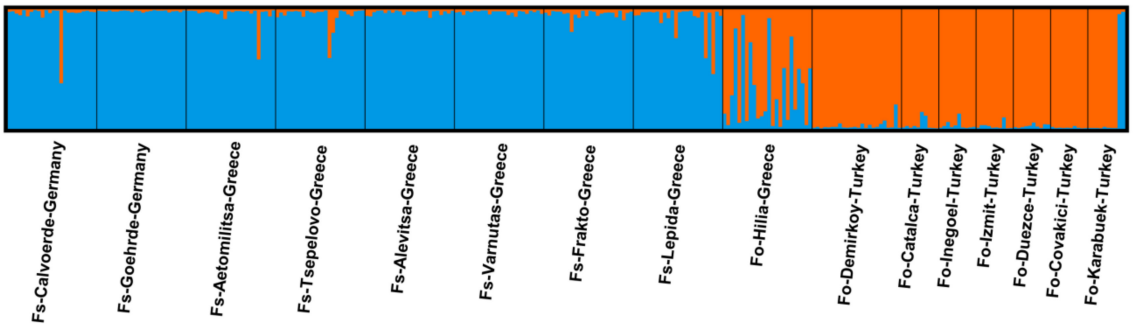

Figure 4. Clustering of individuals for $K = 2$.

4. Discussion

High genetic diversity was revealed for all analyzed beech populations (mean A_R: 3.5, mean H_o: 0.515, mean H_e: 0.498), and no signs of inbreeding were detected. Genetic diversity values were similar to other studies based on EST-SSRs in beech [18,39,40]. Among the diversity indices, only allelic

richness (A_R) was significantly higher in *F. orientalis* (mean A_R: 3.7) compared to *F. sylvatica* (mean A_R: 3.3). Higher A_R in *F. orientalis* compared to *F. sylvatica* populations was also found in a previous study based on isozyme markers [12]. Very low genetic differentiation was detected among *F. sylvatica* populations (mean G_{ST}: 0.005) as well as among *F. orientalis* populations (mean G_{ST}: 0.008). Low genetic differentiation among beech populations in southeast Europe was also found by Gömöry et al. [11] based on isozymes (mean G_{ST}: 0.019 for *Fagus moesiaca*, a putative hybrid form between *F. sylvatica* and *F. orientalis*). Papageorgiou et al. [9] found a genetic differentiation of G_{ST}: 0.089 among beech populations (*F. sylvatica* and *F. orientalis*) in the Rodopi Mountains in northeastern Greece based on AFLPs. These results indicate high gene flow levels among populations. As expected, higher genetic differentiation was detected based on maternally inherited cpDNA markers [8,9].

In the present study, substantial genetic differentiation was found between *F. orientalis* and *F. sylvatica* (mean G_{ST}: 0.122). Even the German reference *F. sylvatica* populations showed substantially lower differentiation to the Greek *F. sylvatica* populations (mean G_{ST}: 0.025) compared to differentiation values between subspecies (mean G_{ST}: 0.167) (Table 4). High genetic differentiation between the two subspecies was also reflected by the NJ dendrogram and the STRUCTURE analysis. In both analyses, two clusters were formed, one comprising *F. sylvatica* populations and the other one comprising *F. orientalis* populations. Two Greek populations located in West Rodopi (Fs-Frakto-Greece and Fs-Lepida-Greece), which previous studies based on chloroplast DNA markers have considered as intermediate or closer to *F. orientalis* [8], clearly group with *F. sylvatica* in both analyses, indicating that they are actually *F. sylvatica*. The population Fo-Hilia-Greece could not be assigned to one of the two subspecies. Previous studies have reported a morphological resemblance of trees from this population with *F. orientalis* [7,9,17]. In the NJ dendrogram, this population is located in an intermediate position between the two clusters, and in the STRUCTURE analysis, it showed a high degree of admixture. Defining individuals with assignment probabilities of ≥0.9 in one cluster as pure species, 0.4 to 0.6 in one cluster as hybrids, and 0.61 to 0.89 as introgressive forms [41], a total of 10 individuals are classified as pure (sub)species, 11 individuals are introgressed forms, and three individuals are hybrids in the population Fo-Hilia-Greece. In combination with intermediate frequencies of *F. sylvatica* and *F. orientalis* specific alleles (see below) in this population, these results indicate hybridization between both subspecies. The population is geographically located in East Rodopi, between *F. sylvatica* populations in the west and *F. orientalis* populations in the east (Figure 1), making contact via gene flow between the two subspecies very likely. Hybridization between *F. sylvatica* and *F. orientalis* has been suggested before for this area [9] and could be confirmed in the present study.

To investigate whether some of the EST-SSRs used in the present study are potentially under selection, outlier analyses were conducted based on grouping of populations into *F. sylvatica* and *F. orientalis*. The Fdist approach [42] implemented in the LOSITAN software [30] revealed four loci (FgSI0006, FS_C2361, FS_C6785, and FS_C7377) to be potentially under directional selection, whereas the Bayesian method implemented in the BayeScan software [31] revealed one potential outlier (FS_C1968), with indications of balancing or purifying selection. Thus, both methods revealed contrasting results. Recently, it was shown that F_{ST}-heterozygosity outlier methods such as the one implemented in LOSITAN are not working reliably if only few populations are compared [43]. In these cases, other methods such as BayeScan may reveal a lower number of false positive results. In the present study, two pooled demes (*F. sylvatica* and *F. orientalis*) were compared with each other, and hence, BayeScan should be the more suitable method in this case. The potential outlier locus (FS_C1968) revealed by BayeScan is located in a sequence annotated as a putative *auxin-response protein* [18]. Auxin-response factors have been shown to be involved in abiotic adaptation (e.g., precipitation/drought, bud burst) in different tree species [44–47], and it has been proposed that beech morphology is related to environmental conditions at its growing sites [7,17]. Furthermore, Varsamis et al. [48] detected significant differences in adaptive traits such as bud burst timing and survival under drought conditions between beech populations from West and East Rodopi in a provenance test and a growth chamber experiment. Thus, this locus may be involved in adaptation in *F. sylvatica* and *F. orientalis*. Albeit,

the LOSITAN results may be inflated by false positive results, the outlier locus Fs_C6785 located in a sequence annotated as a putative *ribosomal protein* [18], and has not been detected in other outlier or environmental association analyses before, is worth noting. Based on a more relaxed *q*-value of 12% (compared to 5% used in the present study), this locus would also be revealed as an outlier by BayeScan. The locus showed a high genetic differentiation between *F. sylvatica* and *F. orientalis* (G_{ST}: 0.504). Strikingly, the allele 189 at this locus showed a high frequency (0.849) in *F. sylvatica*, whereas the allele frequency was low in *F. orientalis* (0.070) (Figure 2). In contrast, allele 192 showed a much lower frequency in *F. sylvatica* (0.148) compared to *F. orientalis* (0.842). The potential hybrid population Fo-Hilia-Greece showed intermediate frequencies for both alleles (allele 189: 0.458, allele 192: 0.500). Thus, this locus may be involved in genetic differentiation of the two subspecies and could be used as diagnostic marker to discriminate *F. sylvatica* and *F. orientalis*.

5. Conclusions

In the present study, we found indications of hybridization between *F. sylvatica* and *F. orientalis* in the transition zone of the two subspecies in northeastern Greece. Based on our marker set, it was possible to discriminate both subspecies. One of the markers (Fs_C6785) showed distinct allele frequencies between *F. sylvatica* and *F. orientalis* and can be used as a diagnostic marker to distinguish both subspecies. This study might be helpful for future studies to further narrow down the hybrid zone of the two subspecies. Future studies may take advantage of genotyping by sequencing approaches to investigate which genomic regions are involved in differentiation of *F. sylvatica* and *F. orientalis*.

Supplementary Materials: The following are available online at http://www.mdpi.com/1424-2818/11/6/90/s1, Figure S1: Electropherogram showing alleles 189 and 192 of EST-SSR locus Fs_C6785, Figure S2: Plots of delta K (a) and log likelihood for each K (b), Table S1: Primer characteristics, data file S1: Genotypic data used in the study.

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