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Hyrcanian forests—Stable rear-edge populations harbouring high genetic diversity of *Fraxinus excelsior*, a common European tree species

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Abstract

Aim: The Hyrcanian forests, in a region of lowland and montane temperate pure and mixed broadleaf forests located in Iran, near the southern shores of the Caspian Sea, form part of the Caucasus biodiversity hotspot. In this region, species experienced suitable and stable environmental conditions over historic periods and even some Arcto-Tertiary relict species could survive. Although the ranges of several European tree species expand to the Hyrcanian forests, its role has mostly been overlooked in phylogeographic studies so far. Here, we used common ash (*Fraxinus excelsior*) to study the genetic diversity, population genetic structure and time of divergence between European and Hyrcanian populations.

Location: Six populations from the Hyrcanian forests at the southern shore of the Caspian Sea (Iran) and three selected populations in Europe (Norway, Denmark and Italy).

Method: We amplified four genomic, seven genic and four plastid SSR (simple sequence repeat) markers in 268 *Fraxinus excelsior* samples.

Results: In particular, EST-SSRs (expressed sequence tag–SSRs), that is genic markers, showed significantly higher genetic diversity in the Hyrcanian forests than in European populations. Population divergence between European and Hyrcanian populations dated back to the end of the middle to upper Pleistocene. A recent reduction in population size was detected in all study populations. Within the Hyrcanian region, a substructure was detected at nuclear and plastid SSRs, with a western to central and a smaller eastern subcluster. Two new plastid haplotypes were described in the easternmost Hyrcanian populations.

Main conclusions: Our results confirm that the Hyrcanian forests harbour high genetic diversity that might be of great value for the evolutionary potential of *Fraxinus excelsior* currently facing global climate change and ash dieback. We argue that the Hyrcanian forests could be an important genetic reservoir also for other European tree species.

*Both authors contributed equally to this work.

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KEYWORDS
divergence time, *Fraxinus excelsior*, genetic diversity, Hyrcanian forests, microsatellites, plastid haplotypes

1 | INTRODUCTION

Geographically peripheral populations of a species' range are highly valuable for conservation purposes and often harbour unique genetic resources (reviewed in Lesica & Allendorf, 1995; Hampe & Petit, 2005; Fady et al., 2016). These populations are often adapted to local environmental conditions at the edge of the species' ecological amplitude (ecologically peripheral). Nowadays, species are challenged by global warming and have to adapt, migrate or will go extinct if they cannot cope with the new environmental conditions (Aitken, Yeaman, Holliday, Wang, & Curtis-McLane, 2008). During climate change, species will experience substantial environmental changes and geographically and ecologically peripheral populations are potentially important resources that could contribute genetic novelty and reinforce standing genetic variation through gene flow over the species range (reviewed in Fady et al., 2016). The Hyrcanian forests at the southern shore of the Caspian Sea harbour the south-easternmost populations of several European tree species, such as *Carpinus betulus*, *Fraxinus excelsior*, *Sorbus torminalis* and *Ulmus glabra* (Akhani, Djamali, Ghorbanalizadeh, & Ramezani, 2010). However, in phylogeographic studies, the role of this region has long been omitted (but see Mayol et al., 2015).

Hampe and Petit (2005) stated that especially "stable" rear-edge populations, those located at the low-latitude margins of current species' distributions and characterized by long-term persistence, are of special importance for the conservation of genetic diversity, phylogenetic history and evolutionary potential of species. These populations are typically located in areas (so-called refugia) with rugged topography where they could survive adverse climatic conditions in the past by slight altitudinal range shifts. In Europe, many temperate species endured the Pleistocene glacial oscillations mainly in southern refugia, typically on the Iberian, the Italian and the Balkan Peninsulas (Bennett, Tzedakis, & Willis, 1991; Taberlet, Fumagalli, Wust-Saucy, & Cosson, 1998; Hewitt, 1999). Palynological, biogeographic, phylogeographic and climate modelling studies aimed to identify and characterize glacial refugia and recolonized areas. In a typical manner, refugia are more species-rich due to climatic long-term continuity and often harbour endemic species (Dynesius & Jansson, 2000). Phylogeographic studies detected high genetic diversity and high levels of allele or haplotype endemism in contemporary populations located in Pleistocene refugia and strong genetic divergence between distinct refuge areas. In contrast, recolonized regions show lower differentiation but often high genetic diversity if they accumulated genetic lineages from different refugia (Comps, Gömöry, Letouzey, Thiébaut, & Petit, 2001; Widmer & Lexer, 2001; Petit et al., 2003). Apart from southern European regions, recently, areas around the Black Sea and south of the Caspian Sea have also been proposed as glacial refugia for temperate forest trees based on climate modelling (Leroy & Arpe, 2007).

The Hyrcanian forests extend from the southern shores of the Caspian Sea to the northern slopes of the Alborz mountain range and stretch approximately over 800 km east-west and 100 km north-south and cover an area of around 1.85 million ha (Sagheb-Talebi, Sajedi, & Pourhashemi, 2014). These highly diverse mixed forests form part of the Caucasus biodiversity hotspot (Mittermeier, van Dijk, Rhodin, & Nash, 2005) and have been affected to a much lesser extent by Pleistocene glacial periods than, for example deciduous forests in northern Europe. While northern parts of Europe were covered repeatedly by ice sheets, the entire Hyrcanian region was free of glaciers during the Pleistocene, although climatic oscillations led to slight contractions and expansions of the forest cover (Sagheb-Talebi et al., 2014 and references therein). The Hyrcanian forests contain a number of endemic tree species, such as *Acer velutinum*, *Quercus castaneifolia* and *Pyrus boissieriana*. Even some Arcto-Tertiary relict species, such as *Zelkova carpinifolia*, *Pterocarya fraxinifolia* or *Parrotia persica* which became extinct in other regions, survived here (Akhani et al., 2010). Despite human impacts in this region have been documented during the last millennium and increased since the 19th century, this region is characterized by a long-term continuity in forest cover (Ramezani, Mohadjer, Knapp, Ahmadi, & Joosten, 2008).

Fraxinus excelsior L. (Oleaceae), the common ash, is an ecologically and economically important tree species used for tools, wood flooring and furniture. It has a wide distribution range and can be found in mixed deciduous forests in most of Europe, except the northern and southernmost parts, and also in northwest Asia, with the Hyrcanian forests as its south-easternmost occurrence (FRAXIGEN, 2005). The species is light-dependent, although young trees are shade tolerant, and can be classified as intermediate between a pioneer species and a permanent forest component (Beck, Caudullo, Tinner, & de Rigo, 2016). *Fraxinus excelsior* is anemophilous and anemochorous (fruits are winged and single-seeded, called samaras) and has a polygamous reproductive system with male, female and hermaphrodite trees (Acles, Burczyk, Lowe, & Ennos, 2005). In the Hyrcanian forests, common ash can reach a diameter of up to 80 cm, a height of 40 m and an age of almost 250 years (Sabeti, 1976; Sagheb-Talebi et al., 2014). The species only accounts for 0.5% of the total stem number in the Hyrcanian Forests (Rasaneh, Kahnamoie, & Salehi, 2001) and usually grows intermixed with other broad-leaved species (*Fraxino-Alnetum* community in alluvial lowlands and *Fraxino-Aceretum* community in wet habitats) but can also be found as individual trees or in small groups on moist soils from the plateau up to 2,200 m a.s.l. A number of studies have examined the genetic diversity and population genetic structure of *F. excelsior*

spanning most of the species distribution range except Hyrcanian populations. Common ash in Europe has persisted through glacial periods in refugia in the Iberian, Italian and Balkan Peninsulas, and the eastern Alps (Heuertz, Fineschi, et al., 2004). Postglacial recolonization and extensive pollen-mediated gene flow led to a large Central European gene pool (for neutral, biparentally inherited SSR markers) ranging from Great Britain to Lithuania, while distinct gene pools and higher population differentiation were found especially in south-eastern Europe (Heuertz, Hausman, et al., 2004). The species exhibits low genetic diversity and high fixation levels at plastid DNA (Heuertz, Hausman, et al., 2004; Tollefsrud et al., 2016) but nuclear genetic diversity is high, particularly in Central Europe (Heuertz, Hausman, et al., 2004). Of late, common ash populations in Europe are in decline due to ash dieback caused by the invasive and spreading fungus *Hymenoscyphus fraxineus* (Helotiaceae). This pathogen is causing severe tree mortality of *F. excelsior* especially in central and eastern Europe (Kjær, McKinney, Nielsen, Hansen, & Hansen, 2012; Landolt, Gross, Holdenrieder, & Pautasso, 2016; McKinney, Nielsen, Hansen, & Kjær, 2011). And yet another threat is likely approaching the European ashes in the nearest future. The Emerald ash borer (*Agrilus planipennis*, Buprestidae), an invasive, Asian-native beetle that feeds on ash species was introduced to Russia (Moscow) in 2003 and with an expansion rate of 13–41 km/year the beetle is likely to settle in Central Europe within 15–20 years (Valenta, Moser, Kapeller, & Essl, 2017). While Asian ash species are resistant, the beetle is highly destructive to ash trees native to Europe and America (Villari, Herms, Whitehill, Cipollini, & Bonello, 2015) including *F. excelsior* (Orlova-Bienkowskaja, 2014).

The general objective of our study was to characterize the genetic diversity and population genetic structure of common ash in the Hyrcanian forests, at the south-eastern margin of the distribution range of the species, to compare with selected European populations, and to estimate the divergence time between different gene pools. We sampled six populations from the Hyrcanian forests and three populations from Europe in Italy, Denmark and Norway. The populations from Denmark, Norway and Italy belong to two different gene pools and are located in regions that were recolonized after the Last Glacial Maximum (Heuertz, Fineschi, et al., 2004; Heuertz, Hausman, et al., 2004). Our specific objectives were (1) to estimate and compare the genetic diversity at genomic simple sequences repeats (SSRs), expressed sequence tag (EST)-SSRs and plastid SSRs in Hyrcanian and European populations, (2) to investigate the patterns of genetic structure and maternal lineages among the study populations and (3) to estimate the divergence time between European and Hyrcanian gene pools. We believe that especially with regard to ongoing population decline and loss of genetic diversity due to ash dieback, the identification of genetically diverse common ash populations will be important for future conservation management and the establishment of breeding programmes for resistance against pests and pathogens in this tree species.

2 | MATERIALS AND METHODS

2.1 | Plant material

Plant material of 268 trees was collected in nine different populations in four countries—Iran, Denmark, Italy and Norway; six populations in Iran and three populations in Europe (Table 1, Figure 1). The samples were collected from late spring to early summer 2011. The population genetic structure and genetic diversity of *F. excelsior* in Europe are well known and have been described in a number of publications (see Heuertz, Hausman, Tsvetkov, Frascaria-Lacoste, & Vekemans, 2001; Heuertz, Fineschi, et al., 2004; Heuertz, Hausman, et al., 2004; Heuertz et al., 2006; Sutherland et al., 2010; Tollefsrud et al., 2016) while common ash populations from the Hyrcanian forests have never been studied before. We therefore focused our sampling effort mainly on the Iranian populations which cover the distribution range of the Hyrcanian forests. Populations from two gene pools in Europe were sampled, a southern one (Italy, representing gene pool P1 described by Heuertz, Hausman, et al., 2004) and two northern ones (Norway and Denmark, representing gene pool P2 described by Heuertz, Hausman, et al., 2004). Collected leaves were dried in silica gel, except for the samples from Denmark, which were kept cool and fresh until DNA extraction.

TABLE 1 Details of the sampled *Fraxinus excelsior* populations in Europe and Iran

Location	*n*	Country	Region	Latitude	Longitude	Elevation (m.a.s.l.)
Kharjegil	30	Iran	Gilan	37°41N	48°54E	500–800
Shafarood	30	Iran	Gilan	37°31N	49°01E	200–900
Kheirood	30	Iran	Mazandaran	36°32N	51°38E	800–900
Nour	30	Iran	Mazandaran	36°33N	52°04E	10
Afratakhte	30	Iran	Golestan	36°48N	55°00E	900–1600
Loveh	30	Iran	Golestan	37°17N	55°41E	1600–1700
Stasevang	30	Denmark	Northern Zealand	55°53N	12°27E	35
Tronstad	30	Norway	Lier	59°53N	10°14E	110
Vallombrosa	28	Italy	Tuscany	43°43N	11°33E	900–1100

Note. n, number of sampled trees.

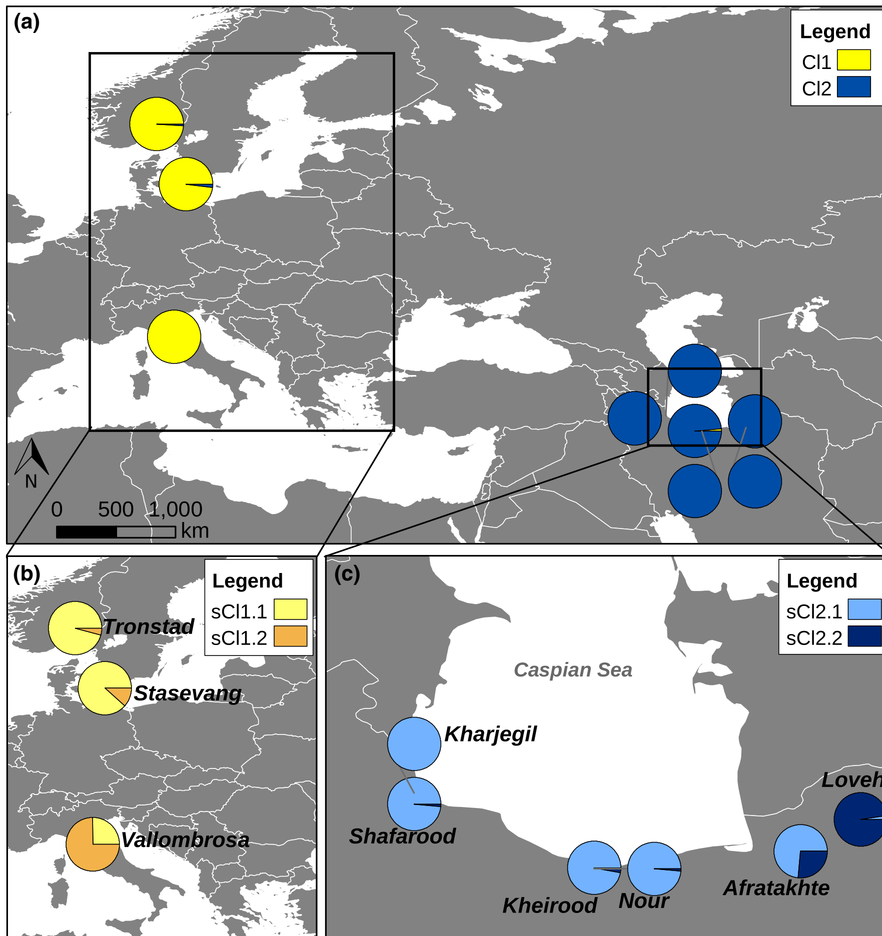

FIGURE 1 Ancestry proportion for each of two main clusters (Cl) and subclusters (sCl) plotted per *Fraxinus excelsior* population based on STRUCTURE analysis using 11 SSRs. Maps show the results from (a) the combined data set of European and Iranian populations, (b) only European populations and (c) only Hyrcanian populations. Subclusters in Europe and Iran were only detected if the sample location was taken into account (locprior) [Colour figure can be viewed at wileyonlinelibrary.com]

2.2 | SSR amplification and genotyping

The Qiagen DNeasy™ Plant mini Kit protocol (Germany) was used for DNA extractions from roughly 20 mg dried or 100 mg fresh leaves. All samples were genotyped at nuclear microsatellites (SSRs), namely seven EST-SSRs, that is FREST-279, FREST-308, FREST-326, FREST-353, FREST-427, FREST-520 and FREST-528, selected based on the level of polymorphism (Sannier, Bertolino, Frascaria-Lacoste, & Fernández-Manjarrés, 2011) and at four genomic SSRs, that is Femsatl4, Femsatl11, Femsatl19 (Lefort, Brachet, Frascaria-Lacoste, Edwards, & Douglas, 1999) and M230 (Brachet, Jubier, Richard, Jung, & Frascaria-Lacoste, 1999). Genomic SSRs are assumed to be neutral and located in unexpressed regions of the genome, while EST-SSRs derive from expressed, potentially functional parts of the genome and are usually less polymorphic than genomic SSRs due to lower mutation rates (Ellis & Burke, 2007; Varshney, Graner, & Sorrells, 2005). In addition, a subset of ten samples per population was amplified at four plastid SSRs (maternally inherited), namely ccmp3, ccmp6, ccmp7 and ccmp10 (Weising & Gardner, 1999), following the protocol in Heuertz, Fineschi, et al., 2004. Forward primers were 5′ end-labelled with fluorochromes.

The Qiagen multiplex PCR kit was used, following the manufacturer's instructions, with three different primer mixes for nuclear SSRs: (mix 1) FREST-279, FREST-308, FREST-326 and FREST-353; (mix 2) Femsatl4, Femsatl19 and M230; (mix 3) FREST-427, FREST-520, FREST-528 and Femsatl 11. Reaction volumes were 10 μl. Applied Biosystems thermo cycler model 2700 and BioRad C1000 were used for amplification of SSRs that was carried out under the following conditions: initial denaturation for 15 min at 94°C, 30 cycles of denaturation at 94°C for 30 s, annealing at 58°C for 90 s and extension at 72°C for 60 s, with a final extension step at 60°C for 30 min. For FREST-520, some re-runs were carried out with an annealing temperature of 68°C, to force more specific amplification, as a substantial amount of the individuals showed three alleles after the first run. Plastid SSRs were also amplified using the Qiagen multiplex PCR kit with a slightly modified protocol with initial denaturation at 95°C for 15 min, and 25 cycles of 94°C for 60 s, an annealing temperature of 55°C for 60 s and a fragment extension at 72°C for 60 s followed by the final extension at 60°C for 30 min.

To determine the SSR sizes, capillary electrophoresis was run on an ABI3130XL sequencer from Applied Biosystems (USA). The GZ500LIZ size standard was used. Fragment sizes were analysed with the GENEMAPPER software version 4.0 (Applied Biosystems). The coding of plastid SSR haplotypes followed previous work in common ash (Heuertz, Fineschi, et al., 2004; Sutherland et al., 2010; Tollefsrud et al., 2016).

2.3 | Data analysis

2.3.1 | Genetic diversity

Null alleles at SSRs in *F. excelsior* have previously been suspected by Heuertz, Hausman, et al., 2004 and were detected, for example by Hebel, Haas, and Dounavi (2006) and Tollefsrud et al. (2016). We therefore calculated the frequency of null alleles per population and locus by applying the expectation–maximization algorithm (Dempster, Laird, & Rubin, 1977) using FREENA (Chapuis & Estoup, 2006). Due to the presence of null alleles at some loci, we used a Bayesian procedure implemented in INEST v.2.0 (Chybicki & Burczyk, 2008) which is robust to the presence of null alleles to estimate inbreeding coefficients (F_{IS_INest}) on the full 11 SSR data sets. A significant contribution of inbreeding was evaluated by comparing the deviance information criterion (DIC) values of a full model including null alleles, genotyping errors and inbreeding to a model where apart from taking into account null alleles and genotyping errors, random mating was assumed.

Genetic diversity in the sampled populations was evaluated by calculating the gene diversity (H_E) and the allelic richness (R_S, expected in a sample of 27 individuals per population) using FSTAT 2.9.3.2 (Goudet, 1995), and private allelic richness (A_p) expected for 54 gene copies using rarefaction in HP-Rare1.1 (Kalinowski, 2005). Stronger association of EST-SSRs with functional genes, due to their position in expressed regions, and lower observed mutation rates make them less polymorphic than genomic SSRs. These characteristics are likely to affect diversity estimates. To compare the different SSR marker types, all genetic diversity estimates were calculated on the combined data set of 11 nuclear SSRs and separately for four genomic SSRs and seven EST-SSRs, respectively.

2.3.2 | Genetic differentiation

To elucidate the genetic structure of the study populations, a Bayesian clustering algorithm implemented in STRUCTURE 2.2 (Pritchard, Stephens, & Donnelly, 2000) was used on the combined SSR data of all populations. We ran an admixture model with correlated allele frequencies between clusters. Ten runs were performed for each number of clusters $K = 1$ to $K = 6$ with a burn-in length of 100,000 and a run length of 200,000 iterations. This analysis was first performed for all study populations and then separately for European and Iranian populations, respectively. In the separate analyses of European and Iranian populations, we also tried the same conditions in combination with a spatial prior (locprior) taking into account sample locations because no structure was detected without this spatial prior. STRUCTURE HARVESTER (Earl & von Holdt, 2012) was used to evaluate the STRUCTURE results and determine the best number of clusters (Evanno, Regnaut, & Goudet, 2005). STRUCTURE results per *K* were compiled using CLUMPP (Jakobsson & Rosenberg, 2007). Maps showing the inferred ancestry proportions for each cluster (Cl) and subcluster (sCl) were displayed in ArcMap 10.2.2. (ESRI).

To further analyse the genetic structure of the study populations, we applied analysis of molecular variance (AMOVA, Excoffier, Smouse, & Quattro, 1992) using GENALEX vers. 6.5 (Peakall & Smouse, 2012). The explained variance in percentage was estimated for three levels in the AMOVA for (1) regions (two; Iran and Europe), (2) populations (nine in total—six in Iran; three in Europe) and (3) within populations (30 individuals in all populations except the Italian with only 28). To rule out the possible effect of unbalanced sampling sizes in Europe and northern Iran, we created five subsets of our data randomly including only three Hyrcanian populations and the three European populations and repeated the AMOVA analyses. Pairwise population differentiation, F_{ST} (Wright, 1949) and Jost's *D* (Jost, 2008), was also calculated in GENALEX. The significance of the differentiation estimates was assessed through standard permutation (1,000 permutations) tests implemented in GENALEX. Also, R_{ST}, an analogue of F_{ST} accounting for allele size (Slatkin, 1995), was assessed for the 11 SSRs in SPAGeDI-1.3.d (Hardy & Vekemans, 2002). Significance of R_{ST} was tested against the null expectation of the absence of population structure using 10,000 permutations. R_{ST} can reveal a phylogeographic signal. If stepwise-like mutations have contributed to population differentiation, the permuted R_{ST}, obtained from 10,000 permutations of allele sizes among alleles, should be significantly lower than the observed R_{ST} (Hardy, Charbonnel, Fréville, & Heuertz, 2003). All analyses were carried out for all 11 nuclear SSRs and then subsequently for the seven EST-SSRs and four genomic SSRs separately.

2.3.3 | Comparing genetic diversity between clusters (Europe vs. Hyrcanian forests)

We conducted two-sided differences tests using 1,000 permutations in FSTAT 2.9.3.2 to evaluate whether gene diversity and allelic richness differed significantly between Hyrcanian and European populations using all 11 nuclear SSRs, as well as separate analyses for genomic and EST-derived SSRs to compare different marker types. To avoid possible effects of unbalanced sampling in the two regions, we have repeated the analyses on ten subsets of data comprising six populations, three randomly chosen Hyrcanian and the three European populations. In addition, to specifically compare allelic richness between the two regions (Iranian vs. European populations) taking into account different sampling sizes, a rarefaction technique accommodated to hierarchical sampling (Kalinowski, 2004) implemented in HP-RARE 1.0 (Kalinowski, 2005) was used. As rarefaction sample sizes, we used the number of genes from the population with the lowest number of samples (54 gene copies in Italy) and the number of populations from the region with the lowest number of populations (three populations in Europe).

2.3.4 | Demography

To investigate the demographic history of the study populations, the Cornuet and Luikart (1996) T_2 statistic which reflects the deviation of gene diversity from expectations based on the number of alleles

in a population at demographic equilibrium was computed using the two-phase model with default parameter settings in BOTTLENECK 1.2.02 (Piry, Luikart, & Cornuet, 1999). Positive T_2 values indicate an excess of gene diversity, which reflects a recent founder event, while negative T_2 values rather point to a recent population expansion. The null expectation was computed using 10,000 iterations, and the significance of T_2 was tested using Wilcoxon signed-rank test (Cornuet & Luikart). To correct for multiple tests, *q*-values were calculated using the R-package *q*-value (Storey, 1995) in R version 3.1.1. (R Core Team 2014).

2.3.5 | Time of divergence

The time of divergence between the two main gene pools (Iran and Europe) identified by STRUCTURE was estimated following an approximate Bayesian computation (ABC) procedure (Beaumont, Zhang, & Balding, 2002) implemented in DIYABC 2.0 (Cornuet et al., 2014). This ABC method does not consider gene flow (only admixture); however, it is appropriate as our case study showed a high genetic differentiation between the two main clusters (F_{ST} = 0.226, *p*-value=0.001, based on 11 nuclear SSRs).

This analysis was performed using genomic SSR and EST-SSR to estimate the divergence time of the two groups from an ancestral population in a simple split model (Figure 2). In the scenario, *t#* refers to time-scale (scaled by generation time), *N#* refers to the effective population size of the corresponding populations (N_1 = Iran; N_2 = Europe), and N_a is the effective population size of the ancestral population, which was assumed to be bigger than the effective population size of the two groups, because a past population decrease was hypothesized.

The generalized stepwise mutation model (GSM; Estoup, Jarne, & Cornuet, 2002) with single nucleotide indels (SNI) was used for all simulations. Default priors were changed to obtain better posterior distributions based on the results from the pilot runs (Table S3.1). The minimum and maximum priors for nSSR and EST-SSR mutation rates were set to 5×10^{-4}–5×10^{-3} and 3×10^{-5}–3×10^{-4} (Table S3.1), respectively. Mean number of alleles and mean genetic diversity were used as summary statistics for single populations. For each pair of populations, we calculated mean number of alleles, mean genetic diversity and F_{ST}. One million simulations were performed. Goodness-of-fit was assessed by model checking using the principal component analysis (PCA) approach implemented in DIYABC, which measures the discrepancy between simulated and real data.

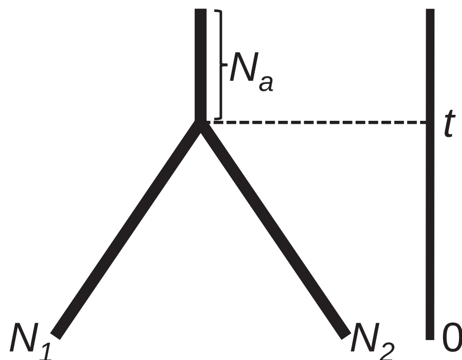

FIGURE 2 Demographic split model used in DIYABC analysis to estimate the divergence time between Hyrcanian and European populations. N_1, effective population size of Iran; N_2, effective population size of Europe; N_a, effective population size of ancestral population; *t*, divergence time scaled by generation time

3 | RESULTS

3.1 | Genetic diversity at population level

Null alleles were present in nuclear SSRs in several populations (Table S1.1). A significant but low contribution of mating among related individuals was detected in two Iranian and all three European stands (F_{IS_INest}; Table 2). The gene diversity (H_E) based on 11 nuclear SSRs was similar in all populations, except for Norway and Italy with slightly lower values (overall range: 0.52–0.69; Table 2). The allelic richness (R_S) ranged from 6.57 in Italy to 9.88 in Denmark and the private allelic richness (A_P) was highest in Kharjegil (Iran) with 0.99 and lowest in Italy with 0.21. At plastid SSRs, we detected a total of six different haplotypes. The number of haplotypes per population varied between one and two (Figure 3 and Table S2).

3.2 | Population genetic structure and maternal lineages

The STRUCTURE results showed a hierarchical genetic structure in the study populations. Based on the full data set, including all populations and both nuclear genetic marker types, a clear differentiation between European and Hyrcanian populations was detected (best *K* = 2, Figure 1a). Separate STRUCTURE runs for European and Hyrcanian populations did not identify a substructure unless the locprior, taking into account the sampling location, was used. In the data from the Hyrcanian forests alone, two subclusters (best *K* = 2) were found using the locprior. The first one comprised the four western and central populations (Kharjegil, Shafarood, Kheirood and Nour) and another one contained the easternmost population (Loveh), while individuals in Afratakhte appeared to be admixed (Figure 1c). The same analyses performed on the European populations only and introducing the locprior to take into account sample sites, revealed two subclusters (best *K* = 2) which comprised Italy on one hand and Denmark and Norway on the other hand (Figure 1b).

The AMOVA showed that most of the genetic variation was found within populations but to a much larger extent for genomic SSRs (91.7%) than for EST-SSRs (64.3%, Table 3). A third of the genetic variation (33.4%) at EST-SSRs was actually found among regions while it was only 4% for genomic SSRs (Table 3). The AMOVA results were very similar over all randomly chosen subsets (including three Hyrcanian and three European populations) confirming the results based on all samples (Table S1.2). Genetic divergence based on F_{ST} and Jost's *D* values calculated on 11 SSRs confirmed the patterns detected by the STRUCTURE analysis. Highest differentiation was found between European and Iranian populations; congruent

TABLE 2 Estimates of population genetic parameters in *Fraxinus excelsior* for all 11 SSRs (combining EST-SSRs and genomic SSRs), four genomic SSRs and seven EST-SSRs

Location	Country	n	11 SSRs H_E	R_S	A_P	F_{IS_INest}	Four genomic SSRs H_E	R_S	A_P	Seven EST-SSRs H_E	R_S	A_P
Kharjegil	Iran	30	0.62	9.20	0.99	0.05	0.84	15.82	2.96	0.49	5.42	0.01
Shafarood	Iran	30	0.63	8.63	0.48	**0.06**	0.81	14.65	1.24	0.53	5.19	0.04
Kheirood	Iran	30	0.61	7.35	0.25	0.04	0.80	12.11	0.45	0.50	4.63	0.14
Nour	Iran	30	0.63	8.32	0.30	0.04	0.86	14.05	0.77	0.50	5.05	0.04
Afratakhte	Iran	30	0.69	8.89	0.55	**0.06**	0.84	14.20	1.22	0.61	5.86	0.17
Loveh	Iran	30	0.65	6.80	0.36	0.02	0.77	10.51	0.49	0.58	4.68	0.28
Stasevang	Denmark	30	0.63	9.88	0.94	**0.02**	0.92	20.49	2.36	0.46	3.81	0.13
Tronstad	Norway	30	0.58	8.51	0.50	**0.03**	0.89	16.53	1.06	0.41	3.94	0.18
Vallombrosa	Italy	28	0.52	6.57	0.21	**0.06**	0.80	12.00	0.32	0.37	3.27	0.15
Iran all			0.64	8.20			0.82	13.56		0.53	5.14	
Europe all			0.58	8.32			0.87	16.34		0.41	3.67	
p-value (EU vs. Iran)			**0.03**	0.87			0.17	0.22		**0.01**	**<0.01**	

Note. *p*-value (EU vs. Iran), is the *p*-value of the two-sided differences test of diversity estimates from Iran and Europe—significant values in bold. A_P, private allelic richness expected for 54 gene copies per population; F_{IS_INest}, inbreeding coefficient calculated using the Bayesian procedure implemented in INEST 2.0, which is robust to the presence of null alleles, F_{IS_INest} values in bold indicate significant inbreeding based on the comparison of DIC values; H_E, gene diversity; *n*, sample number; R_S, allelic richness standardized for a sample of 27 individuals per population.

for F_{ST} values and Jost's *D* values (Table 4). Global differentiation among study regions was $F_{ST} = 0.154$ ($p < 0.0001$) and $R_{ST} = 0.103$ ($p < 0.0001$), and there was a marginally nonsignificant phylogeographic signal ($R_{ST}>R_{ST[permuted]}$, $p = 0.083$) among study populations. Global differentiation among European ($F_{ST} = 0.036$, $p = 0$; $R_{ST} = 0.017$, $p = 0.067$) and Iranian ($F_{ST} = 0.035$, $p = 0$; $R_{ST} = 0.030$, $p = 0.067$) populations, respectively, was lower, and no phylogeographic signal was detected.

The distribution of plastid SSR haplotypes showed the distribution of distinct maternal lineages. Three haplotypes were detected in European populations. In Tronstad (Norway) and Stasevang (Denmark), haplotype H01 was present, and in Stasevang (Denmark) and Vallombrosa (Italy), H02 was detected. In Vallombrosa, we also found haplotype H03. In the western and central populations of the Hyrcanian forests, haplotype H4 was found and two new haplotypes, H23 and H24, in the easternmost Iranian populations (Figure 3, Table 5).

3.3 | Regional comparison of genetic diversity (Europe vs. Hyrcanian forests)

The two-sided permutation tests of differences in genetic diversity between European and Iranian populations performed in FSTAT using 11 SSRs were significant for gene diversity ($H_{E-Iran} = 0.64 > H_{E-EU} = 0.58$; *p*-value = 0.032, Table 2) while the allelic richness (R_S) did not differ significantly. The ten subsets corroborated this result in six of ten tests and never indicated significantly higher gene diversity in European populations (Table S1.3). The regional levels of allelic richness after rarefaction using hierarchical sampling in HP-RARE were 12.20 in Iran and 12.45 in Europe.

For the four genomic SSRs, H_E and R_S were highest in the two Scandinavian populations (Table 2), but European and Iranian populations were not significantly different based on these statistics (Table 2). In accordance with this, testing the ten subsets of six populations of the four genomic SSRs revealed no significant differences

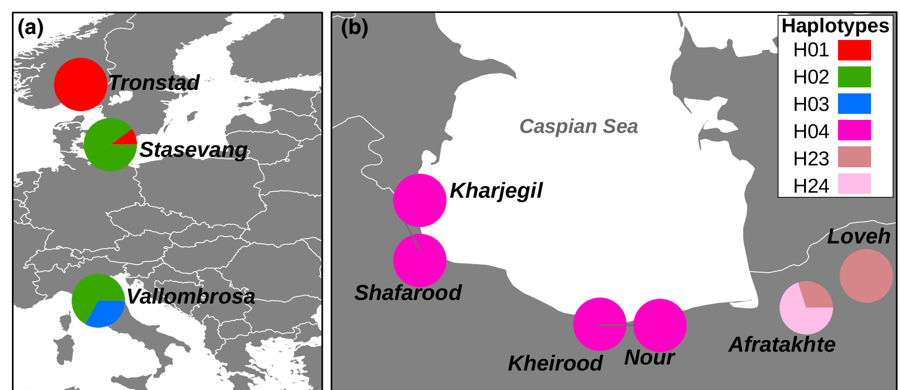

FIGURE 3 Distribution of plastid SSR haplotypes in the *Fraxinus excelsior* study populations in Europe (a) and the Hyrcanian forests (b). Colours for H01-H04 match the colour pattern in Heuertz, Fineschi et al. (2004) [Colour figure can be viewed at wileyonlinelibrary.com]

TABLE 3 Results from analysis of molecular variance (AMOVA) comprising nine *Fraxinus excelsior* populations using 11 SSRs (combining EST-SSRs and genomic SSRs), four genomic SSRs and seven EST-SSRs

	11 SSRs	Four genomic SSRs	Seven EST-SSRs
Among regions	21.7%	4.6%	33.4%
Among populations	2.9%	3.7%	2.4%
Within populations	75.4%	91.7%	64.3%

Note.The distribution of variance in percentage is reported among the three levels in the AMOVA: regions (two; Iran and Europe), populations (nine in total—six in Iran; three in Europe) and within populations (30 individuals in all populations except the Italian with 28).

TABLE 4 Pairwise differentiation of *Fraxinus excelsior* populations

	Kharjegil	Shafarood	Kheirood	Nour	Afratakhte	Loveh	Stasevang	Tronstad	Vallombrosa
Kharjegil	0	0.029	0.019	0.049	0.067	0.104	0.476	0.539	0.468
Shafarood	0.019	0	0.029	0.049	0.042	0.101	0.523	0.577	0.490
Kheirood	0.013	0.018	0	0.043	0.069	0.102	0.505	0.572	0.492
Nour	0.031	0.029	0.027	0	0.095	0.118	0.509	0.572	0.503
Afratakhte	0.036	0.023	0.037	0.048	0	0.059	0.495	0.559	0.528
Loveh	0.058	0.055	0.057	0.063	0.030	0	0.488	0.544	0.497
Stasevang	0.226	0.237	0.238	0.233	0.206	0.217	0	0.043	0.045
Tronstad	0.259	0.268	0.274	0.266	0.238	0.248	0.028	0	0.064
Vallombrosa	0.260	0.264	0.273	0.270	0.254	0.258	0.035	0.051	0

Note. Upper triangle shows Jost′s D, lower triangle shows F_{st} values calculated in the AMOVA. All pairwise values were statistically significant (p < 0.01) after 1,000 permutations conducted in GenAlEx.

TABLE 5 Plastid DNA haplotypes detected in the *Fraxinus excelsior* populations using four plastid SSR markers

Haplotype	ccmp3	ccmp6	ccmp7	ccmp10	Frequency (%)
H01	97	97	118	103	11.49
H02	97	99	117	104	17.24
H03	97	99	117	103	3.45
H04	97	98	118	104	44.83
H23	97	98	118	106	14.94
H24	97	98	118	107	8.05

Note. The haplotype definitions follow Heuertz, Fineschi, et al., 2004, Sutherland et al. (2010) and Tollefsrud et al. (2016), except for the novel haplotypes H23 and H24. Allele sizes for each of the loci (ccmp3, ccmp6, ccmp7 and ccmp10) are given in base pairs.

between European and Hyrcanian populations (Table S1.4). Average regional levels of allelic richness after rarefaction using hierarchical sampling were 21.79 in Iran and 25.62 in Europe for the genomic SSRs.

Contrary to genomic SSRs, gene diversity estimates and allelic richness calculated on EST-SSRs were consistently higher in the Iranian populations. These differences were statistically significant (H_{E-Iran} = 0.53 > H_{E-EU} = 0.41; p-value = 0.01) or highly significant (R_{S-Iran} = 5.14 > R_{S-EU} = 3.67; p-value = 0.006, Table 2). This result was confirmed using data subsets in nine of ten tests for R_S and seven of ten tests for H_E, while diversity estimates were never significantly higher in European populations (Table S1.5). The average allelic richness for EST-SSRs in the two regions, after rarefaction using hierarchical sampling, was 6.72 for Iran and 4.92 in Europe.

When analysing the distribution and overlap of alleles between the two regions, 75 genomic SSR alleles were shared between Iran and Europe, while 41 and 30 were only found in Iran or Europe, respectively (data not shown). For EST-SSRs, 28 alleles were shared among regions, 26 were found in Iran only, while merely seven alleles were private to European populations.

3.4 | Demographic history and time of divergence

An excess of heterozygosity was observed for all study populations using the combined marker set and the seven EST-SSRs (Table 6). All T_2 values were significant, indicating a recent reduction in effective population size. The DIYABC analysis suggested that the European and the Hyrcanian gene pools diverged about 6,740 generations ago (Table 7). Assuming 15/20 years as generation time (Whittle & Johnston, 2003), divergence time scaled to 101,100/134,800 years BP (95% Cis: 38,100/50,800–147,300/196,400). Furthermore, the effective population size of the ancestral population was bigger

TABLE 6 Heterozygosity excess in the studied *Fraxinus excelsior* populations

	11 SSRs		Seven EST-SSRs		Four genomic SSRs	
Location	T_2	*p* (one-tailed for H_E excess)	T_2	*p* (one-tailed for H_E excess)	T_2	*p* (one-tailed for H_E excess)
Kharjegil	1.845	**0.002**	2.284	**0.004**	0.070	0.906
Shafarood	2.503	**0.003**	3.069	**0.004**	0.088	0.723
Kheirood	2.530	**0.003**	2.615	**0.008**	0.731	0.281
Nour	3.161	**0.001**	2.753	**0.004**	1.570	0.141
Afratakhte	2.682	**0.003**	2.887	**0.004**	0.601	0.352
Loveh	3.929	**0.001**	3.579	**0.004**	1.768	0.141
Stasevang	2.983	**0.003**	3.833	**0.004**	-0.112	0.723
Tronstad	2.055	**0.042**	2.778	**0.004**	-0.285	0.906
Vallombrosa	2.689	**0.005**	3.300	**0.004**	0.062	0.723

Note. T_2, bottleneck statistic estimated under the two-phase (TPM) model using 11 SSRs (combining EST-SSRs and genomic SSRs), four genomic SSRs and seven EST-SSRs.
Significance values (in bold) of Wilcoxon signed-rank tests reported as q-values after multiple test correction.

TABLE 7 Approximate Bayesian computation parameter estimates in a simple split model to assess the divergence time between Hyrcanian and European *Fraxinus excelsior* populations

				Quantiles			
Parameter	Mean	Median	Mode	2.5%	5%	95%	97.5%
N_1	7.52×10^3	7.64×10^3	7.98×10^3	4.50×10^3	5.08×10^3	9.58×10^3	9.78×10^3
N_2	6.37×10^3	6.41×10^3	6.52×10^3	3.41×10^3	3.85×10^3	8.84×10^3	9.28×10^3
t	6.61×10^3	6.74×10^3	7.01×10^3	2.54×10^3	3.11×10^3	9.61×10^3	9.82×10^3
N_a	1.19×10^4	1.16×10^4	1.08×10^4	4.43×10^3	5.20×10^3	1.90×10^4	1.95×10^4
µmic_EST-SSR	1.18×10^{-4}	1.06×10^{-4}	6.98×10^{-5}	4.11×10^{-5}	4.73×10^{-5}	2.30×10^{-4}	2.56×10^{-4}
pmic_EST-SSR	2.21×10^{-1}	2.28×10^{-1}	3.00×10^{-1}	1.13×10^{-1}	1.22×10^{-1}	2.96×10^{-1}	3.00×10^{-1}
snimic_EST-SSR	1.25×10^{-6}	2.96×10^{-7}	1.00×10^{-8}	1.15×10^{-8}	1.34×10^{-8}	6.12×10^{-6}	7.69×10^{-6}
µmic_gSSR	3.22×10^{-3}	3.26×10^{-3}	3.47×10^{-3}	1.28×10^{-3}	1.51×10^{-3}	4.74×10^{-3}	4.84×10^{-3}
pmic_gSSR	2.41×10^{-1}	2.54×10^{-1}	3.00×10^{-1}	1.21×10^{-1}	1.35×10^{-1}	3.00×10^{-1}	3.00×10^{-1}
snimic_gSSR	2.25×10^{-6}	7.78×10^{-7}	1.24×10^{-8}	1.45×10^{-8}	2.02×10^{-8}	8.78×10^{-6}	9.54×10^{-6}

Note. N_1, effective population size in Iran; N_2, effective population size in Europe; N_a, effective population size in the ancestral population; *t*, time of divergence; µ*mic*, mean mutation rate; *pmic*, the parameter of the geometric distribution to generate multiple stepwise mutations; *snimic*, mean mutation rate of single nucleotide indels for both nuclear genetic marker types (EST-SSRs or genomic SSRs).

(N_a = 11,600) than those of Iran (N_1 = 7,640) and Europe (N_2 = 6,410), suggesting a contraction event at time "*t*."

The parameter values drawn from the posterior distribution (Figure S3.1), together with the summary statistics (Table S3.2) and the principal components analysis comparison implemented in DIYABC (Figure S3.2), suggested a high goodness-of-fit of the simulated scenario to the observed data.

4 | DISCUSSION

4.1 | Genetic diversity

This is the first study addressing genetic diversity and divergence patterns of common ash in the south-easternmost populations and one of the first studies comparing genetic diversity between Hyrcanian and European populations in a common tree species. Gene diversity of common ash at genomic SSRs was high ($H_{E[gSSRs]}$ = 0.77–0.92, this study), even higher than in previous publications (H_E = 0.64–0.81, Heuertz et al., 2001; Heuertz, Hausman, et al., 2004). As expected for EST-SSRs, these markers displayed less polymorphisms and lower gene diversity ($H_{E[EST\text{-}SSRs]}$ = 0.37–0.61, this study) than genomic SSRs. However, gene diversity at EST-SSRs was higher than in three French populations ($H_{E[mean]}$ = 0.31) previously reported by Sannier et al. (2011) for the same EST-SSRs and significantly higher in Hyrcanian populations compared to European populations in our study. This result in combination with two new plastid haplotypes in the easternmost Hyrcanian populations reveals that *F. excelsior* populations in this region harbour genetic novelty and high genetic diversity. A third of the genetic variation at EST-SSRs was found among regions while it was only 4% for genomic SSRs which was

probably due to size homoplasy (Estoup et al., 2002) in the faster mutating latter markers (Ellis & Burke, 2007). However, differences between the two nuclear marker types have to be interpreted with caution as the low number of only four genomic SSRs implied high stochasticity and low statistical power.

Several studies have evaluated genetic diversity in species from the Hyrcanian forests, (for example Maharramova, Safarov, Kozlowski, Borsch, & Muller, 2015; Naderi et al., 2014; Salehi Shanjani, Vendramin, & Calagari, 2010). However, to our knowledge, only one other study compared genetic diversity in populations from the Hyrcanian forests and Europe. In accordance with our results, the Hyrcanian region was identified as a hotspot of genetic diversity also in *Taxus baccata* (English yew) (Mayol et al., 2015).

4.2 | Population genetic structure

The genetic structure of the study populations revealed strong differentiation between Iranian and European common ash populations with a weak phylogeographic signal indicating ancient divergence. We identified two distinct subclusters along the southern shore of the Caspian Sea: one widespread subcluster covering most of the Hyrcanian forests and a smaller one in the eastern part. A similar pattern of eastern and western populations clustering respectively has also been described for oriental beech (*Fagus orientalis*, Salehi Shanjani et al., 2010). Indeed, the region was not completely unaffected by glacial periods as during the last glacial period, arboreal pollen was very rare, except for birch, indicating a contraction of the forest cover (Leroy, Tudryn, Chalié, López-Merino, & Gasse, 2013). The climate was cooler and drier and steppe-like ecosystems expanded (Sagheb-Talebi et al., 2014; and references therein). Our results could suggest retraction of common ash in at least two refugia in the Hyrcanian region during glacial periods. Different lineages possibly adapted to distinct environmental conditions might have been maintained, as also nowadays, the Hyrcanian forests are characterized by marked environmental differences with a strong precipitation decrease from west to east (Domroes, Kaviani, & Schaefer, 1998) coinciding with the genetic structure.

In Europe, the genetic structure at genomic, genic and chloroplast SSRs corresponded to previous phylogeographic research and fossil pollen data (Gliemeroth, 1997; Heuertz, Fineschi et al., 2004; Heuertz, Hausman, et al., 2004; Munaut, 1986; Tollefsrud et al., 2016). In the Hyrcanian forests, the distribution of plastid haplotypes corroborated the nuclear structure, as the four western and central Iranian populations shared haplotype H04 while the two easternmost populations showed endemic haplotypes H23 and H24. Haplotype sharing is common between *Fraxinus* species; however, the new haplotypes described here have not been detected before in *F. excelsior*, *F. angustifolia* or *F. ornus* (Heuertz et al., 2006). Haplotype H04 was previously described as common in populations from Spain and the British Isles (Heuertz, Fineschi, et al., 2004; Sutherland et al., 2010); however, the presence of this haplotype in Iran was probably due to homoplasy (see also Heuertz, Fineschi, et al., 2004). The distribution of already known haplotypes in European populations coincides with previous studies (Heuertz, Fineschi, et al., 2004; Tollefsrud et al., 2016). The divergence between European and Hyrcanian common ash populations dated back to the end of the middle to late Pleistocene which was characterized by climate oscillations that have shaped population divergence and genetic lineages in a number of species (e.g., Hewitt, 2000). The connectivity and colonization history between European and Hyrcanian populations of common ash remain unknown, and to our knowledge, this topic has only been studied in one other species. Mayol et al. (2015) revealed that Hyrcanian populations of *Taxus baccata* already diverged during the Neogene and represented an independent gene pool, which did not contribute to admixture of lineages in Central Europe. In the future, additional common ash samples collected in populations between the Black and the Caspian Sea and analysed in a combined data set comprising all samples including European and the Hyrcanian populations could shed light on this topic.

4.3 | Implications and conservation

The high conservation value of the species-rich ecosystems in the Hyrcanian forests has already been recognized as part of the Caucasus biodiversity hotspot (Mittermeier et al., 2005). Our results also identified the Hyrcanian forests as a diverse source of genetic variation for *F. excelsior* indicating a high conservation value of these populations for this species. In times of climate change and ash dieback, European populations face strong selective pressures (Kjær et al., 2012; Landolt et al., 2016; McKinney et al., 2011). The invasive pathogenic fungus *H. fraxineus* has already caused severe damage in natural common ash populations across Europe during the last two decades, diminishing standing genetic variation. Only few individual trees show resistance against the pathogen (Lobo, Hansen, McKinney, Nielsen, & Kjær, 2014). Of late, the emerald ash borer, native to Asia, was introduced to Russia where it caused severe damage on *F. excelsior* and from where it could possibly spread to Europe (Baranchikov, Mozolevskaya, Yurchenko, & Kenis, 2008; Musolin, Selikhovkin, & Baranchikov, 2017; Orlova-Bienkowskaja, 2014). Under these circumstances, diverse stable rear-edge populations in the Hyrcanian forests might be important for the conservation of genetic diversity and the evolutionary potential of this species. Further population genomic studies targeting the phylogeographic relationships between European and Hyrcanian populations (also including populations south and east of the Black Sea) and landscape genomic studies aiming to understand environmental adaptations are needed to elucidate the role and importance of the Hyrcanian forests. With the aim of mitigating maladaptation in natural populations due to climate change assisted gene flow has been discussed (Aitken & Whitlock, 2013). In this line, adaptive introgression, that is the movement of genes between closely related species or highly divergent populations of the same species, has recently been proposed as tool in conservation management (Hamilton & Miller, 2015) and might be especially important for species that are not only facing climate change but also threatened by invasive pests and diseases. This might increase the adaptive

potential of species or populations with limited standing genetic variation caused by population decline. Our results provide useful information for the establishment of field trials testing Hyrcanian provenances of *F. excelsior* under European environmental conditions and/or trials testing the susceptibility to pests and pathogens. Such trials could carefully assess the adaptive potential of Hyrcanian common ash trees in European environments, as well as its potential evolutionary role in relation to specific threats, such as ash dieback or the emerald ash borer.

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DATA ACCESSIBILITY

The data are available from the data storage facility of the University of Copenhagen at the following URL: http://www.erda.dk/public/archives/YXJjaGl2ZS1DeDN3ejQ=/published-archive.html.

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BIOSKETCH

Eva. O. Erichsen made fieldwork, laboratory work, data analyses and the first draft of the manuscript was her Master thesis. She is currently a PhD student working on aspects of *genetic diversity and adaptability of forest trees in relation to climate change, including the potential role of refugia*.

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Author contributions: OKH and KST conceived the ideas; EOE and KST collected the samples; EOE did laboratory work, first data analyses and wrote a first draft; KBB, OKH, FB and GGV did the final data analyses and KBB led the writing to which all co-authors contributed.

SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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