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ORIGINAL ARTICLE

Genomic diversity of major tree species in the Eurasian relict forests of northern Iran

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Abstract

We investigated population genetics of the native tree species *Acer velutinum* Boiss., *Fagus orientalis* Lipsky, and *Quercus castaneifolia* C.A. Mey. in the Hyrcanian forests of northern Iran and also *F. orientalis* populations in the Euro-Siberian and Colchic subregions of northern Türkiye. We used the double-digest RADseq method and genotyped 90 populations and 1,589 individuals across the distribution range of the species. Genome-wide SNPs from 28 populations of *A. velutinum*, 32 populations of *F. orientalis*, and 30 *Q. castaneifolia* revealed higher genetic differentiation among *A. velutinum* populations than among *F. orientalis* and *Q. castaneifolia*. The global F_{ST} value was lowest for *F. orientalis* populations and highest for *A. velutinum* populations, while the global F_{IS} value was negative for *A. velutinum*. Demographic history analysis revealed a bottleneck during the last glacial period (11,500—115,000 Kya) for the *A. velutinum* populations with reduced effective population size (*Ne*). All three species show multiple bottlenecks and reduced *Ne* during the Quaternary. Pronounced genetic divergence among *A. velutinum* populations in the Hyrcanian forests compared to the other two species suggests cryptic speciation. Conversely, *F. orientalis* and *Q. castaneifolia* populations showed low levels of genetic structure, suggesting that species-specific factors, such as pollen production and pollination efficiency, may have influenced the genetic patterns within these species in similar environments. The nucleotide diversity of *F. orientalis* populations in Iran is negatively correlated with altitude ($p = 0.046$). In contrast, *A. velutinum* populations show a significant correlation between nucleotide diversity and longitude ($p = 0.008$). Furthermore, the *F. orientalis* populations from Türkiye showed a distinct west–east genetic structure and were highly diverged from the Iranian *F. orientalis* populations.

Keywords Adaptation potential · Hyrcanian forests · Cryptic speciation · Future forests · Quaternary climatic oscillations · Refugia

Introduction

Forests are threatened by human-related influences causing natural habitats to lose native species. Habitat destruction, invasive species, and novel diseases are introduced through human intervention, pushing natural forests to degradation or extinction (Wilcove et al. 1998; Malhi et al. 2014; Popkin 2021). To overcome current or future threats and altered climatic conditions, species with a richer gene pool and higher genetic diversity that can adapt to new conditions are desired (Bennett 1990; Ledig 1992; Aitken and Bemmels 2016). The Quaternary ice ages shaped the present genetic diversity and population genetic structure of most living organisms in the northern hemisphere (Newton et al. 1999; Hewitt 2000). The populations of southern Europe are considered refugial areas for many European forest tree species,

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possessing higher genetic diversity than northern European forest tree populations (Taberlet et al. 1998, p. 199; Hewitt 2000). The northern populations might have experienced genetic bottlenecks and smaller effective population sizes resulting from the climatic oscillations and environmental shifts in the refugial period (Petit et al. 2003; Magri et al. 2006). The re-immigration to the north might have resulted in lower genetic diversity due to founder effects (Lumibao et al. 2017).

The Hyrcanian mixed forests in northern Iran (Sagheb-Talebi et al. 2014) and the Colchic forests in Georgia (Nakhutsrishvili 2013) are two natural Eurasian relict forests in the northern hemisphere, which could be considered the gene pool of most Eurasian forest trees. These forests represent the refugia for many Eurasian endemic and rare flora elements around the Black and the Caspian Sea regions (Velichko et al. 1984; Leroy 2007; Nakhutsrishvili et al. 2011; Vanderplank et al. 2014; Ghorbanalizadeh and Akhani 2022). The Hyrcanian forests (a.k.a. Caspian forests) are at the extreme south-eastern end of the "Euxino-Hyrcanian forests" and are listed as a World Heritage site by UNESCO (UNESCO ID No. 1584 – inscribed 2019). The Hyrcanian forests in Iran are between the Talesh and Alburz mountains in the south and the Caspian Sea in the north, with a stretch of 800 km northwest to the east (Fig. 1, Fig. 2 A-C). In the northwest, they extend into Azerbaijan, and in the east, near the borders with Turkmenistan. The forests have high species diversity and endemism from the Neogene relicts and are part of the Caucasus biodiversity hotspot (Mittermeier et al. 2004; Akhani et al. 2010). These forests comprise over 80 broadleaved tree species, of which the same species or congeneric species are also distributed in European forests, such as *Alnus subcordata* C.A. Mey., *Carpinus betulus* L., *Fraxinus excelsior* L., *Fagus orientalis* Lipsky, *Taxus baccata* L., *Tilia platyphyllos* Scop., and *Ulmus glabra* Huds. The Hyrcanian forests are also the home to well-known relict

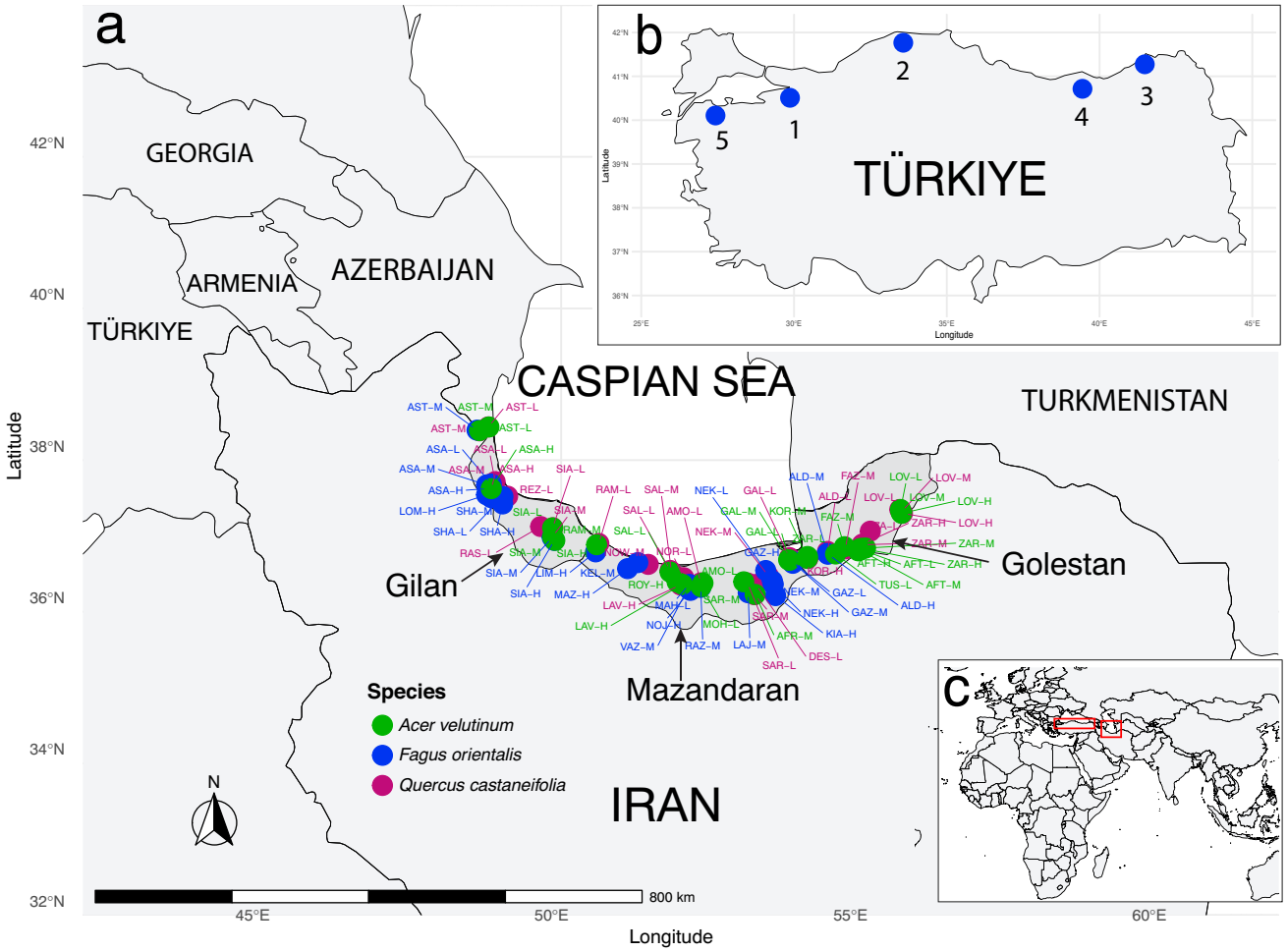

Fig. 1 Sampling locations for the studied species in Iran and Türkiye. **a** Sampling locations for *A. velutinum* (green), *F. orientalis* (blue), and *Q. castaneifolia* (purple) in the Hyrcanian forests of Iran. **b** Sampling locations for *F. orientalis* in Türkiye (populations 1–5). **c** Red boxes in the inset map represent the overall study location. For the population names, see Table 1

Fig. 2 Hyrcanian forests **a** View facing the Caspian Sea from Derazno Mountain, Golestan (2,424 m.a.s.l). **b** & **l**: High elevation view of the forest's vegetation. **c** Mixed forests at high elevation, Livan, Gorgan. **d** Pure stands of *A. velutinum* at Lavij, Mazandaran (human included for scale). **e** and **f** Fresh leaves and samaroid fruits of *A. velutinum*. **g** *F. orientalis* stands. **h** Leaves of *F. orientalis* at Neka, Mazandaran. **i** *Q. castaneifolia* tree with young acorns. **j** Leaves of *Q. castaneifolia* at Shafaroud, Gilan. **k** The vegetation of the southern slope of the Alborz Mountains. Mohammad Vatanparast took all the photos in 2018–2019

Arcto-Tertiary species, such as *Parrotia persica* (DC.) C.A. Mey. (Persian Ironwood), *Gleditsia caspica* Desf. (Caspian honey locust), *Zelkova carpinifolia* (Pall.) K. Koch., and *Pterocarya fraxinifolia* (Poir.) Spach (Nakhutsrishvili et al. 2011; Sagheb-Talebi et al. 2014).

Our study focuses on three deciduous broadleaved tree species of the Hyrcanian forests: *Acer velutinum* (Velvet maple), *Fagus orientalis* (Oriental beech), and *Quercus castaneifolia* C.A.May (Chestnut-leaved oak) (Sabeti 1966). We selected these species due to their abundance and wide distribution across Hyrcanian forests. Existing studies on population genetics and phylogeography in the region (e.g., *Fraxinus excelsior*, *F. orientalis*, *Taxus baccata*, *Acer campestre*, *Populus caspica* Bornm., *Sorbus aucuparia* L., and *Pterocarya fraxinifolia* (Lam.) Spach) have provided valuable insights into the genetic diversity and historical biogeography of these forests (Salehi Shanjani et al. 2010; Grimm and Denk 2014; Erichsen et al. 2018; Maharramova et al. 2018; Ahmadi et al. 2020; Alipour et al. 2021; Yousefzadeh et al. 2021). However, a comprehensive analysis of these three key species is still lacking.

The velvet maple is widely distributed across the entire Hyrcanian forests but is also found in the Alazani River Valley in Georgia (Nakhutsrishvili 2013; Siahkolaee et al. 2017). It is insect-pollinated; it can grow 20–40 m high (Fig. 2 D-F) and reach over two meters in diameter (Mohtashamian et al. 2017). It is a sister species of the sycamore (*Acer pseudoplatanus* L.) with their most recent common ancestor (MRCA) younger than 11 Mya (Li et al. 2019). The oriental beech is the most common species of

the Hyrcanian forests, covering over 15% of the total forest area, where pure and mixed oriental beech communities are distributed between 200 and over 2000 m.a.s.l (Sabeti 1966). It is wind-pollinated, and its distribution has its border limit in the eastern part near Gorgan because of the relatively drier nature of the forests towards the east (see sampling locations in Fig. 1). The chestnut-leaved oak is one of Iran's most crucial native oak species, endemic to the Hyrcanian Forests, wind-pollinated and pure and mixed stands of the species cover 6% of the Hyrcanian forests (Fig. 2 I-J) (Panahi et al. 2011).

We hypothesized that genetic patterns among these species would be influenced by topography in an area 110 km wide and approximately 800 km long, decreasing precipitation from west to east, higher temperatures in the east, past climate changes such as Caspian Sea level fluctuations, and other environmental factors such as altitude. Using the double-digest RADseq (ddRAD) method (Peterson et al. 2012; Andrews et al. 2016), our objectives were to (1) analyze the genetic structure, diversity, and demographic history of the three dominant tree species of the Hyrcanian Forest, (2) explore the effects of longitudinal range and altitudinal gradients on their genetic diversity, and (3) determine whether the observed genetic patterns are consistent across the three species in the similar environment.

Materials and methods

Sample collection

Leaf materials from natural populations of the three species, *A. velutinum*, *F. orientalis*, and *Q. castaneifolia*, were collected in three provinces of Iran (Gilan, Mazandaran, and Golestan) in 2018 and 2019. A total of 85 populations and 1,523 individuals were sampled and preserved in zip-lock plastic bags with silica gel (Table 1 and Fig. 1). To avoid collecting closely related trees (clones), sampled individuals were at least 20–50 m apart. In some locations, populations from three elevational ranges (0–800 m, 800–1500 m, and 1500 m and beyond) were collected (Table 1) to assess the impact of altitudinal gradients on the population's genetic structure (Körner 2007). Leaf materials from five Turkish *F. orientalis* populations (15 trees per population) were also collected to cover the species' distribution range in northern Türkiye.

DNA Extraction, Library Preparation, and Sequencing

Genomic DNA was extracted from 12 to 25 µg dried leaf materials with the Qiagen DNeasy 96-plate or single-column kit using the plant mini protocol with minor adjustments. We quantified genomic DNA with the Qubit dsDNA Broad Range Assay Kit (Invitrogen). Since the initial amount of extracted genomic DNA varied among the individuals, we normalized the final DNA concentration for the next steps. To get genome-wide Single Nucleotide Polymorphism (SNPs), we followed the ddRAD protocol after Peterson et al. (2012) for library preparation. After an initial screening of multiple pairs of restriction enzymes, the pair of EcoRI-MseI was selected for the digestion step in all three species. Each sample was digested with high-fidelity restriction enzymes (New England Biolabs). The double-digest reactions of 20 µl volume used 10–15 µl of DNA, 10 units of EcoRI, 10 units of MseI, and 1 × Cut-Smart buffer (New England Biolabs) for 3 h at 37 °C, and 20 min of heat inactivation at 65 °C. The double-digested reactions were purified with the Agencourt AMPure XP beads (Beckman Coulter) following the manufacturer's protocol, with elution in 40 µl Nuclease-free water. The adapter ligations were carried out in a total volume of 20 µl, combining 30 ng DNA, 0.40 µmol of a sample-specific EcoRI double-strand adaptor, 0.20 µmol of a common MseI adaptor for all samples, 1U of T4 DNA ligase (New England BioLabs), and 2 µl of T4 ligase buffer. The reactions were incubated at 23 °C for 30 min and then heat-killed at 65 °C for 10 min, followed by a slow cooling step to room temperature. A total of 48 EcoRI double-stranded barcodes with five unique base pair sequences were used. Adapter-ligated samples were pooled into sets, each containing 16 samples. The pooled samples were purified using the Agencourt AMPure XP beads, and the DNA was quantified using the Qubit dsDNA High Sensitivity kit. Automatic size selection was conducted using the BluePippin DNA Size Selection instrument (Sage Science, Beverly, MA, USA) with 2% agarose cassettes with internal standards and the selection of genomic fragments from 275 to 475 bp. Each library's size distribution and quantity were measured on the 2100 Bioanalyzer (Agilent Technologies) using the Agilent DNA 1000 Kit. Size-selected libraries were amplified using Phusion High-Fidelity PCR Kit in 25 µl volume, with 0.25 µl Phusion DNA Polymerase, five µl 5X Phusion HF, 1.25 µl of each of 10 µM forward and reverse primers, 0.5 µl of 10 mM dNTPs, and 12 µl of the ligation products with adjustments of nuclease-free water to 25 µl. The PCR protocol was: 1 cycle of 98 °C for 30 s, 12 cycles of 98 °C for 30 s, 60 °C for 30 s, and 72 °C for 30 s, followed by a final extension at 72 °C for 10 min. PCR reactions were done in a Bio-Rad T100 thermal cycler (Bio-Rad Laboratories). The PCR reactions were cleaned with the Agencourt AMPure XP beads and quantified in the 2100 Bioanalyzer. Each species was sequenced in two lanes of Illumina HiSeq X flow cell (150 bp paired-end reads), with 288 pooled individuals per lane.

Table 1 The list of sampled species and populations. Population genetic statistics are calculated using STACKS for each population. The range column represents elevation gradients, which have been broadly classified as low elevations (L) ranging from 0 to 800 m.a.s.l., medium (M) ranging from 800 to 1500 m.a.s.l., and high (H) including 1500 m and beyond. Observed Heterozygosity (Obs Het) is the proportion of heterozygotes in a population. Expected heterozygosity (Exp Het) is the heterozygosity expected under Hardy–Weinberg equilibrium. The π is nucleotide diversity. F_{IS} is the inbreeding coefficient of an individual relative to the subpopulation

Species	Country	Province	Location	Code	Range	Elevation (m)	Coordinates	N	Obs Het	Exp Het	π	F_{IS}
Acer velutinum Boiss	Iran	Gilan	Astara	AST-L	L	154–156	38.437/48.792	20	0.248	0.22	0.226	-0.028
	Iran	Gilan	Astara	AST-M	M	575–700	38.389/48.636	20	0.257	0.214	0.221	-0.071
	Iran	Gilan	Asalem	ASA-H	H	1188–1200	37.624/48.831	19	0.348	0.255	0.264	-0.167
	Iran	Gilan	Siahkal	SIA-L	L	260	37.107/49.858	19	0.197	0.194	0.201	0.026
	Iran	Gilan	Siahkal	SIA-M	M	725	36.990/49.857	20	0.241	0.217	0.225	-0.017
	Iran	Gilan	Siahkal	SIA-H	H	1281–1417	36.942/49.894	19	0.231	0.206	0.213	-0.021
	Iran	Mazandaran	Ramsar	RAM-M	M	574	36.884/50.593	19	0.15	0.149	0.154	0.027
	Iran	Mazandaran	Mohammad Abad	MOH-L	L	650	36.331/52.341	16	0.229	0.19	0.198	-0.052
	Iran	Mazandaran	Salahadin Kola	SAL-L	L	538	36.524/51.823	18	0.2	0.172	0.179	-0.018
	Iran	Mazandaran	Lavij	LAV-H	H	1400	36.361/52.029	18	0.229	0.193	0.2	-0.04
	Iran	Mazandaran	Royan	ROY-H	H	1556	36.393/51.941	20	0.234	0.211	0.218	-0.006
	Iran	Mazandaran	Amol	AMO-L	L	390	36.379/52.373	19	0.216	0.192	0.198	-0.017
	Iran	Mazandaran	Sari	SAR-M	M	800	36.395/53.055	20	0.225	0.203	0.21	-0.014
	Iran	Mazandaran	Afrachal	AFR-M	M	850	36.229/53.246	20	0.231	0.203	0.21	-0.031
	Iran	Mazandaran	Galogah	GAL-L	L	390	36.678/53.819	19	0.212	0.216	0.224	0.044
	Iran	Mazandaran	Galogah	GAL-M	M	780	36.686/53.806	18	0.258	0.232	0.241	-0.025
	Iran	Golestan	Tuskestan	TUS-L	L	880	36.762/54.592	20	0.244	0.228	0.236	0.01
	Iran	Golestan	Kordkuy	KOR-M	M	1105–1785	36.704/54.121	20	0.266	0.237	0.245	-0.025
	Iran	Golestan	Zarrin Gol	ZAR-L	L	350–650	36.729/54.122	20	0.266	0.239	0.248	-0.025
	Iran	Golestan	Zarrin Gol	ZAR-M	M	1179–1203	36.840/55.093	20	0.273	0.264	0.273	0.051
	Iran	Golestan	Zarrin Gol	ZAR-H	H	1490–1530	36.837/55.023	20	0.282	0.247	0.256	-0.03
	Iran	Golestan	Fazel Abad	FAZ-M	M	924	36.858/54.740	19	0.257	0.239	0.247	0.006
	Iran	Golestan	Afratakhteh	AFT-L	L	492	36.828/54.991	19	0.293	0.255	0.263	-0.052
	Iran	Golestan	Afratakhteh	AFT-M	M	1077	36.813/55.004	17	0.275	0.243	0.252	-0.034
	Iran	Golestan	Afratakhteh	AFT-H	H	1486	36.804/54.971	17	0.26	0.23	0.24	-0.02
	Iran	Golestan	Loveh	LOV-L	L	806	37.348/55.678	20	0.272	0.24	0.248	-0.036
	Iran	Golestan	Loveh	LOV-M	M	1259	37.315/55.686	18	0.282	0.23	0.239	-0.09

Table 1 (continued)

Species	Country	Province	Location	Code	Range	Elevation (m)	Coordinates	N	Obs Het	Exp Het	π	F_{IS}
	Iran	Golestan	Loveh	LOV-H	H	1689	37.298/55.697	20	0.266	0.231	0.239	-0.034
Subtotal			**28**					**534**				
Fagus orientalis Lipsky	Türkiye	Bursa	İznik	T1	H	1100	40.585/29.821	15	0.077	0.083	0.087	0.03
	Türkiye	Sinop	Kastamonu	T2	M	858	41.776/33.579	15	0.082	0.085	0.089	0.021
	Türkiye	Çanakkale	Biga	T5	M	750	40.119/27.382	15	0.08	0.08	0.084	0.008
	Türkiye	Artvin	Arhavi	T3	H	1718	41.291/41.477	15	0.149	0.156	0.162	0.044
	Türkiye	Trabzon	Maçka	T4	H	1250	40.797/39.485	15	0.153	0.159	0.166	0.036
	Iran	Gilan	Astara	AST-M	M	848	38.395/48.593	16	0.209	0.207	0.216	0.032
	Iran	Gilan	Shafaroud	SHA-L	L	225	37.520/49.026	18	0.223	0.225	0.233	0.04
	Iran	Gilan	Shafaroud	SHA-M	M	830	37.505/48.829	18	0.208	0.217	0.225	0.055
	Iran	Gilan	Shafaroud	SHA-H	H	985	37.422/49.022	20	0.218	0.219	0.226	0.042
	Iran	Gilan	Lomer	LOM-H	H	1678	37.543/48.752	19	0.221	0.225	0.232	0.042
	Iran	Gilan	Asalem	ASA-L	L	380	37.685/48.832	17	0.226	0.228	0.235	0.039
	Iran	Gilan	Asalem	ASA-M	M	1090	37.672/48.753	17	0.207	0.217	0.225	0.065
	Iran	Gilan	Asalem	ASA-H	H	1600	37.626/48.802	17	0.215	0.216	0.224	0.034
	Iran	Gilan	Siahkal	SIA-M	M	906	36.979/49.851	20	0.215	0.217	0.224	0.045
	Iran	Gilan	Siahkal	SIA-H	H	1447	36.941/49.887	20	0.205	0.218	0.225	0.067
	Iran	Mazandaran	Limakdeh	LIM-H	H	1593	36.790/50.578	18	0.2	0.21	0.218	0.066
	Iran	Mazandaran	Kelarabad	KEL-M	M	541	36.648/51.283	18	0.203	0.215	0.222	0.068
	Iran	Mazandaran	Mahan	MAH-L	L	824	36.361/52.144	20	0.209	0.218	0.224	0.054
	Iran	Mazandaran	Mazichal	MAZ-H	H	1620	36.569/51.112	18	0.204	0.208	0.215	0.046
	Iran	Mazandaran	Vaz	VAZ-M	M	1152	36.310/52.123	19	0.218	0.225	0.233	0.055
	Iran	Mazandaran	Nojomeh	NOJ-H	H	1958	36.282/52.163	19	0.206	0.208	0.215	0.039
	Iran	Mazandaran	Lajim	LAJ-M	M	876	36.251/53.130	18	0.215	0.223	0.23	0.053
	Iran	Mazandaran	Razekeh	RAZ-M	M	587	36.321/52.336	20	0.215	0.219	0.226	0.047
	Iran	Mazandaran	Kiasar	KIA-H	H	1611	36.213/53.588	20	0.223	0.225	0.232	0.041
	Iran	Mazandaran	Neka	NEK-L	L	653	36.544/53.423	15	0.227	0.229	0.238	0.039
	Iran	Mazandaran	Sika, Neka	NEK-M	M	888	36.408/53.533	13	0.221	0.22	0.231	0.033
	Iran	Mazandaran	Sika, Neka	NEK-H	H	1450	36.362/53.549	14	0.217	0.22	0.229	0.041

Table 1 (continued)

Species	Country	Province	Location	Code	Range	Elevation (m)	Coordinates	N	Obs Het	Exp Het	π	F_{IS}
	Iran	Golestan	Alang dareh	ALD-M	M	854	36.768/54.453	18	0.211	0.219	0.227	0.056
	Iran	Golestan	Alang dareh	ALD-H	H	1311	36.759/54.463	20	0.209	0.215	0.222	0.044
	Iran	Golestan	Bandar Gaz	GAZ-L	L	241	36.664/53.868	19	0.221	0.224	0.231	0.041
	Iran	Golestan	Bandar Gaz	GAZ-M	M	352	36.653/53.875	19	0.201	0.213	0.219	0.067
	Iran	Golestan	Bandar Gaz	GAZ-H	H	1484	36.639/53.876	20	0.229	0.229	0.236	0.032
Subtotal			**32**					**565**				
Quercus castaneifolia C.A.May	Iran	Gilan	Astara	AST-L	L	35–150	38.438/48.783	20	0.166	0.213	0.222	0.178
	Iran	Gilan	Astara	AST-M	M	700–715	38.386/48.634	15	0.182	0.236	0.247	0.19
	Iran	Gilan	Asalem	ASA-L	L	163–272	37.716/48.896	17	0.18	0.22	0.23	0.155
	Iran	Gilan	Asalem	ASA-M	M	650–688	37.680/48.796	19	0.209	0.251	0.259	0.163
	Iran	Gilan	Asalem	ASA-H	H	1188–1200	37.620/48.828	18	0.188	0.231	0.241	0.166
	Iran	Gilan	Siahkal	SIA-L	L	187–255	37.108/49.859	16	0.191	0.24	0.25	0.184
	Iran	Gilan	Siahkal	SIA-M	M	725	36.990/49.857	19	0.216	0.244	0.253	0.117
	Iran	Gilan	Rasht	RAS-L	L	88	37.124/49.657	15	0.217	0.248	0.26	0.13
	Iran	Gilan	Rezvanshahr	REZ-L	L	90–120	37.524/49.105	17	0.199	0.241	0.251	0.161
	Iran	Mazandaran	Ramsar	RAM-L	L	300	36.901/50.631	20	0.163	0.222	0.231	0.22
	Iran	Mazandaran	Salahadin Kola	SAL-L	L	210	36.545/51.823	10	0.161	0.22	0.237	0.192
	Iran	Mazandaran	Salahadin Kola	SAL-M	M	350	36.529/51.846	9	0.142	0.183	0.198	0.146
	Iran	Mazandaran	Nuwshahr	NOW-M	M	360	36.625/51.457	20	0.209	0.241	0.25	0.126
	Iran	Mazandaran	Noor	NOR-L	L	180	36.449/52.051	19	0.2	0.243	0.252	0.163
	Iran	Mazandaran	Lavij	LAV-H	H	1986	36.353/51.998	18	0.193	0.242	0.253	0.168
	Iran	Mazandaran	Amol	AMO-L	L	230	36.381/52.335	10	0.186	0.209	0.224	0.098
	Iran	Mazandaran	Sari	SAR-L	L	600	36.380/53.118	10	0.148	0.193	0.209	0.157
	Iran	Mazandaran	Sari	SAR-M	M	920	36.225/53.233	16	0.175	0.227	0.238	0.189
	Iran	Mazandaran	Desele	DES-L	L	351	36.360/53.257	14	0.208	0.245	0.259	0.143
	Iran	Mazandaran	Neka	NEK-M	M	720	36.474/53.466	17	0.203	0.25	0.258	0.182
	Iran	Mazandaran	Galogah	GAL-L	L	180	36.714/53.821	12	0.178	0.212	0.223	0.135
	Iran	Golestan	Kordkuy	KOR-H	H	1990	36.703/54.128	20	0.177	0.23	0.239	0.193
	Iran	Golestan	Allang Darreh	ALD-L	L	800	36.796/54.457	18	0.142	0.188	0.196	0.189
	Iran	Golestan	Fazel Abad	FAZ-M	M	924	36.830/54.754	20	0.174	0.238	0.247	0.229
	Iran	Golestan	Zarrin Gol	ZAR-M	M	1179	36.884/55.032	18	0.19	0.234	0.245	0.164
	Iran	Golestan	Zarrin Gol	ZAR-H	H	1500	36.895/55.048	19	0.183	0.227	0.236	0.166
	Iran	Golestan	Azadhsahr	AZA-L	L	387	37.063/55.173	19	0.168	0.198	0.206	0.132
	Iran	Golestan	Loveh	LOV-L	L	806	37.349/55.666	20	0.204	0.249	0.257	0.175
	Iran	Golestan	Loveh	LOV-M	M	1259	37.319/55.694	17	0.172	0.205	0.214	0.136

Table 1 (continued)

Species	Country	Province	Location	Code	Range	Elevation (m)	Coordinates	N	Obs Het	Exp Het	π	F_{IS}
	Iran	Golestan	Loveh	LOV-H	H	1689	37.298/55.711	17	0.224	0.258	0.268	0.138
Subtotal			**30**					**499**				
Total			**90**					**1,589**				

De novo *assembly and genotyping*

We used the de novo assembly method of STACKS v1.48 (Catchen et al. 2011, 2013) to identify genome-wide SNPs. Raw reads were demultiplexed with the process_radtags package of STACKS to assign the reads to the individuals, filtering low-quality reads and dropping reads missing the expected EcoRI cut site (options -c -q -r). The FASTP software v0.20 (Chen et al. 2018) was used to discard reads shorter than 40 bp and a quality score below 20 with over-represented sequence analysis enabled. Then ustacks package was used to produce consensus sequences of RAD tags with a minimum coverage depth of five and an alpha value of 0.05 for the SNP model. The cstacks package created a set of consensus loci and merging alleles. Using the sstacks package, sets of stacks (putative loci) identified by ustacks were searched against a catalog. The populations package was run to get the loci present in at least 80–65% of the individuals and half of the populations (-p), with a minimum minor allele frequency of 0.05. Only the first SNP per locus was called.

Genetic diversity and population structure

Genetic diversity within populations in each species, such as expected (H_e) and observed (H_o) heterozygosity, nucleotide diversity (π), average inbreeding coefficients (F_{IS}), and the corresponding standard errors (SE), were estimated using the STACKS. The global F_{ST} and F_{IS} for each species were calculated separately using the hierfstat R package (Goudet 2005) applying the Weir and Cockerham (1984) method. Given our large-scale sampling covering the entire distribution range of species in the Hyrcanian forests, we evaluated the correlations between elevations (altitudinal gradients) and longitude with the genetic diversity parameters like π and H_o. For each species, robust linear regressions were performed to model the relationship between elevation and longitude with genetic diversity parameters using R v4.3.2. Regression statistics, including R^2 values and p-values, were extracted to assess the strength and significance of these relationships.

It is common practice to exclude loci under positive selection from population genetic structure and demographic history analysis (Storz 2005; Beaumont 2005) unless the objectives differ. We used two approaches to identify these loci: principal component analysis (PCA) and fixation index (F_{ST}) based methods. For the PCA method, pcadapt R package v4.3.3 was used (Luu et al. 2017). Using the Scree Plot option, we set the K = 10, and following the package recommendation, multiple cutoff values were set for outlier detection using the R package qvalue v2.26 (https://github.com/StoreyLab/qvalue). For the F_{ST}-based outlier detection, the OutFLANK R package v0.2 (Whitlock and Lotterhos

2015) was selected, which uses the distribution of F_{ST} values to infer outlier markers and has been shown to result in a low number of false positives (Luu et al. 2017). Outlier loci were excluded from subsequent analysis using VCFtools (Danecek et al. 2011). The extent of Linkage Disequilibrium (LD) might not be pervasive in RADseq data because of the random selection of markers, low SNP density, and various levels of LD among species (Lowry et al. 2017). Regardless, LD evaluation was performed using the pcadapt R package v4.3.3 (Luu et al. 2017).

The genetic structure of the populations was assessed by a Bayesian clustering algorithm using STRUCTURE v2.3.4 (Pritchard et al. 2000; Falush et al. 2003), assuming Hardy–Weinberg equilibrium (HWE) and LD, assigning individuals into clusters from K = 1–10, with ten iterations of each *K* and 1,000,000 Markov chain Monte Carlo (MCMC) generations after 100,000 burn-in. We used the StrAuto tool to automate the analysis and make STRUCTURE runs in parallel (Chhatre and Emerson 2017). The optimal number of clusters (*K*) was identified using the Δ K method (Evanno et al. 2005) using the STRUCTURE HARVESTER program (Earl and vonHoldt 2012). The postprocessing of STRUCTURE results was done using CLUMPAK, which identifies sets of highly similar runs, separating distinct groups (Kopelman et al. 2015).

We also utilized fineRADstructure, a tool designed explicitly for RADseq data, to infer the population's genetic structure. To convert the output of STACKS into the required fineRADstructure format, we employed the finerad_input.py Python script from the fineRADstructure package (Malinsky et al. 2018). We refined the dataset by selecting only unlinked loci with a minimum sample size of 10 individuals. Subsequently, the loci were sorted using the sampleLD.R script, and the coancestry matrix was calculated utilizing the RADpainter package with default parameters. The results were visualized using the fineRADstructurePlot script in R (R Core Team, 2020). Additionally, we constructed a haplotype network to represent the genetic distance among the selected populations using SplitsTree v4.19.0 (Huson and Bryant 2006).

Demographic and evolutionary history of populations

The effective population sizes (*Ne*) were estimated using NeEstimator v2.1 based on the LD method with random mating, heterozygote-excess, and molecular coancestry methods (Do et al. 2014). We used filtered SNP data sets after OutFLANK and pcadapt with outlier loci removed. We screened out rare alleles with frequencies below 0.050, 0.020, and 0.010 (PCrit) for all methods mentioned above as they can bias the estimation of *Ne* (Do et al. 2014). All calculations were run at 95% confidence intervals using the jackknife on the samples (Waples and Do 2008).

To infer the patterns of population splits and mixtures and gene flow among populations, a maximum-likelihood tree with migration events among Western (Gilan province), Central (Mazandaran), and Eastern (Golestan) populations, was reconstructed using TreeMix v1.13, which applies genome-wide allele frequency estimation and a Gaussian approximation to the genetic drift (Pickrell and Pritchard 2012). To reduce computations and because TreeMix is sensitive to missing data, we selected a subset of populations from Western, Central, and Eastern regions. We ran the populations package of STACKS to get shared SNPs among 90% of individuals (r = 0.9). TreeMix was run in parallel with ten independent runs to estimate the optimum number of migrations and calculate 500 bootstrap replicates with the R package BITE (Milanesi et al. 2017) and scripts from https://github.com/carolindahms/TreeMix.

The demographic history of populations was inferred by Stairway Plot 2 software (Liu and Fu 2020) using the Site Frequency Spectrum (SFS) approach described by Nielsen et al. (2012). We mapped all individuals' trimmed sequences to reference genomes to retrieve SFS for each species independently. For the *A. velutinum*, *F. orientalis*, and *Q. castaneifolia*, we used draft genomes of *Acer yangbiense* Chen & Yang, 2003 (Yang et al. 2019), *Fagus sylvatica* L. (Mishra et al. 2018), and *Quercus robur* L. (Plomion et al. 2018), respectively. The mapping to reference genomes was done using the BWA (Li 2013), and then SAM files were converted and sorted into BAM files using the SAMtools (Li et al. 2009). ANGSD v0.397 (Korneliussen et al. 2014) calculated the site allele frequency likelihoods based on individual genotype likelihoods assuming HWE (dosaf = 1). We filtered out the low qscore bases and mapQ reads (options -minMapQ 1 -minQ 20), followed by an optimization method that estimates the unfolded SFS using the realSFS package of ANGSD with 100 iterations. For the Stairway Plot 2 runs, the SFS output file from ANGSD was used as input, and singletons were excluded. According to the software manual, 0.67% of the sites were used for training with four random breakpoints and 200 estimations. The mutation rate per site per generation was assumed to be 2.5e-9 (Gossmann et al. 2012), with an average generation time of 20 years for each species (Fusco and Minelli 2019).

Results

Summary of genomic data

The genomic data obtained for the three species in this study consisted of a total of 5.3 billion raw reads. Short and low-quality reads, PCR duplicates, and reads without

Table 2 Summary of the sequencing and measures of population differentiation (F_{ST} and F_{IS}) for the Hyrcanian forests tree species

Species	N. Pops	N. Indv	N. reads (raw)	N. reads **(trimmed)**	N. of Loci	F_{ST} **(pop/total)**	F_{IS} **(ind/pop)**
Acer velutinum	28	534	1,999,189,324	1,585,116,676	1,347	0.120	-0.095
Quercus castaneifolia	30	499	1,738,619,546	904,939,358	8,881	0.029	0.228
Fagus orientalis (Iran)	27	490	1,657,536,282	1,366,986,368	2,091	0.019	0.063

RAD tags were trimmed. After these steps, 3.8 billion reads were retained for subsequent analyses. The mean coverage depth calculated by ustacks for *A. velutinum*, *F. orientalis,* and *Q. castaneifolia* samples was 12.4 (6.2—67), 11.2 (5.0–31), and 10.4 (4.47—70), respectively. A total of 1,347 genome-wide SNPs were identified for *A. velutinum*, 2,091 SNPs for *F. orientalis*, and 8,881 SNPs for *Q. castaneifolia* (Table 2), from 28 populations of *A. velutinum*, 32 populations of *F. orientalis*, and 30 populations of *Q. castaneifolia* (Table 1). A total of 12, 118, and 408 loci were identified as outliers for *F. orientalis*, *A. velutinum*, and *Q. castaneifolia*, respectively, using a false discovery rate threshold of 10% by the pcadapt (Figures S4 and S5), and were subsequently excluded from the analyses.

Genetic diversity and population structure

The genetic diversity of the studied species is summarized in Tables 1 and 2 and Fig. 3a. For *A. velutinum*, π values ranged from 0.154 to 0.273, with a mean value of 0.227. *He* values ranged from 0.149 to 0.264 (mean = 0.22), while *Ho* values ranged from 0.150 to 0.348 (mean = 0.248). For *F. orientalis*, the values of π ranged from 0.084 to 0.238 (mean = 0.209), *He* ranged from 0.08 to 0.229 (mean = 0.2), and *Ho* ranged from 0.08 to 0.227 (mean = 0.197). For *Q. castaneifolia*, the values of π ranged from 0.196 to 0.268 (mean = 0.238), *He* ranged from 0.188 to 0.258 (mean = 0.228), and *Ho* ranged from 0.142 to 0.224 (mean = 0.185). When comparing observed and expected heterozygosity, *F. orientalis* populations had nearly identical values, while *Q. castaneifolia* populations had lower observed heterozygosity than expected values. In contrast, *A. velutinum* populations had higher observed heterozygosity than expected. Notably, all but six of the populations of *A. velutinum* had negative F_{IS} values.

The global F_{ST} values indicate the degree of genetic differentiation among the populations. *Fagus orientalis* had the lowest global F_{ST} value (0.018), indicating relatively low genetic differentiation among its populations. On the other hand, *A. velutinum* had the highest F_{ST} value (0.119), which was six times higher than that of *F. orientalis* and four times higher than that of *Q. castaneifolia* (0.029) (Table 2). Among the *A. velutinum* populations, the ASA-H population from the Asalem highland region of the Western Hyrcanian forests (1200 m.a.s.l.) showed the highest *Ho* value (0.347) and the lowest F_{IS} value (-0.166) (Table 1). In addition, this population had the second highest nucleotide diversity (π = 0.264) after the ZAR-M population (π = 0.272). Six populations of *A. velutinum* showed positive F_{IS} values: SIA-L from Gilan, RAM-M, and GAL-L from Mazandaran, and ZAR-M, FAZ-M, and TUS-L from Golestan. The global F_{ST} value for *Q. castaneifolia* populations was 0.029 (Table 2). Among the *Q. castaneifolia* populations, the LOV-H population from Loveh (1689 m.a.s.l.) had the highest *Ho* value (0.223) and nucleotide diversity (π = 0.267).

The *F. orientalis* populations from Türkiye showed significantly lower genetic diversity than those from Iran. The GAZ-H population from Eastern Mazandaran in Iran had the highest *Ho* value of 0.228 and the second highest π value of 0.235, followed by the NEK-L population with a π value of 0.237. Among the Turkish populations, the western populations (T1, T2, and T5) had significantly lower *Ho* and π values than the eastern populations (T3 and T4). These results indicate notable differences in genetic diversity between the western and eastern *F. orientalis* populations in Türkiye and between them and the Iranian populations. The Turkish populations showed lower levels of genetic diversity, as reflected in their lower *Ho* and π values, especially in the western regions. *Fagus orientalis* populations in Iran show a moderate negative correlation between altitude and nucleotide diversity ($R^2 = 0.15$, $p = 0.046$). However, we did not observe significant correlations between nucleotide diversity and elevation for *A. velutinum* and *Q. castaneifolia* (Fig. 3b). In addition, significant correlations ($R^2 = 0.24$, $p = 0.008$) were found between nucleotide diversity and longitude of *A. velutinum* populations (Fig. 3c). The other two species didn't show a significant correlation.

The STRUCTURE analysis identified six distinct genetic clusters as the best K (K = 6, see Figures S2 and 4b) for *A. velutinum*. In contrast, *F. orientalis* and *Q. castaneifolia* populations were grouped into two clusters (best K = 2, see Fig. 4). In the case of *A. velutinum*, the K = 2 clustering showed a clear separation between populations from Gilan and Mazandaran provinces, except for the Galogah populations in eastern Mazandaran, which showed about 50% admixture between the two clusters. When K = 3, the Gilan populations formed a distinct genetic cluster in the western

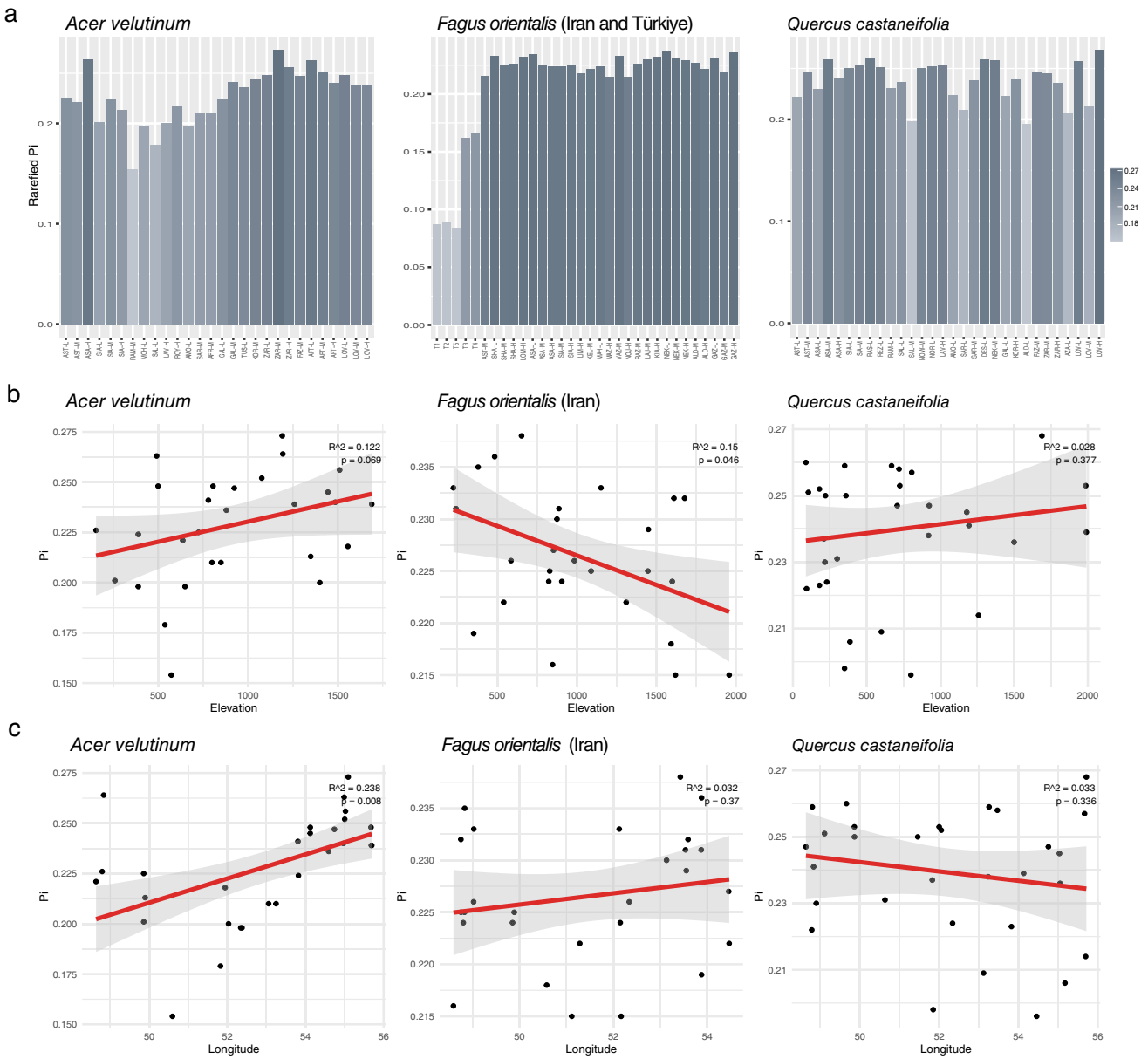

Fig. 3 Genetic diversity of three species based on nucleotide diversity (π) after performing rarefaction. **a** Each bar represents the π value for a specific population. The x-axis represents the population names, and the y-axis represents the rarefied nucleotide diversity (π) values. Populations are ordered from west to east. Correlation of altitudinal gradients with π (**b**), and longitude with π (**c**). Red lines show the robust linear regressions. R^2 and p-values are shown for each species in the upper right corner of each plot

Hyrcanian forests, which gradually merged with the neighboring Mazandaran populations (e.g., RAM-M, as shown in Fig. 4b). As the number of clusters increased to K = 4, the Gilan and Golestan populations separated into distinct clusters, while four clusters were mixed in the central part of the forests (SAR-M, AFR-M, GAL-M). This pattern was also revealed by the TreeMix analysis (red arrow in Fig. 7a). At K = 5, the remote populations of *Acer* from the Golestan National Park in Loveh formed a distinct cluster. Finally, at K = 6, greater levels of admixture were observed in the Mazandaran populations compared to the western and eastern populations (Fig. 4b).

The best number of clusters for *F. orientalis* and *Q. castaneifolia* was K = 2. The Mazandaran and Golestan populations clustered together, unlike *Acer* populations with K = 2. Additionally, some admixture was observed in the western population of Mazandaran. The coancestry matrix heat map of the fineSTRUCTURE analysis is shown in Fig. 5. The fineSTRUCTURE analysis also shows a clear genetic subdivision among the *A. velutinum* populations compared to the *F. orientalis* and

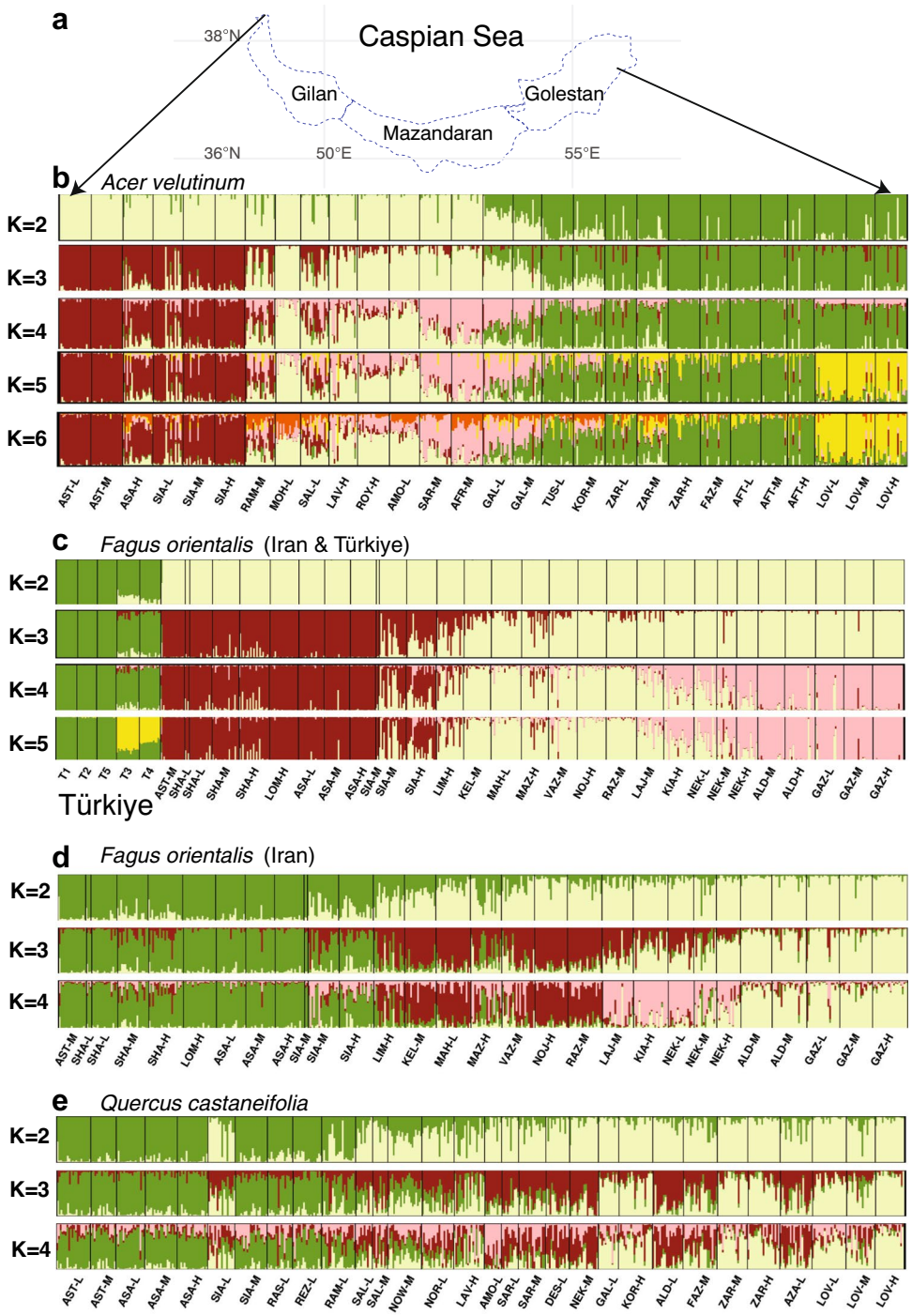

Fig. 4 Population clustering analysis using STRUCTURE software. Each color represents a different cluster assignment for individuals. Solid vertical black lines indicate population boundaries. Population names are shown below the corresponding bars for each species. The order of populations, from left to right, follows the west–east distribution range of each species. **a** Three provinces (Gilan, Mazandaran and Golestan) in northern Iran cover the entire Hyrcanian forests range in Iran. **b** *A. velutinum*, **c** *F. orientalis* from Iran and Türkiye, **d** *F. orientalis* Iranian populations, **e** *Q. castaneifolia*

Q. castaneifolia populations. The fineSTRUCTURE analysis also shows the clear genetic divergence of Turkish *F. orientalis* populations from Iranian populations (Fig. 5c).

Demographic and evolutionary history

The Stairway plot analysis revealed *Ne* declines and population bottlenecks for three species in the past 1—2 million years, but the timing of these fluctuations is slightly different for each species (Fig. 6). The estimated *Ne* for *A. velutinum* is less than 2 million, half that of *Q. castaneifolia* and one fifth that of *F. orientalis* (Fig. 6). During the last glacial period, the *Ne* of *A. velutinum* was estimated to be less than 800,000, while that of *F. orientalis* and *Q. castaneifolia*, it exceeded 10 million. The TreeMix analysis revealed significant genetic drift within the populations of *A. velutinum* compared to those of *F. orientalis* and *Q. castaneifolia*. Specifically, the populations of *A. velutinum* populations in the

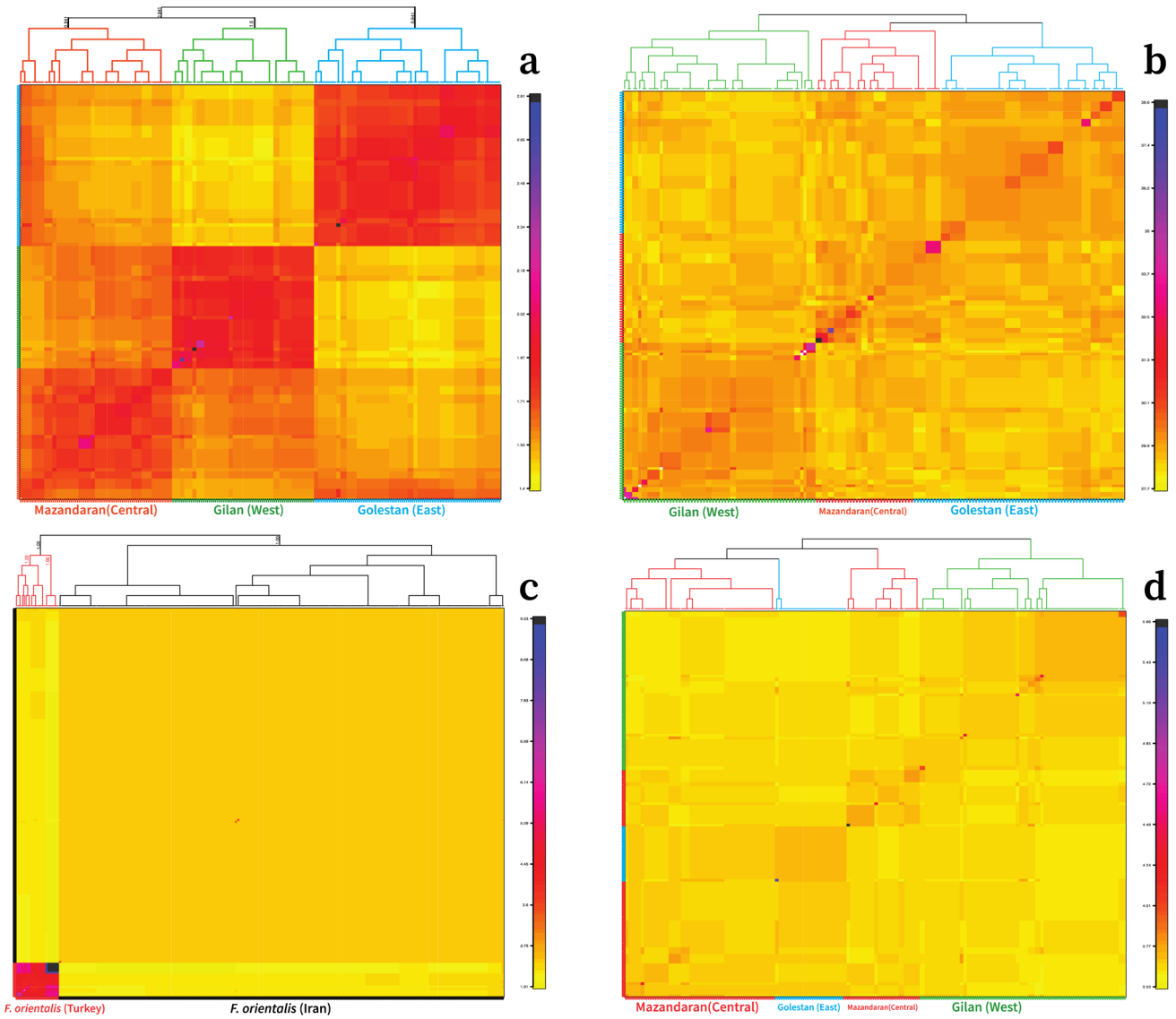

Fig. 5 A coancestry matrix heat map from the fineSTRUCTURE analysis. **a** *A. velutinum*, **b** *Q. castaneifolia*, **c** *F. orientalis* from Iran and Türkiye, **d** *F. orientalis* of Iranian populations. Individuals of *A. velutinum* show a visible substructure with a coancestry cluster of the west-central (**a**). The coancestry matrix of *Q. castaneifolia* shows that the eastern and central populations are clustered (**b**). *F. orientalis* from Türkiye is highly isolated, with the arrow indicating the populations from the Colchic region of Türkiye (**c**). Eastern populations of *F. orientalis* from the Hyrcanian forests are nested within the central cluster (**d**). The vertical bars represent the estimated coancestry of the RAD loci

Gilan and Mazandaran formed a distinct clade, which was also supported by the network analysis (Figs. 6, 7 & S1) and showed significant differentiation from the populations in Golestan populations (Fig. 7a). These results are consistent with those obtained from the K = 2 structure analysis. In the case of *F. orientalis* and *Q. castaneifolia*, the Gilan populations showed an ancestral state, with the *Q. castaneifolia* population showing the least drift among the populations compared to *F. orientalis* and *A. velutinum* (Fig. 7).

Discussion

In this study, our goal was to investigate the genomic diversity of three dominant and ecologically and economically important tree species in the Hyrcanian forests of northern Iran and to understand how paleoclimatic events and ecological factors have shaped the genetic diversity of the species and whether different tree species show different genetic patterns in the similar environments where they all coexist.

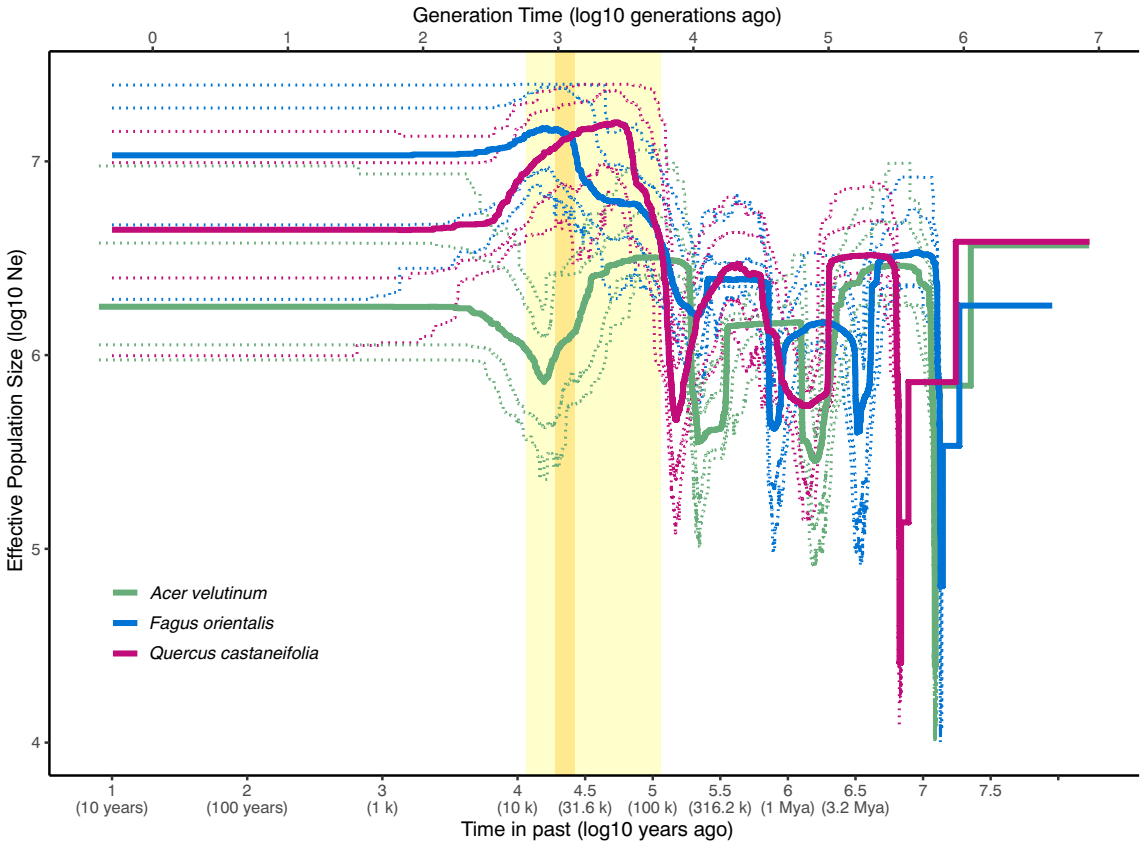

Fig. 6 Inferred demographic history of *A. velutinum*, *F. orientalis* (Iranian populations), and *Q. castaneifolia* presented in a multilayer plot. Demographic histories were inferred from 534 individuals of *A. velutinum*, 490 individuals of *F. orientalis* (Iranian populations), and 499 individuals of *Q. castaneifolia*, using Stairway Plot 2 with unfolded SFSs. The bold line represents the median of 200 inferences obtained by subsampling. The dotted lines represent the confidence intervals around the median Ne estimates at the 75% and 95% confidence intervals, respectively. The lower x-axis represents the logarithmic time scale (log 10 years ago) and shows the shift in *Ne* in response to climatic and environmental changes. The top x-axis represents the logarithmic scale of generations before the present. The y-axis represents a logarithmic scale of effective population size (log10 *Ne*). The broad vertical column represents the last glacial period, approximately 11,500 to 115,000 years ago, and the narrow column outlines the Last Glacial Maximum (LGM), approximately 27,000 to 19,000 years ago

Quaternary glacial and interglacial cycles have influenced the genetic diversity of many European forest trees. However, the impact of these cycles in the Hyrcanian forests of the Caucasus biodiversity hotspot has only received attention in recent years and has been overlooked in many European studies. This study is one of the first genome-wide studies on the native tree species in the Hyrcanian forests of Iran, and it uses extensive sampling to better understand the demographic history of these valuable tree species.

Genetic diversity and population structure in the hyrcanian forests

The low F_{ST} values of *F. orientalis* populations in our study (Table 2) are consistent with previous studies, indicating high levels of gene flow between neighboring populations (Salehi Shanjani et al. 2004, 2010; Bijarpasi et al. 2020). *Fagus orientalis* and *Q. castaneifolia* are both wind-pollinated tree species. This trait can facilitate long-distance gene flow between populations and potentially lead to lower levels of genetic differentiation (Hamrick et al. 1992). Similarly, low levels of genetic differentiation have been reported for wind-pollinated forest trees such as the Japanese larch (Nishimura and Setoguchi 2011), European larch (Nardin et al. 2015), *Quercus serrata* (Ohsawa et al. 2008), and Norway spruce (Unger et al. 2011). In contrast, *A. velutinum* is insect-pollinated, which may result in more significant genetic differentiation than in wind-pollination species (Friedman and Barrett 2009; Wessinger 2021). While wind dispersal of *A. velutinum* samara fruits can theoretically travel long distances, the dense forest environment may limit and obscure this long-distance dispersal (Pandey et al. 2012). Of the three species studied, *A. velutinum* populations had negative F_{IS} values (Table 2). Negative F_{IS} values indicate excess heterozygosity, possibly due to assortative mating or selection

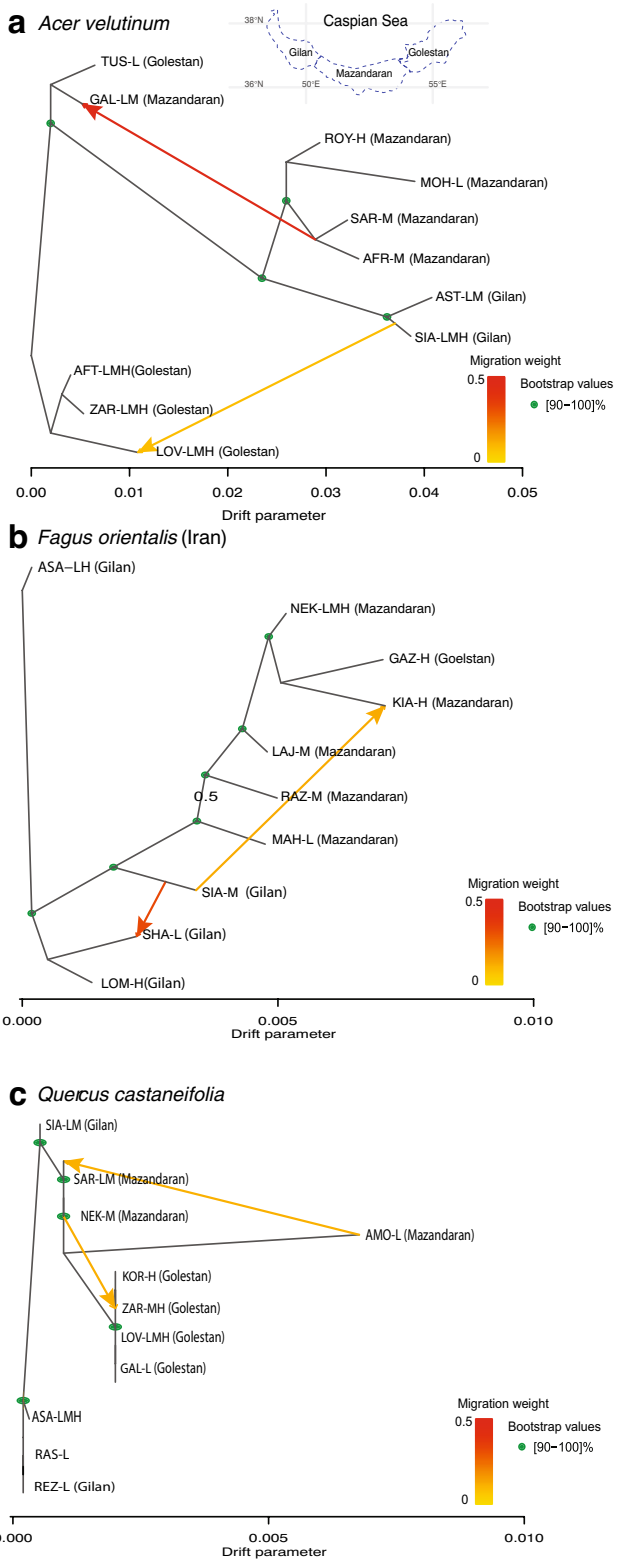

Fig. 7 Maximum likelihood tree inferred by TreeMix, showing the evolutionary relationships among selected populations of each species in the Hyrcanian forests. **a** *A. velutinum*, **b** *F. orientalis* and **c** *Q. castaneifolia*. The green circle shows bootstrap values above 90% based on 500 bootstrap replicates. Estimated migration edges are labeled and represented by arrows, with the direction of admixture indicated. The color of the arrows corresponds to the migration weight. The branch length reflects the genetic drift from the most recent common ancestor. Additional information on residual, drift matrices and models can be found in Supplementary Fig. S6

against homozygotes, suggesting avoidance of inbreeding (Stoeckel et al. 2006). The low *Ne* estimated for *A. velutinum* compared to the other two species (Fig. 6) could be due to the solitary growth habit of *A. velutinum* trees in the Hyrcanian forests, with occasional occurrences in small groups (Sagheb-Talebi 1999; Mohtashamian et al. 2017). This could lead to a small census population size (N) over time and subsequently lower *Ne*. Other factors contributing to the negative F_{IS} and low *Ne* (see Sect. "Demographic history of tree species in Hyrcanian forests" below) cannot be excluded and require further investigation.

Our study revealed a phylogeographic pattern for the studied tree species in the Hyrcanian forests. For *F. orientalis* and *Q. castaneifolia*, the STRUCTURE analysis indicated a western (Gilan) versus a central-eastern genetic cluster (best K = 2, Fig. 4), with a contact/admixture zone in the central (Mazandaran). *Acer velutinum* showed a west-central versus eastern (Golestan) structure when K = 2, although the ΔK method of Evanno (2005) suggested K = 6 (Fig. 4b). Considering the K = 2 clusters for *A. velutinum* (Fig. 4b), similar genetic structures have also been identified in other species, such as *Populus caspica*, *Fraxinus excelsior*, and *Pterocarya fraxinifolia* in the Hyrcanian forests (Erichsen et al. 2018; Maharramova et al. 2018; Alipour et al. 2021), suggesting multiple refugial regions across the Hyrcanian forests. However, a complex phylogeographic structure was observed when K = 6 was considered for *A. velutinum,* with the far eastern Loveh population in the Golestan National Park emerging as a distinct cluster (Figures S1.1 and 4b). These results suggest the influence of multiple factors in shaping the divergence and genetic structure of these populations, such as the drier and warmer climate of the eastern Hyrcanian forests compared to the western (Gilan) and central (Mazandaran) regions (Khatibi and Saberi 2020), the presence of multiple refugia and cryptic speciation, similar to that reported for *Acer campestre* in the Colchic region (Grimm and Denk 2014), and different precipitation and soil patterns between the western and eastern Hyrcanian forests (Khalili and Rahimi 2014; Waez-Mousavi 2018). Moreover, *Acer* species have low pollen productivity and poor pollen and seed dispersal (Andersen 1970; Pandey et al. 2012; Chybicki et al. 2014), which may contribute to the strong population genetic structure observed in *A. velutinum* in the Hyrcanian forests. Pollen diagrams of the late Holocene epoch from Nowshar in Mazandaran also reflect this pattern (Ramezani et al. 2008). The Hyrcanian region has high species diversity in the genus *Acer* compared to the genera

Fagus and *Quercus* (Mohtashamian et al. 2017). Considering our genetic diversity and structure analysis, the *A. velutinum* populations may consist of multiple cryptic lineages triggered by ecological factors. Cryptic speciation also has been reported for animal species in the region (Ahmadzadeh et al. 2013; Parvizi et al. 2018).

We found negative correlations between π and elevation in *F. orientalis* populations in the Hyrcanian forests (Fig. 3b). These results do not agree with those of Salehi Shanjani (2011), who found no correlation between elevation and genetic diversity for *F. orientalis* in the Hyrcanian forests. This may be because their sample size was limited to only two populations, one each in Mazandaran and Golestan provinces, and no material from the Gilan. Our sampling of *F. orientalis* in Iran ranges from 225–1958 m.a.s.l. Such a negative correlation may correspond to the decrease in air temperature along the higher elevation in the Hyrcanian forests, reflected in the lower dominance of the *F. orientalis* at higher elevations (Esmailzadeh and Soofi 2022). Altitude and related climatic variables have been identified as the main ecological drivers of forest vegetation in the Hyrcanian region (Moradi et al. 2020). The local climate has also affected the genetic differentiation of oriental beech in Georgia and Azerbaijan (Sękiewicz et al. 2022). Beech species, such as *Fagus crenata* Blume, prefer humid and misty environments for optimal growth, with a minimum of 800 mm precipitation as a limiting factor (Tsukada 1982). We also found a significant correlation between π and longitude for *A. velutinum* populations (Fig. 3c). Like *Q. castaneifolia*, *A. velutinum* is a light-demanding species (Sagheb-Talebi 1999). The correlation of the velvet maple with longitude may reflect the ecological preferences, however, more in-depth research is needed to confirm or refute these results.

Demographic history of tree species in Hyrcanian forests

The Hyrcanian forests owe their existence to the Caspian Sea and the Talesh and Alborz mountain ranges, which extend over 800 km south of the Caspian Sea from Azerbaijan to Turkmenistan (Fig. 1). These mountains, with many peaks over 4000 m, create an orographic effect that causes moisture from the Caspian Sea to cool and condense, resulting in year-round precipitation unlike the rest of Iran (Ramezani et al. 2023). This stable, humid environment supports a diverse ecosystem in the region. However, the fluctuations in the Caspian Sea level (±50 m) from the Mikulino Interglacial to the Holocene (Roshan et al. 2012; Anderson et al. 2013) and climatic changes such as warming and cooling conditions have likely influenced the demographic history of species around the Caspian Sea (Trifonov et al. 2024).

The SFS plots showed a decrease in *Ne* for *A. velutinum* during the last glacial period 11,500 to 115,000 years ago and a slight reduction in *Ne* for oriental beech (Fig. 6). In contrast, *Q. castaneifolia* populations show a consistent, stable population size without experiencing a decrease in *Ne* during this period. According to the SFS plots, the current *Ne* of *A. velutinum* is less than two million, half the *Ne* of *Q. castaneifolia* and one-fifth of that of *F. orientalis*. The Hyrcanian forests and the northeastern part of Iran have been estimated to have been drier during the final stage of the last glacial period (Wells 1989). The blowing sand (loess) and sand deserts were prevalent around the Caspian Sea, particularly on the eastern, northern, and western sides during the ice ages (Cox et al. 2020). As a result, sea level fluctuations may have contributed to the population bottlenecks, which have also been reflected in multiple genetic clusters as refugia for the velvet maple in these regions, but the exact extent and magnitude remain to be determined.

Multiple bottlenecks and reductions in *Ne* for the studied species were revealed during the Pleistocene epoch (Fig. 6). Such bottlenecks could be attributed to the drier climatic conditions with reduced precipitation and a decrease in the level of the Caspian Sea (Anderson et al. 2013; Krijgsman et al. 2019). Palynology and sedimentology of the Pliocene from eastern Azerbaijan show pollen elements of common present-day trees that prefer warm climates (such as *Quercus* and *Pterocarya*), which are well represented throughout (Richards et al. 2021). The environmental fluctuations of the South Caspian basin during the Early-Middle Pleistocene may have influenced the genetic diversity and population size of the tree species (Trifonov et al. 2024). The MRCA of *A. velutinum*, *F. orientalis*, and *Q. castaneifolia* is about 11, three, and five Mya, respectively (Renner et al. 2016; Hipp et al. 2020; Jiang et al. 2022). These timelines suggest that these species and their ancestors have experienced multiple climatic and environmental changes, such as warming and cooling conditions in the region over millions of years, which may have led to population fragmentation and speciation.

Evolutionary divergence of *F. orientalis*

Our results (Figs. 3a, 4, 5, and S1.2) showed genetic differentiation between the Turkish and Iranian populations of *F. orientalis*. The Iranian populations had almost three times higher π than the Turkish populations (Table 1 and Fig. 3a). The populations from the Colchic forests (T3 and T4) had twofold higher π than the Western Anatolian populations (T1, T2, and T5) (Figs. 1 and 3–5). The T3 and T4 populations from the Colchic region are east of the Anatolian diagonal, whereas the T1, T2, and T5 populations are west. The T3 and T4 populations are distributed at higher

elevations than the other three Turkish populations and are topographically in conditions where forest access and management are more difficult. This may result in less anthropogenic impact on the T3 and T4 populations. In addition, genetic diversity in the Turkish populations of *F. orientalis* increases from west to east, reflecting the Pleistocene refugia in this region (Ledig 2001). This west-to-east increase in genetic diversity is also reflected in the *F. sylvatica* and *F. orientalis* populations in Europe and Asia using allozyme markers with higher allelic richness than in European populations (Gömöry et al. 2007). This westward gradient of decreasing genetic diversity has also been found in other tree species, including ash (*F. excelsior*) and yew (*Taxus baccata*) (Mayol et al. 2015; Erichsen et al. 2018). These results support the hypothesis that the east–west colonization of multiple Irano-Turanian floristic elements was crucial in introducing Mediterranean, Saharo-Arabian, Balkan, and subsequently European elements during the Oligocene and Miocene (Oosterbroek and Arntzen 1992; Manafzadeh et al. 2014; Stojilkovič et al. 2022). On the other hand, the results of whole-genome sequencing and nuclear microsatellite markers of *F. orientalis* in the Caucus and Hyrcanian region of Azerbaijan show that the studied populations in the Hyrcanian region have reduced genetic diversity in comparison to the populations in the Greater Caucasus (Sękiewicz et al. 2022; Capblancq et al. 2024).

Our results suggest distinct genetic divergence of the *F. orientalis* populations in Hyrcanian forests compared to the Western and Caucasian populations. Previous studies have revealed heterogeneity within *Fagus* lineages in Iran, Georgia, and Türkiye, calling for taxonomic revisions (Denk 1999; Denk et al. 2002; Gömöry and Paule 2010; Renner et al. 2016; Gomory et al. 2018; Cardoni et al. 2022). The genetic differentiation in this study suggests its potential status as a separate species, a conclusion supported by existing research (e.g., Cardoni et al. 2022). The *F. orientalis* is distributed from the Balkan Peninsula to northern Iran, with the type species from Türkiye. Divergence time analyses estimated the MRCA of *F. orientalis* and *F. sylvatica* to be more than 10 million years old (Renner et al. 2016; Jiang et al. 2022). Current taxonomic classifications, such as the two subspecies concept of *F. sylvatica* and the three subspecies of *F. orientalis* (Greuter and Raus 1981; Duty 1985; Shen 1992), are inconsistent with current genetic and morphological knowledge. We therefore recommend further studies to address the taxonomic uncertainties surrounding these lineages.

The potential of Hyrcanian forests in shaping future forests

In contrast to European forests, the Hyrcanian forests have experienced relatively stable environmental conditions (Djamali et al. 2011). This stability has likely contributed to higher genetic diversity and the ability of taxa to adapt to their environment over time (Carnaval et al. 2009; Carvalho et al. 2017). A recent study comparing the genetic diversity of the European ash tree (*Fraxinus excelsior*) revealed significantly higher genetic diversity in the Hyrcanian forests than in European populations (Erichsen et al. 2018). On the other hand, forests in Europe have lower genetic diversity, partly due to glaciation (Gömöry et al. 2007; Lumibao et al. 2017). Therefore, the Hyrcanian forests have great potential for the development of future forests. By implementing assisted migration (Aitken et al. 2024) of seed sources and species from the Hyrcanian forests, it is possible to increase the resilience and resistance of future European forests to ongoing climate change (Mellert and Šeho 2022; Kurz et al. 2023; Budde et al. 2023). For example, the projected future climate seriously threatens the forests of the European beech (*F. sylvatica*) (Martinez Del Castillo et al. 2022). As *F. orientalis* occurs on warmer and drier sites (Kandemir and Kaya 2009), using this species in some parts of future European beech forests is a realistic possibility. Field trials have been established in Denmark using Caspian plant materials to assess their adaptive potential and suitability for northern European conditions (Stanturf et al. 2018). Similar trials are being conducted in Iran using European materials, and both countries are conducting comparative plantings with local plant materials. Traits related to fitness, such as phenology, are among the first traits to be evaluated in these trials. It is worth noting that the translocation of species outside their natural range carries its own biotic and abiotic risks, such as reduced genetic diversity, introduction of pests and pathogens, impacts on sink ecosystems, and many unknowns.

The present phylogeographic study serves as a genetic characterization of the valuable genetic resource that the Hyrcanian forests represent. It may guide the selection of seed sources for future field trials by identifying regions with distinctive (differentiated) genetics or avoiding subpopulations with low genetic diversity. Despite significant progress in the floristic and phytogeographic studies of the region (Akhani 1998; Manafzadeh et al. 2017; Noroozi et al. 2019; Gholizadeh et al. 2020; Ghorbanalizadeh and Akhani 2022), additional research is needed from a genetic and ecological perspective to fully understand the evolutionary history of the forests, community dynamics, and their potential to adapt to current and future risks, including climate change, and human activities. The Hyrcanian forests face serious threats, including habitat destruction from agricultural practices, logging, human settlements (Heshmati 2007; Naqinezhad et al. 2015), and impaired regeneration (Scharnweber et al. 2007). The decline in the understory forest area by more than 50% is alarming and highlights the urgent need

for conservation efforts (Salehi Shanjani et al. 2010; Müller et al. 2017). Therefore, further studies, conservation measures, and management strategies are essential to protect and preserve the unique ecosystem of the Hyrcanian forests for the benefit of future generations.

Conclusions

Our study is the first comprehensive sampling and population genetic analysis of multiple key tree species in the Hyrcanian forests. *Acer velutinum* populations show more significant genetic differentiation than *F. orientalis* and *Q. castaneifolia*, suggesting cryptic speciation within the species. *Fagus orientalis* populations in Iran show a moderate negative correlation between altitude and genetic diversity, and significant correlations were found between genetic diversity and longitude in *A. velutinum* populations. Genetic differentiation is pronounced between Iranian and Turkish populations of *F. orientalis*, with the latter exhibiting lower genetic diversity. The Quaternary glaciations alter the demographic history of the three species. Further research is recommended to explore the evolution of flora and fauna using innovative approaches and dense sampling to understand the gene pool in these valuable forests.

Data Archiving Statement

Raw DNA sequences will be submitted to the Sequence Read Archive (SRA) of GenBank. Scripts used to analyze RADseq data for the current study are available on GitHub (https://github.com/vatanparast/Genomic_Diversity_Hyrcanian_Forests/).

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Author Contributions MV and OKH contributed to the conception and design of the study. PM and OKH secured the funding. MV performed leaf material collection, RADseq library preparation, formal analysis, data curation, and visualization. KST applied for fieldwork permits in Iran. SA provided oriental beech samples from Turkey. MV wrote the first draft of the manuscript. OKH offered many comments on numerous drafts of the manuscript. All authors have commented on earlier versions of the manuscript. All authors read and approved the final version of the manuscript.

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Data Availability Raw data will be deposit to the SRA of GenBank.

Declarations The authors declare no conflicts of interest.

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