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Chloroplast microsatellite diversity of *Pinus brutia* Ten. and *Pinus halepensis* Mill. populations across the Mediterranean basin: Inferences of their distributions

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Abstract

Aim of study: To characterize and compare the genetic resources and gain some insights into the evolutionary history of Aleppo pine (*Pinus halepensis* Mill.) and Brutia pine (*Pinus brutia* Ten.) species which are both distributed across more than 8 million hectares of area in the Mediterranean Basin.

Area of study: Fifty-six populations from eight Mediterranean basin countries where *P. halepensis* and *P. brutia* species are located.

Materials and methods: We analyzed 1344 seeds belonging to 56 populations using five cpSSR primers (Pt15169, Pt30204, Pt41093, Pt87268, and Pt110048).

Main results: The analysis of molecular variance (AMOVA) revealed that the genetic diversity among the Brutia pine populations was slightly higher than that of Aleppo pine (27.06% and 24.27%, respectively). The Aleppo pine populations separately displayed a clear east-west differentiation across the Mediterranean Basin, confirming previous results using other markers. Although the Brutia pine populations showed no spatial genetic pattern, geographically close populations and/or populations from their continual distribution range were genetically closer than the fragmented and/or ecologically marginal populations.

Research highlights: The seven Aleppo pine populations from the eastern range (Türkiye, Greece, and Italy) were more than two-fold diverse than the 13 populations from the western range (Spain and Morocco). The eastern range of Aleppo pine and Brutia pine populations had similar levels of genetic diversity parameters. These results suggested that the Eastern Mediterranean Basin is a possible genetic diversity center for the two pine species.

Additional key words: halepensis-complex; simple sequence repeats; tree genetics

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Introduction

Genetic diversity is key to survival, adaptation, and evolution in response to abiotic and biotic stresses. Adapting to changing environmental conditions is especially important in the Mediterranean region, which is characterized by heterogeneous environmental conditions. Many species including Pinus species face a loss of adaptation ability owing to potential natural and artificial events like climate change, human activities, and fires (Richardson et al., 2007; StClair & Howe, 2011). Intraspecific variability is essential to modulate adaptive responses to changing local conditions, and forest tree species (particularly Mediterranean pines, except for *P. pinea*) usually harbor extremely large intraspecific genetic diversity (Heuertz et al., 2010; Fady, 2012).

Two conifers play a key role in Mediterranean coastal forests (Fady et al., 2003; Boydak, 2004). Aleppo pine (*Pinus halepensis* Mill.) and Brutia pine, also known as Calabrian or Brutian pine (*Pinus brutia* Ten.) cover together more than 8 million hectares (Daskalakou & Thanos, 2010). The natural distribution of Aleppo pine is in southern Europe (Spain, Italy, and Greece) and northern Africa (Morocco, Algeria, and Tunisia). Brutia pine is predominantly distributed in Türkiye, but some Brutia pine populations are also found in Crete, Cyprus, Syria, and Lebanon (Mauri et al., 2016). Aleppo and Brutia pines are vicariant species; the former is mainly found in the coastal areas of the western Mediterranean region while the latter is native to the eastern Mediterranean region, both of which play important roles in local economies (Fady et al., 2003; Boydak, 2004). The evolutionary and ecological characteristics of Aleppo and Brutia pines are closely related (Tozkar et al., 2009). These pines live in a Mediterranean-type climate, which is characterized by warm, dry summers and cool, mild winter conditions, and thrive in a complex range of ecological habitats from sea level up to 2,000 meters (Fady et al., 2003; Boydak, 2004; Daskalakou & Thanos, 2010). Both species are known to adapt to the Mediterranean climate. Natural and artificial hybridization between these two species has been described in many studies. Viable seeds were only obtained when Aleppo pine was used as the pollen donor and Brutia pine was used as the maternal parent (Schiller et al., 1986; Korol et al., 1995; Bucci et al., 1998). Despite their closeness, the two pines are recognized as separate species occupying different geographical ranges and bioclimates (Fady et al., 2003). Both pines can be identified using morphological, palynological, biochemical traits and DNA markers (Bucci et al., 1998; Korol et al., 2002a; Tozkar et al., 2009).

Characterizing the genetic resources of these two pines is essential to predict their adaptive potential, thereby implementing adequate strategies for the conservation, management, and improvement of these species. Aleppo and Brutia pines, separately or together were previously analyzed for their background levels of diversity, using morphological (Panetsos et al., 1997; Isik & Isik, 1999; Isik et al., 1999), biochemical traits (Korol et al., 2002a,b; Kaya et al., 2006) and DNA markers (Bucci et al., 1998; Kandemir et al., 2004; İçgen et al., 2006; Lise et al., 2007; Grivet et al., 2011; Kurt et al., 2012; Ruiz Daniels et al., 2018; Olsson et al., 2021). The results of several studies have suggested that both species are distinct and that Brutia pine is more diverse than Aleppo pine (Bucci et al., 1998; Kurt et al., 2012).

However, researchers have rarely analyzed these two pines simultaneously using the same markers. To date, only one study (Bucci et al., 1998) has used chloroplast simple sequence repeats (cpSSR) markers to analyze the genetic diversity of these two closely related species (Aleppo pine and Brutia pine). The latter study was limited by the number of populations analyzed within each species, limiting the inferences. The cpSSR primers developed by Vendramin et al. (1996) from the *Pinus thunbergii* Parl. chloroplast genome have been used in many pine species (Bucci et al., 1998; Robledo-Arnuncio et al., 2005; Grivet et al., 2009; Heurtz et al., 2010; Kurt et al., 2012). cpSSR markers are very useful tools for determining genetic diversity and phylogeographic analysis of closely related species due to their high polymorphism levels and easy optimization for related species (Bucci et al., 1998; Kurt et al., 2012).

Here we analyzed a comprehensive set of Aleppo and Brutia pine populations across the Mediterranean Basin using common chloroplast microsatellite markers. The main goal of this study was to characterize the genetic diversity of these two key Mediterranean pines to gain insights into the evolutionary history of the Aleppo-Brutia species complex.

Material and methods

Studied populations and DNA extraction

A comprehensive sampling was performed from the natural distribution of *P. halepensis* and *P. brutia* by collecting samples from 56 populations. Twenty-one populations of *P. halepensis* (488 individuals) and 35 populations of *P. brutia* var. *brutia* Ten. (836 individuals), and one population of *P. brutia* var. *eldarica* Medw. (Khorasan-Razavi, 20 individuals) were mostly sampled from the natural distribution range (Fig. 1 and Table 1). Few of the sampled *P. brutia* populations were outside the natural distribution range. Hereinafter, the *P. halepensis* populations and *P. brutia* var. *brutia* Ten. populations are referred to as Aleppo pine and Brutia pine, respectively, while the Khorasan-Razavi (*P. brutia* var. *eldarica* Medw.) population is referred to in the text as Eldarica pine. Previously collected bulked seeds from each population were used for analyses.

Table 1. Geographic information and descriptive genetic parameters of studied populations.

No	Population	Country	Altitude (m)	Latitude	Longitude	n	A	Ne	P	R^h	H_e	D^2_{sh}
				Brutia pine								
1.	Adana-Pos	Türkiye	745	37.541667°	35.416667°	24	8	4.24	0	5.95	0.80	0.51
2.	Adapazari-Geyve	Türkiye	530	40.386667°	30.420278°	24	5	3.60	0	3.84	0.75	0.3
3.	Amasya-Amasya	Türkiye	430	40.895278°	36.355278°	23	23	23.00	15	16.00	1.00	15.4
4.	Amasya-Bafra	Türkiye	50	41.651111°	35.456111°	24	23	22.15	16	15.51	1.00	16.24
5.	Ankara-Nallihan	Türkiye	750	40.148889°	30.855556°	24	12	7.02	0	8.77	0.89	1.1
6.	Antalya-Antalya	Türkiye	275	36.995833°	30.552778°	24	12	10.29	2	10.84	0.94	0.97
7.	Antalya-Alanya	Türkiye	350	36.610000°	31.965278°	24	15	9.29	0	9.21	0.93	1.21
8.	Antalya-Gundogmus	Türkiye	1000	36.710000°	32.181944°	24	11	6.86	0	8.08	0.89	1.25
9.	Antalya-Kas	Türkiye	1050	36.408333°	29.500000°	24	5	1.72	0	3.26	0.44	0.17
10.	Antalya-Kumluca	Türkiye	250	36.433333°	30.250000°	24	12	6.70	1	8.37	0.89	0.96
11.	Balikesir-Ayvacik	Türkiye	300	39.883333°	26.416667°	24	19	14.40	9	13.18	0.97	12.54
12.	Bolu-Goynuk	Türkiye	750	40.405000°	30.659722°	23	11	5.94	3	8.06	0.87	1.73
13.	Bursa-Orhaneli	Türkiye	650	40.011111°	28.925000°	24	7	3.23	2	4.68	0.73	0.54
14.	Denizli-Acipayam	Türkiye	750	37.181667°	29.213889°	23	5	2.91	0	4.54	0.68	0.4
15.	Isparta-Sutculer	Türkiye	1100	37.513889°	30.869444°	19	9	1.45	1	2.96	0.32	2.19
16.	Isparta-Bucak	Türkiye	800	37.500000°	30.683333°	21	10	6.06	1	8.07	0.88	1.1
17.	Istanbul-Kanlica	Türkiye	10	40.850556°	29.123333°	23	7	6.81	2	7.57	0.90	0.39
18.	Izmir-Bergama	Türkiye	620	39.235556°	27.146389°	20	9	2.51	0	5.04	0.63	0.77
19.	Izmir-Izmir	Türkiye	150	38.240556°	26.600556°	22	7	5.41	0	7.35	0.86	5.12
20.	Kastamonu-Duragan	Türkiye	220	41.419722°	35.106667°	24	14	3.97	1	5.27	0.78	1.1
21.	K.Maras-Antakya	Türkiye	480	35.900000°	36.016667°	24	9	7.20	1	9.71	0.90	0.52
22.	K.Maras-K.Maras	Türkiye	800	37.778889°	36.706944°	24	19	4.57	3	6.39	0.82	18.21
23.	Kutahya-Tavsanli	Türkiye	700	39.490278°	29.291389°	24	11	6.26	1	7.23	0.88	1.86
24.	Mersin-Bozyazi	Türkiye	350	36.226389°	33.105556°	24	11	3.89	2	6.43	0.78	1.41
25.	Mersin-Tarsus	Türkiye	1000	37.091667°	34.558333°	24	15	14.40	8	13.18	0.97	1.63
26.	Mersin-Gulnar	Türkiye	650	36.241667°	33.255556°	24	11	5.14	0	7.80	0.84	1.24
27.	Mersin-Silifke	Türkiye	100	36.216667°	33.716667°	23	11	7.78	0	8.49	0.91	2.42
28.	Mugla-Marmaris	Türkiye	60	37.004722°	28.328333°	24	9	6.86	0	8.08	0.89	8.48
29.	Mugla-Yilanli	Türkiye	750	37.288333°	28.563889°	24	8	10.94	8	12.63	0.96	0.64
30.	S.Urfa-Adiyaman	Türkiye	1000	37.886667°	37.674722°	24	13	6.22	0	8.10	0.88	4.47
31.	S.Urfa-Sirnak	Türkiye	600	37.483056°	41.888333°	24	12	8.00	4	10.56	0.91	1.1
32.	Kibris1	Cyprus	126	35.262778°	33.039167°	24	9	4.36	4	6.49	0.80	0.59
33.	Kibris2	Cyprus	157	35.350833°	32.982500°	24	10	3.74	0	5.74	0.76	0.86
34.	Mersin-Mut	Türkiye	1150	36.839167°	33.303333°	19	14	4.88	7	8.93	0.83	8.17
35.	Bursa-MKP	Türkiye	400	39.928611°	28.625278°	20	6	9.00	1	9.06	0.93	0.91
	Mean					**23**	**11.23**	**7.17**	**2.63**	**8.85**	**0.84**	**3.33**
				Eldarica pine								
36.	Khorasan-Razavi	Iran	1063			19	8	4.31	5	7.00	0.82	4.07
				Aleppo pine								
37.	Mugla-Gokova	Türkiye	50	36.954722°	29.208333°	23	5	1.76	1	2.78	0.45	0.42
38.	Izmir-Urla	Türkiye	50	38.255556°	26.709167°	22	11	8.02	6	7.76	0.92	8.65
39.	Cabanellas-Alta	Spain	210	42.248294°	2.783798°	27	9	3.78	0	5.14	0.76	0.65
40.	Tivissa-Cataluna	Spain	400	41.059193°	0.760224°	25	4	1.52	0	2.05	0.36	0.09
41.	Zuera-Monegros	Spain	575	41.918800°	-0.921611°	25	6	2.62	0	3.49	0.64	0.29
42.	Alcantud-Alcarria	Spain	950	40.564133°	-2.313436°	27	5	2.65	0	2.81	0.65	0.8
43.	Tuejar-Maestrazgo	Spain	600	39.819100°	-1.159188°	24	7	4.00	1	4.68	0.78	1.94
44.	Tibi-Levante Interior	Spain	1010	38.519440°	-0.648611°	26	4	2.33	0	2.08	0.59	0.45
45.	Benicassim-Litoral Levantino	Spain	430	40.077655°	0.025914°	24	4	2.09	0	2.17	0.54	1.02
46.	Villajoyosa-Sudeste	Spain	70	38.496100°	-0.303656°	25	6	2.99	1	3.50	0.69	1.21
47.	Monovar-Betica Septentrioal	Spain	700	38.385360°	-0.957389°	26	7	3.22	1	3.95	0.72	1.82
48.	Benamaurel-Betica Meridional	Spain	920	37.702100°	-2.738858°	26	8	3.25	1	4.23	0.72	1.12
49.	Frigiliana-Sur	Spain	570	36.818198°	-3.920522°	23	6	2.86	1	3.43	0.68	1.16
50.	Alcotx-Mallorca Menorca	Spain	100	39.971779°	4.168438°	25	6	3.14	1	3.09	0.71	0.81
51.	Amfilohia	Greece	25	38.883652°	21.283507°	28	12	8.52	4	7.48	0.92	6.93
52.	Kassandra	Greece	25	40.091078°	23.881487°	24	18	12.52	8	10.18	0.96	7.16
53.	Litorale Tarantino	Italy	10	40.619829°	17.116000°	22	11	6.21	3	7.26	0.88	2.68
54.	Gargano Marzini	Italy	200	41.902422°	15.941800°	22	10	3.56	1	6.21	0.75	2.4
55.	Gargano Monte Pucci	Italy	100	41.547383°	15.857200°	14	6	2.72	2	5.00	0.68	1.46
56.	Tabarka	Tunisia	144	36.505600°	9.075704°	15	12	8.33	6	9.40	0.94	6.93
57.	Zaouia Ifrane	Morocco	1512	33.570000°	-5.140000°	23	4	2.07	0	2.46	0.54	0.17
	Western populations mean (Spain, Tunisia and Morocco)					**24.36**	**6.14**	**3.20**	**0.86**	**3.75**	**0.67**	**1.32**
	Eastern populations mean (Türkiye, Greece and Italy)					**22.00**	**10.29**	**6.19**	**3.57**	**6.67**	**0.79**	**4.24**
	Mean					**23.57**	**7.52**	**4.20**	**1.76**	**4.72**	**0.71**	**2.29**
	Grand mean for all populations					**23.18**	**9.79**	**6.02**	**2.19**	**6.16**	**0.79**	**2.97**

n: sample size. A: number of haplotypes. Ne: effective number of haplotypes. P: number of private haplotypes. R^h: haplotypic richness. H_e: genetic diversity. D^2_{sh}: mean genetic distance among individuals within populations according to stepwise mutation model (Goldstein et al., 1995)

Randomly selected 30-35 seeds from each population were germinated for DNA extractions. DNA was extracted according to Doyle & Doyle (1990) protocol. The average number of analyzed samples from each population was 24, ranging from 19 to 28 individuals per population (Table 1).

Chloroplast microsatellites and PCR products

For a preliminary analysis, ten primer pairs were selected from Vendramin et al. (1996) and tested on 16 individuals from eight geographically distant populations. Based on the preliminary analysis results, only five primer pairs (Pt15169, Pt30204, Pt41093, Pt87268, and Pt110048) were used to analyze all populations, while the others were discarded as they did not give any product in PCR amplification or were not polymorphic. PCR amplifications were performed in a total volume of 10 µL containing 5 ng of template DNA, 2.5 mM $MgCl_2$, 0.3 mM dNTP mix, 0.5 U Taq polymerase, 1X reaction buffer (Thermo Fisher Scientific), and different amounts of forward and reverse primers for studied primer pairs (1 µM for Pt15169, Pt41093 and Pt87268; 2 µM for Pt110048; and 2.5 µM for Pt30204). PCR reactions were carried out on an Applied Biosystems 9700 thermal cycler according to the following conditions: initial denaturation at 95°C for 5 min, followed by 25 cycles of 1 min at 94°C, 45 s at 55°C, and 1 min at 72°C, and a final extension step of 8 min at 72°C. The amplified fragments were resolved on an ABI 310 genetic analyzer (Applied Biosystems). The size of fragments was determined by GeneScan® Analysis Software 3.1 (Thermo Fisher Scientific) concerning the GeneScan-500 LIZ size standard (Applied Biosystems).

Data analysis

The combination of size variants across five chloroplast microsatellite regions was defined as haplotypes. The chloroplast haplotype variation parameters including the total number of haplotypes within populations, effective number of haplotypes, number of private haplotypes, haplotypic richness, and haplotypic Nei's genetic diversity (Nei, 1987) were calculated using HaplotypeAnalysis 1.05 (Eliades & Eliades, 2009). A median-joining network including potential median vectors was constructed using Network 10 with cpSSRs data (Bandelt et al., 1999). The average genetic distance among individuals within populations (Goldstein et al., 1995) was estimated according to the stepwise mutation model, as defined by Vendramin et al. (1998). Analysis of molecular variance (AMOVA) was performed using Arlequin 3.1 to estimate the neutral genetic differentiation of species and populations within species (Excoffier et al., 2005). The unweighted pair group method with arithmetic mean (UPGMA) dendrogram of population pairs was obtained from Nei's (1987) genetic distance values, which were obtained from haplotype data using FigTree 1.4.4 Software (Rambaut, 2018).

The spatial and non-spatial genetic mixture analyses were applied to the haplotypes of populations (Corander et al., 2003, 2008). The program Structure 2.3.4 (Pritchard et al., 2000) was used to evaluate the admixture structure patterns of the populations within each species. The analysis was performed according to the MCMC algorithm with 50000 Burn-in periods, 500,000 replications, and 10 iterations. Results of the structure analysis were evaluated using the web-based program Structure Harvester (Earl & von Holdt, 2012) and the number of clusters (K) was

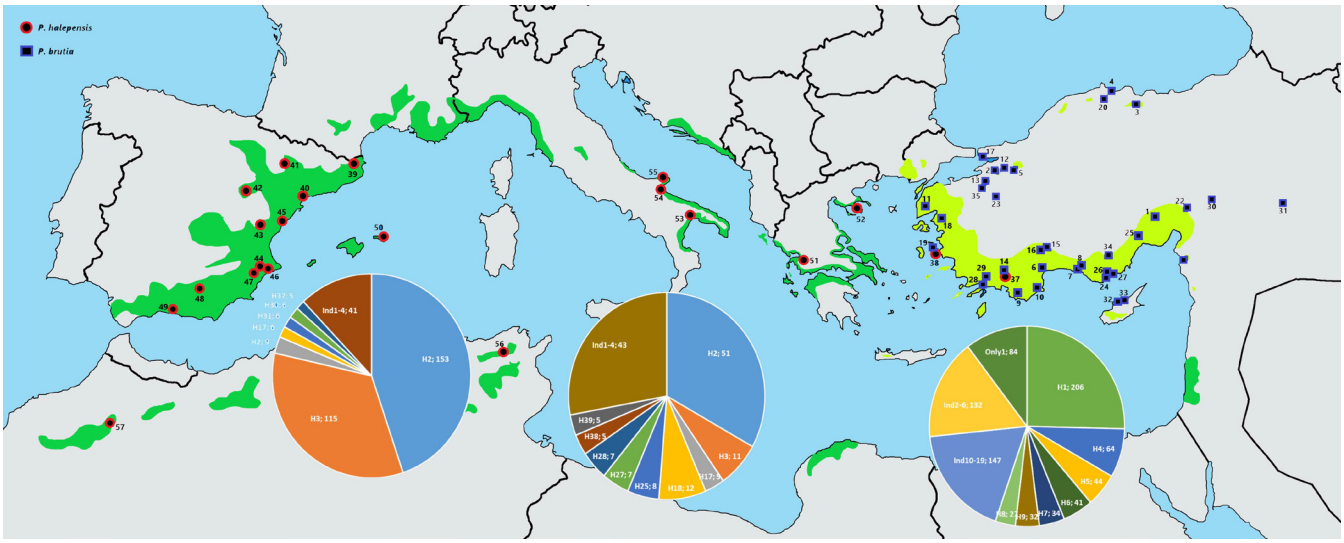

Figure 1. Distribution map of 56 studied populations and their haplotypes in different colors (red dot: Aleppo pine; blue square: Brutia pine (see Table 1 for more information about populations). The haplotypes of 14 western populations (left) and 7 eastern populations (center) of Aleppo pine are presented above. The Brutian pine populations' haplotypes are presented in the right part of the figure. Ind: ındividuals. Ind1-4: total haplotypes of individuals (from 1 to 4 individuals). The green and yellow areas indicate the natural distribution range of Aleppo and Brutia pine, respectively.

obtained by employing Evanno et al. (2005) and Jakobsson & Rosenberg (2007) computations in Structure Harvester.

Results

Chloroplast microsatellite variation

All analyzed primers were polymorphic for Aleppo, Brutia, and Eldarica pines. Overall, 51 size variants were detected from the five primers in the studied species. Almost half of the size variants (24 alleles) were species-specific (private alleles) and each species had private alleles. The total number of size variants for Aleppo pine and Brutia pine were 33 and 42, respectively (Fig. S1 [suppl]). Most private alleles were found in Brutia pine populations (Table S1 [suppl]). The common alleles of primers for each species had distinct size variants. Eldarica pine size variants were found to be closer to the Brutia pine than the Aleppo pine alleles. All the studied primers showed mutational steps among alleles from 1-bp to 5-bp in the Brutia pine populations. Mutational steps of the size variants of alleles were observed in three primers (Pt15169, Pt41093, and Pt87268) for Aleppo pine (Fig. S1).

The detected 51 alleles were combined into 204 different haplotypes. The frequency of the fourteen (H1-H14) haplotypes was greater than 1%, and the total frequency of these haplotypes corresponded to 65.8%. The distribution of haplotypes in the species was 63 and 150 haplotypes in Aleppo and Brutia pines, respectively. The Eldarica pine population had four private haplotypes. The most common haplotypes of each species were found at much lower frequencies in the other species. Ten haplotypes were shared between Aleppo and Brutia pine populations. The Aleppo pine populations of the eastern and western Mediterranean Basin had different haplotypes, except for two common haplotypes (H2 and H3) (Figs. 1 and 2). The number of haplotypes ranged from 4 to 18, and the effective haplotype number (Ne) was between 1.52 and 12.52 in Aleppo pine populations. In Brutia pine populations, the number (A) of haplotypes ranged from 5 to 23, and Ne was between 1.45 and 23 (Table 1). If all populations were analyzed together, the mean A and the mean Ne were 9.79 and 6.02, respectively (Table 1). Haplotypic richness values ranged from 2.05 (Tivissa) to 10.18 (Kassadra) in Aleppo pine populations. In the Brutia pine populations, the haplotypic richness values ranged from 2.96 (Isparta-Sutculer) to 15.51 (Amasya-Bafra). Nei's gene diversity values (h) showed similarity with the haplotypic richness value of populations. The mean genetic distance among individuals within populations for each species was 2.29 and 3.33 for Aleppo pine and Brutia pine, respectively. If the eastern and western populations of Aleppo pine were considered separately (except for the Tabarka population from Tunisia, which was grouped with Brutia pine populations), the mean genetic distance among individuals within populations changed dramatically (4.24 vs 0.89) (Table 1). The values of genetic diversity parameters were more similar between the Aleppo pine populations of the eastern Mediterranean Basin and Brutia pine populations than the western Aleppo pine populations (Table 1). The Aleppo pine populations from the eastern range (Türkiye, Greece, and Italy) were more than two-fold more diverse than the 13 populations from the western range (Spain and Morocco). When the level of genetic diversity between

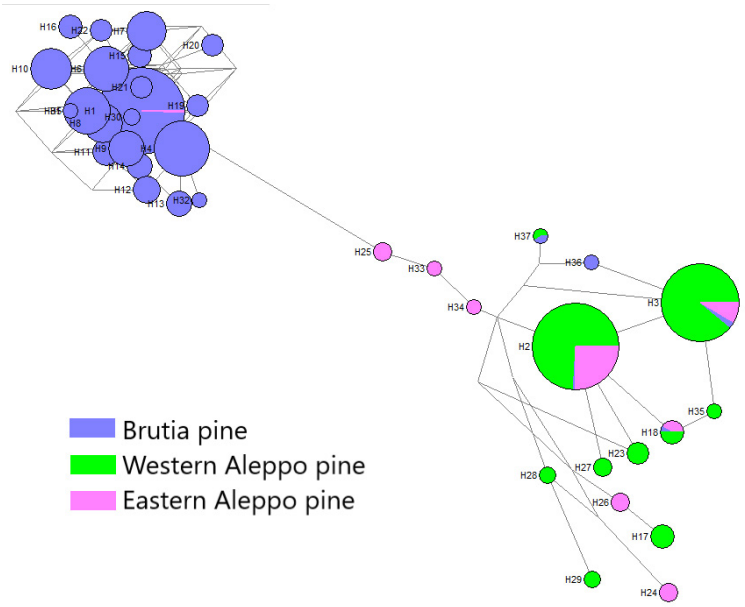

Figure 2. Median-joining network for the 37 most frequent (>5 frequency) cpSSR haplotypes of Aleppo and Brutian pines.

Table 2. The results of molecular variance analysis (AMOVA) based on assuming no population structure

Source of variation	df	Variance components	Variation (%)	Fixation indices	p*
Between species	1	1.130	51.98	F_{CT}=0.247	<0.0001
Among populations within species	55	0.258	11.87	F_{SC}=0.638	<0.0001
Within populations	1266	0.786	36.15	F_{ST}=0.519	<0.0001
Total	1322	2.174			

*Significance tests were based on 10,000 permutations.

the eastern range Aleppo pine and Brutia pine populations were compared, the eastern range Aleppo pine populations had slightly higher genetic diversity than Turkish red pine populations (Table 1).

A median-joining network was constructed to understand the relationships between the 37 most common cpSSR haplotypes detected in Aleppo pine and Brutia pine (Fig. 2). The haplotype network showed a minimum number of evolutionary events that separated each haplotype. The network indicated that H1 was centered in Brutia pine haplotypes, while H2 and H3 were centered in the Aleppo pine haplotypes. Although the divergent center is not clear for Brutia pine, H2 might be considered the divergent center of Aleppo pine.

Phylogeographic comparison of species and populations

The molecular variance analysis based on the stepwise mutation model showed that between species and among populations within species variation based on assuming no population structure were 51.98 and 11.87% of the total variation, respectively (Table 2).

AMOVA results showed that haplotypic differentiation among species was statistically significant. However, the genetic diversity of Brutia pine populations was higher than among Aleppo pine populations (Table 3). The dendrogram indicated that the genetic distance between the Aleppo and Brutia pine populations was generally compatible with geographic separation at the species level, except for a few populations. The Amasya-Amasya, Amasya-Bafra, K.Maras-K.Maras and Balikesir-Ayvacik populations of Brutia pine clustered with the Tunisian population (Tabarka) of Aleppo pine. The Eldarica pine population (Khorasan-Razavi) clustered with the Bursa-MKP population of Brutia pine (Fig. 3). Brutia pine populations did not present any clear geographic structure within their natural distribution range. However, the Aleppo pine populations showed clear east-west differentiation across the Mediterranean Basin. The two populations of Aleppo pine from Türkiye were in the same group as those from Greece and Italy. The Aleppo pine populations from the western Mediterranean clustered together, except for the Tivissa population from Spain. The Tivissa population also clustered with the Greek and Turkish populations in the eastern groups (Fig. 3 and Table 1). The eastern Mediterranean

Table 3. AMOVA results for Aleppo and Brutia pine populations.

Source of variation	df	Variance components	Variation (%)	Fixation indices	p*
Aleppo pine populations					
Western and Eastern population groups	1	0.137	15.74	F_{CT}=0.102	<0.0001
Among populations within groups	19	0.075	8.63	F_{SC}=0.244	<0.0001
Within populations	474	2.657	75.63	F_{ST}=0.157	<0.0001
Total	494	0.869			
Brutia pine populations					
Among populations	34	0.321		F_{ST}=0.271	<0.0001
Within populations	776	0.866			
Total	810	1.187			

*Significance tests were based on 10,000 permutations.

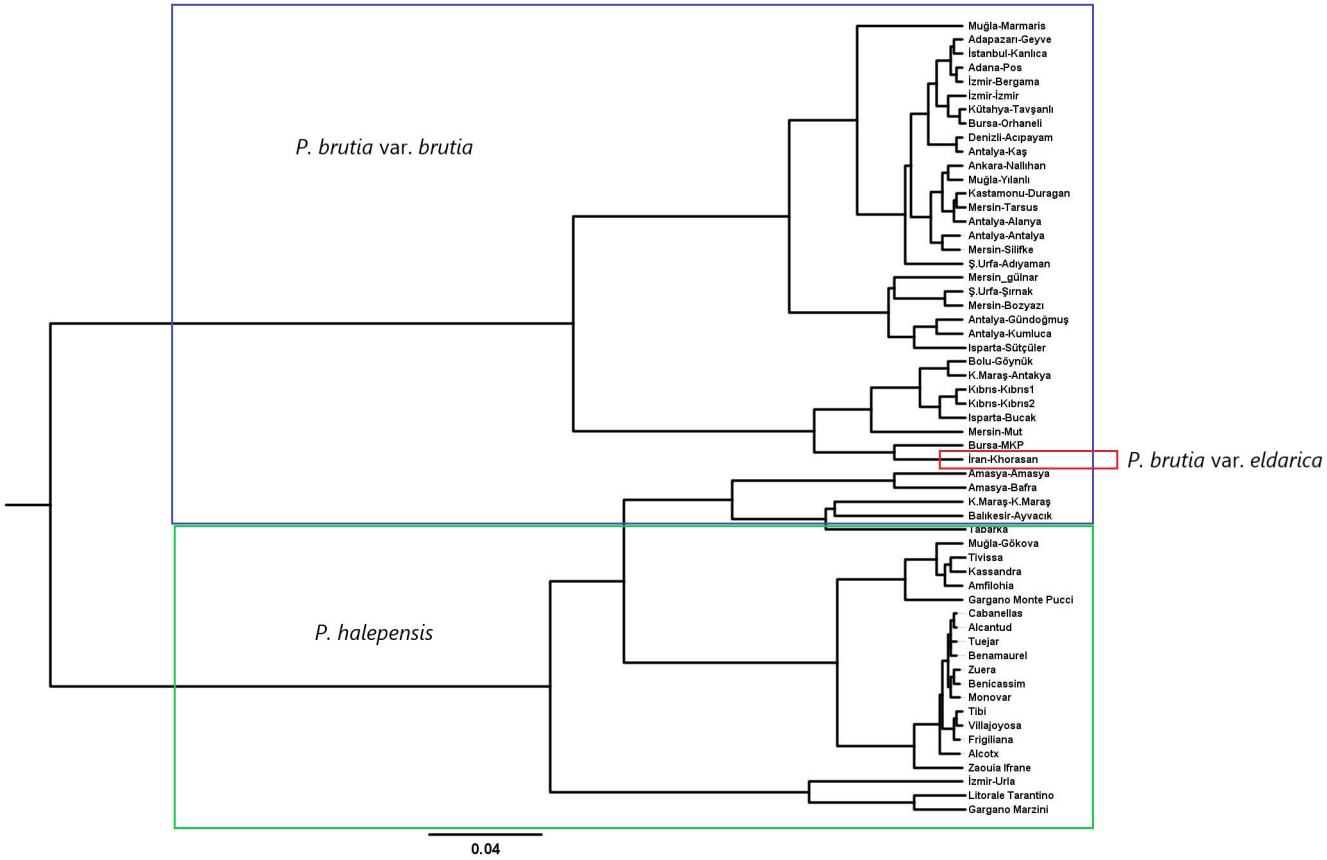

Figure 3. UPGMA dendrogram of studied populations based on Nei's genetic distance

group of Aleppo pine is composed of Turkish, Greek, and Italian populations while the western Mediterranean group is composed of Spanish and Moroccan populations.

In the structural analysis, Δ*K* showed clear peaks for K=2 in both Aleppo and Brutia pine populations, and most of the populations were admixtures of two clusters, while a few populations had specific population structures according to K=2. For Brutia pine, Cluster 1 was reported to be predominantly in Adapazari-Geyve, Amasya-Amasya, Amasya-Bafra, Ankara-Nallıhan, Antalya-Antalya, Antalya-Gündoğmuş, Isparta-Sütçüler, İstanbul-Kanlıca, İzmir-İzmir, Kastamonu-Durağan, Kmaraş-Antakya, Kmaraş-Kmaraş, Mersin-Bozyazi, Mersin-Tarsus and Kibris1 (>90%); Cluster 2 was mainly detected in Balıkesir-Ayvacık, Bolu-Göynük, Denizli-Acipayam, Muğla-Marmaris and S.Urfa-Adiyaman (>90%) (Fig. S2). For Aleppo pine, Cluster 1 was observed to be predominantly in western Mediterranean populations (>90%), while Cluster 2 was generally detected in eastern Mediterranean populations (>90%) (Fig. S3).

Discussion

The studies with the same molecular markers, especially with paternally inherited chloroplast microsatellites, are limited for both Aleppo and Brutia pine and have been rarely studied (Olsson et al., 2021). According to our literature knowledge, halepensis-complex pine species (Turkish red, Aleppo, and Eldarica pines) were analyzed only in the study of Bucci et al. (1998) using cpSSR markers. Our study is based on the first extensive sampling of two species through their natural distribution range across Mediterranean Basin countries (Fig. 1 and Table 1). This study had almost three-fold population numbers (20 vs 57) and more than five-fold (247 vs 1338) individual numbers (Table 1) more than Bucci et al. (1998). Therefore, our study provides comprehensive data contributing to Aleppo and Brutia pine genetic diversity, phylogeographic structure, conservation actions, and phylogenetic relationships.

In this study, we selected ten primer pairs for preliminary analysis, and the five most polymorphic primers were used for all analyses. We found a high number of haplotypes for only five cpSSR loci in two species (Table 1). The Eldarica pine population (Khorasan-Razavi) alone had four haplotypes. Bucci et al. (1998) reported 27 alleles for the same SSR markers in the studied Aleppo and Brutia pine populations. The higher number of alleles (51) detected in the present study could be explained by various reasons such as sampling comprehensive populations, analyzing a large number of individuals, the inclusion of ecologically marginal populations (Fig. 1 and Table 1), and effective population size of the eastern Mediterranean tree species (suggested for Mediterranean conifers by Fady (2005) and

for Aleppo pine by Grivet et al. (2009)). We found 63 and 150 haplotypes, and 33 and 44 alleles for Aleppo and Brutia pine, respectively. Twenty-six out of 51 alleles were common for both species. Of the 204 haplotypes, only nine were shared by the two species. As previously reported by different studies (Bucci et al., 1998; Kurt et al., 2012), similar to our study, the Brutia pine populations analyzed had higher levels of genetic diversity compared to those of Aleppo pine populations. Olsson et al. (2021), combining molecular and fossil information, suggested that at the beginning of the Pleistocene (~ 2 million years), *P. brutia* may have expanded in the eastern Mediterranean, while *P. halepensis* populations went through bottlenecks. The suggestion by Olsson et al. (2021) clarifies our results.

The haplotypic and allelic variations observed in this study are consistent with the results of cpSSR analysis in other pine species, *P. pinaster* (25 alleles and 108 haplotypes for six cpSSR loci, Ribeiro et al., 2001), *P. sylvestris* (29 alleles and 139 haplotypes for six cpSSR loci, Robledo-Arnuncio et al., 2005), *P. uncinata* (62 alleles and 174 haplotypes for ten cpSSR loci, Dzialuk et al., 2009) and *P. cembra* (22 alleles and 41 haplotypes for six cpSSR loci, Hohn et al., 2005). The allelic and haplotypic differentiation of cpSSR markers depends on the number of loci, populations, individuals, and polymorphism levels of the related loci and species (Robledo-Arnuncio et al., 2005; Kurt et al., 2012). Moreover, the species-specific alleles reported by Bucci et al. (1998) were 21 for Aleppo and one for Brutia pine, compared to 7 and 18 respectively in the present study (Table S2). There were three population-specific alleles for Eldarica pine (Khorasan-Razavi). These results suggest that the five cpSSR analyzed are optimal for species delimitation in Brutia pine, but more loci should be included in the analyses in Aleppo pine.

The allele size variants and allele range between the minimum and maximum in this study (Table S1) were also more diverse than those reported by Bucci et al. (1998). All the studied loci in the Brutia pine populations showed 1 to 5-bp mutational steps (Fig. S1). Those steps were seen from 1 to 7-bp only in a few loci of the Aleppo and Eldarica pine populations. However, Kurt et al. (2012) reported the 1 and 2-bp gap in six cpSSR loci (four of them are the same as in this study) of Brutia pine populations. Also, Robledo-Arnuncio et al. (2005) detected the 2-bp gap in the Pt71936 region, and they reported that size variants were due to a 5-bp deletion in the microsatellite flanking region according to sequence analysis. The chloroplast microsatellites in this study are mononucleotide-repeats (Vendramin et al., 1996) like the aforementioned studies (Bucci et al., 1998; Robledo-Arnuncio et al., 2005; Kurt et al., 2012). Therefore, individuals carrying all mutational steps in this study should be sequenced to reveal the source of mutations.

The analysis of molecular variance showed that population-level differentiation was reduced for both species, while most of the variation (83% and 73% for Aleppo and Brutia pine, respectively) was found among individuals within populations (Table 2). Therefore, tree improvement programs and conservation studies need to consider mainly individuals within a population. Although Western Aleppo pine populations have lower genetic variation than their eastern counterparts, they still maintain historical demography. This situation can be explained as the Western Aleppo pine populations have potentially adapted gene pools to their environment. For this reason, Western Aleppo pine populations should be conserved more effectively than their eastern counterparts. The Eastern Aleppo pine populations might be preferable for studies on tree improvement and especially the investigation of adaptive traits. In addition, Brutia pine and Aleppo pine populations of the eastern Mediterranean are important to constitute a reservoir of genetic diversity for improvement and conservation programs. Conservation of both pine forests is not only crucial for related species but is also very vital for the biodiversity of all living organisms related to forests and individual trees. Conservation and sustainable use of both pine forests by *ex-situ* and *in-situ* conservation efforts and protected areas are one of the most important steps to prevent biodiversity loss. The global temperature rises and increase in human activities will probably affect Mediterranean forests harsher than other ecosystems, especially at the lower altitudinal distribution of both pine species (StClair & Howe, 2011). Therefore, conservation and protection strategy studies should be increased at the local, regional and international levels to maintain sustainable biodiversity levels for the two pine forests and related species (Climent et al., 2021).

Aleppo pine populations showed clear geographic differentiation, while the Brutia pine populations did not show a clear geographic pattern like Aleppo pine; however, geographically close populations and/or populations from their continual distribution range were genetically closer than fragmented and/or ecologically marginal populations (Figs. 1 and 3; Table 1). As expected, the Eldarica pine population was genetically closer to the Turkish red pine populations (Fig. 3). The same results for Eldarica pine were reported by Bucci et al. (1998). Some populations of Brutia pine (Amasya-Amasya, Amasya-Bafra, K. Maras-K.Maras and Balikesir-Ayvacik) clustered with the Tabarka population of Aleppo pine from Tunisia (Fig. 3). These populations have been sharing a haplotype (H3), which is a common second haplotype for Aleppo pine. This phenomenon could be explained by unidirectional mating (Aleppo pine as pollen donor and Brutia pine as a female parent not reciprocally) between two species reported by different researchers using morphological traits (Panetsos et al., 1997), allozymes (Korol et al., 1995, 2002a) and DNA markers (Bucci et al., 1998; Tozkar et al., 2009). Kremer et al. (2012) reported that airborne pollens from forest trees have the potential to be transported in considerable amounts over hundreds to thousands of kilometers based on the results of aerobiological studies. The

evolutionary history (derived from a common ancestor) and recent colonization of both species (in the last 10,000 years) in the Mediterranean Basin could be also explained by the aforementioned phenomenon (Conkle et al., 1988; Bucci et al., 1998; Tozkar et al., 2009).

The present study indicated that Aleppo pine populations were divided into two different groups: one included the populations from Spain (except Tivissa), and the other included Turkish, Greek, and Italian populations (Fig. 3). The structure analysis results also supported the dendrogram (Figs. S2 and S3). Our findings showed similarity with Mediterranean conifers (Fady, 2005) and Aleppo pine populations (Grivet et al., 2009). The clear east-west differentiation of Aleppo pine populations was previously described by Schiller et al. (1986) as an Eastern Mediterranean race and Western Mediterranean race by their isozymes which was also confirmed by our results. Bucci et al. (1998) reported two main groups for Aleppo pine populations: a central Mediterranean group (Italian and Spanish populations) and a southern Mediterranean group (Greek and Algerian populations). The different results observed in the study of Bucci et al. (1998) could be related to sampling from individual trees in Aleppo pine populations and a small number of populations. The genetic diversity patterns from east to west and the high variation levels in the eastern population could be attributed to various reasons: (i) climatic conditions at the beginning of the Quaternary in Europe and the Mediterranean Basin (Tzedakis et al., 2002; Petit et al., 2005); (ii) demographic bottlenecks and founder effects with a small population size of Aleppo pine in the western basin (Morgante et al., 1998; Grivet et al., 2009; Ruiz Daniels et al., 2018; Olsson et al., 2021); (iii) limited gene flow with ancestor populations and accumulation of new mutations (Petit et al., 2005); (iv) the effect of different selection pressures at various environments (Kremer et al., 2002; Kurt et al., 2012); and (v) the more and larger refugia of eastern Mediterranean and to be a possible genetic diversity center for the two pine species (Fady, 2005; Grivet et al., 2009; Fady & Conord, 2010), either alone and/or by combinations of those.

As a result, this study adds new information about cpSSR variation of an extensive sampling of Aleppo and Brutia pine populations across the Mediterranean Basin. The Brutia pine populations are much more diverse than the Aleppo pine populations and have no clear geographical differentiation in Türkiye. Aleppo pine populations show a clinal east-west variation across the Mediterranean Basin. Eastern populations are more diverse than western ones and the Eastern Mediterranean Basin could be considered the genetic diversity center of both pine species.

Authors' contributions

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Software: Not applicable
Supervision: N. Kaya, Y. Kurt
Validation: N. Kaya, Y. Kurt
Visualization: N. Kaya, Y. Kurt
Writing – original draft: N. Kaya, Y. Kurt
Writing – review & editing: N. Kaya, Y. Kurt, B. Çengel, E. Velioğlu, D. Grivet, S. C. Gonzalez-Martinez

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