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Genetic analysis of selected high-yielding oleoresin trees of Aleppo pine (*Pinus halepensis* Mill.)

Maria Tsaktsira[1], Christoforos Karanikas[1], Dimitrios Mitras[1], Peter Zhelev[2], and Apostolos Scaltsoyiannes[1*]

[1]School of Forestry and Natural Environment, Aristotle University of Thessaloniki, 54124 Thessaloniki, Greece. *E-mail: skaltsoy@for.auth.gr
[2]University of Forestry, 1797 Sofia, Bulgaria.

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Abstract

Oleoresin, a quantitative trait with great variability, is the most important non-wood product of Aleppo pine (*Pinus halepensis* Mill.). That being so, and the fact that oleoresin's added value has risen lately, the primary objective of this research was the selection of high-yielding (plus) and low-yielding oleoresin (control) Aleppo pine trees from two Greek populations (Chalkidiki and Euboea) and their genetic analysis with isozymes. The hypothesis tested was whether certain genetic parameters such as heterozygosity and fixation index *F*, are different between plus and control trees in the two populations. Horizontal starch gel electrophoresis was used in order to analyze the endosperms of the selected trees. Eleven enzyme systems were applied which resulted in 17 loci with 33 alleles. The mean expected heterozygosity was high for all groups of trees. No differences were found among mean fixation indices *F* across all groups of trees but a tendency was detected at certain loci between the two populations. Furthermore, a first analysis for the discrimination and the fingerprinting of certain selected trees was performed with molecular markers (RAPDs). In total, six primers were applied which resulted in 68 loci. The discrimination and fingerprinting of the selected trees were successful using just 9 loci. The results imply that since there is great variability in oleoresin yield and high expected heterozygosity among the plus trees of the Greek Aleppo pine populations, a breeding program in order to increase the oleoresin yield should be conducted.

Key words: high-yielding trees, isozymes, Mediterranean pine, RAPDs, resin production.

Introduction

Aleppo pine (*Pinus halepensis* Mill.) is a tree widely distributed alongside the Mediterranean basin. In Greece, its range extends from Chalkidiki, in the north, to Peloponnesus, in the south and it can also be found in certain islands of Aegean and Ionian Sea. It is of great ecological importance since it is a very drought tolerant species, very well adapted to the semiarid sites of Mediterranean and probably will be of special value in the future due to climate change and temperature increase. In many countries it is used for recreational purposes and its wood is also useful as firewood. However, the most important non-wood forest product of Aleppo

pine is oleoresin. In Greece, oleoresin is being tapped from the bole of the tree after wounding the trunk.

Oleoresin plays an important ecological role since it protects the wounded trees from invading insects and associated fungal pathogens (Croteau and Johnson 1985). In addition, its components (Karanikas et al. 2010) are very important feedstocks for industrial use. The volatile fraction, turpentine, is commonly used as paint thinner. Monoterpene and sesquiterpene flavor and fragrance agents are added to foods, beverages, perfumes, soaps, toothpaste, tobacco and other products (Verlet 1993). Recent studies have revealed the great importance of oleoresin in the improvement of diesel fuel (oil) as a scavenger of moisture (Tsanaktsidis et al. 2014a,b) or its use in advanced biofuels (Harvey et al. 2010), while more recently, the effect of oleoresin in pharmacology and on climate change is being investigated (Scaltsoyiannes et al. 2015, 2018). In Greece, many people residing in villages neighboring Aleppo pine forests make their living by oleoresin tapping which though is a difficult job with small profit, attracts more and more people due to the recent economic crisis. Due to the above-mentioned reasons, oleoresin production is of great importance.

Great variability in oleoresin flow and in subsequent annual yield has been observed among the individuals of a pine population. The environment contributes in some degree to this variability (Papaioannou and Megalophonos 1966, Coppen and Hone 1995, Westbrook et al. 2013), but of great importance is the genetic heritability. Many studies have revealed that oleoresin yield is a trait with medium to high heritability (Squillace and Bengston 1961, Hanover 1966, Squillace 1971, McReynolds et al. 1989). In Greece, the annual average oleoresin yield from a tree is about 3 kg, but many individuals yielding more than 10 kg·yr^{-1} have been reported (Tsoumis 1995, Karanikas et al. 2011).

Due to its importance in the Mediterranean basin, Aleppo pine has been the objective of many genetic analyses (e.g. Panetsos 1975, 1981, Schiller and Grunwald 1987). Isozyme studies have been conducted by Korol et al. (1995), Teisseire et al. (1995), Korol and Schiller (1996) and Agúndez et al. (1997). A couple of Greek populations have been studied by Loukas et al. (1983), Schiller et al. (1986) and Korol et al. (2002).

The calculation of mean expected heterozygosity ($\overline{H_e}$) or gene diversity of a certain species contributes to its protection since the allelic variation is critical to the survival of a species and allows organisms to adapt to changing environments. Furthermore, measuring genetic variation of a population is a first step in evaluating the tree improvement potential of the species (White et al. 2007). Aleppo pine's oleoresin yield is a quantitative trait and thus high gene diversity is necessary in order to proceed to a breeding program. A breeding program which uses selected high yielding oleoresin pines will result in progenies that yield larger quantities of oleoresin. Even if these selected trees are vegetatively propagated and then established in artificial plantations, the annual oleoresin yield would be larger and this would lead to a more profitable profession for the oleoresin tapping workers (Susaeta et al. 2014). In this frame Westbrook et al. (2013) associated SNPs with oleoresin flow in loblolly pine and developed a genomic prediction model to accelerate breeding for enhanced oleoresin flow.

Thus, the first objective of this research was to confirm the large phenotypic variation of oleoresin yield in the Greek popula-

tions of Aleppo pine and more specifically to locate and select individuals that yield large (plus trees) and small (control trees) quantities of oleoresin. The second objective was to acquire a detailed knowledge of allelic variation and heterozygosity of the 'populations' under consideration in order to evaluate the potential of a breeding program. A biochemical (isozyme) analysis of the selected trees was performed in order to calculate the gene diversity. In addition, heterozygosity and fixation indices were compared between high yielding and low yielding oleoresin trees aiming to unveil any differences that could lead to an association with oleoresin flow. The final objective was the fingerprinting of some selected genotypes using molecular markers and more specifically the technique of Random Amplified Polymorphic DNA (RAPD). These high-yielding trees, given a high diversity in their populations, can be vegetatively propagated through grafting to establish a seed orchard.

Materials and Methods

The two Aleppo pine populations included in the study were selected based on the following two criteria: (a) to represent different site and climate conditions and (b) to contain trees that are currently being tapped for commercial purposes. The two Aleppo pine populations that met these two criteria, were in Chalkidiki (40°01' N, 23°25' E) and in Euboea (38°49' N, 23°26' E), Greece (Fig. 1). The two areas

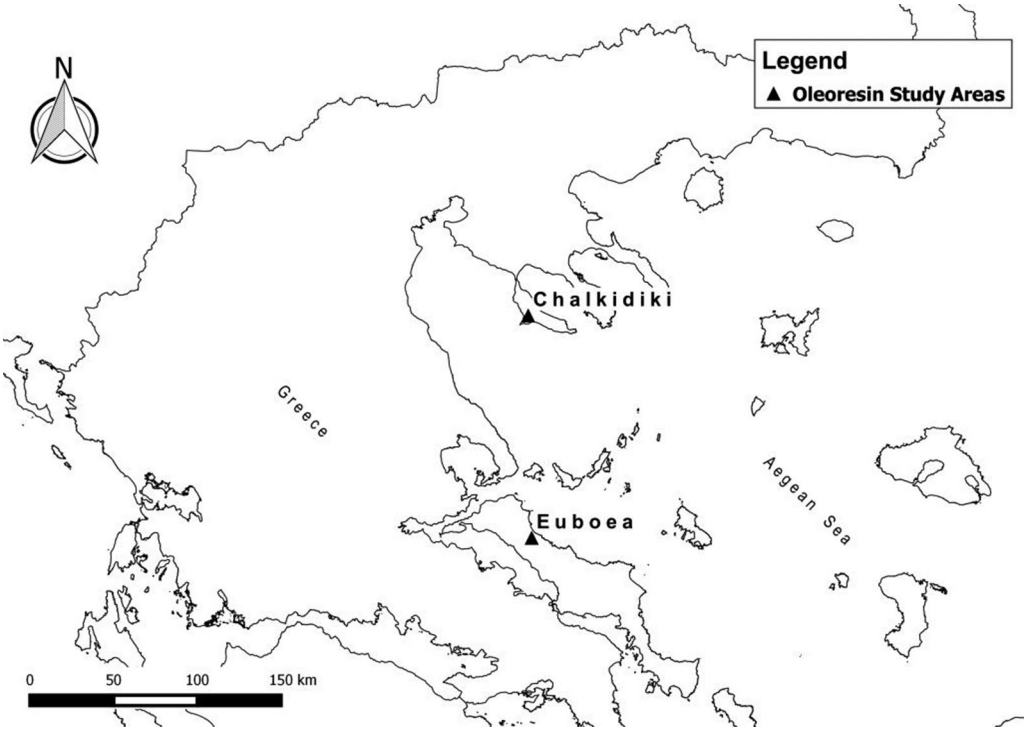

Fig. 1. Two Aleppo pine populations located in Chalkidiki (Northern Greece) and Euboea (Central Greece) respectively, were selected for this study.

are approximately 130 km apart. In these two selected areas, after consulting with the oleoresin tapping workers, we located trees that yield more than 10 kg·yr^{-1} (plus trees) and trees that yield less than 2 kg·yr^{-1} (control trees). The oleoresin yield of these individuals was measured for two successive years in order to confirm their annual yield. From the first northern population we selected 19 plus and 11 control trees, while from the second southern population we selected 11 plus and 9 control trees. In total, 50 individuals, 30 plus and 20 control trees, were selected. Female cones were collected from all the selected trees and were transferred to the lab. Then, the cones were put in the oven at 40 °C until they opened (about 10 days). The seeds were put in wet Petri dishes and when germinated, the endosperms were extracted and used for further laboratory analyses.

The plant tissue was ground and homogenized according to Conkle et al. (1982) and the isozyme analyses were conducted on horizontal starch gel electrophoresis. In total, three buffer systems were used; *Lithium-Borate-Tris-Citrate* (LBTC) and *Histidine-HCl* (H) according to Cheliak and Pitel (1985) and *Morpholine-Citrate* (D) according to Conkle et al. (1982), while the following eleven enzyme systems were applied: 6-phosphogluconate dehydrogenase, 6PGD (EC 1.1.1.44); menadione reductase, MNR (EC 1.6.99.2); isocitrate dehydrogenase, IDH (EC 1.1.1.42); leucine aminopeptidase, LAP (EC 3.4.11.1); phosphoglucose isomerase, PGI (EC 5.3.1.9); Acid phosphatase, ACP (EC 3.1.3.2); β-esterase, β-EST (EC 3.1.1.3); malate dehydrogenase, MDH (EC 1.1.1.37); phosphoglucomutase, PGM (EC 5.4.2.2); glucose 6-phosphate dehydrogenase, G6PD (EC 1.1.1.49); *g*lutamate dehydrogenase GDH (EC 1.4.1.2). Since the gymnosperms' endosperm is haploid tissue, seven (7) endosperms per tree were used in order to detect all the alleles of each locus (Morris and Spieth 1978). The probability of missing an allele when analyzing haploid seed tissue is calculated as 0.5^{n-1}, where n is the number of endosperms analyzed, and therefore, using seven seeds reduced this probability to 1.6 %. Frequency-based genetic analyses were applied using the cross-platform package GenAlex 6.41 (Peakall and Smouse 2006). More specifically, the following population genetic characteristics were calculated: the allele frequency per locus (A), the observed heterozygosity (H_o), the expected heterozygosity (H_e) and the fixation index F.

For additional statistical analysis, four groups of trees were denoted as conditional populations, 1 and 3 consisting of plus trees in Chalkidiki and Euboea, respectively, and 2 and 4 – of control trees. The significance of differentiation among the population pairs based on the genotypes (genotypic differentiation) was tested by exact G-test on contingency tables (Goudet et al. 1996). Additionally, allelic differentiation was tested for comparison. Unbiased estimates were obtained as described by Raymond and Rousset (1995a). The analysis was done in two steps – first the test for significant genotypic differentiation was performed for all population pairs, and the second step was done on pooled data – populations 1 and 3 (plus trees) were pooled and so were the populations 2 and 4 (control trees). Software Genepop v. 4.7 (Raymond and Rousset 1995b) was used for the analysis.

Furthermore, the fingerprinting of 4 high-yielding oleoresin pine trees was performed through the Random Amplified Polymorphic DNA (RAPDs) markers.

To avoid dominance, DNA was extracted from haploid tissue which in the case of conifers is the seed megagametophyte. Two of the fingerprinted trees were from Chalkidiki and the other two from Euboea. Seven megagametophytes from each tree were isolated by the same method, used in the case of isozymes. DNA extractions were performed by using the CTAB protocol (Doyle and Doyle 1987). DNA amplifications by Polymerase Chain Reaction (PCR) were carried out in a Mastercycler Eppendorf ep Gradient S Thermocycler using the profile proposed by Gómez et al. (2001). The volume of the reaction mixture was 25 μl and contained: 2.5 μl 10× Buffer, 2 mM Mg^{2+}, 200 μM dNTPs, 200 nM primer, 0.75 U Taq polymerase, 10 ng DNA. Total 30 oligonucleotide primers (Operon kits OPA01-10, OPE01-10, OPH01-10) were tested in amplification, polymorphisms and Mendelian inheritance. The chosen 6 (OPA01, OPA07, OPE02, OPH05, OPH07, OPH09) were used in the amplifications of DNAs from the four selected trees. The reactions were visualized under UV light, after running in 1.5% agarose gels in 0.5× TBE buffer and ethidium bromide staining. The GenAlex 6.41 (Peakall and Smouse 2006) software was used for the fingerprinting of the selected genotypes.

Results

The genetic analyses of 11 enzyme systems resulted in a total of 17 loci with 33 alleles (Table 1). Among them, 4 loci (Idh-A, Acp-A, Pgm, Gdh) were found monomorphic and the rest 13 were polymorphic. At the polymorphic loci, the detected number of alleles per locus was two, with the exception of three alleles at β-Est and four

Table 1. Loci and allele frequencies for each group of trees.

Locus	Allele	Chalkidiki		Euboea	
		CH_P	CH_C	E_P	E_C
6pgd-A	1	0.711	0.818	0.818	0.722
	2	0.289	0.182	0.182	0.278
6pgd-B	1	0.763	0.773	0.909	0.611
	2	0.237	0.227	0.091	0.389
Mnr	1	0.737	0.818	0.682	0.556
	2	0.263	0.182	0.318	0.444
Idh-A	1	1	1	1	1
Idh-B	1	0.289	0.227	0.045	0
	2	0.711	0.773	0.955	1
Lap	1	0.447	0.318	0.682	0.778
	2	0.553	0.682	0.318	0.222
β-Est	1	0.395	0.136	0	0
	2	0.368	0.455	0.864	0.813
	3	0.237	0.409	0.136	0.187
Pgi	1	0.105	0.136	0.409	0.056
	2	0.895	0.864	0.591	0.944
Mdh-A	1	0	0.091	0	0
	2	1	0.909	1	1
Mdh-B	1	1	0.955	0.909	0.944
	2	0	0.045	0.091	0.056
Mdh-C	1	0	0	0.045	0.056
	2	0.079	0	0.045	0
	3	0.105	0.091	0	0.056
	4	0.816	0.909	0.909	0.889
Mdh-D	1	0.868	0.636	0.591	0.722
	2	0.132	0.364	0.409	0.278
Acp-A	1	1	1	1	1
Acp-B	1	0.053	0	0.091	0.222
	2	0.947	1	0.909	0.778
Pgm	1	1	1	1	1
G6pd	1	0.579	0.636	0.682	0.667
	2	0.421	0.364	0.318	0.333
Gdh	1	1	1	1	1

Note: CH_P = Chalkidiki plus trees, CH_C = Chalkidiki control trees, E_P = Euboea plus trees and E_C = Euboea control trees.

alleles at Mdh-C. The frequency of the most common allele was always higher than 0.5 (minor polymorphism, *sensu* Lewontin 1985), with two exceptions concerning the locus β-Est-1. The trees from Chalkidiki exhibited a slightly larger polymorphism P_X = 76.47 % than the trees from Euboea P_E = 70.59 %. In total, the mean polymorphism was P = 73.53 %. Unique or private alleles were detected at β-Est-1 and Mdh-A-1 loci for the Chalkidiki individuals and at Mdh-C-1 locus for the Euboea individuals.

The allele frequencies per locus were similar across all the groups with certain exceptions (Table 1). The frequency of Idh-B-1 was 0.289 and 0.227 for the plus and control trees of Chalkidiki, respectively, while for the plus and the control trees of Euboea was just 0.045 and zero (0), respectively. The same pattern was found for the β-Est-1 locus where the frequency of Chalkidiki plus trees was 0.395, whereas the corresponding frequencies for the Euboea were zero (0). Thus, the frequency of these specific alleles could be used as a tool for the differentiation of the two populations, since high frequencies would indicate a sample from Chalkidiki and low (or even zero) frequencies would indicate a sample from Euboea. In contrast, the allele Mdh-C-1 was not detected at all among the selected trees of Chalkidiki while it was detected with small frequencies among the trees of Euboea. An interesting pattern can be found at the LAP enzyme system. The first allele Lap-1 was more frequent among the plus and control trees of Euboea than the trees o Chalkidiki where the second allele Lap-2 was more frequent. Furthermore, the allele Mdh-C-2 was detected (in small frequencies) only among the plus trees of both Chalkidiki and Euboea. This allele shows a tendency for differentiation between plus and control trees.

For both populations (Chalkidiki and Euboea), the mean observed heterozygosity was $\overline{H_o}$ =0.255 and the mean expected heterozygosity was $\overline{H_e}$ =0.244. As for the Chalkidiki trees, both plus and control ones, the largest observed heterozygosity (H_o) was detected at the G6pd locus (0.660) and the largest expected heterozygosity (H_e) at the β-Est locus (0.660). The maximum value of fixation index F was calculated for the Pgi locus (0.515) and its minimum value for the G6pd locus (–0.250) indicating large differences between H_o and H_e in both cases. The largest H_o for the Euboea trees was detected in the same locus as for the Chalkidiki trees, that is G6pd (0.650) but the largest H_e was detected on a different locus, namely, the Mnr locus (0.469). For Euboea, the F was maximum at the β-Est locus (0.650) revealing an excess homozygosity and on the other hand the minimum value was calculated at the G6pd locus (-0.481) revealing an excess of heterozygosity.

The comparison of plus and control trees at the two selected areas based on H_o, H_e and F index is presented at Table 2.

The mean H_o across all four groups was quite similar ranging from 0.250 to 0.267. That was also the case for the mean H_e which ranges from 0.224 to 0.254. Between the plus trees of the two populations, the highest H_o and H_e were detected at different loci. In the population of Chalkidiki, the highest H_o and H_e were observed at the β-Est locus while in the Euboea population the highest H_o and H_e were found at the Pgi locus. The mean fixation index F for each of the four groups was close to zero, ranging from –0.066 to +0.022, but with great variability within each group, since the corresponding SE was quite large. The comparison between

Table 2. Observed (H_o) and expected (H_e) heterozygosities and fixation index *F* for plus and control trees from Chalkidiki and Euboea.

Locus	Plus trees						Control trees					
	Chalkidiki			Euboea			Chalkidiki			Euboea		
	H_o	H_e	*F*	H_o	H_e	*F*	H_o	H_e	*F*	H_o	H_e	*F*
6pgd-A	0.579	0.411	–0.407	0.182	0.298	0.389	0.364	0.298	–0.222	0.333	0.401	0.169
6pgd-B	0.263	0.361	0.272	0.182	0.165	–0.100	0.455	0.351	–0.294	0.556	0.475	–0.169
Mnr	0.316	0.388	0.186	0.455	0.434	–0.048	0.364	0.298	–0.222	0.667	0.494	–0.350
Idh-A	0.000	0.000	ND	0.000	0.000	ND	0.000	0.000	ND	0.000	0.000	ND
Idh-B	0.474	0.411	–0.152	0.091	0.087	–0.048	0.455	0.351	–0.294	0.000	0.000	ND
Lap	0.474	0.494	0.042	0.455	0.434	–0.048	0.636	0.434	–0.467	0.444	0.346	–0.286
B-Est	0.632	0.671	0.059	0.09	0.254	0.64	0.364	0.650	0.440	0.125	0.339	0.631
Pgi	0.105	0.188	0.441	0.818	0.483	–0.692	0.091	0.236	0.614	0.111	0.105	–0.059
Acp-A	0.000	0.000	ND	0.000	0.000	ND	0.000	0.000	ND	0.000	0.000	ND
Acp-B	0.105	0.102	–0.029	0.182	0.173	–0.053	0.000	0.000	ND	0.222	0.375	0.407
Mdh-A	0.000	0.000	ND	0.000	0.000	ND	0.182	0.165	–0.100	0.000	0.000	ND
Mdh-B	0.000	0.000	ND	0.182	0.165	–0.100	0.091	0.087	–0.048	0.111	0.105	–0.059
Mdh-C	0.263	0.317	0.170	0.182	0.169	–0.073	0.182	0.165	–0.100	0.222	0.204	–0.091
Mdh-D	0.263	0.229	–0.152	0.636	0.483	–0.316	0.364	0.463	0.214	0.556	0.401	–0.385
Pgm	0.000	0.000	ND	0.000	0.000	ND	0.000	0.000	ND	0.000	0.000	ND
G6pd	0.526	0.488	–0.080	0.636	0.434	–0.467	0.727	0.463	–0.571	0.667	0.444	–0.500
Gdh	0.000	0.000	ND	0.000	0.000	ND	0.000	0.000	ND	0.000	0.000	ND
Mean	0.250	0.254	0.022	0.256	0.224	–0.057	0.267	0.247	–0.066	0.251	0.231	–0.043
SE	0.058	0.055	0.048	0.065	0.046	0.075	0.059	0.050	0.076	0.063	0.049	0.071

Note: SE = standard error and ND = not defined.

the two plus groups, at 6pgd-A locus, demonstrates that the Chalkidiki trees have their minimum *F* value (–0.407) and the Euboea trees have their maximum *F* value (0.389) while at Pgi locus the Chalkidiki trees have their maximum *F* value (0.441) and the Euboea trees have their minimum *F* value (–0.692). A similar pattern at the two aforementioned loci can be found between the control trees from the two provenances. Thus, at two loci we find the maximum and minimum value alternately between the two groups of plus trees.

The exact tests of population differentiation at the different allozyme loci revealed that there were 12 significant p-values out of 75 possible (Table 3). Three significant cases of differentiation were detected between population pairs CHp and Ec, between CHp and Ep, and between CHc and Ec. Two significant p-values were found between the pairs CHc-Ep and one – between the populations Ep and Ec. The differentiation between Ep and Ec at the locus 6pgd-B was at the threshold level of significance ($p = 0.051$). When all loci were included in the analysis, there was significant differentiation between three population pairs – CHp-Ep, CHp-Ec, and CHc-Ec. No significant differentiation was found when the plus and control trees from the two localities were pooled (CHp+Ep vs. CHc+Ec).

The results of the genic differentiation (i.e., differentiation calculated based on allelic structure, Table 4) revealed practically the same picture: 12 significant

Table 3. *P*-values (unbiased estimate) of the genotype differentiation among populations tested by Fisher's exact test.

Population pairs	6Pgd-A	6pgd-B	Mnr	Idh-B	Lap	Est	Pgi	Acp-B	Mdh-A	Mdh-B	Mdh-C	Mdh-D	G6pd	All loci
CHp-CHc	0.288	1.000	0.566	0.742	0.398	0.149	1.000	0.520	0.126	0.367	0.568	0.068	1.000	0.586
CHp-Ep	0.349	0.245	0.784	**0.021**	0.125	**0.0003**	**0.006**	0.612	**–***	0.127	0.212	**0.015**	0.571	**0.00004**
CHp-Ec	1.000	0.381	0.236	**0.009**	**0.029**	**0.003**	0.743	0.234	–	0.321	0.288	0.210	0.760	**0.006**
CHc-Ep	1.000	0.362	0.478	0.148	**0.024**	0.051	0.065	0.476	0.477	1.000	0.477	1.000	1.000	0.245
CHc-Ec	0.719	0.276	0.064	**0.038**	**0.002**	0.161	0.738	0.075	0.478	1.000	0.770	0.742	1.000	**0.040**
Ep-Ec	0.746	**0.051**	0.492	1.000	0.716	**1.000**	**0.003**	0.460	**–**	1.000	0.850	0.440	1.000	0.462
CHp+Ep vs CHc+Ec	0.809	0.241	1.000	0.411	1.000	0.145	0.196	0.744	0.156	1.000	0.459	0.353	1.000	0.786

Legend: The meaning of CHp, CHc, Ep and Ec is explained in Table 1. *P*-values indicating significant differentiation are in bold.
Note: * Test was not possible because there was only one genotype.

Table 4. *P*-values (unbiased estimate) of the allelic differentiation among populations tested by Fisher's exact test.

Population pairs	6Pgd-A	6Pgd-B	Mnr	Idh-B	Lap	Est	Pgi	AcpB	Mdh-A	Mdh-B	Mdh-C	Mdh-D	G6pd	All loci
CHp-CHc	0.381	1.000	0.542	0.764	0.419	0.094	1.000	0.527	0.130	0.367	0.367	0.053	1.000	0.494
CHp-Ep	0.383	0.189	0.768	**0.023**	0.107	**0.0001**	**0.009**	0.619	**–***	0.130	0.150	**0.025**	0.560	**0.00003**
CHp-Ec	1.000	0.345	0.224	**0.011**	**0.024**	**0.0008**	0.663	0.162	–	0.322	0.209	0.264	0.771	**0.002**
CHc-Ep	1.000	0.413	0.485	0.184	**0.034**	**0.009**	0.089	0.488	0.489	1.000	0.488	1.000	1.000	0.198
CHc-Ec	0.705	0.312	0.093	0.053	**0.005**	0.055	0.613	**0.033**	0.491	1.000	0.773	0.739	1.000	**0.036**
Ep-Ec	0.705	0.054	0.517	1.000	0.724	1.000	**0.013**	0.381	**–**	1.000	0.848	0.508	1.000	0.647
CHp+Ep vs CHc+Ec	0.816	0.227	1.000	0.418	1.000	0.067	0.175	0.708	0.158	1.000	0.361	0.363	1.000	0.664

Legend: The meaning of CHp, CHc, Ep and Ec is explained in Table 1. *P*-values indicating significant differentiation are in bold.
Note: * Test was not possible because there was only one genotype.

cases of differentiation: four for the pair CHp-Ep (loci Idh-B, Est, Pgi and Mdh-D), three – CHp-Ec (loci Idh-B, Lap and Est), two for the pairs CHc-Ep (Lap, Est) and CHc-Ec (Lap and Acp-B) and one for the pair Ep-Ec (Pgi). At overall level, when all loci were considered, significant differentiation was detected between the pairs CHp-Ep, CHp-Ec, and CHc-Ec.

The genetic profiles of the individuals from Chalkidiki and Euboea that took place in this experiment are presented in Table 5. According to the 68 loci, we have done a check for the possible existence of identical genotypes among the selected trees. Based on the analyses all four individuals that were studied posed a different genotype.

Table 6 shows the number of individuals which exhibited the same genotype according to the number of loci that are included in the analysis.

Table 5. Genotypes at RAPD loci of 4 plus trees.

LOCUS	E3	CH5	E5	CH6	LOCUS	E3	CH5	E5	CH6	LOCUS	E3	CH5	E5	CH6
OPA01-250	11	00	11	00	OPA07-1720	11	00	00	00	OPH07-620	11	11	11	00
OPA01-400	11	11	11	11	OPA07-2000+	11	00	00	00	OPH07-700	00	00	11	11
OPA01-490	11	11	11	00	OPE02-600	11	00	11	00	OPH07-750	00	11	11	11
OPA01-500	00	11	00	11	OPE02-850	00	00	00	10	OPH07-850	11	11	11	11
OPA01-550	11	00	11	00	OPE02-900	00	11	00	00	OPH07-880	00	00	11	00
OPA01-620	00	11	00	00	OPE02-980	00	11	10	11	OPH07-980	11	11	11	11
OPA01-700	11	11	11	00	OPE02-1100	11	11	11	11	OPH07-1100	00	11	11	11
OPA01-750	00	11	00	11	OPE02-1230	11	11	11	11	OPH07-1470	00	11	11	00
OPA01-900	11	00	00	11	OPE02-1350	00	00	10	10	OPH07-1600	00	11	00	11
OPA01-1050	00	11	11	00	OPE02-1470	11	11	11	11	OPH07-1720	11	11	11	00
OPA01-1170	00	00	00	11	OPE02-1850	11	11	11	11	OPH07-2000	11	11	11	11
OPA01-1300	00	00	00	11	OPH05-300	11	11	11	11	OPH09-500	11	00	11	11
OPA01-1400	00	00	11	00	OPH05-550	11	11	00	11	OPH09-580	00	11	00	00
OPA07-400	11	00	00	00	OPH05-650	11	11	11	11	OPH09-620	00	11	00	00
OPA07-490	00	11	00	00	OPH05-700	00	11	11	00	OPH09-650	00	11	00	00
OPA07-750	00	00	11	11	OPH05-750	00	11	00	00	OPH09-700	00	11	00	11
OPA07-850	11	11	00	00	OPH05-860	00	00	11	11	OPH09-850	11	11	11	11
OPA07-980	00	11	11	00	OPH05-980	00	11	00	11	OPH09-980	00	11	00	11
OPA07-1050	00	00	00	11	OPH05-1100	00	00	00	11	OPH09-1100	11	00	11	00
OPA07-1230	00	11	11	11	OPH05-1350	11	11	11	11	OPH09-1230	00	11	00	11
OPA07-1350	11	00	00	00	OPH05-2000	11	11	11	11	OPH09-1600	11	11	11	00
OPA07-1400	00	00	00	11	OPH05-2000+	11	11	11	11	OPH09-2000+	11	11	11	11
OPA07-1600	00	00	11	00	OPH07-550	00	00	00	11					

Table 6. Matching and unique genotypes for the loci combination of 4 trees.

Number of loci	# with matching genotype	# with unique genotype	# matching except 1 locus	# matching except 2 loci	# matching except 3 loci
1	4	0	0	0	0
1+2	4	0	4	0	0
1+2+3	2	2	4	3	0
1+2+3+4	2	2	2	3	3
1+2+3+4+5	2	2	2	0	3
1+2+3+4+5+6	2	2	0	2	0
1+2+3+4+5+6+7	2	2	0	0	2
1+2+3+4+5+6+7+8	2	2	0	0	2
1+2+3+4+5+6+7+8+9	0	4	2	0	0
1+2+3+4+5+6+7+8+9+10	0	4	0	2	0
…	…	…	…	…	…
1+2+3+4+...+67+68	0	4	0	0	0

Note: # = Number of trees.

Complete separation of the selected individuals can be achieved if we take under consideration 9 loci. The possibility of finding two individuals with identical genotype is $7.3 \cdot 10^{-4}$, whereas for the total amount of the 68 loci the possibility is $2.7 \cdot 10^{-20}$.

Discussion

The isozyme literature for *P. halepensis* concerning the presence or absence of a locus and its corresponding alleles presents great variability. This becomes evident with the following discussion. Loukas et al. (1983) detected two polymorphic 6pgd loci for a Greek population in Attica which is in accordance with the current study but that wasn't the case for the rest of the studies reviewed. While in most of the studies there were detected two loci, the first of them (6pgd-A) was monomorphic (Schiller et al. 1986, Teisseire et al. 1995, Korol and Schiller 1996, Agúndez et al. 1997). Korol et al. (1995) detected just one monomorphic locus, whereas Schiller et al. (1986) and Korol et al. (2002) detected three loci with the first of them monomorphic. A similar difference among the current study and the previews studies can be found for the Mnr locus where we report two alleles in contrast with the other studies which detected two loci either monomorphic or polymorphic (Schiller et al. 1986, Korol and Schiller 1996, Korol et al. 2002).

In the case of β-Est the findings of the current study deserve a special mention. To the best of our knowledge this is the first time that this particular locus is reported for Aleppo pine. Three alleles were detected for the Chalkidiki population and two alleles for the Euboea population. The β-Est enzyme system was reported in other pines (*Pinus lawsonii* Roz. and *Pinus montezumae* Lamb.) where three loci were detected with 3, 2 and 2 alleles, respectively (Vargas et al. 2002).

Furthermore, we report for the first time the detection of four alleles at the Mdh-C locus in *P. halepensis*. Most stud-

ies detected four loci, mostly monomorphic (Schiller et al. 1986, Korol et al. 1995, Teisseire et al. 1995, Agúndez et al. 1997). Korol et al. (2002) reported four polymorphic loci with two alleles at Mdh-C locus while we detected four alleles at Mdh-C for the first time.

Studies with isozyme analyses of *P. halepensis* populations from all around the Mediterranean basin reported mean expected heterozygosity ($\overline{H}_e$) ranging from 0.020 to 0.143, smaller than the current study (Teisseire et al. 1995, Korol and Schiller 1996, Agúndez et al. 1997). Loukas et al. (1983) analyzed the Greek population of Attica and found high $\overline{H}_e$ = 0.179. On the other hand, Schiller et al. (1986) reported low levels of $\overline{H}_e$ for two Greek populations, the first in Peloponnesus ($\overline{H}_e$ = 0.056) and the second in Euboea ($\overline{H}_e$ = 0.065). More recently, Korol et al. (2002) analyzed one population from Chalkidiki and another from Euboea. The mean expected heterozygosity was $\overline{H}_e$ = 0.177 and $\overline{H}_e$ = 0.188, respectively. In the last two studies, the two Greek populations had the highest values among 17 and 20 Mediterranean populations, respectively. In the current study we detected even higher $\overline{H}_e$ values with $\overline{H}_e$ = 0.232 for Euboea and $\overline{H}_e$ = 0.255 for Chalkidiki. These high $\overline{H}_e$ values of the Greek populations demonstrate their high gene diversity and the level of adaptation they have acquired through the years. Probably, the environmental factors favour the presence of the species in the north-east Mediterranean. Moreover, the species adaptation will be tested in the case of Euboea where almost the entire Aleppo pine population was burned during the wildfires of August 2021. The level of natural regeneration and the subsequent survival of the species will be an indicator of the population's high gene diversity. It is also worth mentioning that the Chalkidiki population has very high gene diversity although it is situated close to the northern limits of the species.

As for the mean fixation indices *F*, we could say that for the three out of four groups, there is an excess of heterozygotes. One possible explanation for these excesses in heterozygotes is the fact that the trees that comprise these groups are selected for their oleoresin yield and therefore, are not selected randomly (White et al. 2007).

Significant differentiation between the population pairs was detected in 9 cases out of 63 possible (Table 3). They were in four loci out of the eleven studied and concerned different combinations of population pairs. Only in one case (Ep-Ec, locus Pgi) there was significant differentiation between plus- and control trees within the same locality. When all loci were included in the analysis, there was significant differentiation between three population pairs – CHp-Ep, CHp-Ec, and CHc-Ec. No significant differentiation was found when the plus and control trees from the two localities were pooled (CHp+Ep vs. CHc+Ec). Evidently, more advanced markers and more advanced statistical methods (Zou et al. 2016) would be necessary to reveal the genetic background of the oleoresin production and to proceed further to successful marker-assisted selection.

The application of six primers of RAPD markers was a simple and safe procedure for the identification and discrimination of four selected individuals. This specific technique can be used for the fingerprinting and separation of a plurality of further individuals, as demonstrated by the very low possibility of identical genotype calculated to specific loci on the analyzed individuals.

In conclusion, we could say that there

is great variability in oleoresin yield and high heterozygosity among the selected plus trees. These trees can form the basic material, through vegetative propagation, for the establishment of either seed orchards or artificial plantations aiming to multiple oleoresin yield and income of landowners. However, new, more informative markers and approaches could be put into use in order to identify the prospective individuals for oleoresin production. Besides the selection of plus trees and establishment of seed orchards, development of genome-wide association studies could help to identify candidate genes or genome regions contributing to the traits of interest. The seed orchards could provide opportunities for performing of controlled crosses necessary for the genetic association studies. Experience and analogies with other pine species could also facilitate such studies (Lauer et al. 2022).

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