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Quantitative genetic parameters of heartwood and its chemical traits in a black pine (*Pinus nigra* J.F.Arnold) clonal seed orchard established in Greece

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Abstract

Background: Black pine (*Pinus nigra* J.F.Arnold) is one of the most productive conifers species for timber production in southern Europe, the Mediterranean region and Greece. Recently, the interest for its heartwood extractives content due to their medicinal properties has been renewed. Black pine can be used to produce high added value products, such as bioactive compounds produced from wood and wood waste materials.

Methods: Quantitative genetic parameters were estimated for heartwood chemical traits and heartwood percentage in a 44-year-old *Pinus nigra* clonal seed orchard, established in Peloponnese, Greece.

Results: Significant variation was found among clones and among provenances for all studied traits. Heritability on a clone mean basis was extremely high for total acetone extractives, total resin acids as for all resin acids (≥0.85), except levopimaric acid (0.47) and very high for total stilbenes, pinosylvins as for dehydroabietic acid and heartwood percentage (0.69-0.79). On an individual basis, the genetic control was moderate to high (0.53-0.62) for total acetone extractives, total resin acids as for most of resin acids (≥0.85) with dehydroabietic acid presenting low value (0.39) while levopimaric acid very low (0.15). Total stilbenes, pinosylvins and its ether derivatives as heartwood percentage exhibited low values of heritability on individual basis (0.31-0.43). The phenotypic correlation (r_p) between total acetone extracts and total stilbenes was negatively weak (≤-0.173) and significant (p≤0.01) while the genetic correlation (r_g) was moderate to strong (≤-0.502). The r_p values between several pinosylvins were significantly (p≤0.01) moderate to strong (0.529-0.975) as were genetic correlations (0.583-0.975). Between the studied resin acids, both r_p and r_g values were mostly medium to strong ($r_p \geq 0.8$ and $r_g \geq 0.7$) and significant (p≤0.01) in the case of phenotypic correlations, with minor exceptions (levopimaric acid). Phenotypic and genetic correlations between heartwood percentage and its chemical traits were positive (being in most cases significant), except for dehydroabietic and levopimaric acid.

Conclusions: The studied clones, comprising the clonal seed orchard, can be used in clonal forestry and subsequent breeding cycles, indicating high potential for advanced breeding, especially for heartwood extractives that are of high pharmaceutical and economic value.

Keywords: black pine; clonal seed orchard; stilbenes; pinosylvin; resin acids; heartwood; heritability.

Introduction

Black pine (*Pinus nigra* J.F.Arnold) is one of the most economically important native conifers in southern Europe and the Mediterranean region (Isajev et al. 2004), and is one of the main timber species in Greece. The species has a wide geographical distribution, extending from the Caucasian coast of the Black Sea to the Atlas Mountains in northwest Africa. This terrestrial extent has led to the existence of many forms among the subspecies and high levels of genetic variation among and within populations (Mirov 1967; Vidakovic 1974, 1991). The species' importance is attributed to its ability to develop under dry and poor environmental conditions (as a low demanding species), on a variety of soil substrates, being wind resistant but shade intolerant, and to the natural durability of its wood (Isajev et al. 2004). Due to its ecological value and silvicultural significance, it has been planted in several regions outside its natural range, including New Zealand, Great Britain, France, Argentina and the United States (Cown 1972; Lee 1968; Wheeler et al. 1976; Wilcox & Miller 1975).

Silviculturally, black pine has been extensively used in reforestation programs throughout Greece because it is well-adapted to a wide range of sites, can grow on degraded land, is easy to establish even with minimal care, grows rapidly and produces high quality wood, which is in high demand (Matziris 1989). To satisfy the demand for large quantities of quality seed, breeding efforts have focused on conducting provenance trials (Varelides et al. 2001), progeny tests and establishing clonal seed orchards (Matziris 2005). The aim has been to produce material that is genetically improved for traits of interest, mostly growth.

Recently, there has been renewed research interest in pine extracts, particularly in stilbenes, i.e., pinosylvin and its ether derivatives, as well as in resin acids, i.e., abietic, dehydroabietic, neoabietic, palustric, levopimaric, pimaric, isopimaric and sandaracopimaric acid. These bioactive compounds are high added value products which can be produced from wood and wood waste (Ioannidis et al. 2017, 2019; Pietarinen et al. 2006).

Stilbenes, also called phytoalexins, are polyphenolic compounds produced by various plants in response to environmental stresses such as weather changes, injuries from insects or infections by pathogens (Celimene et al. 1999, 2001; Dixon 2001; Gref et al. 2000; Hart 1981; Kennedy et al. 1995; Schultz et al. 1992; Schultz et al. 1990) . Due to their fungal toxicity and water repellent properties, they affect wood natural durability (Gref et al. 2000; Hart 1981; Kennedy et al. 1995). Pinosylvin is a stilbene with many medicinal properties, including antibacterial and antifungal (Lee et al. 2005; Lindberg et al. 2004), antioxidant (Willför et al. 2003), antimetastatic (Park et al. 2012), potential cancer chemopreventive activity (Park et al. 2013), antiapoptotic and cardiovascular protective (Jeong et al. 2013) and anti-inflammatory effects (Laavola et al. 2015).

In addition, pine resin and its derivatives have been widely applied in the chemical industry in uses such as polymer additives, emulsifiers in synthetic rubber, in adhesives, surface coatings, printing inks, chewing gums etc. and in the Greek wine industry (Langenheim 2003; Rezzi et al. 2005). More importantly, several medicinal uses concerning the treatment of abscesses, boils, cancers, toothache and others have been reported even from antiquity (Langenheim 2003). In recent years, a renewed scientific interest in pine resin extracts has arisen (Reveglia et al. 2018) due to their medicinal properties, i.e. antimicrobial (Spessard et al. 1995), anti-inflammatory (Fernandez et al. 2001; Takahashi et al. 2003), cardiovascular (de Oliveira et al. 2008; Gonzalez et al. 2009), cytotoxic (Schmeda-Hirschmanna et al. 2005a) and others (Schmeda-Hirschmann et al. 2005b; Talevi et al. 2007; Ulusu et al. 2002).

Several genes are involved in the formation of both groups of bioactive compounds. Rapidly activated stilbene synthase-encoding genes take part in the formation of stilbenes (Chong et al. 2009; Donnez et al. 2009; Ebel 1986). Respectively, diterpene synthase-encoding genes act on the geranylgeranyl diphosphate (GGPP) substrate to form diterpenes, following two independent transformations by other enzymes (Keeling & Bohlmann 2006). This explains why pinosylvin and its ether derivatives (Fries et al. 2000; Partanen et al. 2011) as well as the diterpene resin acids (Ericsson et al. 2001; Fries et al. 2000) are highly heritable properties. Genetic control of extractive production has considerable economic potential in some species (Taylor et al. 2002).

The aim of the present work was to estimate for the first time, genetic effects on regulation of the traits of specific heartwood extractives in *Pinus nigra*. We investigated the qualitative and quantitative variations in two major heartwood group of substances (stilbenes and resin acids) in 52 *Pinus nigra* L. subsp. *pallasiana* plus-tree clones, originating from four provenances from the Peloponnese region of southern Greece (Figure 1). The populations from which the selection of clones were made are the most southern or borderline populations of the species in Greece as well as in Europe. Clonal repeatability, on individual and on clone mean basis, of the target bioactive compounds was evaluated, indicating the magnitude of their inheritance. The heartwood of these trees produced an exceptionally high extractive content, approximately 30% w/w (Ioannidis et al. 2017), making it particularly interesting for the present analysis. The target compounds were the stilbenes pinosylvin, monomethyl and dimethyl ether of pinosylvin, the abietane type resin acids, abietic, dehydroabietic, neoabietic, palustric and levopimaric, and the pimarane type resin acids, pimaric, isopimaric and sandaracopimaric.

Methods

Plant Material

In Greece, black pine's significant effort has focused on selective breeding. In 1978, a 10-ha clonal seed orchard was established in the area of Koumani in the western part of the Peloponnese, Greece. The orchard comprises 52 clones derived from intensively selected plus trees in the natural black pine forest of the Peloponnese and a total number of 2,700 grafts (Matziris 1989). The elite trees originated from four provenances (Figure 1): Mt. Zarouhla, Mt. Feneos, Mt. Parnonas and Mt. Taigetos. Ramets of each clone were randomly located within the clonal seed orchard under the condition that two ramets of the same clone were planted at least 30 m apart from each other (single tree plot design).

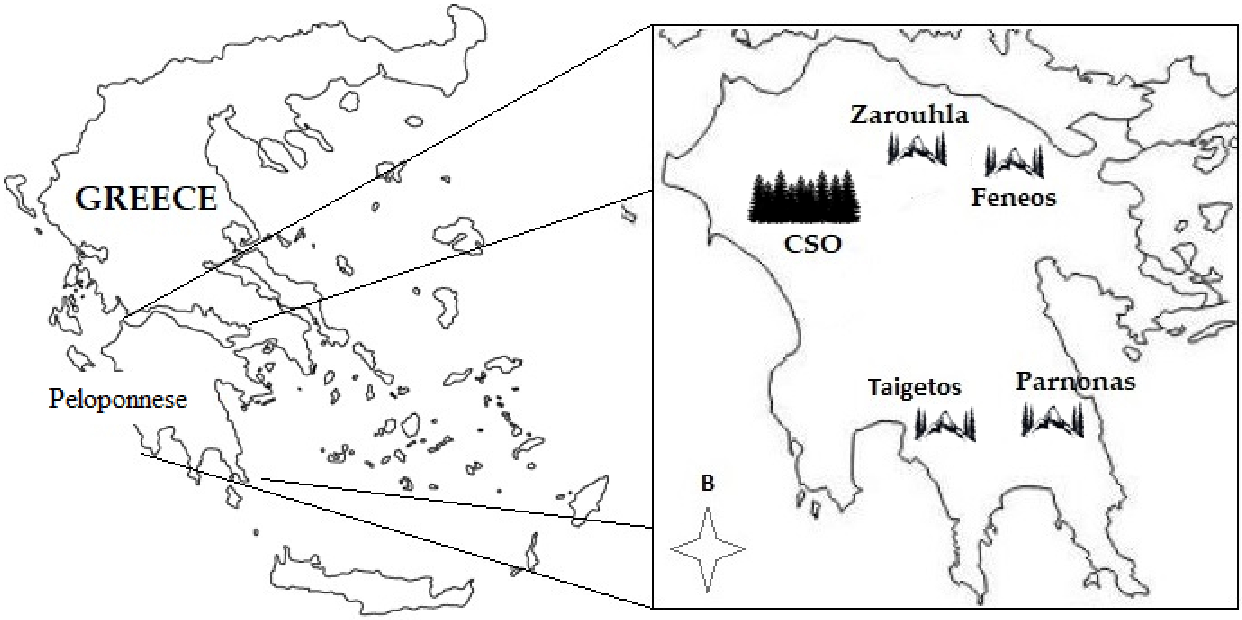

FIGURE 1: Map showing the location of the four black pine provenances (Mt. Zarouhla, Mt. Feneos, Mt. Parnonas and Mt. Taigetos) and the clonal seed orchard (CSO).

Sampling - Heartwood discrimination and orientation - Extraction protocol

Sampling, coring, heartwood discrimination and orientation and extraction protocol, ^{1}H-NMR spectra analysis, calibration curves, validation method and quantitation of pinosylvins and resin acids are described extensively in Ioannidis et al. (2017) and Ioannidis et al. (2019). In brief, 12mm-diameter increment cores including the pith were extracted, 30 cm above ground and in a north-south orientation from a total of 260 healthy individuals, covering all 52 clones planted in the clonal seed orchard (five ramets per clone). Heartwood was separated from the rest of the core based on a visual assessment and milled to produce ≤0.75 mm particles. Pinosylvins and resin acids were extracted from freeze-dried ground heartwood using acetone as solvent. The extract from each sample was dissolved in 600 μL of deuterated chloroform ($CDCl_3$) and the solution was transferred to a 5 mm NMR tube. ^{1}H-NMR spectra were recorded at 400 MHz (Bruker DRX400). One-dimensional (1D) and two-dimensional (2D) quantitative nuclear magnetic resonance (qNMR) were used permitting rapid quantification of the analytes. Concentrations were based on freeze-dried heartwood (dhw) and expressed in mg/g_{dhw}.

Studied traits

The following traits were studied in the Koumani clonal seed orchard: heartwood concentration of total acetone extracts (TAE), total stilbenes (TS), pinosylvin (P), monomethyl (PMME) and dimethyl ether (PDME) of pinosylvin, total resin acid (TRA), the abietane-type resin acids, i.e. abietic acid (AA), dehydroabietic acid (DAA), neoabietic acid (NAA), palustric acid (PlA) and levopimaric acid (LPmA), the pimarane-type resin acids i.e. pimaric acid (PmA), isopimaric acid (IPmA) and sandaracopimaric acid (SPmA), and finally the heartwood proportion (HW). The heartwood proportion was calculated as the ratio of heartwood radius to total under bark radius and expressed as a percentage.

Statistical analysis

Analyses were based on values obtained from individual trees and were performed at a significance level of $a=0.05$. The following linear model was used in the analysis:

$$y_{ijk} = \mu + p_i + c_j(p_i) + e_{ijk} \qquad (1)$$

where y_{ijk} is the phenotypic measurement for a trait measured on the k^{th} tree, j^{th} clone and i^{th} provenance, μ is the fixed population mean of all trees averaged across the clonal seed orchard, p_i is the random effect of the i^{th} provenance (i=1,2,3,4), $c_j(p_i)$ is the random effect of the j^{th} clone (j=1,2,...,52) nested within the i^{th} provenance, and e_{ijk} is the random residual error of k^{th} tree, j^{th} clone and i^{th} provenance. The variance components were estimated by the restricted maximum likelihood (REML) method. Descriptive statistics, analysis of variance (ANOVA) as well as variance component estimates based on the 0.05 level of significance were calculated using SPSS v.20 software (IBM SPSS Statistics 2011, IBM Corp.).

Heritabilities

The following variance components were estimated: σ^2_e the variance component due to error, σ^2_p the variance component due to provenances and $\sigma^2_{c(p)}$ the variance component due to clones within provenances. Estimates of broad sense heritability were obtained on an individual tree (H^2_i) and clone mean (H^2_c) basis, respectively, as follows:

$$H^2_i = \sigma^2_{c(p)}/(\sigma^2_e + \sigma^2_{c(p)}) \qquad (2)$$

$$H^2_c = \sigma^2_{c(p)}/((\sigma^2_e / r) + \sigma^2_{c(p)}) \qquad (3)$$

Where H^2_i, H^2_c, σ^2_e, σ^2_p and $\sigma^2_{c(p)}$ are as above, and r is the number of ramets sampled per clone, (i.e., r=5). Standard errors for heritability estimates were calculated, where feasible, using Dickerson's approximation (Dickerson 1969).

Phenotypic and genetic correlations

For all the studied traits, phenotypic correlation coefficients and their significance level were assessed using Pearson's correlation coefficient (Snedecor & Cohran 1980):

$$r_p = \sigma_{p_{xy}}/(\sigma_{p_x} \times \sigma_{p_y}) \qquad (4)$$

where σ_{p_x} and σ_{p_y} are the square roots of the phenotypic variance from an analysis of variance of each trait, and $\sigma_{p_{xy}}$, is the phenotypic component from analysis of covariance of x and y traits. Broad sense genetic correlations (r_g) between all paired traits were calculated as follows (Falconer 1960):

$$r_g = \sigma_{g_{xy}}/(\sigma_{g_x} \times \sigma_{g_y}) \qquad (5)$$

where σ_{g_x} and σ_{g_y} the square roots of the clonal variance components from the analysis of variance of each trait, and $\sigma_{g_{xy}}$ is the clonal component from analysis of covariance of x and y traits. These correlations as well as heritability estimates are biased if any genotype by environment interaction exists (Lambeth et al. 1994).

Results

The overall mean values for all traits assessed in the Koumani clonal seed orchard along with their minimum and maximum values, standard deviations and coefficients of variation are presented in Table 1. More details about stilbenes and resin acids can be found in Ioannidis et al. (2017) and Ioannidis et al. (2019), respectively. The average heartwood content was 11.2% and ranged from 1.2% up to 27.0%. The visualised results of the traits assessed on the sampled trees in the clonal seed orchard are presented in the boxplots in additional files (Figures A1 to A4).

The variation among clones for the studied traits is evident from the analysis of variance. The analysis showed that there were statistically significant ($p<0.001$) differences among clones for all examined traits. Concerning provenances, the analysis showed significant differences in concentrations of TAE ($p<0.05$), P ($p<0.01$), PDME ($p<0.05$), TRA ($p<0.05$), AA ($p<0.01$), NAA ($p<0.05$), PmA ($p<0.01$) and SPmA ($p<0.01$). The variance components for the studied traits are presented in Figure 2.

The individual (H^2_i) and clone (H^2_c) broad sense heritabilities and their standard errors for all studied traits are shown in Figure 3 and Table A1 in Additional File. TAE had the highest heritability on an individual tree basis (0.86) and moderate heritability on a clone mean basis (0.55). The heritability values for stilbene concentration on individual mean basis (H^2_i) were moderate to low and ranged from 0.33 (TS) to 0.79 (PDME). The genetic control on a clone mean basis (H^2_c)

TABLE 1: Descriptive statistics of the traits assessed in the total clonal seed orchard sample (n=260).

Trait (units of measurement)	Minimum	Maximum	Mean	Std. Dev.	CV (%)
Total acetone extracts (mg/g$_{dhw}$)	81.79	480.28	304.15	96.02	31.57
Total stilbenes (mg/g$_{dhw}$)	10.99	128.22	59.92	21.79	36.37
Pinosylvin (mg/g$_{dhw}$)	1.19	40.23	17.07	6.76	39.60
Pinosylvin monomethyl ether (mg/g$_{dhw}$)	8.94	94.28	40.32	15.55	38.58
Pinosylvin dimethyl ether (mg/g$_{dhw}$)	0.21	7.91	2.54	1.22	48.09
Total resin acid (mg/g$_{dhw}$)	30.05	424.70	219.98	96.20	43.73
Abietic acid (mg/g$_{dhw}$)	7.00	181.75	76.77	37.39	48.70
Dehydroabietic acid (mg/g$_{dhw}$)	2.56	38.59	11.69	5.73	49.02
Neoabietic acid (mg/g$_{dhw}$)	2.91	101.82	39.34	21.21	53.91
Palustric acid (mg/g$_{dhw}$)	9.76	105.22	47.94	23.31	48.62
Levopimaric acid (mg/g$_{dhw}$)	0.08	64.91	8.07	11.38	141.02
Pimaric acid (mg/g$_{dhw}$)	2.20	59.42	22.54	11.28	50.04
Isopimaric acid (mg/g$_{dhw}$)	0.50	34.09	10.91	6.53	59.85
Sandaracopimaric acid (mg/g$_{dhw}$)	0.16	6.67	2.72	1.49	54.78
Heartwood proportion (%)	1.20	27.04	11.15	4.39	39.37
Diameter at breast height (cm)	18.30	48.80	35.33	5.0351	14.25
Total tree height (m)	15.20	21.90	18.64	1.3733	7.37

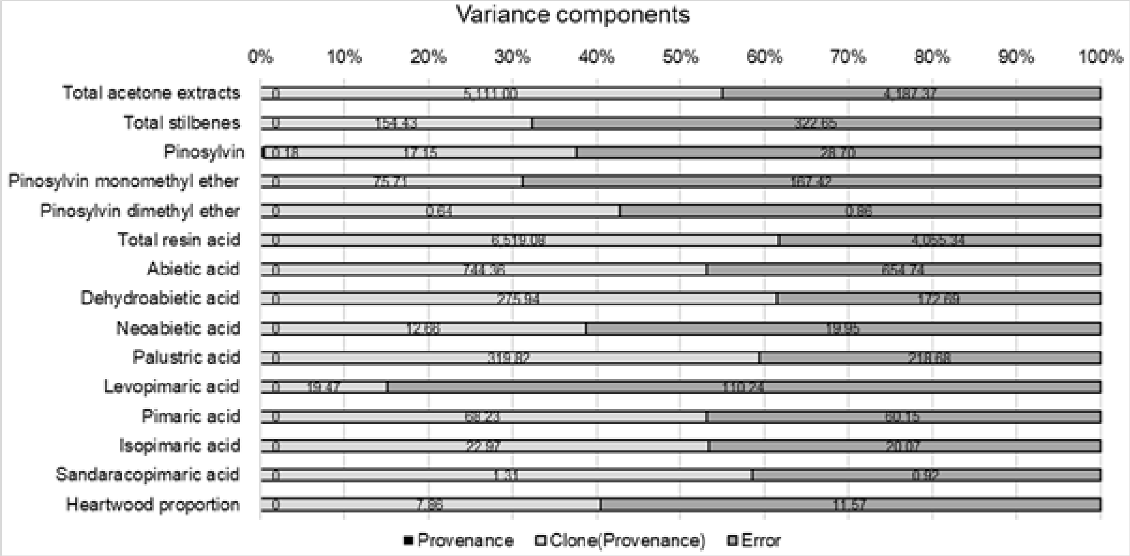

FIGURE 2: Results from the variance components analysis for the studied traits. The numbers in each section of the bars represent the variance estimates calculated by the restricted maximum likelihood method using the $y_{ijk} = \mu + p_i + c_j(p_i) + e_{ijk}$ general linear model.

was low to moderate strong and ranged from 0.37 (P) to 0.71 (TS). The clone mean heritability values (H^2_c) for resin acids were higher comparing those for stilbenes, ranging from 0.47 (LPmA) to 0.89 (NAA and TRA). The H^2_i values of the studied resin acids were lower and ranged from 0.15 (LPmA) to 0.62 (NAA and TRA). Heritability values on individual and clone mean basis for heartwood percentage were estimated at 0.44 and 0.77, respectively (Figure 3 and Additional file Table A1).

Pearson's phenotypic correlation (r_p) and genetic correlation (r_g) coefficients between all pairs of traits are shown in Table 2 and Table 3. Phenotypically, TAE was negatively correlated with all types of stilbenes, i.e., with pinosylvin r_p=-0.173 (*p<0.01*), with PMME r_p=-0.236 (*p<0.01*) and with PDME r_p=-0.359 (*p<0.01*). TAE showed a slight positive correlation with heartwood percentage (r_p=0.188, *p<0.01*). Stilbenes were positively, strongly and significantly correlated with each other (r_p=0.529-0.770, *p<0.01*), and in most cases, negatively (range from -0.233 to -0.088) and significantly (*p<0.01* or *p<0.05*) correlated with heartwood percentage, except for the very loose positive and non-significant correlation between P and heartwood percentage (r_p=0.30).

Strong phenotypic correlations were identified between TAE and TRA (r_p=0.918, *p<0.01*) and TAE with all acids (r_p=0.702-0.918, *p<0.01*) except LPmA (r_p=0.452, *p<0.01*). Resin acids were positively, significantly and in most cases strongly (r_p=0.622 to 0.964, *p<0.01*) correlated with each other. Weak to medium phenotypic correlations were identified between all resin acids with LPmA (0.172-0.502, *p<0.01*). Some r_p values, concerning correlations for IPmA and SPmA with several resin acids, were moderate (Table 2).

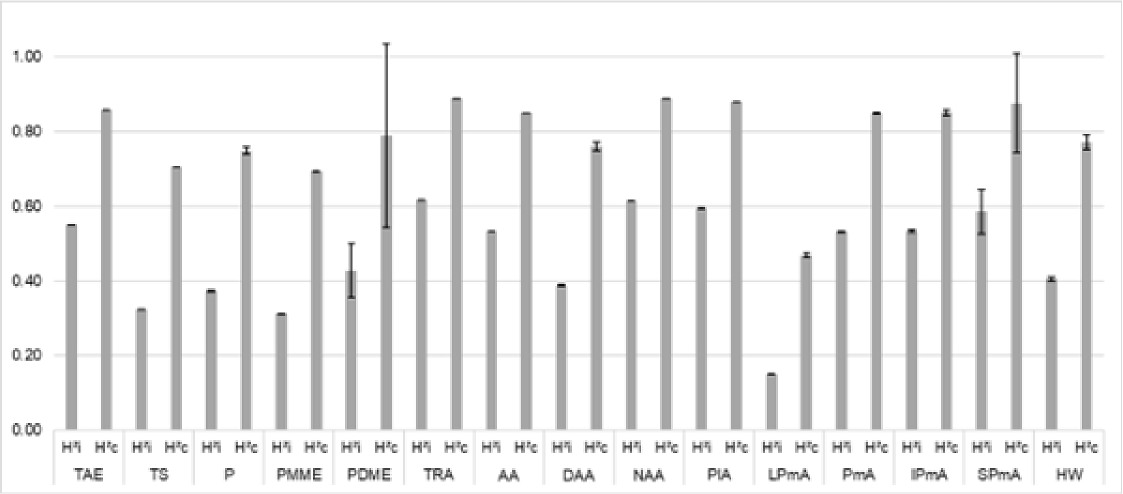

FIGURE 3: Broad sense heritability estimates at individual and clone mean basis and their standard errors (red whiskers) for the studied traits. Full descriptions of the trait abbreviations are given in the text.

TABLE 2: Pearson's phenotypic correlation coefficients (above diagonal) and broad sense genetic correlations (below diagonal) among all pairs of traits.

		Phenotypic correlation coefficients					
		TAE	**TS**	**P**	**PMME**	**PDME**	**HW**
Genetic correlation coefficients	TAE		-0.233**	-0.173**	-0.236**	-0.359**	0.176**
	TS	-0.745**		0.838**	0.975**	0.737**	-0.093
	P	-0.502**	0.864**		0.697**	0.529**	0.030
	PMME	-0.758**	0.975**	0.686**		0.770**	-0.123*
	PDME	-0.757**	0.791**	0.583**	0.747**		-0.233**
	HW	0.315**	0.137*	0.310**	0.062	-0.134**	

Correlation is significant at the ** p<0.01, * p<0.05

Total resin acids correlated slightly positively with heartwood percentage (r_p=0.168, *p<0.01*). Low positive correlations were estimated between abietane type resin acids with heartwood percentage (r_p=0.136-0.224, *p<0.01* and *p<0.05* regarding pimaric acid), and in two cases i.e. DAA and LPmA, were negative (-0.059 and -0.034 respectively) but non-significant. Likewise, weak positive correlations were estimated between pimarane type resin acids with heartwood percentage (r_p=0.129-0.150, *p<0.05*).

The genetic correlations between TAE and stilbenes were always negative and significant (r_g=-0.758 to -0.502, *p<0.01*), while they were positive with heartwood percentage (0.315, *p<0.01*). Between pinosylvins, moderate to strong genetic correlations were observed (r_p=0.583-0.975, *p<0.01*), while those between heartwood percentage and stilbenes were lower (0.062-0.315), and in the case of PDME, negative (-0.134). Moderate to strong and significant genetic correlations were identified between TAE and all resin acids (0.478-0.959) except for SPmA for which r_g was negative (-0.131). Resin acids were positively and strongly correlated with each other (r =0.692-0.989, *p<0.01*). However, the positive correlations between heartwood percentage and resin acids were much weaker (r_g=0.151-0.340) and were negative with DAA and LPmA, i.e. -0.006 and -0.269 respectively (Table 3).

Discussion

Despite the extensive spread and importance of black pine, few studies have been conducted internationally, either into its heartwood extractives or their proportion. Especially for Black pine's stilbenes content, the literature is very poor. Likewise, there are limited previous studies that have estimated the genetic control, heritabilities in broad and narrow sense, as well as phenotypic and genetic correlations between the mentioned traits. Consequently, black pine is not considered a well-characterised species concerning heartwood stilbenes, resin acids and percentage content.

Heritabilities

Heartwood extractives

The content of extractives is usually less than 10%, but it can vary from trace amounts up to 40% of the dry wood weight (Sjöström 1993). Miller (1999) reported that the

TABLE 3: Pearson's phenotypic correlation coefficients (above diagonal) and broad sense genetic correlations below diagonal) among all pairs of traits.

		Pearson correlation coefficients										
		TAE	TRA	AA	NAA	DAA	PlA	LpmA	PmA	IPmA	SPmA	HW
Genetic correlation coefficients	TAE		0.918**	0.848**	0.877**	0.702**	0.837**	0.452**	0.834**	0.732**	0.829**	0.176**
	TRA	0.959**		0.907**	0.964**	0.748**	0.930**	0.502**	0.901**	0.802**	0.915**	0.168**
	AA	0.933**	0.980**		0.839**	0.650**	0.754**	0.172**	0.806**	0.693**	0.832**	0.197**
	NAA	0.888**	0.985**	0.955**		0.622**	0.912**	0.472**	0.867**	0.807**	0.887**	0.224**
	DAA	0.478**	0.861**	0.838**	0.795**		0.729**	0.485**	0.637**	0.462**	0.655**	-0.059
	PlA	0.876**	0.971**	0.917**	0.958**	0.858**		0.530**	0.765**	0.722**	0.825**	0.136*
	LPmA	0.571**	0.939**	0.887**	0.901**	0.912**	0.954**		0.480**	0.397**	0.457**	-0.034
	PmA	0.730**	0.929**	0.921**	0.921**	0.751**	0.851**	0.796**		0.697**	0.857**	0.150*
	IPmA	0.590**	0.889**	0.835**	0.868**	0.692**	0.868**	0.891**	0.796**		0.738**	0.133*
	SPmA	-0.131*	0.989**	0.953**	0.983**	0.819**	0.969**	0.924**	0.949**	0.876**		0.129*
	HW	0.315**	0.225**	0.340**	0.291**	-0.006	0.167*	-0.269**	0.164*	0.151*	0.194**	

Correlation is significant at the ** p<0.01, * p<0.05

variation in extractives content depends on factors such as species, growth conditions, and the time of year when a tree is cut. While extractives are present in sapwood, they mainly occur in the heartwood and knots (Ioannidis et al. 2017; Partanen et al. 2011; Taylor et al. 2002; Venäläinen et al. 2003; Willför et al. 2003; Willför et al. 2004a; Willför et al. 2004b).

The concentrations of total acetone extractives, pinosylvin and its ether derivatives in black pine have described extensively by Ioannidis et al. (2017), while information on resin acids is contained in Ioannidis et al. (2019). The findings in the study presented here are important as the mean recorded amount of total stilbenes, for the studied genetic material, was five times higher than the highest ever reported result for black pine, although the number of studies focusing on the specific species and topic is rather limited. Concerning resin acids, they predominate in conifer heartwood extracts, while a wide range of compounds occur in angiosperms, although the range varies within each species (Gutiérrez et al. 2001). Moreover, there are differences in resin content and composition between different parts of the tree, depending, apart from genetic and environmental factors, on the age of tree and the growing conditions (Back 2000). In *Pinus sylvestris* L. older trees had higher concentrations of extractives (Hovelstad et al. 2006) while in *Picea abies* (L.) H.Karst. lower concentrations could be attributed to the young age of the clones (Fengel & Wegener 2003). In contrast, Fries et al. (2001), in several *Pinus sylvestris* L. experiments, showed that older trees generally had lower extractives content compared to younger trees, indicating that differences in environmental conditions and tree age may cause differences in the amounts of heartwood extracts. Substantially, the differences among clones could be attributed to the different environments in which they are grown and evolve. Generally, clones originating from the most southeastern origin (Mt. Parnon provenance), growing under xerothermic environmental conditions (Tselepidakis & Theoharatos 1989), predominated in most characteristics. Conversely, clones from the Zarouhla provenance, which is the most north-western origin of the four tested, had the lowest values. Clones from Feneos (northeastern origin) and Taygetos (southwestern origin) provenances, had intermediate values.

The high clonal repeatability estimates found for the studied traits is evident. Both the high heritabilities of heartwood chemical traits and of its proportion demonstrate they are under strong genetic control. Heritability on a clone mean basis was extremely strong, while it was moderate on an individual tree basis. High broad sense heritabilities on an individual and clone mean basis indicate high potential for advanced breeding, for stilbenes and resin acids, both being of high pharmaceutical and economic value. Despite these high estimates, it should be emphasised that extractive content generally depends, apart from a genetic component, on factors such as species, growth conditions, and the specific period of the year where the analysis is done. Heartwood extractives, as mentioned above, are strongly determined genetically (Zobel & Jett 1995) with a mild environmental effect, e.g. site quality (Taylor et al. 2002). Pinosylvin and its ether derivatives are highly heritable heartwood properties (Fries et al. 2000; Partanen et al. 2011). Our results are in accordance to those of Partanen et al. (2011) in Scots pine (*Pinus sylvestris* L.), who estimated high heritability values for pinosylvin concentration (0.81), total stilbenes (0.61) and total phenols (0.74). The heritability of pinosylvin monomethyl ether was lower (0.48), as in the genetic material of the Peloponnese. In both studies, clones with high or low concentrations of pinosylvin could be clearly identified despite the within clone variation. Strong genetic control is expected because the formation of typical pine species phytoalexins (e.g. pinosylvin) is based on the rapidly activated stilbene synthase-encoding genes (Chong et al. 2009). Pinosylvin formation in the genus *Pinus* is catalysed by stilbene synthase (STS), while pinosylvin-O-methyltransferase gene (PMT), is involved in the pinosylvin metabolism and the formation of the pinosylvin monomethylether (Chiron et al. 2000; Kodan et al. 2002). It may be noted that heritability estimates are probably biased upward due to the consideration of only one experimental site, compared with a more general estimate for a set of environments (Fries et al. 2000).

Heartwood percentage

Considering the age of the trees in the clonal seed orchard at the time of sampling (35 years old), the small proportion of heartwood was expected. Heartwood formation varies with provenance and site, and also depends largely upon tree age (Fries 1999). In *Pinus sylvestris* L. provenance trials, the lowest heartwood percentage values were found in younger trials while the highest in trials that were close to harvest age. According to Fries (1999), these data indicate that the time of initiation of heartwood formation may be a limiting factor for high heartwood production. This conclusion perhaps explains the small percentage of heartwood observed in the clonal seed orchard. Heartwood formation in black pine depends largely upon tree age and the trees in the clonal seed orchard were relatively young. The rotation age of the black pine in Greece can reach and exceed 120 years, depending on the objectives of the management, the site quality, etc., so higher amounts of heartwood would be expected from harvested stands than were found in this study. Comparing black pine's heartwood percentage (11.5% at age 35 years) to other species such as Scots or radiata (*Pinus radiata* D.Don) pines has led to the assumption that the formation of black pine's heartwood starts at an older age, increases as the age progresses, as it is formed at a constant rate of annual growth rings and is expanding at a slow rate (Cown 1972). On the contrary, heartwood percentage in *Pinus sylvestris* L. ranges at higher values depending on the origin, location and age of trees (Fries 1999). The same applies to radiata pine (Cown et al. 1992). Heartwood size is generally under moderate genetic control and with some environmental influence (Zobel & Jett 1995). Pâques (2001) observed high heartwood percentage in

Larix sp., denoting statistically significant differences among the origins of families and clones. Broad sense heritabilities at an individual basis of the Peloponnesian *Pinus nigra* L. heartwood percentage were quite similar to the findings in *P. sylvestris* L. (Partanen et al. 2011) and *P. radiata* D. Don (Nicholls & Brown 1974). On the other hand, Venäläinen et al. (2006) estimated lower broad sense heritability on an individual basis in *Larix sibirica* Ledeb.

Phenotypic and genetic correlations

Total acetone extractives correlated positively but weakly to heartwood percentage, showing a combined selection potential for these traits. The correlations between different stilbene types are expected to be strongly positive because the formation of pinosylvin and its ether derivatives in pine species are due to rapidly activated encoding stilbene related genes that act in the heartwood and they are involved in their metabolism and formation (Chiron 2000; Chiron et al. 2000; Ebel 1986; Kodan et al. 2002; Schanz et al. 1992). Venalainen et al. (2003) found a positive and significant correlations between P and PMME in both outer and inner heartwood. Ericsson et al. (2001), found that environmental and genetic correlations in *P. sylvestris* L., between stilbenes types, as well as with the resin acids were extremely high.

Sehlstedt-Persson and Karlsson (2010) estimated that phenolic concentration in *P. sylvestris* L. exhibits a positive phenotypic correlation with total extracts. In the same species, Venalainen et al. (2003) estimated that although phenotypic correlations between resin acids and both stilbenes and total phenols were generally positive, they were not statistically significant. The results of our study in black pine from Peloponnese, indicate an opposite relationship between total acetone extracts and stilbenes. The negative correlation of total acetone extractives with all types of stilbenes essentially indicates that stilbene output is independent of total extractives production. A similar result was obtained by Ericsson et al. (2001) who observed negative genetic correlation between stilbene and total acetone extracts.

Negative environmental correlations of various extracts, including pinosylvin monomethyl ether and total pinosylvins, with heartwood proportion were found by Ericsson et al. (2001) in *P. sylvestris* L., although genetic correlations between stilbenes and heartwood were not detected. Ericsson et al. (2001) also estimated that there were positive environmental (pimaric and abietic acid) and genetic (pimaric acid) correlations with heartwood diameter as well as negative ones (fatty acids and sterols). Essentially, the results concerning stilbenes and heartwood proportion indicate that black pine from the Peloponnese could not be selected in combination for these traits, at least for the clones and the provenances that were in the Koumani clonal seed orchard. On the contrary, when a positive correlation was estimated between some heartwood extractives and heartwood percentage, the selection of trees with more resistant wood should be efficient, resulting in increase of both the content of heartwood and the concentration of extractives. Despite the negative correlations between several of the traits studied, clones representing correlation breakers are able to be identified, (i.e. clones that performed well for traits negatively correlated). In addition, exceptional clones for positively correlated traits were also identified. These clones could be selected and might be the base to produce improved reproductive material.

Conclusions

The heartwood of the selected black pine phenotypes originating from Peloponnese are rich in extracts such as stilbenes and resin acids. The great variation among clones and provenances for the studied traits led to the identification of specific clones that outperform for several chemical traits. The genetic control of the studied traits was moderate to high, indicating the high potential for effective selection and breeding, and especially for the pinosylvins and several types of resin acids that are of high pharmaceutical and economic value. The deployment of selected clones in clonal forestry plantations may yield immediate and significant revenues.

List of abbreviations

^{1}H-NMR: proton nuclear magnetic resonance
ANOVA: analysis of variance
AA: abietic acid
CSO: clonal seed orchard
DAA: dehydroabietic acid
dhw: dried heartwood
GGPP: geranylgeranyl diphosphate
HW: heartwood proportion
IPmA: isopimaric acid
NAA: neoabietic acid
LPmA: levopimaric acid
MRT: multiple range test
P: pinosylvin
PmA: pimaric acid
PMME: monomethyl ether of pinosylvin
PDME: dimethyl ether of pinosylvin
PlA: palustric acid
PMT: pinosylvin-O-methyltransferase gene
qNMR: quantitative nuclear magnetic resonance
REML: restricted maximum likelihood
TAE: total acetone extracts
SPmA: sandaracopimaric acid
TRA: total resin acid
TS: total stilbenes

Competing interests

The authors declare no competing interests.

Authors' contributions

KI designed the methodology, experimental work, performed the statistical analysis, and contributed to writing, reviewing and editing of the manuscript, PK carried out experimental work, data curation, helped in formal analysis and participated in original draft preparation. All authors read, revised and approved the final manuscript.

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Additional File

FIGURES A1 to A4: Boxplots of studied traits for the sampled trees of the CSO. TABLE A1: Broad sense heritability estimates at individual and clone mean basis and their standard errors for the studied traits.

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Additional File

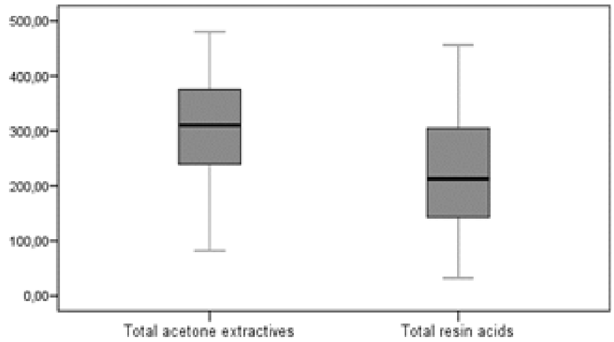

FIGURE A1:
Boxplots of total acetone extracts (mg/g_{dhw}) and total resin acid (mg/g_{dhw}) for the sampled trees of the CSO.

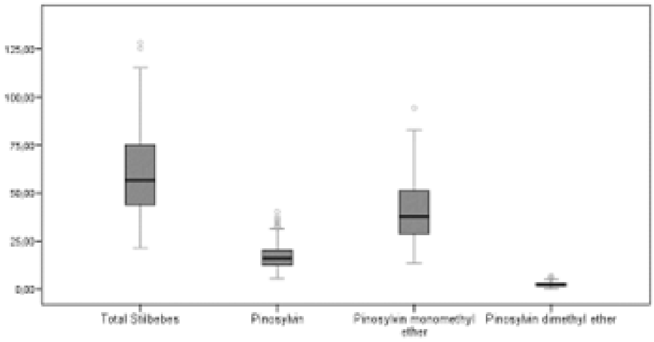

FIGURE A2:
Boxplots of total stilbenes (mg/g_{dhw}), pinosylvin (mg/g_{dhw}), monomethyl (mg/g_{dhw}) and dimethyl ether mg/g_{dhw}) of pinosylvin for the sampled trees of the CSO. (The circles present the outliers.)

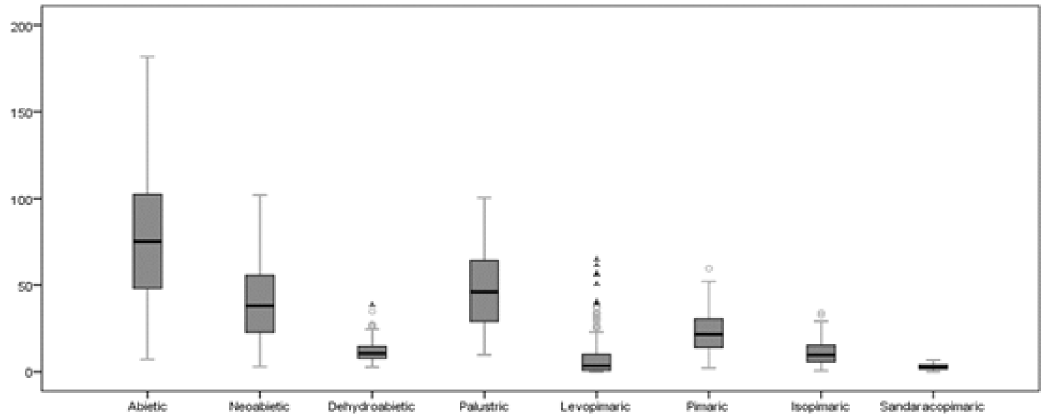

FIGURE A3:
Boxplots of the studied resin acids (mg/g_{dhw}) for the sampled trees of the CSO. (The circles and the stars represent the outliers the extreme values respectively.)

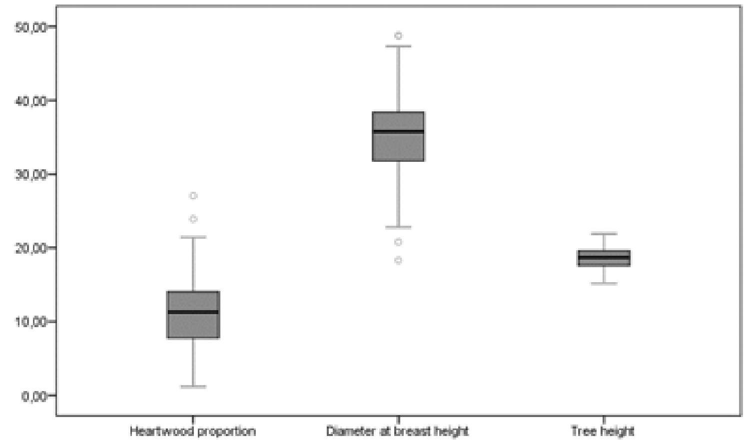

FIGURE A4:
Boxplots of heartwood proportion (%), diameter at breast height (cm) and tree height (m) of the sampled trees of the CSO. (The circles present the outliers.)

TABLE A1: Broad sense heritability estimates at individual and clone mean basis and their standard errors for the studied traits.

Estimate	**TAE**	**TS**	**P**	**PMME**	**PDME**	**TRA**	**AA**	**DAA**	**NAA**	**PlA**	**LPmA**	**PmA**	**IPmA**	**SPmA**	**HW**
H^2_i	**0.55**	**0.32**	**0.37**	**0.31**	**0.43**	**0.62**	**0.53**	**0.39**	**0.62**	**0.59**	**0.15**	**0.53**	**0.53**	**0.59**	**0.40**
$SE_{\mathrm{H}^2_i}$	0.00001	0.00019	0.00222	0.00037	0.07206	0.00001	0.00009	0.00312	0.00031	0.00025	0.00051	0.00097	0.00289	0.05958	0.00537
H^2_c	**0.86**	**0.71**	**0.75**	**0.69**	**0.79**	**0.89**	**0.85**	**0.76**	**0.89**	**0.88**	**0.47**	**0.85**	**0.85**	**0.88**	**0.77**
$SE_{\mathrm{H}^2_c}$	0.00003	0.00091	0.00892	0.00183	0.24522	0.00003	0.00023	0.01198	0.00064	0.00055	0.00493	0.00247	0.00736	0.13313	0.01959