

RESEARCH ARTICLE

Genetic, epigenetic and metabolite variation in peripheral European Yew (*Taxus baccata* L.) populations at an unexplored part of the species natural distribution

Eleftheria Dalmaris¹✉, Evangelia Avramidou²✉, Eirini Sarrou³, Aliki Xanthopoulou³, Salvatore Multari⁴, Stefan Martens⁴, Filippas A. Aravanopoulos¹*

1 Laboratory of Forest Genetics, Faculty of Agriculture, Forestry and Natural Environment, Aristotle University of Thessaloniki, Thessaloniki, Greece, **2** Laboratory of Forest Genetics and Biotechnology, Institute of Mediterranean Forest Ecosystems, Hellenic Agricultural Organization DIMITRA, Athens, Greece, **3** Institute of Plant Breeding and Genetic Resources, Hellenic Agricultural Organization DIMITRA, Thessaloniki, Greece, **4** Centro Ricerca e Innovazione, Fondazione Edmund Mach, San Michele all'Adige, Trento, Italy

✉ These authors contributed equally to this work

* aravanop@for.auth.gr



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Abstract

Taxanes form effective anticancer agents, which are found in the leaves and bark of the yew tree (*Taxus* L.). Paclitaxel (Taxol®) and related taxanes are widely used in cancer therapy. Due to the high demand of taxanes, there is strong pharmaceutical interest in evaluating unexplored population diversity as a potential genetic and biochemical resource. Three peripheral Greek *Taxus baccata* L. populations (Mt Cholomon, Mt Olympus and Mt Vourinos) were investigated to assess genetic (microsatellite markers), epigenetic (methylation sensitive amplified markers) and chemodiversity (targeted LC-MS/MS analysis of five major taxanes) variation. Taxane concentration varied significantly among populations and seasons. The dominant compound in needles was 10-deacetylbaccatin III (DAB), ranging from 267.8 (Mt Vourinos) to 517.6 (Mt Olympus) mg kg⁻¹ dw. Substantial genetic diversity (AR=5.00; He=0.537) and significant population differentiation (Fst=0.153) were detected, while epigenetic analyses showed moderate haploid epigenetic diversity (H_{epi}=0.051) and comparable levels of DNA methylation across populations. Multivariate analyses indicated clear population structuring in genetic and metabolomic profiles, whereas epigenetic variation was less strongly structured. Together, these results demonstrate pronounced spatial and seasonal variation in taxane production, alongside considerable genetic differentiation, and sufficient levels of total methylation, suggesting a potential capacity for responses to future climatic change. Our findings highlight peripheral Greek populations as valuable genetic resources for conservation and breeding aimed at sustainable taxane production.

manuscript be published. The respective links are provided below: Metabolite Data: <https://zenodo.org/records/18107976> SSR data: <https://zenodo.org/records/18083581> MSAP data: <https://zenodo.org/records/18093836>.

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Introduction

Paclitaxel, the active ingredient of Taxol®, is a bioactive compound found in the foliage and bark of several *Taxus* (yew) species [1]. The poisonous activity of the yew tree was described by Dioscorides, Pliny the Elder, Galen and Julius Cesar who reported the “fortuitous accidents brought about by this poison [2]. Shakespeare mentions the “magic power” of the yew tree in “Macbeth” (Macbeth, Act IV, Scene 1c). Paclitaxel is one of the three most used chemotherapeutic agents with global demand continuing to increase [3,4]. Paclitaxel biosynthesis involves a complex multigene pathway [5], recently resolved at the genomic level [6]. Originally paclitaxel was extracted from the bark of the Pacific yew tree (*Taxus brevifolia*), which proved to be unsustainable, as harvesting the bark ultimately kills the tree, while large amounts of bark are needed for small amounts of taxol [2,7]. Although it is possible to chemically synthesize paclitaxel, the method is considered very expensive [8,9]. Other taxanes, such as baccatin III and 10-deacetylbaccatin III have been identified as important precursors of paclitaxel and are used for paclitaxel semi-synthesis [10,11]. Taxane concentrations vary widely among species, populations, individuals, tissues and seasons, with reported values spanning several orders of magnitude [9,12–20].

The European yew (*Taxus baccata*), a dioecious conifer with a scattered distribution in Europe and the Mediterranean, is currently a principal source for sustainable paclitaxel production via semi-synthesis [7,21,22]. To increase environmental sustainability and economic feasibility alternative taxol production methods are investigated, including artificial cultivation in plantations [23–25]. Factors like geographical location, climate, and glaciation have influenced the genetic structure of *T. baccata* [26]. Results indicate a reduction of genetic diversity and an increase of genetic differentiation moving from northwest to southeast [27]. Furthermore, DNA methylation studies in *T. baccata*, a prerequisite for understanding plasticity, phenotypic variation, and adaptation to ongoing climatic change [28–31], have been implicated in the regulation of paclitaxel biosynthesis [32]. Beyond variation in the genome and epigenome levels, chemodiversity across species and populations has also been seen. For instance, in three *Taxus* species, including *T. baccata*, distinct profiles were found by analyzing about 2,250 metabolites across various pathways, which emphasizes the genetic basis of chemodiversity and its impact on taxane biosynthesis [33]. Therefore, an integrated investigation of the multifaceted genetic, epigenetic and chemodiversity patterns in unexplored marginal and peripheral *T. baccata* populations, is essential for understanding the biological basis of taxane variability and identifying invaluable genetic resources for sustainable paclitaxel production.

In parallel, *T. baccata* has one of the highest declining rates in Europe resulting in fragmented and marginal small populations, some of which are becoming locally extinct [34]. The European Union has designated specially protected areas [Habitats Directive 92/43/EEC; 35], but in Greece, the protection of the species is limited, since only two populations were identified as a priority habitat type (code 9580: “Mountainous coniferous woods with *Taxus baccata*”). Greece lies at the south-eastern margin of the species’ natural distribution and hosts fragmented rear-edge populations that likely persisted through Pleistocene climatic fluctuations. These

populations span steep gradients of altitude temperature and water availability, which shape genetic structure, as well as epigenetic and metabolite variation. Accordingly, these populations constitute an informative system for integrative studies with comprehensive diversity assessments being essential for identifying genetic resources relevant for paclitaxel production and for guiding *in situ* and *ex situ* conservation strategies.

The objective of this study was to characterize diversity at the genetic, epigenetic and metabolite levels in peripheral Greek populations of *T. baccata*. In particular we quantified within and between population chemodiversity focusing on five main taxanes (paclitaxel, 10-deacetylbaccatin III, baccatin III, 10-deacetyltaxol, and cephalomannine), assessed seasonal variation in paclitaxel, 10-deacetylbaccatin III, and baccatin III, evaluated the extent and structure of genetic diversity within and among populations, and characterized population-level DNA methylation patterns as a measure of epigenetic variation. We further integrated genetic, epigenetic and metabolite datasets to examine their relationships and relevance for *in situ* and *ex situ* conservation strategies.

Based on these objectives we tested the following null hypotheses: (a) taxane concentrations do not differ significantly within and among peripheral Greek populations of *T. baccata*, (b) paclitaxel, 10-deacetylbaccatin III, and baccatin III do not exhibit significant seasonal variation, (c) peripheral populations' genetic diversity is within the range reported for central ones, (d) genetic diversity is not significantly structured within and among populations, (e) DNA methylation patterns do not significantly differ within and among populations, and (f) taxane chemodiversity is not significantly associated with genetic or epigenetic variation.

Materials and methods

Experimental sites and collections

Leaf tissue was collected from healthy *T. baccata* individuals. Sampling was random while maintaining a minimum of 25 m distance between trees to avoid sampling filial structures. Three natural populations were sampled: (1) Mt Cholomon (Chalkidiki, N=27), (2) Mt Olympus (Pieria, N=29) and (3) Mt Vourinos (Kozani, N=38; Fig 1 and S1 Table). Needle samples were collected at 2m height from north-facing and well shaded branches. From each tree, tree height and diameter on breast height (DBH; S1 Fig) were recorded. Needle collection was conducted in three different seasons: (1) in early May, after the end of the flowering period (spring); (2) in late August, at the end of the growing season (summer) and (3) in early December, at the beginning of the winter period (winter).

Metabolomic analysis

Chemicals. Laboratory reagents were purchased from Sigma-Aldrich (Milan, Italy) and Fisher Scientific (Milan, Italy). Deionized water was purified *in loco* with the Arium® purification system (Sartorius AG, Goettingen, Germany). Authentic standards of taxanes, namely paclitaxel (PAC), 10-deacetylbaccatin III (DAB), baccatin III (BAC), 10-deacetyltaxol (DEAC), and cephalomannine (CEPH), were purchased from TransMIT (Gießen, Germany). Stock solutions of taxanes were prepared by dissolving the standards in methanol (LC-MS grade).

Taxanes extraction. The extraction of taxanes was performed according to the method of Mubeen et al. [36] with some modifications. The freshly collected needles were freeze-dried (Freeze-dryer Alpha 1–2 LD plus, Christ, Germany; at –24 °C) and milled using a laboratory Mill IKA A11 into homogenous powder. Samples (0.5 g) were mixed with 4 mL of 60% acetone, stirred on an orbital shaker for 20 mins, and centrifuged (5 min; 1800 × g; 4 °C) (procedure repeated thrice). The supernatants were pooled, and acetone was evaporated under reduced pressure at T ≤ 38 °C. The remaining aqueous phase was mixed with 4 mL of dichloromethane. The mixture was vortexed for about 1 min and centrifuged (2 min; 900 × g; 4 °C) (procedure repeated twice). The organic layers were collected and dried under reduced pressure at T ≤ 38 °C. The dry residue was dissolved in 2 mL of LC-MS methanol, filtered through PTFE 0.22 µm membranes into glass vials, and stored at –80 °C until further analysis. Results are expressed as mg kg^{–1} dw (dry weight).

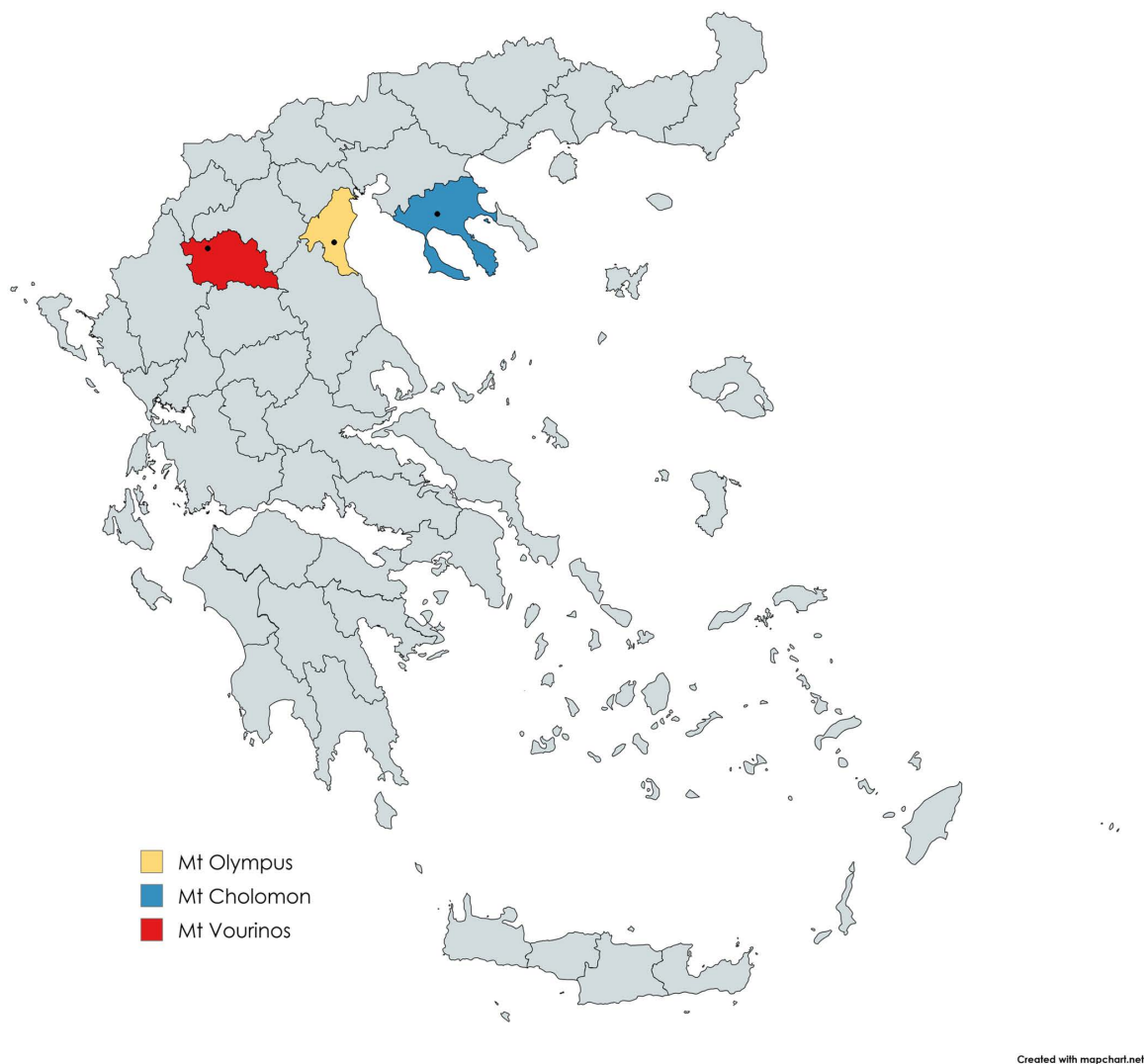


Fig 1. Locations of studied populations of *T. baccata* in Greece. Reprinted from MapChart (<https://www.mapchart.net/greece.html>) under a CC BY license, with permission from Minas Giannakas, founder and developer of MapChart, original copyright 2025).

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Ultra performance liquid chromatography mass spectrometry (UPLC-MS/MS). The UPLC-MS analysis was adapted from Prokopowicz et al. [37] and performed on a UHPLC Dionex 3000 (Thermo Fisher Scientific Germany), equipped with a binary pump, an online vacuum degasser, and a column compartment. The separation of taxanes was carried out on a Kinetex C18 100Å column (2.1 mm × 100 mm, 2.6 μ), equipped with a column guard (Phenomenex, U.S.A.), kept at 35°C. Samples were injected using an autosampler (Dionex Thermo Fisher Scientific Germany) set at 4°C. The mobile phase was composed of water with 0.1% v/v formic acid (A) and acetonitrile with 0.1% v/v formic acid (B). Separation was carried out as follows: 80% A (0–0.5 min), 80–50% A (0.5–7 min), 50–28% A (7–10 min), 20–0% A (10–10.2 min), 0% A (10.2–12 min), 0–80% A (12.1–15 min). The flow rate was 0.3 mL min⁻¹ and the injection volume was 5 μL.

The MS/MS analysis was performed on an API 5500 triple-quadrupole mass spectrometer (Applied Biosystems/MDS Sciex, Toronto, Canada). The instrument was operated using an electrospray source in positive ion mode. The ESI parameters were as follows: the spray voltage was set at 5500 V for positive mode, the source temperature was set at 250 °C,

the nebulizer gas (Gas 1) and heater gas (Gas 2) at 40 and 20 psi respectively. UHP nitrogen (99.999%) was used as both curtain and collision gas (CAD) at 20 and 9 psi respectively.) Analyst™ software version 1.6.1 (Applera Corporation, Norwalk, CT, USA) was used for instrument control and data acquisition. Compounds were identified by comparing the retention time and the spectral characteristic of the peaks with those of authentic standards. Multiple reactions monitoring (MRM) was used for quantification based on the peak area of the samples. The limit of detection (LOD) and limit of quantification (LOQ) were identified (Table 1), and calibration curves of the five targeted compounds were injected using external standards according to Dalmaris et al. [38] (Table 1).

Microsatellite analysis

Twelve microsatellite loci were selected for genotyping: TAX23, TAX36, TAX60, TAX31, TAX09, TAX50, TAX26, TAX92, and TAX86 described by Dubreuil et al. [39], and: TB56, TB58, TB40 described by Mahmoodi et al. [40]. Polymerase chain reaction (PCR) conditions followed Dubreuil et al. [39] and Mahmoodi et al. [40], respectively. The following conditions were used according to Dubreuil et al. [39]: 5 min at 94° C followed by 10 touchdown cycles of 30s at 94° C, 40s at 60° C (1 C lower per cycle) and 40s at 72° C, and 25 cycles of 30s at 94° C, 30s at 50 C and 40s at 72° C, with a final extension step of 7 min at 72° C. According to Mahmoodi et al. [40], the following reaction conditions were used: 5 min at 95° C, followed by ten touchdown cycles of 30s at 95° C, 45s at 60° C (1 C lower per cycle) and 40s at 72° C, and 25 cycles of 30s at 95° C, 30s at 50° C and 40s at 72° C, with a final extension step of 7 min at 72° C. One µL of PCR product was added to 10 µL HiDi™ formamide (Applied Biosystems) and 0.15 µL 500 ROX Size Standard (Applied Biosystems), and capillary electrophoresis was run on an ABI3730 DNA Analyzer (Applied Biosystems). Results were analyzed using the GeneMapper Software v4.1 (Life Technologies), and allelic profiles were scored by automatic binning and visual checking.

MSAP analysis

For the MSAP assay, double digests were initially performed using a combination of either *EcoRI/HpaII*, or *EcoRI/MspI* restriction enzymes. Digestion of 200-ng aliquots of genomic DNA was carried out in 20 µL containing 1X Cut smart buffer, 4 U *EcoRI* (New England, Biolabs) and 4 U of either *HpaII* or *MspI* enzyme (New England, Biolabs) for 3 h at 37 °C. Two different adaptors designed to avoid reconstruction of restriction sites, one for the *EcoRI* sticky ends and one for the *HpaII/MspI* sticky ends, were ligated to the DNA after digestion, by adding to each final digestion 5 µL of a mix containing 5 pmol of *EcoRI* adapter, 50pmol of *HpaII/MspI* adapter, 1 mM ATP, 1X “One-for-All” Buffer, and 1 U of T4 DNA ligase (Invitrogen). The ligation mixture was incubated for 3 h at 25°C (S2 Table). Digested and ligated DNA fragments were diluted 5-fold and used as templates for the pre-selective amplification reaction. Pre-amplification reactions were performed using either *MspI/HpaII*-primers in a total volume of 2 µL containing 1X Kapa Taq Buffer, 0.2mM of each

Table 1. Multiple reactions monitoring (MRM) and quantification parameters of the five taxanes infused to the mass for optimization.

Compound	RT ⁽¹⁾ (min)	R ² (2)	ES ⁽³⁾	Molecular ion (m/z)	Quantifier ion	Qualifier ions		LOD ⁽⁴⁾ mg/mL	LOQ ⁽⁵⁾ mg/mL	Slope	Sigma
					Q1 m/z	Q1 m/z	Q2 m/z				
10-deacetylbaicatin III	6.10	0.9955	+	545	105	207	171	0.00013310	0.00040330	245400000	9899.495
baicatin III	6.91	0.9987	+	587	105	121	177	0.00020630	0.00062530	30530000	1909.188
10-deacetyltaol	7.66	0.9964	+	812	286	105	122	0.00001510	0.00004577	15450000000	70710.678
Cephalommanine	9.57	0.9967	+	832	264	105	83	0.00042610	0.00129100	1424000000	183847.763
Paclitaxel	9.81	0.9953	+	854	105	286	77	0.00002692	0.00008159	866700000	7071.068

(1) RT: Retention time; (2) R²: Regression; (3) ES: Electrospray ionization mode; (4) LOD: Limit of detection; (5) LOQ: Limit of quantification

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dNTP, 2.5 mM MgCl₂, 30 ng of each primer *EcoRI*+A, *MspI*//*HPaI*+A, 1 U Taq DNA polymerase (Kapa Biosystems), and 5 µL of diluted fragments (from the digestion and ligation reaction). Subsequently, pre-amplified fragments were diluted 10-fold and used as templates for the selective amplifications. For the selective amplification, only the *EcoRI* primers were labeled. Primer combinations employed and their sequences are shown in [S2 Table](#). Selective PCR were performed in a 10 µL total volume of 1X Kapa Taq Buffer, 2.5 mM MgCl₂, 0.08 mM of each dNTP, 5 ng of labeled *EcoRI* primer, 30 ng of *HpaI*//*MspI* primer, 1 U of Taq DNA polymerase (Kapa Biosystems), and 3 µL of diluted pre-amplified DNA. The cycling program was as follows: initially, a brief 30 s held at 94° C was implemented, followed by 23 cycles of 94° C for 30 s, 56° C for 30 s, and 72° C for 1 min, followed by a final hold at 72° C for 30 min. A 5 µL aliquot of the reaction was electrophoresed on agarose (1.25% w/v + ethidium bromide) to verify amplification; the remaining 15 µL was diluted 10-fold with TE. Selective amplifications were carried out in 10 µL total volumes containing 5 µL of diluted pre-selective template and 0.2 dNTP mix (10 mM), 2.5 mM MgCl₂, 30 ng of *HpaI*//*MspI* primer, 30 ng of *EcoRI* primers and 1 U of Taq DNA polymerase (Kapa Biosystems) per reaction. Selective amplification cycling was performed according to the following program: an initial cycle of 94° C for 30 s, 65° C for 30 s, 72° C for 1 min, and then twelve cycles of 94° C for 30 s, with an annealing temperature starting at 65° C for 30 s, but decreasing by 0.70° C for each cycle, 72° C for 1 min, and finally, 22 cycles of 94° C for 30 s, 56° C for 30 s, 72° C for 1 min, and a final hold at 72° C for 30 min. Eight primer combinations were employed during the selective amplification stage. The whole experiment was repeated twice to only retain for further processing fully reproducible MSAP bands.

Statistical analysis

Metabolites

Analysis was processed using the R package [\[41\]](#). All data were checked for normality (by graphical data visualization and the Shapiro-Wilk test), and homogeneity of variances (by graphical data visualization and Levene's test). A two-way-ANOVA analysis was performed to assess the main effects of the two independent variables and their interactions. Where significant effects were detected, post hoc tests were conducted with Tukey's HSD test method to identify differences between group means while controlling for multiple comparisons. A Biplot PCA analysis of metabolite profiling data derived from samples that were collected during the spring period performed using the XLSTAT statistical software (Version 2014.5.03; Addinsoft Inc., Brooklyn, NY, USA).

Microsatellites

Measures of intra-population and inter-population genetic parameters, number of observed (*N_a*), and effective (*N_e*) alleles; observed (*H_o*) and expected (*H_e*) heterozygosity [\[42\]](#), were calculated using the GENEALEx 6.5 software [\[43,44\]](#). The inbreeding coefficient (*F_{is}*) for each population and each locus was obtained by computing a hierarchical AMOVA using the ARLEQUIN 3.5.2.2 software [\[45\]](#). Statistical significance was determined by a non-parametric approach using 1000 permutations. As the assessment of allelic richness by the measure of allele frequencies needs to take into account the variation in population sizes, allelic richness (*A*) and the number of private alleles (*Pa*) were computed using the rarefaction method with HP-RARE software [\[46\]](#).

Analysis of molecular variance (AMOVA) to partition genetic variance was analysed in Arlequin 3.11. Principal coordinate analysis (PCoA) to analyze genetic structure by a covariance standardized approach of pairwise [\[47\]](#) genetic distances was conducted in GenAlEx version 6.5. The relationships among populations were initially investigated by an unweighted pair group method using arithmetic means (UPGMA) dendrograms based on Nei's [\[47\]](#) genetic distances. Nei's genetic distance was calculated in GenAlEx v. 6.5. FigTree v1.3.1 was used to visualize and edit the tree. To delineate the genetic repartition of yew populations' structure, a principal coordinate analysis (PCoA) was performed based on Nei's unbiased genetic distance [\[42\]](#) using the GENEALEx 6.5 software.

To determine the genetic groups among populations, we used Bayesian clustering method in STRUCTURE V2.3.4. This analysis was run at 5 independent runs per K Value (K1–8) with a burn-in period of 100,000 iterations and 100,000 Markov chain Monte Carlo (MCMC). Structure Harvester was used to visualize the best K value based on delta K (ΔK). We used Mantel tests to determine the pattern of isolation by distance at 1000 permutations using GenAlEx version 6.537. The probability of identity (P_{ID}) was estimated in GenAlEx [43] to evaluate the discriminatory power of each locus. Deviations from Hardy-Weinberg equilibrium (HWD) were estimated using Genepop v4.2 [48].

MSAP

An AFLP Excel Macro [49] was used to convert allele size data from GeneMapper4.0 (Applied Biosystems, USA) into binary form and to indicate the presence “1” or absence “0” of fragments. Only reproducible fragments ranging from 150 to 500 bases were counted and further analyzed in order to reduce the impact of potential size homoplasy [50]. For MSAP analyses, comparison of the banding patterns of *EcoRI/HpaII* and *EcoRI/MspI* reactions, results in four conditions of a particular fragment: I: fragments present in both profiles (1/1), indicating an unmethylated state (n subepiloci); II: fragments present only in *EcoRI/MspI* profiles (0/1), indicating hemi or fully methylated CG-sites (h-subepiloci); III: fragments present only in *EcoRI/HpaII* profiles (1/0), indicating hemimethylated CHG-sites (m-subepiloci); and IV: absence of fragments in both profiles (0/0), representing an uninformative state caused either by different types of methylation, or due to restriction site polymorphism [51]. To separate unmethylated and methylated fragments and to test for the particular impact of the methylated conditions II and III, we used the ‘Mixed-Scoring 2’ approach [52].

Epigenetic diversity within populations was quantified using the R script MSAP_calc.r [52] as: (i) number of total and private bands (polymorphic subepiloci), (ii) percentage of polymorphic subepiloci (P_{epi}) and (iii) mean Shannon’s information index (I_{epi}). GenAlEx 6 [43] was employed to compute haploid epigenetic diversity (H_{epi}) within populations. The significance of population differences were assessed by Kruskal–Wallis tests as epigenetic diversity metrics derived from dominant markers often violate assumptions of normality and homoscedasticity. GenAlEx was also used to conduct Analysis of Molecular Variance (AMOVA) – separately for each subepiloci class – to study the variation of CCGG methylation states (epiloci) among the five populations. A series of Principal Coordinate Analyses (PCoA) were employed to assess the ordination in multivariate space revealed by different types of epiloci.

Results

Dendrometric and climatic variables

The Mt Olympus population presented the tallest trees (7.9–31.4 m) and the largest DBH (35–115 cm) of all populations (S1 Fig), while the Mt Vourinos population had the shortest individuals (0.4–11 m) with the smallest DBH (0.2–20 cm) (S1 Fig). The latter population is grown in an area that presents the highest altitude and lowest annual precipitation of all populations (S1 Table). The other two populations grow at similar altitudes and precipitation levels.

Identification and seasonal variation of taxanes in *T. baccata* needles

A targeted LC-MS/MS method was adopted for the determination of the content of five taxanes, PAC, DAB, BAC, DEAC and CEPH. The phytochemical analysis conducted on an individual tree basis and the respective concentrations of the three populations are presented in S3 Table. Metabolite data from two populations (Mt Olympus and Mt Cholomon) have already been published [38]. Based on spring-collected samples, DAB is the main taxane compound, with concentrations varying from 267.8 (Mt Vourinos) to 517.6 (Mt Olympus) mg kg⁻¹ dw. On the contrary, PAC, BAC, CEPH, and DEAC were detected in the extracts at lower amounts (12.9–24.4 mg kg⁻¹ dw, 0.1–10.1 mg kg⁻¹ dw, 11.8–16.2 mg kg⁻¹ dw and 11.5–22.7 mg kg⁻¹ dw, respectively). Significant differences were observed between populations (Table 2 and S4 Table). According to the Biplot PCA analysis (Fig 2), the first two components provided a very strong resolution and explained 73.79% of

Table 2. Statistical comparison of the *T. baccata* populations and Tukey HSD pairwise multiple comparison test.

(1) Significance after a two-way ANOVA			
	DAB	PAC	BAC
Season	***	**	***
Pop	***	***	***
Season * Pop	**	*	***
(2) Tukey HSD pairwise multiple comparison test			
i) For the Season effect			
	DAB	PAC	BAC
Spring	A	A	A
Summer	B	A	B
Winter	B	B	C
ii) For the Population			
	DAB	PAC	BAC
Mt Olympus	A	A	B
Mt Cholomon	A	B	A
Mt Vourinos	B	C	B

(1) Statistical comparison of the selected *T. baccata* populations for each of the three taxanes (DAB: 10 Deacetylbaecatin III, PAC: Paclitaxel, BAC: Baccatin III) after a two-way ANOVA with Populations (3 levels; Mt Olympus, Mt Cholomon and Mt Vourinos), Season (3 levels; Spring, Summer and Winter). Significant differences are represented with asterisks for: *** $p \leq 0.001$, ** $p \leq 0.01$ and * $p \leq 0.05$. (2) Populations and Season showed significant differences in all the measured compounds and therefore Tukey HSD pairwise multiple comparison tests were performed for the: (i) Season and (ii) Population. Sampling period or population with a different uppercase letter are significantly different as determined by Tukey HSD pairwise multiple comparison test for $p \leq 0.05$.

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the total variance. Variables PAC, DEAC and CEPH showed strong positive loadings in PC1 while BAC loaded negatively showing an opposing contribution. PC2 was mainly associated with positive loadings for BAC and DAB, whereas CEPH contributed negatively. Population clustering was consistent with these loadings, although no single compound acted as a strict population discriminator. Individuals from Mt Cholomon and Mt Vourinos populations clustered closer, while individuals of Mt Olympus revealed much higher dispersion (Fig 2). In addition, none of the five taxanes determined in the samples, could characterize the populations as a discriminator compound. However, considering that: (i) DAB was the most predominant compound detected in the needle's samples, (ii) PAC is the commercially valuable bioactive compound, and (iii) BAC serves as a precursor for the semi-synthesis of PAC, the investigation was carried out by focusing on these three taxanes out of the five originally analyzed.

The concentration of DAB, PAC and BAC showed a significant seasonal variation (DAB and BAC $p \leq 0.001$; PAC p -values ≤ 0.01 ; Fig 3, Table 2). The concentration of DAB differed significantly in spring samples in comparison to summer and winter ones, whereas PAC content in winter-collected samples varied significantly from spring and summer collection (post-hoc test, $p \leq 0.05$; Fig 3 and Table 2). Lastly, BAC concentration showed to be significantly different amongst all sampling periods (post-hoc test, $p \leq 0.05$; Fig 3 and Table 2). Besides seasonal variation, significant population variation was apparent as well (for all metabolites $p \leq 0.001$; Table 2). Samples from Mt Cholomon represented significantly higher concentrations for all three taxanes compared to samples from Mt Vourinos, whereas the BAC content was significantly higher compared only with Mt Olympus samples. The concentrations of DAB and PAC were significantly higher in samples from Mt Olympus compared to samples from Mt Vourinos (post-hoc test, $p \leq 0.05$; Fig 3 and Table 2).

The concentrations of all taxanes were categorized in three dissimilar patterns across populations and sampling periods (significant Season*Pop interactions; DAB $p \leq 0.01$; PAC $p \leq 0.05$; BAC $p \leq 0.001$), Table 2, but none of the taxanes

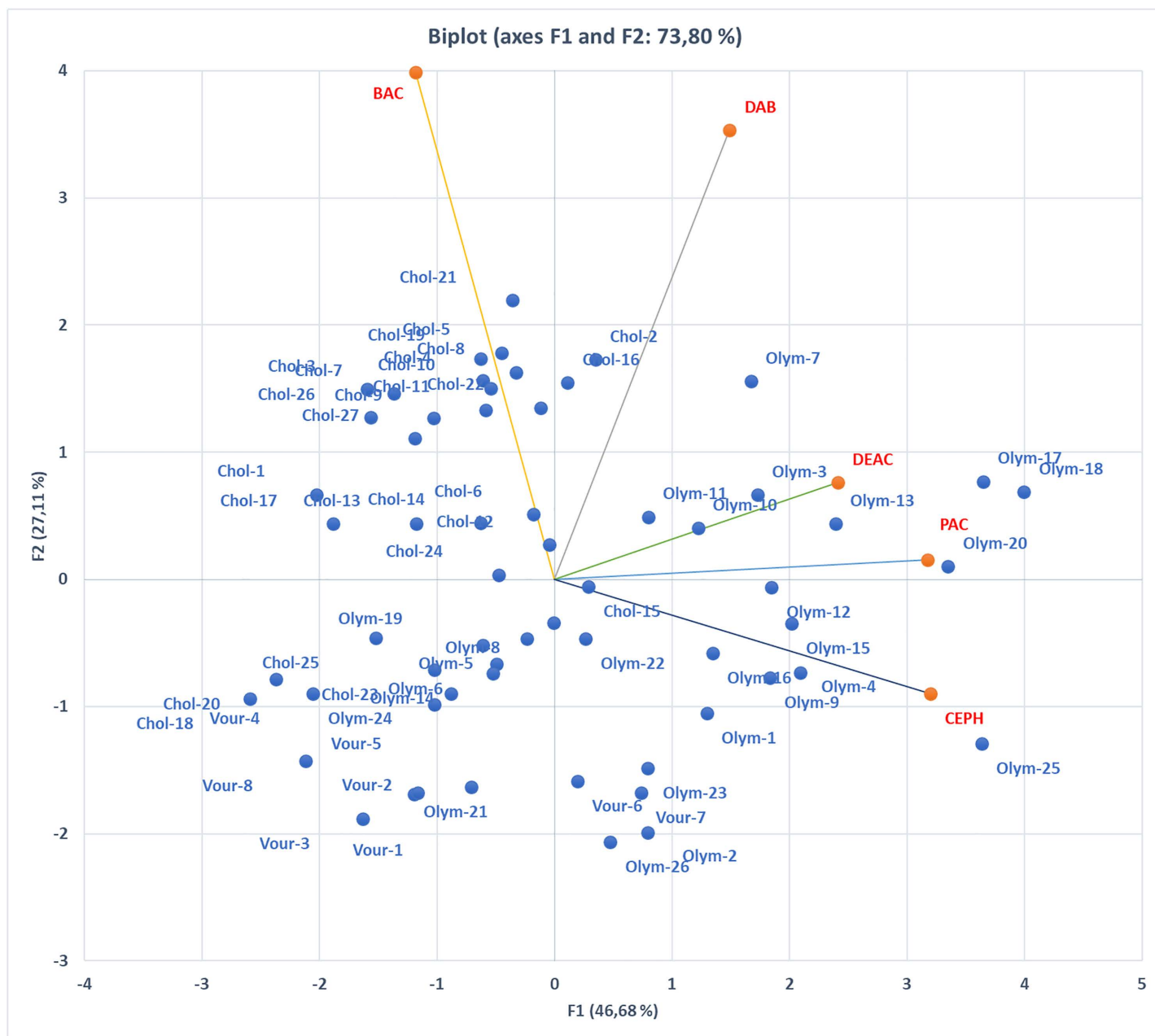


Fig 2. PCA Biplot of LC-MS/MS analysis of taxane metabolite analysis for the *T. baccata* populations of Mt. Olympus, Mt Cholomon, and Mt Vourinos, based on the samples that were collected during the spring period.

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followed a particular trend. The PAC content from Mt Olympus and Mt Cholomon samples and the BAC content from Mt Vourinos samples showed an increase in their concentration from spring to winter. On the other contrary, DAB concentration from Mt Olympus and Mt Vourinos samples, and BAC concentration from Mt Cholomon samples showed a decreasing trend from spring to winter. Lastly, the concentrations of PAC from Mt Vourinos, DAB from Mt Cholomon and BAC from Mt Olympus samples decreased from spring to summer, followed by an increase in the winter (Fig 3 and S3 Table).

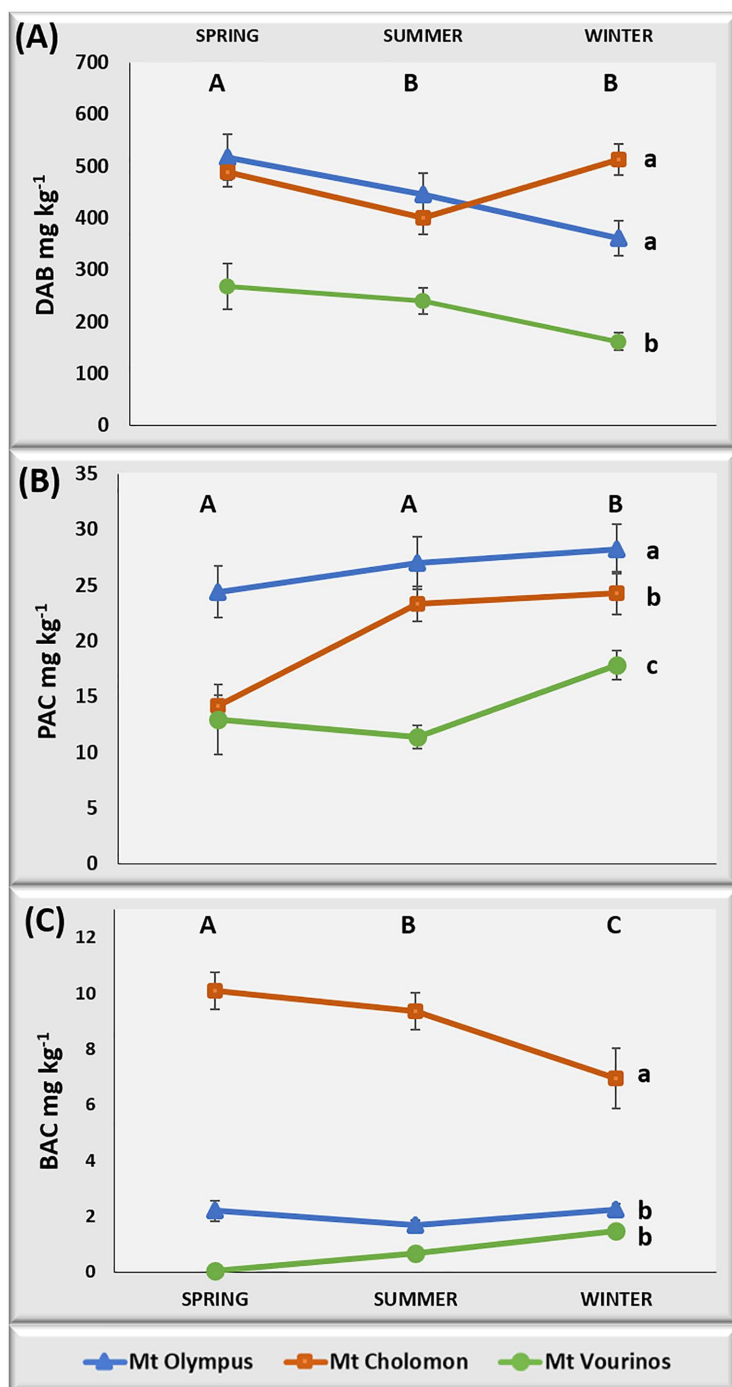


Fig 3. Seasonal variation of Paclitaxel, 10-Deacetylbaccatin III and Baccatin III in dry needles of *T. baccata* populations. (A) Paclitaxel (PAC), (B) 10-Deacetylbaccatin III (DAB), and (C) Baccatin III (BAC). Symbols represent the average of each population for the different compounds. Standard error bars represent variation within each of the populations. Sampling periods with a different uppercase (at top of each figure) and populations with a lowercase (at the side of each figure) letter are significantly different as determined by Tukey HSD pairwise multiple comparison test for $p \leq 0.05$.

<https://doi.org/10.1371/journal.pone.0324582.g003>

Overall, DAB was consistently the most abundant taxane, with the highest concentrations recorded in spring in the Mt Olympus population (517.5 mg kg⁻¹ dw; [S3 Table](#)). The highest PAC concentration was found also in Mt Olympus during the winter collection (28.3 mg kg⁻¹ dw; [S3 Table](#)). Lastly, the maximum BAC concentration was found in Mt Cholomon samples during spring (10.1 mg kg⁻¹ dw; [S3 Table](#)).

Genetic variation of *T. baccata* populations

A total of 94 alleles were observed across the 12 SSR loci studied. The number of alleles per locus ranged from three (TB56) to 17 (TAX50), with a mean value of $A = 7.83$ ([Table 3](#)). The number of alleles per locus independent of sample size AR (the allelic richness) ranged from 4.167 (Mt Cholomon) to 6.083 (Mt Olympus) with a mean value of 5.000 ([Table 3](#)). Private alleles were also detected: private allelic richness (pAR) ranged from 0.583 to 1.500 with a mean 1.000 ([Table 3](#)), and on the average (pAR) amounted to about 15% of total AR. Observed heterozygosity ranged from 0.262 to 0.348 with a mean value of 0.299 for the three populations ([Table 3](#)). The average expected heterozygosity (He) was highest in Mt Olympus (0.612) and lowest in Mt Cholomon (0.441; [Table 3](#)). The probability of identity (P_{ID}) was estimated at 1.6×10^{-6} for the Mt Cholomon samples and at 3.5×10^{-10} for the Mt Olympus genotypes whereas total P_{ID} was 1.5×10^{-10} ([Table 3](#)). The inbreeding coefficient (Fis) ranged from 0.254 (TB40) to 1.000 (TAX36) with a mean value of 0.443 ([S5 Table](#)). At four loci (TAX23, TAX36, TAX09 and TAX50) the Fis value deviated significantly from zero ([S5 Table](#)).

Global genetic differentiation across populations was estimated as F_{ST} and Gst (0.153 and 0.138, respectively, $p < 0.001$; [S5 Table](#)). F_{ST} parameters varied among SSR loci, ranging from 0.004 (TB40) to 0.498 (TAX36; [S5 Table](#)). All pairwise Fst and Gst values were highly significant ($p < 0.001$; [S6 Table](#)). Gene differentiation coefficients (Gst, Fst) suggested significant population differentiation (moderately high Fst values, [S6 Table](#)). The AMOVA of the SSR data set produced congruent results (significant Φ statistics; $p < 0.001$, [Table 4](#)). AMOVA also revealed a high percentage of variation due to population subdivision (76%). Furthermore, the inter-population AMOVA showed highly significant ($p < 0.001$) genetic differences among populations. Thus, AMOVA ($\Phi_{ST} = 0.243$; [Table 4](#)) also supports the results of F- and G-statistics ([S5 Table](#)). Furthermore, deviations from the Hardy-Weinberg equilibrium were found for all populations in many of the studied loci ([S7 Table](#)).

Table 3. Genetic diversity parameters of the *T. baccata* populations studied.

Population	Number of alleles (N)	Allelic Richness (AR)	Private Allelic Richness (pAR)	Effective Number of Alleles (Ne)	Percentage of polymorphism (P)	Shannon index (I)	Observed Heterozygosity (Ho)	Expected heterozygosity (He)	Fixation index (F)	Probability of identity P(ID)
Mt Cholomon	27.000	4.167	0.917	2.031	100	0.847	0.262	0.441	0.479	1.6×10^{-6}
Mt Olympus	29.000	6.083	1.500	3.349	100	1.270	0.348	0.612	0.406	3.5×10^{-10}
Mt Vourinos	38.000	4.750	0.583	2.648	100	1.077	0.287	0.558	0.432	1.5×10^{-8}
Mean	31.333	5.000	1.000	2.676	100	1.064	0.299	0.537	0.439	Total 1.5×10^{-10}

<https://doi.org/10.1371/journal.pone.0324582.t003>

Table 4. Analysis of molecular variance of the *T. baccata* populations based on SSR markers.

Source	Df	SS	MS	Est. Var.	Percentage	Φ_{ST}
Among Populations	2	212.109	106.055	3.111	24%	
Within Populations	91	883.423	9.708	9.708	76%	0.243
Total	93	1095.532		12.819	100%	$p < 0.001$

<https://doi.org/10.1371/journal.pone.0324582.t004>

Relationships among populations were illustrated by a UPGMA Nei's [47] genetic distance dendrogram, which grouped populations into two main clusters (Fig 4). This finding was supported by PCoA (Fig 5) results which depicted two major clusters in the first two principal coordinates that accounted for 27.02% of the total variation (Fig 5). Population samples were distributed distinctly on the ordination space, with the primary separation occurring along the first coordinate. Mt. Cholomon samples were positioned mainly on the positive side of the first coordinate forming one cluster, Mt. Vourinos samples on the negative side and Mt. Olympus samples showing intermediate values with additional separation along the second coordinate. The latter two populations formed a loosely defined cluster.

When a Mantel test was applied, there was a significant but weak correlation between geographic and genetic distances ($p=0.010$, $R^2=0.140$). These results were further corroborated by STRUCTURE analysis which was performed without prior information on the geographic origin of samples. The highest likelihood of the data was obtained for $K=2$. Low levels of admixture were observed in the three populations studied (Fig 6).

Epigenetic variation of *T. baccata* populations

Epi-polymorphism varied among the populations studied (Table 5). The percentage of polymorphic epigenetic bands ranged from 46.16% in Mt Cholomon to 58.97% in Mt Olympus (mean percentage of polymorphic bands 53.21%). The number of private bands diversified from 462 (Mt Cholomon) to 865 (Mt Vourinos) (Table 5). The Shannon index (I_{epi}), based on the frequency of methylation patterns within each marker type ranged from 0.188 (Mt Cholomon) to 0.223 (Mt Vourinos) (mean 0.209; Table 5). The Shannon index values were significantly different among the populations studied (Kruskal–Wallis $\chi^2=24,588$; $p<0.001$). The average index for methylation susceptible polymorphic loci was $I_{epi}=0.209$. Lastly, MSAP mean haploid epigenetic diversity (H_{epi}) was 0.051 and ranged from 0.049 (Mt Cholomon) to 0.053 (Mt Olympus).

AMOVA showed that approximately 86% of the total epigenetic variation was partitioned within populations (Table 6). Considering n-, m- and h-subepiloci separately, Φ_{ST} values were 0.138, 0.133, and 0.132, respectively (Table 6). The relative levels of full methylation (m-subepiloci), hemi-methylation (h-subepiloci) and non-methylation (n-subepiloci) presented an overall mean of 53.74%, 53.36% and 51.19% respectively (Fig 7). The total level of 5'CCGG- methylation (sum of h- and m- subepiloci) ranged from 44% in Mt Cholomon to 62.33% in Mt Vourinos presenting (mean 53.55% across all populations). Population differences of 5'CCGG- methylation were not significant (Kruskal-Wallis $\chi^2=0.622$ $p=0.733$). Pairwise epigenetic F_{ST} values showed that epigenetic differentiation was moderate and ranged from 8.9% to 17.2% (S8 Table). When a Mantel test was applied, no significant relationship was found between the epigenetic distance and the geographical distance ($R^2=0.009$, $p<0.020$).

The PCoA of epigenetic distances revealed varying population differentiation in multivariate space (Fig 8). The PCoA of m- and h- subepiloci failed to separate the populations and explained 21.92% and 20.5% of the total variation respectively. The same pattern was also depicted when all epi-alleles were combined and used for PCoA, where no clear differentiation of populations was evident and 20.32% of the total variation was explained.

Discussion

Metabolomic analyses

This study confirms pronounced spatial and seasonal variation in taxane concentrations, consistent with previous reports [14,16,17,53]. The variance observed in the dendrometric and climatic variables of the populations under study (S1 Table), contributed to this result. Variation in taxane accumulation reflects both biological and environmental factors, complicating direct comparisons among studies [9,54]. Overall, the concentrations of DAB, PAC, and BAC follow three different trends over the three collection periods (Fig 3). Similar results indicating variation and different seasonal fluctuations throughout the year have been reported in several studies [14,16,17], whereas in other studies DAB, PAC, and BAC needle concentration reached their maximum at the same period, e.g., spring for *T. wallichiana* [15] or winter for *T. wallichiana* var. *mairei* [55].

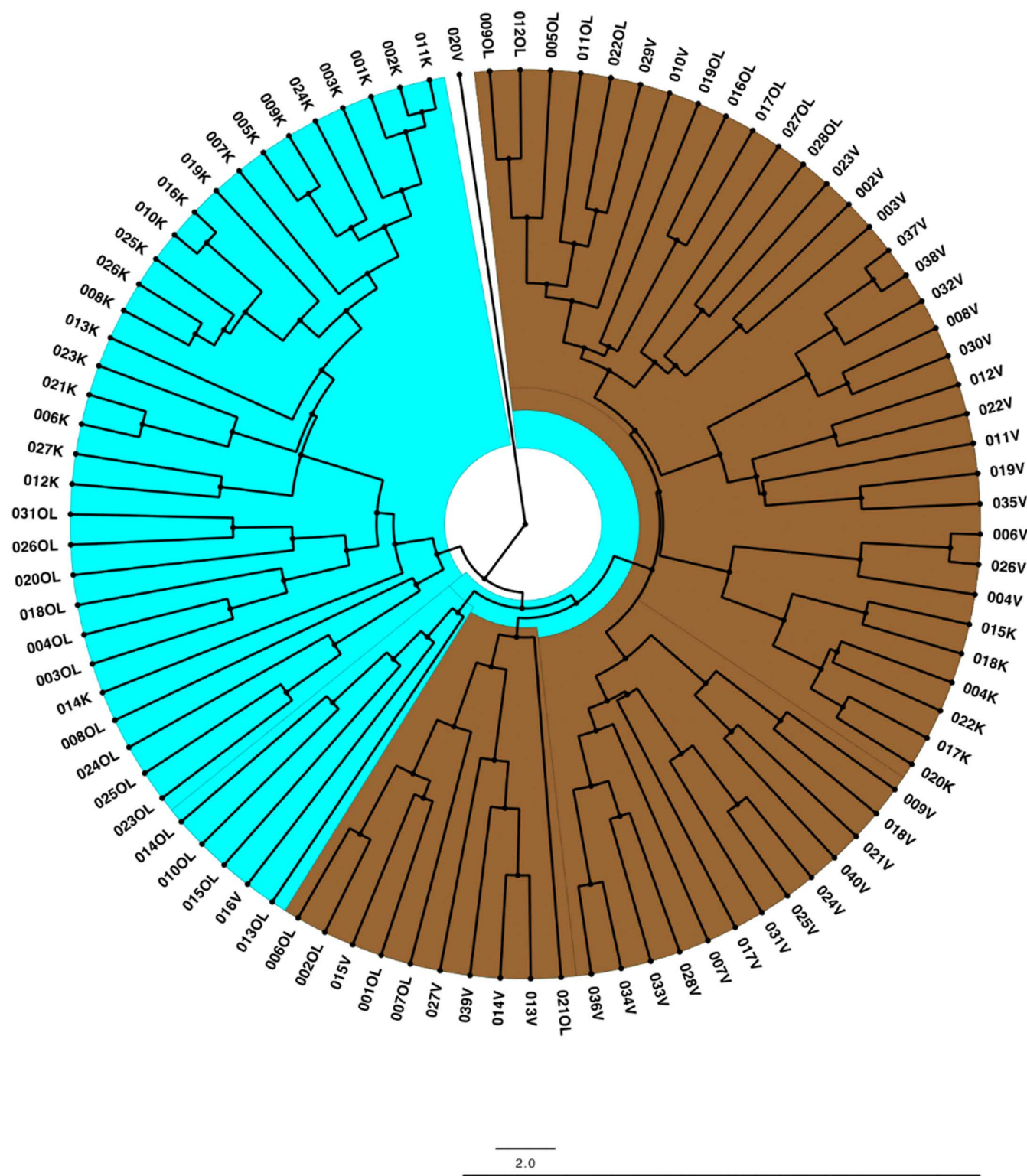


Fig 4. Genetic relationships of *T. baccata* populations based on SSR markers. Unweighted pair-group method using arithmetic average (UPGMA) unrooted tree illustrating genetic relationships among 94 individuals of *T. baccata* analyzed with 12 SSR loci. Samples included are indicated after population name.

<https://doi.org/10.1371/journal.pone.0324582.g004>

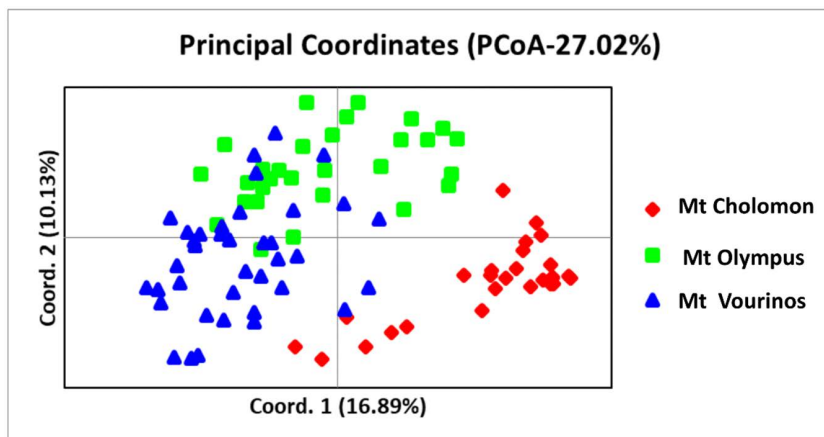


Fig 5. Principal coordinate analysis (PCoA) of 94 individuals in the *T. baccata* studied, based on 12 polymorphic SSR loci. A total of 27.02% of total variance accumulated on the first two components (axis 1 = 16.89%; axis 2 = 10.13%).

<https://doi.org/10.1371/journal.pone.0324582.g005>

Overall, the main taxane in yew trees studied in all seasons was DAB in agreement with several other reports [16,53,55–57], and its highest concentration at the population level was found in Mt Olympus. DAB presented its maximum concentration in the needles collected at the end of the flowering period for Mt Olympus and Mt Vourinos populations, and at the beginning of winter for Mt Cholomon, whereas the maximum BAC needle concentration was found to be the opposite; in winter for Mt Olympus and Mt Vourinos and in spring for Mt Cholomon. Maximum concentration of DAB was found a month after the start of flush (June) in a seasonal study that used two cultivars (*T. x media* ‘Hicksii’ and *T. x media* ‘Dark Green Spreader’; MI, USA) [58], whereas in the same study, BAC concentration of *T. x media* ‘Dark Green Spreader’ had maximum concentration in the same period as DAB, but the maximum for the second cultivar was shown to be three months later, in September. In another seasonal study [17], August was identified to be the month with the highest DAB concentration, a month later than BAC concentration (Tehran Iran, *T. baccata*). In *T. wallichiana* needle samples from West Bengal India, the maximum concentration for both DAB and BAC was recorded during spring (March – April) [15]. Vance et al. [56] showed that both DAB and BAC needle concentration have the same optimum month for *T. brevifolia* (Oregon, USA), but that the maximum was identified to be in October. Lastly, *T. baccata* var. *fastigiata* DAB reached its maximum concentration in June (Ireland) [14] and in *T. canadensis* in August (New Brunswick, Canada) [16].

Needle samples obtained from the three *T. baccata* populations, accumulated higher PAC amounts in December at the beginning of winter when temperatures in Greece start to decrease, in contrast to the findings for the DAB concentration in the current study. Cameron & Smith [16] identified the highest PAC concentration in August–September, the same period as DAB (Canada, *T. canadensis*), Hook et al. [14] between February and April (*T. baccata* var. *fastigiata*), whereas Vance et al. [56] showed that *T. brevifolia* (Oregon, USA) PAC needle concentration expressed limited variation throughout an eight-month period (March to October), with a slight increase in late June. Lastly, the maximum PAC concentration was observed in June for *T. x media* ‘Hicksii’ and *T. x media* ‘Dark Green Spreader’ (MI, USA) [58].

Correlation analysis between the taxanes’ concentrations and climatic conditions (temperature, daylight etc.) has been employed to investigate whether these factors could somehow affect the *in-planta* biosynthesis and accumulation of the metabolites present in the needles [53,55]. A positive and strong correlation was identified in *T. baccata* (Botanical Garden, Karaj, Iran) DAB needle and stem tissue concentrations with maximum, minimum and mean temperature, and light intensity of the day of sampling [53]. On the contrary, for BAC and PAC, no significant strong correlation with climatic conditions was identified [53]. Yang et al. [55], identified strong negative correlations between DAB needle concentration on one hand and monthly mean maximum temperature as well as length of daylight on the other in *T. wallichiana* var. *mairei*

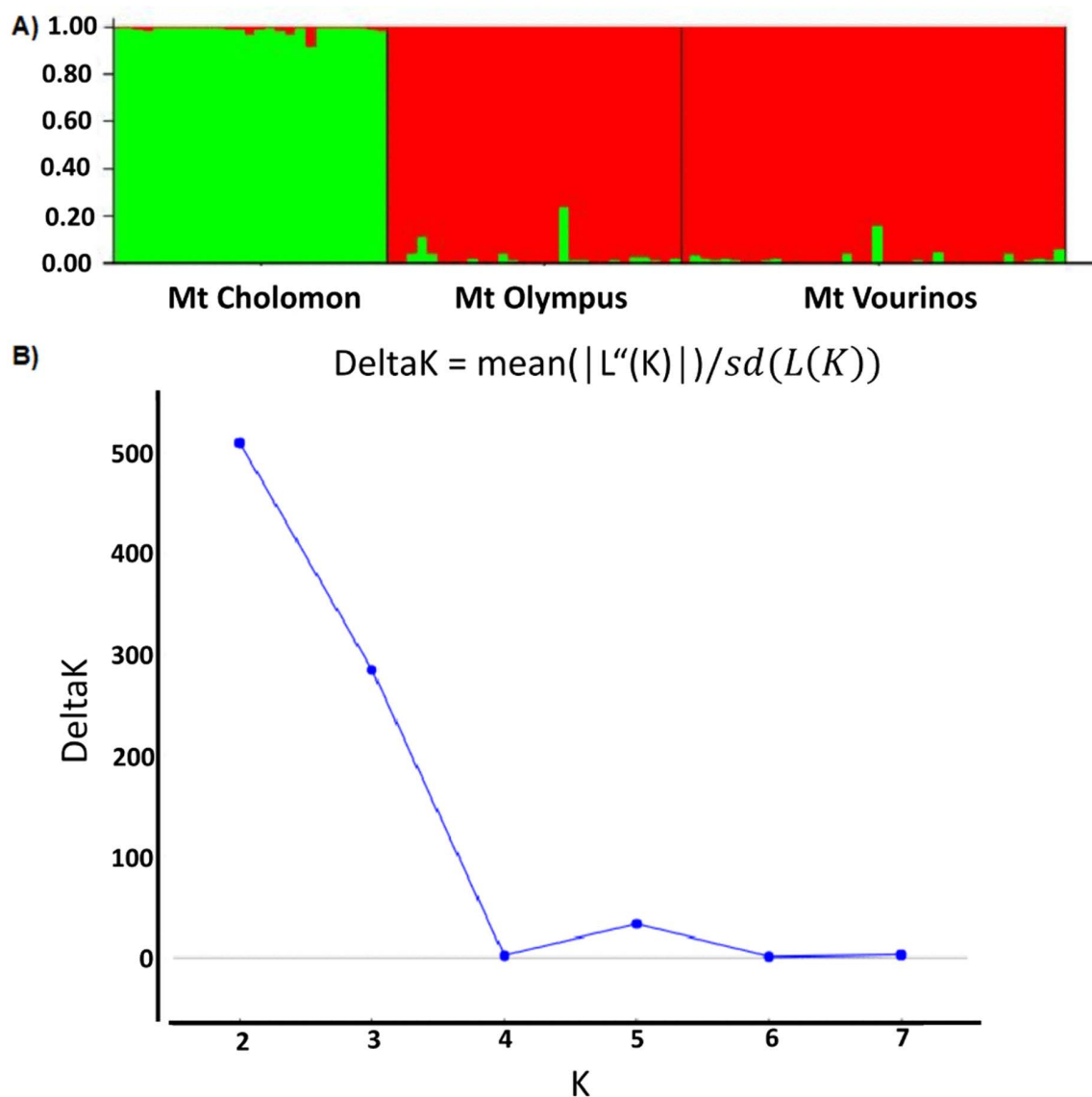


Fig 6. STRUCTURE analysis. (A) STRUCTURE analysis for SSR data with K=2 clusters for the *T. baccata* populations studied. Barplots represent the average estimated membership probability (y axis) of an individual that belongs to a specific cluster. Each cluster is indicated by a different color. (B) Estimation of the number of populations for K ranging from 1 to 7 by calculating delta K values.

<https://doi.org/10.1371/journal.pone.0324582.g006>

Table 5. Total epigenetic diversity parameters (MSAP) of *T. baccata* populations.

Population	Geographic Locality	No. Bands	No. Private bands	P _{epi}	I _{epi}	H _{epi}
Mt Olympus	Pieria	1872	633	54.51	0.214 (SE=0.02)	0.051 (SE=0.001)
Mt Vourinos	Kozani	2025	865	58.97	0.223 (SE=0.02)	0.053 (SE=0.001)
Mt Cholomon	Chalkidiki	1585	462	46.16	0.188 (SE=0.03)	0.049 (SE=0.02)
Mean	—	1828.3	653.3	53.21	0.209	0.051

P_{epi}: percentage of polymorphic subepiloci, I_{epi}: Shannon's information index based on epiloci, H_{epi}: haploid epigenetic diversity.

<https://doi.org/10.1371/journal.pone.0324582.t005>

Table 6. Hierarchical AMOVA for methylation-sensitive amplification polymorphisms (MSAP) data (all subepiloci, as well as different subepiloci classes, m, h and n separately) performed by grouping populations according to regions of origin.

Loci/groups	Source of variation	d.f.	Est. Var.	Total variance (%)	Φ -statistics (Φ_{ST})	P-value
MSAP All subepiloci	Among populations	2	23.515	14		
	Within Populations	91	146.420	86	0.138	<0.001
	Total	93	169.935	100		
MSAP m-subepiloci	Among Populations	2	8.767	13		
	Within Populations	91	59.963	87	0.133	<0.001
	Total	93	65.731	100		
MSAP h-subepiloci	Among Populations	2	10.101	13		
	Within Populations	91	66.476	87	0.132	<0.001
	Total	93	76.577	100		
MSAP n-subepiloci	Among Populations	2	4.647	17%		
	Within Populations	91	22.981	83%	0.168	<0.001
	Total	93	27.628	100%		

<https://doi.org/10.1371/journal.pone.0324582.t006>

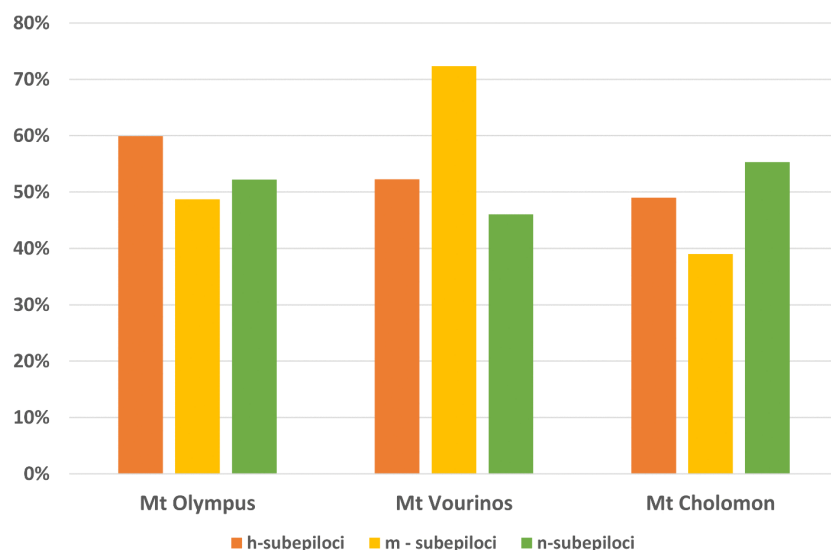


Fig 7. Percentage of polymorphic marker per population calculated from R script MSAP_calc.r for the *T. baccata* populations “h-subepiloci” “m-subepiloci” and “n-subepiloci”. Population differences of 5’CCGG- methylation were not significant (Kruskal-Wallis $\chi^2=0.622$ $p=0.733$).

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(Ningbo, China). The same study found similar correlation between PAC concentration and monthly mean maximum and minimum temperature [55].

Genetic analyses

At the population level, SSR markers indicated high levels of genetic variation in Mt Olympus and Mt Vourinos and relatively high levels in the population of Mt Cholomon. Notable amounts of genetic diversity were detected based on the observed ($H_o=0.299$) and expected ($H_e=0.537$) heterozygosity. The observed heterozygosity of the Greek populations was higher than that of populations from the Eastern Austrian Alps ($H_o=0.178$ – 0.272 ; [59]), lower than that of a population from Montseny Mountains in NE Spain ($H_o=0.353$; [60]), in populations from the northern part of the Czech Republic

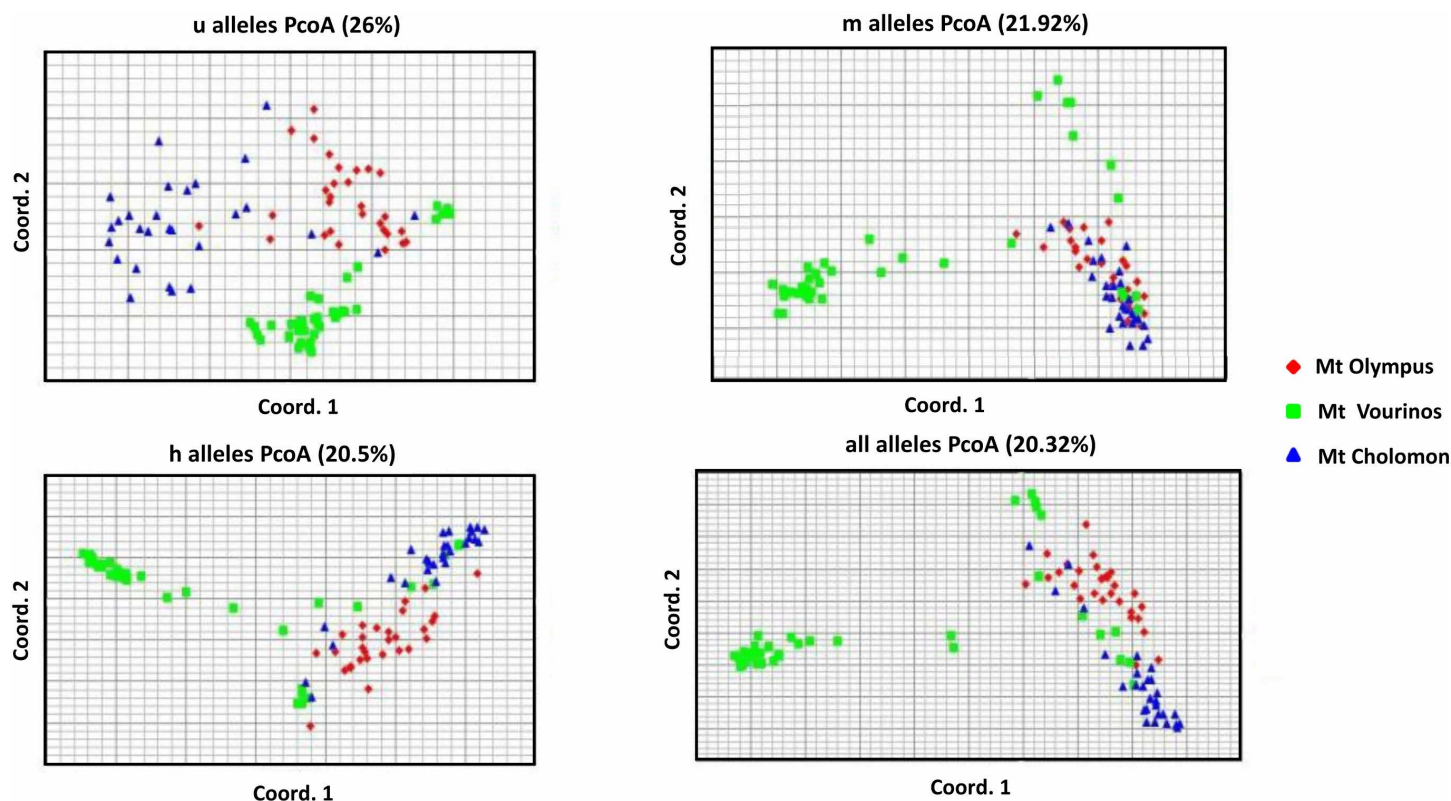


Fig 8. Principal Coordinate Analysis of epigenetic data. PCoAs are partitioned into three methylation types: u alleles, m alleles, h alleles and all epigenetic alleles.

<https://doi.org/10.1371/journal.pone.0324582.g008>

($H = 0.420\text{--}0.570$; [61]) and in western ($H_o = 0.508\text{--}0.622$; [62]), and northern western ($H_o = 0.563\text{--}0.685$; [63]) Polish populations, and lower than the average in a study that covered a large part of the natural range (195 populations, $H_o = 0.171\text{--}0.768$, $\mu = 0.474$; [26]). The expected heterozygosity in populations from the Eastern Austrian Alps was found (similar to H_o) to be lower than our study ($H_e = 0.274$; [59]), whereas comparable values of H_e were discovered in NE Spanish ($H_e = 0.509$; [60]) and western Polish ($H_e = 0.564$; [62]) populations. Nevertheless, several other studies that analyzed populations of *T. baccata* from Spain, Britain, Poland and Czech Republic presented higher H_e values [27,60,61,63–65], including a population from northern Poland that presented the highest H_e of all ($H_e = 0.870$; [63]). Higher average H_e was also found in the study that covered a large part of the natural range (195 populations, $H_e = 0.406\text{--}0.855$, $\mu = 0.701$; [26]). Furthermore, allelic richness ($AR = 5.000$) of the Greek populations was within the range and higher than the average of the study that covered a large part of the distribution of *T. baccata* [195 populations ($AR = 2.243\text{--}5.295$, $\mu = 3.952$; [26]) and higher than populations from Spain ($AR = 3.98, 4.23$; [27,65]) and Poland ($AR = 4.000$; [62]). Overall, genetic analyses revealed relatively high genetic diversity, lower (but within the range) than the average found in range-wide studies, and significant population structure. This result, consistent with expectations for fragmented populations [63], likely reflects long-term isolation.

Results also indicate that there is less gene diversity as expected from HWE (H_o lower than H_e). Furthermore, some inbreeding may be involved ($F_{is} = 0.443$), indicating heterozygote deficiency and non-random mating [38,66–70]. Positive inbreeding coefficient values in *Taxus* species have been shown in several studies [60,66–70]. The understory habitat of *Taxus* may promote inbreeding, as it restricts gene flow (pollen and seed dispersal), thereby favoring mating between

relatives [70,71]. In addition, these populations are characterized by a small number of old, large reproducing trees, a pattern that may contribute to inbreeding. Fragmentation, small census and effective population size and skewed sex ratios, which are common in *T. baccata* [34], can be confounding factors as well.

F- and G- statistics were consistent with AMOVA results ($F_{ST}=0.153$, $G_{ST}=0.138$), which showed that 76% of the genetic variation was partitioned within populations. Results indicate moderately high genetic differentiation among populations. Comparable results were shown in studies of *T. baccata* in the Western Mediterranean Basin ($F_{ST}=0.155$, 88% within population variation; [27]), in western Norway ($F_{ST}=0.166$; [68]), in northern Spain ($F_{ST}=0.129$, 88% within population variation; [65]) and in Poland ($F_{ST}=0.155$, 79.5% within population variation; [70]). Lower values of genetic differentiation were found in populations of *T. baccata* from Czech Republic ($F_{ST}=0.068$; [61]) and in British populations ($F_{ST}=0.05$, 63% within population variation; [64]). These results were further corroborated by the PCoA and STRUCTURE analysis. Both identified two major gene pools one in Chalkidiki peninsula (Mt. Cholomon) and one in southern Macedonia (Mt. Olympus and Mt. Vourinos). This separation is interrelated with the local geography and is likely the result of isolation by distance. These results suggest the establishment of at least two gene conservation units (GCUs) for *T. baccata*.

Epigenetic analyses

Studying methylation patterns in forest tree populations is becoming essential to access adaptation under climatic changes [28]. To the authors' knowledge, this is the first study that involves the epigenetic MSAP method in natural populations of *Taxus* species. In one study MSAP have been employed in long term cell *Taxus media* cv. Hicksii cultures accompanied with HPLC analysis [32], and findings suggest that there was a higher level of DNA methylation in the low-paclitaxel yielding cell line for PAC biosynthesis after long-term culture. Wheeler et al. [19] studied five single trees from five natural populations of *T. brevifolia* with the TCL-HPLC method and suggested that taxol production is affected by epigenetic variation.

In this study, the mean haploid epigenetic diversity (H_{epi}) was 0.051, which was similar to that reported for Greek populations of the conifer *Pinus nigra* [$H_{epi}=0.049$; 72], but lower than Greek populations of the angiosperm *Prunus avium* populations [mean $H_{epi}=0.108$; 73]. The total relative methylation in *Taxus* populations was 53.55% and was lower than *Pinus nigra* (68.02) but higher compared to *P. avium* Greek populations (49.67). In the above studies, population differences are statistically significant, indicating the multifaceted level of methylation for natural populations. Total methylation was also assessed after vegetative propagation for *Pinus pinea* [74] and was higher, compared to our results (64.36% vs. 53.55%). Such differences may reflect methodological variation in MSAP epilocus scoring, the inclusion of vegetatively propagated material and individual variation in genome wide methylation levels. Methylation type PCoAs indicated moderate population structuring.

The epigenetic findings indicate an association between epigenetic variation and taxol production, suggesting that methylation polymorphism may contribute to the plastic regulation of secondary metabolite production [52,75,76]. Our results are in line with evidence that variation in cytosine methylation correlates with environmentally-driven phenotypic variation, which is potentially linked to epigenetically mediated regulation of complex traits, such as secondary metabolites [74]. Moreover, recent work in the Norway spruce (*Picea abies*) epitype system has demonstrated that DNA methylation can underlie environmentally induced epigenetic states stable for years across generations, offering a framework for epigenetic memory in conifers [76]. Nevertheless, MSAP markers have inherent limitations, as they target a restricted fraction of the genome at CCGG sites, lack locus-specific resolution, do not fully resolve methylation context and do not capture additional epigenetic layers [52,75]. Future studies combining whole-genome bisulfite sequencing with reduced representation approaches would provide a more comprehensive view of epigenetic variation.

Comparative analysis of genetic and epigenetic diversity

In the present study, MSAP mean haploid epigenetic diversity was $H_{epi}=0.051$ and differed significantly ($t=7.981$, $p=0.048$) from SSR gene diversity (expected heterozygosity $H_e=0.537$). We have observed a negative non-significant

correlation between genetic (SSR) and epigenetic (MSAP) diversity values regarding H_{epi} ($r=-0.678$, $p=0.188$) and Shannon's epigenetic and genetic index ($r=-0.693$, $p=0.163$). Overall, genetic diversity appears to be decoupled from epigenetic diversity. These results should be interpreted with caution because the markers differ in their dominance levels. Albaladejo et al. [77] used MSAP and SSR markers in a greenhouse experiment for studying epigenetic and genetic correlation in *Pistacia lentiscus* between mother trees and offsprings. They also found that epigenetic variation was mostly decoupled from genetic variation.

Conclusions

Our results demonstrate sufficient genetic diversity and clear population structure in the peripheral Greek *T. baccata* populations studied. The sufficient DNA methylation levels observed may be indicative of a potential for future adaptive responses, assuming that elevated methylation facilitates dynamic epigenetic reprogramming in response to stress. Marked seasonal and population-level variation in taxane production highlights spring as the optimal harvest period, with DAB consistently representing the dominant compound. Considering the importance of *T. baccata* as the natural source of precursor compounds for the semi-synthetic paclitaxel production and the value of peripheral/ marginal populations, future studies should focus on the genomic, epigenomic and metabolomic profile of *T. baccata* in Greece.

These results support breeding strategies based on the identification and clonal propagation of high taxane-producing genotypes, combined with the use of genetically diverse germplasm from multiple populations. Such an approach enables the development of selected planting material, while maintaining evolutionary potential in natural populations.

The preservation of remaining *Taxus* populations in their natural environment and the conservation of their genetic resources are particularly important, given the rarity of yew trees in Greece, their fragmented distribution, small population size (generally less than 50 individuals with limited regeneration), and low representation under statutory protection [34]. Conservation strategies should therefore prioritize: (i) expanding protected areas encompassing *Taxus* woodlands and establishing gene conservation units, (ii) prohibiting yew logging, (iii) introducing genetic monitoring [78], (iv) regulating herbivory and grazing pressure, (v) developing *ex-situ* conservation using germplasm from multiple populations, beginning with high-taxane producing trees and (vi) employing assisted gene flow where appropriate [79].

Supporting information

S1 Fig. Height and DBH range of the *T. baccata* Mt Olympus, Mt Cholomon and Mt Vourinos populations.
(TIF)

S1 Table. Altitude (m), annual rainfall (mm) and coordinates of the *T. baccata* populations employed in this study.
(DOCX)

S2 Table. Primers and adapters used in the f-MSAP analysis of the *T. baccata* populations.
(DOCX)

S3 Table. Descriptive statistics of the concentration of 10 Deacetylbaccatin III (DAB), Paclitaxel (PAC), Baccatin III (BAC), Cephalomannine (CEPH) and 10-deacetylTaxol (10DEAC) of dry shaded needles in the *T. baccata* populations studied.
(DOCX)

S4 Table. Statistical comparison of the *T. baccata* populations and Tukey HSD pairwise multiple comparison test for cephalomannine (CEPH) and 10-deacetylTaxol (10DEAC).
(DOCX)

S5 Table. F- and G- statistics for 12 SSR loci analyzed in the *T. baccata* populations studied.
(DOCX)

S6 Table. Genetic Pairwise F_{ST} Values in the *T. baccata* populations studied.

(DOCX)

S7 Table. Summary of chi-square tests for hardy-weinberg equilibrium of the *T. baccata* populations studied.

(DOCX)

S8 Table. Epigenetic pairwise F_{ST} Values in the *T. baccata* populations studied.

(DOCX)

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Author contributions

Conceptualization: Filippos A. Aravanopoulos.

Data curation: Eleftheria Dalmaris, Evangelia Avramidou, Eirini Sarrou, Aliko Xanthopoulou, Salvatore Multari.

Formal analysis: Evangelia Avramidou, Eirini Sarrou, Aliko Xanthopoulou.

Funding acquisition: Filippos A. Aravanopoulos.

Investigation: Eleftheria Dalmaris, Evangelia Avramidou, Eirini Sarrou, Aliko Xanthopoulou, Salvatore Multari.

Methodology: Eleftheria Dalmaris, Evangelia Avramidou, Eirini Sarrou, Aliko Xanthopoulou, Salvatore Multari, Filippos A. Aravanopoulos.

Project administration: Filippos A. Aravanopoulos.

Resources: Stefan Martens, Filippos A. Aravanopoulos.

Supervision: Filippos A. Aravanopoulos.

Validation: Filippos A. Aravanopoulos.

Visualization: Eleftheria Dalmaris, Evangelia Avramidou, Eirini Sarrou, Aliko Xanthopoulou.

Writing – original draft: Eleftheria Dalmaris, Evangelia Avramidou, Eirini Sarrou, Aliko Xanthopoulou.

Writing – review & editing: Salvatore Multari, Stefan Martens, Filippos A. Aravanopoulos.

References

1. Bhujar S, Gauchan DP. *Taxus wallichiana* (Zucc.), an endangered anti-cancerous plant: a review. Inter J Res. 2018;5(21):12.
2. Le Roux M, Guérin F. From the pacific yew (*Taxus brevifolia*) to the english yew (*Taxus baccata*): steps towards the discovery of docetaxel (Taxotere®). Navelbine® and Taxotere®. Elsevier. 2017. 151–212. <https://doi.org/10.1016/b978-1-78548-145-1.50004-0>
3. Sofias AM, Dunne M, Storm G, Allen C. The battle of “nano” paclitaxel. Adv Drug Deliv Rev. 2017;122:20–30. <https://doi.org/10.1016/j.addr.2017.02.003>
4. Yin J-Y, Lai M, Yu X-Y, Su D-D, Xiong X-Y, Li Y-L. Comprehensive strategies for paclitaxel production: insights from plant cell culture, endophytic microorganisms, and synthetic biology. Hortic Res. 2024;12(3):uhae346. <https://doi.org/10.1093/hr/uhae346> PMID: 40061810
5. Croteau R, Ketchum REB, Long RM, Kaspera R, Wildung MR. Taxol biosynthesis and molecular genetics. Phytochem Rev. 2006;5(1):75–97. <https://doi.org/10.1007/s11101-005-3748-2> PMID: 20622989
6. Xiong X, Gou J, Liao Q, Li Y, Zhou Q, Bi G, et al. The *Taxus* genome provides insights into paclitaxel biosynthesis. Nat Plants. 2021;7(8):1026–36. <https://doi.org/10.1038/s41477-021-00963-5> PMID: 34267359
7. Sati P, Sharma E, Dhyani P, Attri DC, Rana R, Kiyekbayeva L, et al. Paclitaxel and its semi-synthetic derivatives: comprehensive insights into chemical structure, mechanisms of action, and anticancer properties. Eur J Med Res. 2024;29(1):90. <https://doi.org/10.1186/s40001-024-01657-2> PMID: 38291541

8. Collin HA. Secondary product formation in plant tissue cultures. *Plant Growth Regul.* 2001;34(1):119–34. <https://doi.org/10.1023/a:1013374417961>
9. Shao F, Wilson IW, Qiu D. The research progress of taxol in *Taxus*. *Curr Pharm Biotechnol.* 2021;22(3):360–6. <https://doi.org/10.2174/1389201021666200621163333> PMID: [32564747](#)
10. Veselá D, Šaman D, Valterová I, Vaněk T. Seasonal variations in the content of taxanes in the bark of *Taxus baccata* L. *Phytochem Anal.* 1999;10(6):319–21. [https://doi.org/10.1002/\(SICI\)1099-1565\(199911/12\)10:6<319::AID-PCA471>3.0.CO;2-W](https://doi.org/10.1002/(SICI)1099-1565(199911/12)10:6<319::AID-PCA471>3.0.CO;2-W)
11. Frense D. Taxanes: perspectives for biotechnological production. *Appl Microbiol Biotechnol.* 2007;73(6):1233–40. <https://doi.org/10.1007/s00253-006-0711-0> PMID: [17124581](#)
12. Fett Neto AG, DiCosmo F. Distribution and amounts of taxol in different shoot parts of *Taxus cuspidata*. *Planta Med.* 1992;58(5):464–6. <https://doi.org/10.1055/s-2006-961515> PMID: [1361676](#)
13. Griffin J, Hook I. Taxol content of Irish yews. *Planta Med.* 1996;62(4):370–2. <https://doi.org/10.1055/s-2006-957910> PMID: [8792673](#)
14. Hook I, Poupat C, Ahond A, Guénard D, Guéritte F, Adeline M-T, et al. Seasonal variation of neutral and basic taxoid contents in shoots of European Yew (*Taxus baccata*). *Phytochemistry.* 1999;52(6):1041–5. [https://doi.org/10.1016/S0031-9422\(99\)00264-2](https://doi.org/10.1016/S0031-9422(99)00264-2)
15. Mukherjee S, Ghosh B, Jha TB, Jha S. Variation in content of taxol and related taxanes in Eastern Himalayan populations of *Taxus wallichiana*. *Planta Med.* 2002;68(8):757–9. <https://doi.org/10.1055/s-2002-33808> PMID: [12221606](#)
16. Cameron SI, Smith RE. Seasonal changes in the concentration of major taxanes in the biomass of wild Canada yew (*Taxus canadensis* Marsh.). *Pharm Biol.* 2008;46(1–2):35–40. <https://doi.org/10.1080/13880200701729760>
17. Ghassempour A, Rezadoost H, Ahmadi M, Aboul-Enein HY. Seasons study of four important taxanes and purification of 10-deacetylbaccatin III from the needles of *Taxus baccata* L. by two-dimensional liquid chromatography. *J Liq Chromatogr Relat Technol.* 2009;32(10):1434–47. <https://doi.org/10.1080/10826070902901184>
18. Yu C, Luo X, Zhan X, Hao J, Zhang L, L Song Y-B, et al. Comparative metabolomics reveals the metabolic variations between two endangered *Taxus* species (*T. fuana* and *T. yunnanensis*) in the Himalayas. *BMC Plant Biol.* 2018;18(1):197. <https://doi.org/10.1186/s12870-018-1412-4> PMID: [30223770](#)
19. Wheeler NC, Jech K, Masters S, Brobst SW, Alvarado AB, Hoover AJ, et al. Effects of genetic, epigenetic, and environmental factors on taxol content in *Taxus brevifolia* and related species. *J Nat Prod.* 1992;55(4):432–40. <https://doi.org/10.1021/np50082a005> PMID: [1355111](#)
20. van Rozendaal EL, Lelyveld GP, van Beek TA. Screening of the needles of different yew species and cultivars for paclitaxel and related taxoids. *Phytochemistry.* 2000;53(3):383–9. [https://doi.org/10.1016/S0031-9422\(99\)00094-1](https://doi.org/10.1016/S0031-9422(99)00094-1) PMID: [10703062](#)
21. Santoyo-Garcia JH, Valdivia-Cabrera M, Ochoa-Villalreal M, Casasola-Zamora S, Ripoll M, Escrib A, et al. Increased paclitaxel recovery from *Taxus baccata* vascular stem cells using novel in situ product recovery approaches. *Bioresour Bioprocess.* 2023;10(1):68. <https://doi.org/10.1186/s40643-023-00687-8> PMID: [38647629](#)
22. Hayne K, Günter B, Pietzarka U. Dendroklimatologische und dendroökologische Untersuchungen an Gemeiner Eibe (*Taxus baccata* L.) am Kätherstobel, Kanton St. Gallen, Schweiz. *Der Eibenfreund.* 2016;22:59–93.
23. Li S, Fu Y, Zu Y, Sun R, Wang Y, Zhang L, et al. Determination of paclitaxel and other six taxoids in *Taxus* species by high-performance liquid chromatography-tandem mass spectrometry. *J Pharm Biomed Anal.* 2009;49(1):81–9. <https://doi.org/10.1016/j.jpba.2008.10.006> PMID: [19036549](#)
24. Liu Y, Zhao F, Wang Q, Zhao Q, Hou G, Meng Q. Current perspectives on paclitaxel: focus on its production, delivery and combination therapy. *Mini Rev Med Chem.* 2023;23(18):1780–96. <https://doi.org/10.2174/1389557523666230210145150> PMID: [36825714](#)
25. Yang Y-H, Mao J-W, Tan X-L. Research progress on the source, production, and anti-cancer mechanisms of paclitaxel. *Chin J Nat Med.* 2020;18(12):890–7. [https://doi.org/10.1016/S1875-5364\(20\)60032-2](https://doi.org/10.1016/S1875-5364(20)60032-2) PMID: [33357719](#)
26. Mayol M, Riba M, González-Martínez SC, Bagnoli F, de Beaulieu J-L, Berganzo E, et al. Adapting through glacial cycles: insights from a long-lived tree (*Taxus baccata*). *New Phytol.* 2015;208(3):973–86. <https://doi.org/10.1111/nph.13496> PMID: [26096330](#)
27. González-Martínez SC, Dubreuil M, Riba M, Vendramin GG, Sebastiani F, Mayol M. Spatial genetic structure of *Taxus baccata* L. in the western Mediterranean Basin: past and present limits to gene movement over a broad geographic scale. *Mol Phylogenet Evol.* 2010;55(3):805–15. <https://doi.org/10.1016/j.ympev.2010.03.001> PMID: [20211747](#)
28. Balao F, Paun O, Alonso C. Uncovering the contribution of epigenetics to plant phenotypic variation in Mediterranean ecosystems. *Plant Biol (Stuttg).* 2018;20 Suppl 1:38–49. <https://doi.org/10.1111/plb.12594> PMID: [28637098](#)
29. Sow MD, Le Gac A-L, Fichot R, Lanciano S, Delaunay A, Le Jan I, et al. RNAi suppression of DNA methylation affects the drought stress response and genome integrity in transgenic poplar. *New Phytol.* 2021;232(1):80–97. <https://doi.org/10.1111/nph.17555> PMID: [34128549](#)
30. Amaral J, Ribeyre Z, Vigneaud J, Sow MD, Fichot R, Messier C, et al. Advances and promises of epigenetics for forest trees. *Forests.* 2020;11(9):976. <https://doi.org/10.3390/f11090976>
31. Kiselev KV, Tyunin AP, Karetin YA. Salicylic acid induces alterations in the methylation pattern of the VaSTS1, VaSTS2, and VaSTS10 genes in *Vitis amurensis* Rupr. cell cultures. *Plant Cell Rep.* 2015;34(2):311–20. <https://doi.org/10.1007/s00299-014-1708-2> PMID: [25420769](#)
32. Fu Y, He C. Nucleic acid modifications with epigenetic significance. *Curr Opin Chem Biol.* 2012;16(5–6):516–24. <https://doi.org/10.1016/j.cbpa.2012.10.002> PMID: [23092881](#)
33. Zhou T, Luo X, Zhang C, Xu X, Yu C, Jiang Z, et al. Comparative metabolomic analysis reveals the variations in taxoids and flavonoids among three *Taxus* species. *BMC Plant Biol.* 2019;19(1):529. <https://doi.org/10.1186/s12870-019-2146-7> PMID: [31783790](#)

34. Kassioumis K, Papageorgiou K, Glezakos TJ, Vogiatzakis IN. Distribution and stand structure of *Taxus baccata* populations in Greece; results of the first national inventory. *ecmed*. 2004;30(2):159–70. <https://doi.org/10.3406/ecmed.2004.1456>
35. Benham S, Houston Durrant T, Caudullo G, de Rigo D. *Taxus baccata* in Europe: distribution, habitat, usage and threats. 2016.
36. Mubeen S, Li Z-L, Huang Q-M, He C-T, Yang Z-Y. Comparative transcriptome analysis revealed the tissue-specific accumulations of taxanes among three experimental lines of *Taxus yunnanensis*. *J Agric Food Chem*. 2018;66(40):10410–20. <https://doi.org/10.1021/acs.jafc.8b03502> PMID: 30208705
37. Kobusiak-Prokopowicz M, Marciniak A, Ślusarczyk S, Ściborski K, Stachurska A, Mysiak A, et al. A suicide attempt by intoxication with *Taxus baccata* leaves and ultra-fast liquid chromatography-electrospray ionization-tandem mass spectrometry, analysis of patient serum and different plant samples: case report. *BMC Pharmacol Toxicol*. 2016;17(1):41. <https://doi.org/10.1186/s40360-016-0078-5> PMID: 27577698
38. Dalmaris E, Avramidou EV, Xanthopoulou A, Aravanopoulos FA. Dataset of targeted metabolite analysis for five taxanes of hellenic *Taxus baccata* L. populations. *Data*. 2020;5(1):22. <https://doi.org/10.3390/data5010022>
39. Dubreuil M, Sebastiani F, Mayol M, González-Martínez SC, Riba M, Vendramin GG. Isolation and characterization of polymorphic nuclear microsatellite loci in *Taxus baccata* L. *Conserv Genet*. 2008;9(6):1665–8. <https://doi.org/10.1007/s10592-008-9515-3>
40. Mahmoodi P, Khayam Nekouei SM, Mardi M, Pirseyedi SM, Ghaffari MR, Ramroodi M. Isolation and characterization of new microsatellite marker in *Taxus baccata* L. *Conserv Genetics Res*. 2010;2(1):195–9. <https://doi.org/10.1007/s12686-009-9133-5>
41. Team R. R version 3.5.0: A language and environment for statistical computing. Vienna, Austria: R Foundation for Statistical Computing; 2018.
42. Nei M. Analysis of gene diversity in subdivided populations. *Proc Natl Acad Sci U S A*. 1973;70(12):3321–3. <https://doi.org/10.1073/pnas.70.12.3321> PMID: 4519626
43. Peakall R, Smouse PE. GenAIEx 6.5: genetic analysis in Excel. Population genetic software for teaching and research—an update. *Bioinformatics*. 2012;28(19):2537–9. <https://doi.org/10.1093/bioinformatics/bts460> PMID: 22820204
44. Peakall R, Smouse PE. genalex 6: genetic analysis in Excel. Population genetic software for teaching and research. *Mol Ecol Notes*. 2005;6(1):288–95. <https://doi.org/10.1111/j.1471-8286.2005.01155.x>
45. Excoffier L, Lischer HEL. Arlequin suite ver 3.5: a new series of programs to perform population genetics analyses under Linux and Windows. *Mol Ecol Resour*. 2010;10(3):564–7. <https://doi.org/10.1111/j.1755-0998.2010.02847.x> PMID: 21565059
46. Kalinowski ST. hp-rare 1.0: a computer program for performing rarefaction on measures of allelic richness. *Mol Ecol Notes*. 2004;5(1):187–9. <https://doi.org/10.1111/j.1471-8286.2004.00845.x>
47. Nei M. Estimation of average heterozygosity and genetic distance from a small number of individuals. *Genetics*. 1978;89(3):583–90. <https://doi.org/10.1093/genetics/89.3.583> PMID: 17248844
48. Rousset F. genepop'007: a complete re-implementation of the genepop software for Windows and Linux. *Mol Ecol Resour*. 2008;8(1):103–6. <https://doi.org/10.1111/j.1471-8286.2007.01931.x> PMID: 21585727
49. Rinehart TA. AFLP analysis using GeneMapper software and an Excel macro that aligns and converts output to binary. *Biotechniques*. 2004;37(2):186–8. <https://doi.org/10.2144/04372BM01> PMID: 15335205
50. Vekemans X, Beauwens T, Lemaire M, Roldán-Ruiz I. Data from amplified fragment length polymorphism (AFLP) markers show indication of size homoplasy and of a relationship between degree of homoplasy and fragment size. *Mol Ecol*. 2002;11(1):139–51. <https://doi.org/10.1046/j.0962-1083.2001.01415.x> PMID: 11903911
51. Schulz B, Eckstein RL, Durka W. Epigenetic variation reflects dynamic habitat conditions in a rare floodplain herb. *Mol Ecol*. 2014;23(14):3523–37. <https://doi.org/10.1111/mec.12835> PMID: 24943730
52. Schulz B, Eckstein RL, Durka W. Scoring and analysis of methylation-sensitive amplification polymorphisms for epigenetic population studies. *Mol Ecol Resour*. 2013;13(4):642–53. <https://doi.org/10.1111/1755-0998.12100> PMID: 23617735
53. Nasiri J, Naghavi MR, Alizadeh H, Moghadam MRF. Seasonal-based temporal changes fluctuate expression patterns of TXS, DBAT, BAPT and DBTNBT genes alongside production of associated taxanes in *Taxus baccata*. *Plant Cell Rep*. 2016;35(5):1103–19. <https://doi.org/10.1007/s00299-016-1941-y> PMID: 26883228
54. Sinha D. A review on taxanes: an important group of anticancer compound obtained from *Taxus* sp. *IJPSR*. 2020;11(5):1969–85. [https://doi.org/10.13040/IJPSR.0975-8232.11\(5\).1969-85](https://doi.org/10.13040/IJPSR.0975-8232.11(5).1969-85)
55. Yang L, Zheng Z-S, Cheng F, Ruan X, Jiang D-A, Pan C-D, et al. Seasonal dynamics of metabolites in needles of *Taxus wallichiana* var. *mairei*. *Molecules*. 2016;21(10):1403. <https://doi.org/10.3390/molecules21101403> PMID: 27775631
56. Vance NC, Kelsey RG, Sabin TE. Seasonal and tissue variation in taxane concentrations of *Taxus brevifolia*. *Phytochemistry*. 1994;36(5):1241–4. [https://doi.org/10.1016/s0031-9422\(00\)89644-2](https://doi.org/10.1016/s0031-9422(00)89644-2) PMID: 7765363
57. Glowinski K, Mroczek T, Zobel AM. Seasonal changes in the concentrations of four taxoids in *Taxus baccata* L. during the autumn-spring period. *Phytomedicine*. 1999;6(2):135–40. [https://doi.org/10.1016/S0944-7113\(99\)80049-X](https://doi.org/10.1016/S0944-7113(99)80049-X) PMID: 10374254
58. ElSohly HN, Croom EM, Kopycki WJ, Joshi AS, McChesney JD. Diurnal and seasonal effects on the taxane content of the clippings of certain *Taxus cultivars*. *Phytochem Anal*. 1997;8(3):124–9. [https://doi.org/10.1002/\(sici\)1099-1565\(199705\)8:3<124::aid-pca342>3.0.co;2-1](https://doi.org/10.1002/(sici)1099-1565(199705)8:3<124::aid-pca342>3.0.co;2-1)
59. Klumpp R, Dhar A. Genetic variation of *Taxus baccata* L. populations in the Eastern Alps and its implications for conservation management. *Scandinavian J Forest Res*. 2011;26(4):294–304. <https://doi.org/10.1080/02827581.2011.566888>

60. Dubreuil M, Riba M, González-Martínez SC, Vendramin GG, Sebastiani F, Mayol M. Genetic effects of chronic habitat fragmentation revisited: strong genetic structure in a temperate tree, *Taxus baccata* (Taxaceae), with great dispersal capability. *Am J Bot*. 2010;97(2):303–10. <https://doi.org/10.3732/ajb.0900148> PMID: 21622391
61. Komárková M, Novotný P, Cvrčková H, Máchová P. The Genetic Differences and Structure of Selected Important Populations of the Endangered *Taxus baccata* in the Czech Republic. *Forests*. 2022;13(2):137. <https://doi.org/10.3390/f13020137>
62. Stefanowska S, Meyza K, Iszkuło G, Chybicki IJ. Trunk perimeter correlates with genetic bottleneck intensity and the level of genetic diversity in populations of *Taxus baccata* L. *Annal Forest Sci*. 2021;78(3). <https://doi.org/10.1007/s13595-021-01080-1>
63. Chybicki IJ, Oleksa A, Burczyk J. Increased inbreeding and strong kinship structure in *Taxus baccata* estimated from both AFLP and SSR data. *Heredity* (Edinb). 2011;107(6):589–600. <https://doi.org/10.1038/hdy.2011.51> PMID: 21712844
64. Gargiulo R, Saubin M, Rizzuto G, West B, Fay MF, Kallow S, et al. Genetic diversity in British populations of *Taxus baccata* L.: is the seedbank collection representative of the genetic variation in the wild?. *Biological Conservation*. 2019;233:289–97. <https://doi.org/10.1016/j.biocon.2019.01.014>
65. Maroso F, Vera M, Ferreira J, Mayol M, Riba M, Ramil-Rego P, et al. Genetic diversity and structure of *Taxus baccata* from the Cantabrian-Atlantic area in northern Spain: a guide for conservation and management actions. *Forest Ecol Manag*. 2021;482:118844. <https://doi.org/10.1016/j.foreco.2020.118844>
66. El-Kassaby YA, Yanchuk AD. Genetic Diversity, Differentiation, and Inbreeding in Pacific Yew from British Columbia. *Journal of Heredity*. 1994;85(2):112–7. <https://doi.org/10.1093/oxfordjournals.jhered.a111407>
67. Miao YC, Lang XD, Zhang ZZ, Su JR. Phylogeography and genetic effects of habitat fragmentation on endangered *Taxus yunnanensis* in south-west China as revealed by microsatellite data. *Plant Biol (Stuttg)*. 2014;16(2):365–74. <https://doi.org/10.1111/plb.12059> PMID: 23890056
68. Myking T, Vakkari P, Skroppa T. Genetic variation in northern marginal *Taxus baccata* L. populations. Implications for conservation. *Forestry*. 2009;82(5):529–39. <https://doi.org/10.1093/forestry/cpp022>
69. Senneville S, Beaulieu J, Daoust G, Deslauriers M, Bousquet J. Evidence for low genetic diversity and metapopulation structure in Canada yew (*Taxus canadensis*): considerations for conservation. *Can J For Res*. 2001;31(1):110–6. <https://doi.org/10.1139/x00-152>
70. Litkowiec M, Lewandowski A, Wachowiak W. Genetic variation in *Taxus baccata* L.: A case study supporting Poland's protection and restoration program. *Forest Ecology and Management*. 2018;409:148–60. <https://doi.org/10.1016/j.foreco.2017.11.026>
71. Chybicki IJ, Oleksa A, Kowalkowska K. Variable rates of random genetic drift in protected populations of English yew: implications for gene pool conservation. *Conserv Genet*. 2012;13(4):899–911. <https://doi.org/10.1007/s10592-012-0339-9>
72. Katsidi EC, Avramidou EV, Ganopoulos I, Barbas E, Doulis A, Triantafyllou A, et al. Genetics and epigenetics of *Pinus nigra* populations with differential exposure to air pollution. *Front Plant Sci*. 2023;14:1139331. <https://doi.org/10.3389/fpls.2023.1139331> PMID: 37089661
73. Avramidou EV, Ganopoulos IV, Doulis AG, Tsaftaris AS, Aravanopoulos FA. Beyond population genetics: natural epigenetic variation in wild cherry (*Prunus avium*). *Tree Genetics Genomes*. 2015;11(5). <https://doi.org/10.1007/s11295-015-0921-7>
74. Sáez-Laguna E, Guevara M-Á, Díaz L-M, Sánchez-Gómez D, Collada C, Aranda I, et al. Epigenetic variability in the genetically uniform forest tree species *Pinus pinea* L. *PLoS One*. 2014;9(8):e103145. <https://doi.org/10.1371/journal.pone.0103145> PMID: 25084460
75. González-Benito ME, Ibáñez MÁ, Pirredda M, Mira S, Martín C. Application of the MSAP technique to evaluate epigenetic changes in plant conservation. *Int J Mol Sci*. 2020;21(20):7459. <https://doi.org/10.3390/ijms21207459> PMID: 33050382
76. Viejo M, Tengs T, Yakovlev I, Cross H, Krokene P, Olsen JE, et al. Epitype-inducing temperatures drive DNA methylation changes during somatic embryogenesis in the long-lived gymnosperm Norway spruce. *Front Plant Sci*. 2023;14:1196806. <https://doi.org/10.3389/fpls.2023.1196806> PMID: 37546277
77. Albaladejo RG, Parejo-Farnés C, Rubio-Pérez E, Nora S, Aparicio A. Linking DNA methylation with performance in a woody plant species. *Tree Genetics Genomes*. 2019;15(2). <https://doi.org/10.1007/s11295-019-1325-x>
78. Aravanopoulos FA. Genetic monitoring in natural perennial plant populations. *Botany*. 2011;89(2):75–81. <https://doi.org/10.1139/b10-087>
79. Aitken SN, Whitlock MC. Assisted gene flow to facilitate local adaptation to climate change. *Annu Rev Ecol Evol Syst*. 2013;44(1):367–88. <https://doi.org/10.1146/annurev-ecolsys-110512-135747>