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Morpho-Anatomical Characteristics and Volatile Profiles of *Pinus nigra* J.F.Arnold from the Balkan Peninsula and Southern Carpathians

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Abstract: This is the first report on morpho-anatomical and phytochemical differentiation of 19 native populations representing different *Pinus nigra* J.F.Arnold subspecies (*banatica* (Borbás) Novák, *dalmatica* (Vis.) Franco, *nigra*, and *pallasiana* (Lamb.) Holmboe) in the Balkans and Southern Carpathians. The 9 morpho-anatomical characteristics and 10 headspace volatiles of needles were analyzed with multivariate statistical analyses. The combination of results from all multivariate analyses with both types of markers revealed that *P. nigra* is differentiated into three groups within the studied area (the Dalmatian coast, Greece, and the rest of the Balkans with the Southern Carpathians). The first group included the population from an island in Dalmatia that corresponds to *P. nigra* subsp. *dalmatica*. The third group consisted of populations from continental Croatia, Bosnia and Herzegovina, Serbia, Romania, and Bulgaria, which corresponds to *P. nigra* subsp. *nigra.* In light of the recent molecular data that indicated that the Greek populations (the second group) represent a distinct genetic lineage of *P. nigra* placed between the populations from the principal area (*P. nigra* subsp. *nigra*) and Turkey (*P. nigra* subsp. *pallasiana*), one can speculate that there is one more subspecies of *P. nigra* in this region that corresponds to populations from Greece. Extending our analyses to Asia Minor and Crimea could bring additional results that would be valuable for clarifying the intriguing issue of the diversification of *P. nigra* in the eastern part of its range.

Keywords: *Pinus nigra*; Balkans; Southern Carpathians; needle morpho-anatomy; headspace; needle volatiles; multivariate statistical analyses

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1. Introduction

Pinus nigra J.F.Arnold (European black pine) is a member of the section *Pinus* and the subgenus *Pinus* (Diploxylon) within the family Pinaceae [1]. It is considered a Tertiary relict [2], one of the most economically valuable European pines, and one of the species most used for reforestation in Europe since the 19th century [3]. Black pine has a large but patchy natural range that spreads from southwestern Africa through the northern Mediterranean and eastwards to Asia Minor and Crimea [4]. Conforming to the topographic and climatic diversity of this region, as well as the disjunctive distribution, *P. nigra* shows great phenotypic variability in respect of its morpho-anatomical and ecological characteristics [5]. Consequently, numerous classification schemes for *P. nigra* have been proposed, but its infraspecific taxonomy is still regarded as unresolved [6]. Several authors considered it

an example of a collective species [7,8], while others regarded it as a single species that is subdivided into two or several allopatric subspecies with a lack of reproductive isolation [5,9,10]. In the Flora Europaea, Gaussen et al. [10] listed five *P. nigra* subspecies: subsp. *nigra*, subsp. *salzmannii* (Dunal) Franco, subsp. *laricio* (Poiret) Maire, subsp. *dalmatica* (Vis.) Franco, and subsp. *pallasiana* (Lamb.) Holmboe. They retained the status of accepted subspecies in the current floristic databases [11–13].

Although molecular markers are very effective as a tool for resolving the biogeographic history of coniferous taxa [14], the existing molecular data on the impacts of historical fragmentation on the genetic diversity and differentiation of *P. nigra* are somewhat inconsistent. According to the paternally inherited cpDNA, Naydenov et al. [15] have determined the existence of only three genetic groups of *P. nigra* that match three geographic areas (1. Western Mediterranean; 2. Balkan Peninsula; and 3. Asia Minor), estimating that their most recent common ancestor is more than 10 million years old. However, Scotti-Saintagne et al. [16], using a set of different molecular markers, identified six *P. nigra* lineages (1. Northern Africa and Iberian Peninsula; 2. Continental France, Corsica, and Calabria; 3. Central Europe; 4. Dalmatian coast; 5. Crimea with Turkey; and 6. Cyprus) dating from relatively recent divergence (between the Last Glacial Maximum and the onset of the Holocene). Furthermore, these authors propose to modify the currently accepted nomenclature, which acknowledges five subspecies, and name their six recognized genetic lineages using the regionally accepted subspecies-level names. Finally, five genetic groups of *P. nigra* (1. Morocco-Spain; 2. France-Corsica; 3. Western Greece; 4. Northern Turkey; and 5. the remaining part of the natural range of black pine) were revealed according to the maternally inherited mtDNA [17].

The territory of the Balkan Peninsula and Southern Carpathians has been included in numerous classification schemes of *P. nigra* [5,10,18], which distinguished several *P. nigra* subspecies within this area (subsp. *austriaca* (Höss) Vidaković, subsp. *banatica* (Borbás) Novák, subsp. *dalmatica*, subsp. *gocensis* (Đorđ.) Vidaković, subsp. *illyrica* (Vid.) Fukarek, subsp. *nigra*, and subsp. *pallasiana*). As previously listed, three of them (subsp. *dalmatica*, subsp. *nigra*, and subsp. *pallasiana*) were recognized as accepted subspecies in the Flora Europaea [10]. Until the 21st century, only a single comparative taxonomic study of *P. nigra* based on the anatomical characteristics of needles was performed within this area [19]. That study compared *P. nigra* populations by using vastly different numbers of analyzed individuals (for certain populations, only a single individual). After that, to our knowledge, there were two population studies regarding the phenotypic diversity of *P. nigra* that included almost the entire Balkans [20] or a significant part of it [21] and were based on multivariate statistical analysis. Specifically, Mitić et al. [20], using cuticular wax compounds, revealed two phytochemical groups of *P. nigra*: 1. Greece and southernmost North Macedonia that might correspond to subsp. *pallasiana*; and 2. the remaining part of the Balkans with Southern Carpathians that might correspond to subsp. *nigra*. Furthermore, Liber et al. [21] showed the existence of several *P. nigra* groups (subsp. *illyrica*, subsp. *dalmatica*, and transitional population) within the territory of Croatia, based on needle morpho-anatomy and later confirmed with RAPD (randomly amplified polymorphic DNA) markers [22]. If we also consider the aforementioned molecular studies of black pine, it is obvious that the situation is far from simple and that the distribution of infraspecific *P. nigra* taxa in the Balkans is not clearly defined.

There is a substantial amount of research using morpho-anatomical methods to determine population variability and differentiation in pines [23–26]. For instance, Boratyńska et al. [24] used morpho-anatomical needle characteristics to reveal geographic differentiation of the *P. mugo* complex, indicating isolation by distance. Other authors showed variation in the cone morphology and/or needle anatomy of *P. sylvestris* populations according to habitat type and altitude zones [25,26]. The morpho-anatomical characteristics of pine needles are also valuable markers for analyses in other fields, i.e., detection of hybrids [24], environmental pollution studies [27], determining the effects of elevated CO_2 [28], etc.

The chemophenetic significance of the terpenes is validated and widely accepted, as they fulfill the basic criteria proposed by Harborne [29]: very high structural diversity, physiological stability, widespread distribution in plant families, and quite simple identification. According to Hanover [30], terpene systems in conifers offer important opportunities for studying the nature of gene regulation and its evolutionary significance. Several authors have already studied the population variability of terpenes of *P. nigra* in many parts of its natural range [31–35], but there is an observed lack of data covering the entire Balkans and Southern Carpathians. Furthermore, in all these population studies, terpenes were isolated by extraction in nonpolar solvents from cortical oleoresin [31] or needles [32–35]. Nonetheless, in recent times, static headspace (HS) analysis has been preferred for quick and efficient extraction of highly volatile compounds and studying the geographic variation of coniferous species [36–38]. Furthermore, this technique is recommended for the research of threatened and protected plant species as it requires a very small amount of material [39]. Still, the main disadvantage of static HS is its limitation to samples that contain considerable levels of highly volatile compounds.

In the present study, the differentiation of 19 native populations representing four *P. nigra* subspecies (*banatica*, *dalmatica*, *nigra*, and *pallasiana*) from different mountain systems (Dinaric, Carpathian-Balkan, Rhodopean, and Scardo-Pindic) of the Balkans and Southern Carpathians was analyzed based on needle morpho-anatomy as well as HS volatiles. We set the following questions: (1) Is it possible to determine the exact number of infraspecific taxa of *P. nigra* within the studied area by a combination of morpho-anatomical and phytochemical analyses? (2) Is differentiation between geographical regions stronger than between studied taxa? and (3) Are there single characters or groups of characters among the morphological and phytochemical characters that are "good" enough to separate the taxa of *P. nigra*?

2. Materials and Methods

2.1. Plant Material

In this study, we selected 19 native populations, representing four *P. nigra* subspecies (*banatica*, *dalmatica*, *nigra*, and *pallasiana*), from different mountain systems (Dinaric, Carpathian-Balkan, Rhodopean, and Scardo-Pindic) of the Balkans and Southern Carpathians. Figure 1 shows a map of the research area, with the locations of selected populations of the studied taxa. Furthermore, geographical and geological data, date of collection, voucher data, as well as the number of studied individuals for every population are presented in Supplementary Material (Table S1). Thus, plant material was sampled from 10 adult individuals in each population (at least 30 m apart from each other), except for one population of *P. nigra* subsp. *pallasiana* (Greece, Mt. Smolika) and one population of *P. nigra* subsp. *dalmatica* (Croatia, Brač), where 20 and 11 individuals were selected for analysis, respectively. The voucher specimen of each population was deposited in the "Herbarium Moesiacum Niš" (HMN) of the Department of Biology and Ecology, Faculty of Sciences and Mathematics, University of Niš, Niš, Serbia. Identification of plant material was carried out by Dr. B.K. Zlatković, Dr. P.D. Marin, and Dr. Z.S. Mitić.

For morpho-anatomical research, 10 needles were collected from each individual from the central part of a two-year-old shoot increment. After sampling, the needles were conserved in 70% alcohol and saved for further preparation and measurement-taking. For phytochemical analysis, two-year-old needles were collected as previously described, but they were deposited in labeled polyethylene bags, transferred to a freezer, and stored at −20 °C prior to further analysis (no longer than 15 days).

Figure 1. Geographical position of analyzed populations (Layer source: Nikolić et al. [38]). For a description of the location and habitat conditions of the populations, cf. (Supplementary Material Table S1). Mountain systems: (1) Dinaric; (2) Scardo-Pindic; (3) Rhodopean; (4) Carpathian-Balkan.

2.2. Morpho-Anatomical Characteristics of Needles

The set of quantitative morpho-anatomical characteristics, preparation, and measurement procedures were generally adopted from Nikolić et al. [40]. Table 1 contains a list of the studied morpho-anatomical characteristics of the needles. The characteristic needle length was measured with a digital caliper, while other characteristics were observed with a Leica Gallen III light microscope. Needle cross-sections were cut with a razor blade in the central part of the needles.

The total number of examined needles was 2010, representing 201 individuals. The averages of 10 needle measurements for each characteristic per individual were taken as the valid values for each individual.

Table 1. Morpho-anatomical characteristics of the needles of studied *P. nigra* taxa: descriptive statistics, results of ANOVA, and post hoc tests.

No.	Morpho-Anatomical Characteristics	F	*p*	*P. nigra* subsp. *nigra* *n* = 70	*P. nigra* subsp. *dalmatica* *n* = 11	*P. nigra* subsp. *banatica* *n* = 40	*P. nigra* subsp. *pallasiana* *n* = 80
				$X \pm SD$	$X \pm SD$	$X \pm SD$	$X \pm SD$
1.	Needle length (cm)	27.2	***	10.1 ± 2.0^{b}	6.0 ± 1.0^{a}	8.6 ± 1.2^{b}	12.2 ± 1.4^{c}
2.	Needle width (μm)	15.8	***	1658.2 ± 213.9^{a}	1838.0 ± 140.5^{b}	1638.4 ± 153.8^{a}	1731.9 ± 182.6^{b}
3.	Needle thickness (μm)	22.0	***	941.9 ± 105.2^{b}	1086.6 ± 67.2^{c}	850.4 ± 116.0^{a}	989.4 ± 93.2^{c}
4.	Endodermis tube perimeter (μm)	24.5	***	2071.0 ± 290.8^{a}	2455.5 ± 196.7^{b}	1839.7 ± 203.2^{a}	2214.0 ± 262.0^{b}
5.	Number of resin ducts	12.3	***	4.9 ± 2.1^{a}	8.4 ± 2.2^{b}	4.4 ± 2.1^{a}	6.3 ± 2.7^{b}
6.	Resin duct diameter (μm)	6.6	***	114.3 ± 14.3^{b}	121.4 ± 18.6^{b}	115.1 ± 10.5^{b}	107.9 ± 9.9^{a}
7.	Distance between resin duct and endodermis tube (μm)	0.8	ns	101.3 ± 13.4	105.8 ± 19.2	99.7 ± 15.1	108.6 ± 22.1
8.	Epidermis + cuticle thickness (μm)	10.3	***	32.0 ± 3.1^{a}	36.3 ± 1.4^{b}	31.3 ± 3.0^{a}	29.1 ± 6.2^{a}
9.	Hypodermis thickness (μm)	4.3	**	74.5 ± 15.1^{ab}	77.0 ± 7.1^{b}	68.6 ± 11.0^{a}	80.2 ± 18.8^{b}

F: ANOVA F-test. *p*: level of significance (ns: not significant; **: $p < 0.01$; ***: $p < 0.001$). *n*: the number of analyzed individuals. *X*: mean. SD: standard deviation. Means with different superscript letters within the same row (a, b, c) differ significantly (Tukey's HSD for unequal N post hoc test).

2.3. Static Headspace (HS)

Two-year-old needles of each individual were cut into pieces of 2–3 mm long. Five hundred mg of chopped plant material was placed in 20-mL HS vials, and 1 mL of distilled water was added to each vial. Thereafter, the vials were placed in a tray for further automated procedures. Specifically, each sample was heated at 80 °C for 20 min according to the following program: shaking for 5 s, pausing for 2 s. After equilibration, the gas vapor of HS volatiles above the sample was sampled with a gas syringe and then injected into the gas chromatograph analyzer (500 μL, in a split ratio of 10:1).

2.4. Gas Chromatography-Mass Spectrometry/Flame Ionization Detector (GC-MS/FID) Analyses

GC-MS was used for the qualitative analysis of HS volatiles and GC-FID for obtaining quantitative data. Samples were analyzed on an Agilent Technologies 7890B GC equipped with a fused silica capillary column (HP-5MS, 250 μm × 25 m, film thickness 0.25 μm, Agilent Technologies, Santa Clara, CA, USA) and coupled with a 7890A FID detector and 7000B MS/MS spectrometer (operating in MS1 scan mode) from the same company. Both qualitative and quantitative analyses of the samples were conducted according to previously published operating parameters [38].

2.5. Statistical Analyses

Statistical data processing was performed by STATISTICA 8 software (Statsoft, Inc., Tulsa, OK, USA). Statistical matrices included morpho-anatomical characteristics of needles as well as the relative percentage composition of HS volatiles as original variables. Analysis of Variance (ANOVA) was used to test the significance of differences in the mean values of variables among the studied *P. nigra* taxa. Furthermore, the significance of the differences between individual pairs of *P. nigra* taxa, based on mean values of variables, was determined by Tukey's honestly significant difference (HSD) for an unequal N post hoc test.

For the multivariate statistical analysis, we included all 9 studied morpho-anatomical characteristics, but in the case of HS volatiles, only compounds detected in the composition $\geq 0.5\%$ in at least one of the analyzed taxa (10 compounds in total) were considered. Multivariate statistical analysis involved canonical discriminant analysis (CDA) and agglomerative hierarchical clustering (AHC). The CDA was performed to check the hypotheses that the analyzed sample was composed of distinct groups that are morpho-

anatomically and/or phytochemically differentiated from each other. In addition, this analysis was carried out to determine the relative importance of morpho-anatomical characteristics and HS volatiles as discriminators between a priori groups. Overall differences between the compared groups are presented by Mahalanobis distances. The calculated matrix distance was used for AHC with the application of Ward's method.

3. Results

3.1. Morpho-Anatomical Characteristics of Needles

Cross sections of needles in all studied taxa of *P. nigra* were predominantly lunate, with resin ducts located in the mesophyll tissue (median type). In very few cases, there were resin ducts touching the epidermis (external type) or central cylinder (internal type).

Results of descriptive statistics for quantitative morpho-anatomical characteristics of needles were presented per taxon (Table 1) as well as per the mountain system inhabited by each studied population (Supplementary Material, Table S2). The comparison of taxa showed the highest mean value of needle length in *P. nigra* subsp. *pallasiana* and the smallest in *P. nigra* subsp. *dalmatica*. On the other hand, the highest mean values of most other characteristics (needle width, needle thickness, endodermis tube perimeter, number of resin ducts, resin duct diameter, and epidermis + cuticle thickness) were obtained for *P. nigra* subsp. *dalmatica*, followed by *P. nigra* subsp. *pallasiana*. As the studied populations of *P. nigra* subsp. *pallasiana* originated at two different mountain systems (Figure 1), we may also notice a considerable difference between them: the populations from the Scardo-Pindic mountain system were characterized by wider and thicker needles, with a thicker hypodermis, a larger endodermis tube, and a greater number of resin ducts in comparison to the Rhodopean populations (Table S2).

ANOVA with *P. nigra* taxa as a grouping variable (Table 1) revealed significant differences between the mean values of all morpho-anatomical characteristics except for the distance between the resin duct and the endodermis tube. The characteristics of needle length and endodermis tube perimeter stood out with high F values (27.2 and 24.5, respectively), indicating that they may play the most important role in the morpho-anatomical differentiation of the studied *P. nigra* taxa. Verification of the ANOVA results was performed by a post hoc test (Tukey (HSD) for unequal N), to determine specific taxa with significant differences. None of the tested morpho-anatomical characteristics showed significant differences among all four *P. nigra* taxa, pointing to existence of distinct morpho-anatomical groups for each of the tested taxa. The needle width, endodermis tube perimeter, and number of resin ducts indicated differentiation of subspecies *dalmatica* and *pallasiana* vs. *nigra* and *banatica*; the epidermis + cuticle thickness showed divergence of subspecies *dalmatica* vs. other subspecies; and the resin duct diameter suggested the differentiation of subspecies *pallasiana* vs. other subspecies. Moreover, the needle length revealed the differentiation of *P. nigra* subspecies in three groups (1. *dalmatica*; 2. *nigra* and *banatica*; and 3. *pallasiana*). However, when ANOVA with a post hoc test was performed with mountain systems as the grouping variable (Table S2), most (i.e., five) analyzed morpho-anatomical characteristics indicated significant differentiation of the Scardo-Pindic system vs. other systems.

For the multivariate statistical analysis, we used all nine studied morpho-anatomical characteristics of the needles. However, the CDA with 19 populations of *P. nigra* from the Balkans and Southern Carpathians (treated as 19 a priori groups) showed an overlap of all populations, so it was not presented. After that, two additional CDAs were tested (with *P. nigra* taxa and mountain systems as a priori groups). The CDA for four subspecies of *P. nigra* (considered as four a priori groups) showed that the first two canonical axes (CAs) participated in 92.0% of the total discrimination, of which the CA1 had 59.9% (Figure 2A and Table 2). The CA1 was mainly determined by needle length and endodermis tube perimeter, and the CA2 by endodermis tube perimeter (Table 2). The scatter plot obtained by this CDA revealed a certain tendency toward differentiation of *P. nigra* subsp. *dalmatica*, while other *P. nigra* subspecies were mainly overlapping (Figure 2A). Therefore, the morpho-anatomical characteristics analyzed by the CDA with *P.nigra* taxa as priori groups suggested

the existence of two major morpho-anatomical groups: *P. nigra* subsp. *dalmatica* and the rest of *P. nigra* taxa. The dendrogram obtained by AHC (Figure 2B) also showed grouping of *P. nigra* taxa in a similar way: *P. nigra* subsp. *dalmatica* vs. other subspecies.

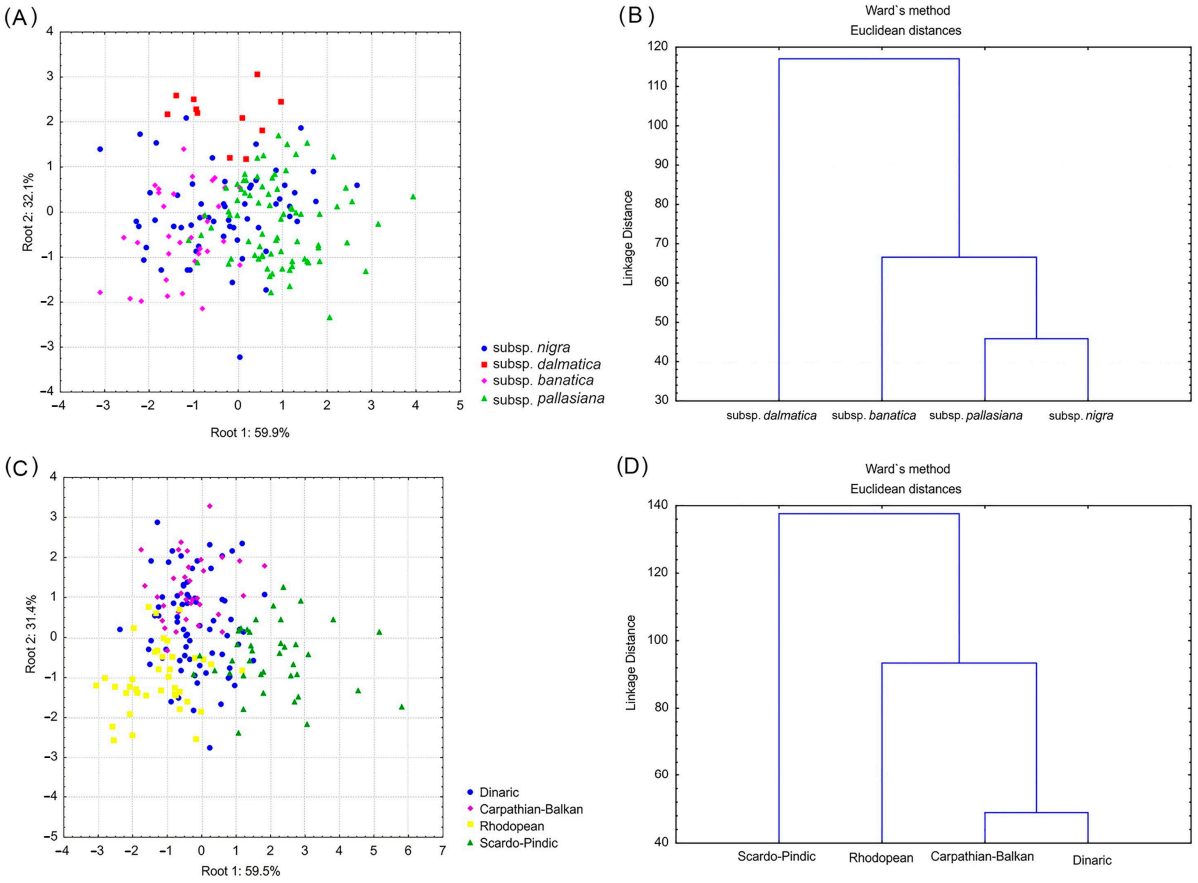

Figure 2. Multivariate statistical analyses based on 9 needle morpho-anatomical characteristics of 201 individuals from 19 populations of *P. nigra*: (**A**) CDA with *P. nigra* taxa as a priori groups; (**B**) AHC with *P. nigra* taxa as a priori groups; (**C**) CDA with mountain systems as a priori groups; (**D**) AHC with mountain systems as a priori groups.

Table 2. Standardized coefficients for the first two canonical axes (CAs) of variation in nine needle morpho-anatomical characteristics from two discriminant functional analyses (with *P. nigra* taxa and mountain systems as a priori groups). Significant coefficients are in boldface.

	A Priori Groups			
	***P. nigra* Taxa**		**Mountain Systems**	
Variables	**CA1**	**CA2**	**CA1**	**CA2**
Needle length	**0.742**	−0.393	**−0.527**	**−0.908**
Needle width	−0.374	−0.014	**−0.518**	0.251
Needle thickness	0.297	0.370	**−0.734**	**−0.520**
Endodermis tube perimeter	**0.636**	**0.603**	**0.909**	−0.409
Number of resin ducts	−0.158	0.066	**0.922**	0.408
Resin duct diameter	−0.271	0.103	0.323	0.369
Distance between resin duct and endodermis tube	0.181	−0.444	0.187	−0.126
Epidermis + cuticle thickness	−0.163	0.411	**−0.816**	−0.051
Hypodermis thickness	−0.371	0.107	**0.780**	**0.571**
Eigenvalue	0.662	0.355	1.205	0.635
Cum.Prop.	0.599	0.920	0.595	0.909

The CDA for four mountain systems of studied *P. nigra* populations revealed that the first two canonical axes participated in 90.9% of the total discrimination, of which the first axis (CA1) accounted for 59.5% (Figure 2C and Table 2). The significant influence of CA1 was the distribution of almost all morpho-anatomical characteristics (except the resin duct diameter and the distance between the resin duct and the endodermis tube; Table 2). The obtained scatter plot (Figure 2C) showed a smaller number of differentiated morpho-anatomical groups than was a priori proposed. Namely, most individuals from the Scardo-Pindic mountain system (Greece) were separated along the CA1, showing a considerable degree of differentiation, while individuals from the other mountain systems were overlapping. Therefore, two morpho-anatomical groups could be distinguished based on the CDA with mountain systems as priori groups: the Scardo-Pindic mountain system as one group and all the remaining mountain systems (Dinaric—Carpathian-Balkan—Rhodopean) as the other. The dendrogram obtained by AHC (Figure 2D) supported the results of the CDA. Given that the previous CDA failed to confirm differentiation of *P. nigra* subsp. *pallasiana*, the result of this CDA can support the fact that only *P. nigra* subsp. *pallasiana* populations from the Scardo-Pindic mountain system were distinguished from other Balkan black pine populations. On the contrary, the Rhodopean populations of this subspecies did not show a signal of significant differentiation based on the applied set of morpho-anatomical markers (Figure 2C,D).

In this way, by combining the results of all multivariate analyses (Figure 2), the morpho-anatomical characteristics of needles suggested the existence of three groups of *P. nigra* within the studied area: 1. Dalmatian coast, distinguished by the shortest needles and the largest endodermis tube; 2. Greece (Scardo-Pindic mountain system), characterized by the longest needles and medium endodermis tube; and 3. the remaining part of the Balkans with the Southern Carpathians, characterized by the needles intermediate in length and the smallest endodermis tube.

Finally, when the three obtained morpho-anatomical groups of *P. nigra* were compared regarding the habitat type and sampling time of plant material (Table S1), it was obvious that there was no concordance between them. Hence, it seems more likely that the observed differentiation of *P. nigra* in the Balkans and Southern Carpathians was genetically conditioned.

3.2. HS Needle Volatiles

GC-MS/FID analyses of HS needle volatiles, isolated from 201 individuals from 19 natural populations of four *P. nigra* taxa, revealed the presence of 12 compounds: 10 monoterpene hydrocarbons and two sesquiterpene hydrocarbons (Table 3). Results of descriptive statistics for all identified HS needle volatiles were presented per taxon (Table 3), as well as per mountain system (Supplementary Material, Table S3). As the most volatile compounds, monoterpene hydrocarbons represented the main compound class in all *P. nigra* taxa, slightly varying from 91.0% (*P. nigra* subsp. *pallasiana*) to 94.1% (*P. nigra* subsp. *dalmatica*). On the other side, concentrations of sesquiterpene hydrocarbons were significantly lower, ranging from 5.8% (*P. nigra* subsp. *dalmatica*) to 8.9% (*P. nigra* subsp. *pallasiana*). Furthermore, in terms of dominant HS volatile compounds, the studied *P. nigra* taxa were also uniform, with obvious domination of α-pinene and β-pinene. α-Pinene had the lowest concentration in *P. nigra* subsp. *pallasiana* (72.6%) and *P. nigra* subsp. *nigra* (72.7%) and the highest in *P. nigra* subsp. *dalmatica* (85.1%), but β-pinene had the lowest concentration in *P. nigra* subsp. *dalmatica* (5.9%) and the highest in *P. nigra* subsp. *pallasiana* (13.2%). Regarding the most abundant sesquiterpene hydrocarbon, germacrene D, its concentration again varied from *P. nigra* subsp. *dalmatica* with 3.2% to *P. nigra* subsp. *pallasiana* with 6.6%. By comparing the Scardo-Pindic and Rhodopean mountain systems (*P. nigra* subsp. *pallasiana*), it was noticed that the Rhodopean populations contained a somewhat lower concentration of α-pinene (71.1%) but a twice-as-high concentration of β-pinene (15.7%) compared to the populations from the Scardo-Pindic system (75.4 and 8.3%, respectively). On the other side, the Scardo-Pindic populations were characterized by the highest concentration of both

detected sesquiterpene hydrocarbons (germacrene D—6.7% and (*E*)-caryophyllene—2.9%) among all mountain systems.

Table 3. HS needle volatiles of studied *P. nigra* taxa: descriptive statistics, results of ANOVA, and post hoc tests.

No.	Compounds	Class of Compounds	RI	LI	F	*p*	*P. nigra* subsp. *nigra* *n* = 70	*P. nigra* subsp. *dalmatica* *n* = 11	*P. nigra* subsp. *banatica* *n* = 40	*P. nigra* subsp. *pallasiana* *n* = 80
							X ± SD	*X* ± SD	*X* ± SD	*X* ± SD
1.	Tricyclene	MH	921	921	0.4	ns	0.0 ± 0.1	0.0 ± 0.0	0.0 ± 0.1	0.0 ± 0.2
2.	α-Thujene	MH	926	924	2.7	ns	1.2 ± 1.0	0.7 ± 0.6	0.8 ± 0.9	0.8 ± 0.7
3.	**α-Pinene**	MH	934	932	7.7	***	**72.7 ± 10.2** ^{a}	**85.1 ± 8.7** ^{b}	**79.5 ± 6.5** ^{a}	**72.6 ± 11.7** ^{a}
4.	Camphene	MH	949	946	1.3	ns	1.1 ± 0.4	0.8 ± 0.4	1.2 ± 0.6	1.1 ± 0.6
5.	Sabinene	MH	971	969	5.4	**	0.1 ± 0.3 ^{b}	0.0 ± 0.0 ^{ab}	0.0 ± 0.1 ^{ab}	0.0 ± 0.0 ^{a}
6.	**β-Pinene**	MH	980	974	2.3	ns	**11.5 ± 10.7**	**5.9 ± 6.3**	**8.0 ± 6.6**	**13.2 ± 13.7**
7.	Myrcene	MH	990	988	9.9	***	1.2 ± 0.4 ^{c}	0.5 ± 0.4 ^{a}	1.0 ± 0.5 ^{bc}	0.9 ± 0.4 ^{ab}
8.	Limonene + β-Phellandrene	MH	1029	1024/1025	9.7	***	2.9 ± 2.1 ^{b}	1.4 ± 0.9 ^{ab}	1.5 ± 0.8 ^{a}	1.7 ± 0.8 ^{a}
9.	(*E*)-β-ocimene	MH	1044	1044	2.8	*	0.4 ± 0.4 ^{b}	0.0 ± 0.0 ^{a}	0.3 ± 0.5 ^{b}	0.5 ± 0.6 ^{b}
10.	Terpinolene	MH	1087	1086	4.5	**	0.7 ± 0.8 ^{b}	0.1 ± 0.2 ^{ab}	0.4 ± 0.6 ^{ab}	0.3 ± 0.4 ^{a}
11.	(*E*)-Caryophyllene	SH	1420	1417	0.4	ns	2.2 ± 1.3	2.6 ± 1.5	2.1 ± 1.4	2.3 ± 1.5
12.	Germacrene D	SH	1485	1484	4.0	**	6.1 ± 3.3 ^{b}	3.2 ± 2.1 ^{a}	5.1 ± 2.7 ^{b}	6.6 ± 3.5 ^{b}
	Total						99.9 ± 0.5	100.0 ± 0.9	99.9 ± 0.3	99.9 ± 0.3
	Monoterpene hydrocarbons (MH)						91.7 ± 3.8	94.1 ± 3.9	92.8 ± 3.4	91.0 ± 4.4
	Sesquiterpene hydrocarbons (SH)						8.2 ± 3.9	5.8 ± 3.2	7.2 ± 3.4	8.9 ± 4.3

RI: Experimental linear retention indices relative to C_8–C_{20} alkanes. LI: Literature indices-Adams' retention indices. F: ANOVA F-test. *p*: level of significance (ns: not significant; *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$). *n*: the number of analyzed individuals. *X*: mean. SD: standard deviation. Means with different superscript letters within the same row (a, b, c) differ significantly (Tukey's HSD for unequal N post hoc test). The HS volatiles recorded in the average content > 10% in at least one of the studied *P. nigra* taxa are in boldface.

Out of 12 tested HS volatiles, ANOVA with *P. nigra* taxa as a grouping variable (Table 3) showed significant differences for seven compounds (α-pinene, sabinene, myrcene, limonene + β-phellandrene, (*E*)-β-ocimene, terpinolene, and germacrene D). Tukey HSD for an unequal N post hoc test supported the significance of the difference between individual pairs of studied *P. nigra* taxa for all compounds, just as ANOVA. None of the tested volatiles showed significant differences among all four *P. nigra* taxa. Three compounds (α-pinene, (*E*)-β-ocimene, and germacrene D) indicated differentiation of subspecies *dalmatica* vs. other subspecies. On the other side, when ANOVA with a post hoc test was performed using mountain systems as the grouping variable (Table S3), two compounds ((*E*)-β-ocimene and (*E*)-caryophyllene) indicated divergence of the Scardo-Pindic system vs. other systems.

For the multivariate statistical analysis, only HS volatiles detected in concentrations $\geq 0.5\%$ in at least one of the analyzed taxa (10 compounds in total) were considered. Once again, the CDA with 19 native *P. nigra* populations (treated as 19 a priori groups) showed an overlap of all populations, so it was not presented. However, the CDA for four *P. nigra* subspecies (considered as four a priori groups) revealed that the first two canonical axes explained 88.9% of the total discrimination, of which the CA1 explained 63.0% (Figure 3A and Table 4). Four compounds (α-pinene, β-pinene, (*E*)-caryophyllene, and germacrene D) showed a significant impact on CA1 that explained the highest percentage of discrimination (Table 4). Like with morpho-anatomical characteristics, this CDA suggested only a slight separation of *P. nigra* subsp. *dalmatica* (Figure 3A), which was also confirmed by the AHC analysis (Figure 3B).

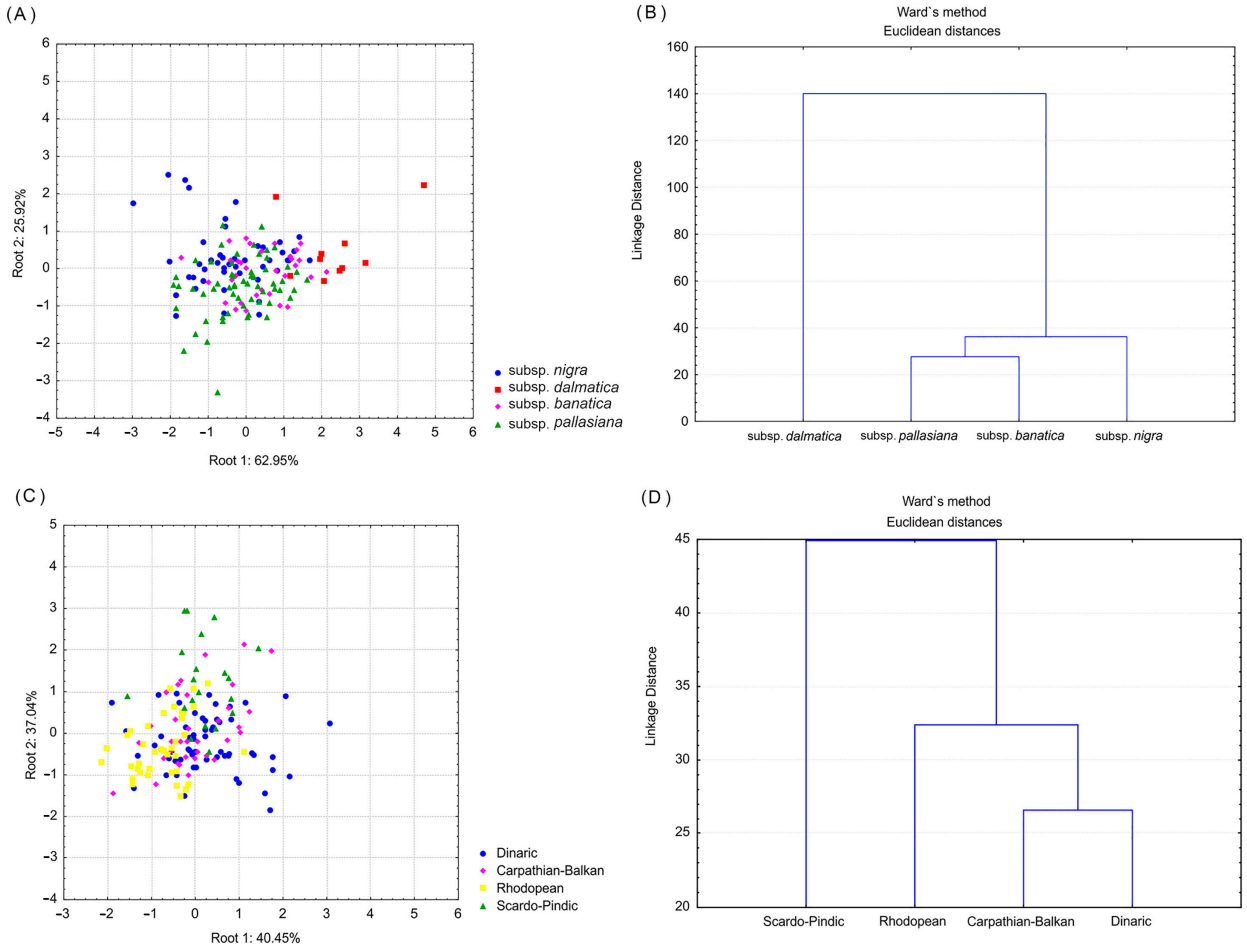

Figure 3. Multivariate statistical analyses based on 10 HS needle volatiles of 201 individuals from 19 populations of *P. nigra*: (**A**) CDA with *P. nigra* taxa as a priori groups; (**B**) AHC with *P. nigra* taxa as a priori groups; (**C**) CDA with mountain systems as a priori groups; (**D**) AHC with mountain systems as a priori groups.

Table 4. Standardized coefficients for the first two canonical axes (CAs) of variation in 10 needle volatiles from two discriminant functional analyses (with *P. nigra* taxa and mountain systems as a priori groups). Significant coefficients are in boldface.

	A Priori Groups			
	***P. nigra* Taxa**		**Mountain Systems**	
Variables	**CA1**	**CA2**	**CA1**	**CA2**
α-Thujene	0.469	0.272	**0.809**	**0.613**
α-Pinene	**5.956**	**6.360**	**8.735**	**0.546**
Camphene	0.232	0.030	**0.524**	**0.649**
β-Pinene	**5.935**	**6.518**	**8.614**	**0.622**
Myrcene	−0.340	0.342	0.426	−0.030
Limonene + β-Phellandrene	0.463	**1.640**	**1.913**	−0.254
(*E*)-β-ocimene	0.202	−0.204	0.251	**0.671**
Terpinolene	0.096	**0.948**	**0.584**	**−0.847**
(*E*)-Caryophyllene	**1.272**	**0.865**	**1.337**	0.329
Germacrene D	**0.904**	**1.789**	**2.461**	0.217
Eigenvalue	0.517	0.213	0.220	0.201
Cum.Prop.	0.630	0.889	0.405	0.775

The CDA for four mountain systems of studied *P. nigra* populations showed that the first two functions participated in 77.5% of the total discrimination, of which the first function was represented by 40.5% (Figure 3C and Table 4). The significant influence of CA1 was the distribution of almost all tested HS volatiles (except myrcene and (*E*)-β-ocimene; Table 4). The obtained scatter plot revealed very slight differentiation of the Scardo-Pindic system from the other mountain systems (Figure 3C), in agreement with the AHC analysis (Figure 3D). Considering that the previous CDA failed to confirm the distinction between *P. nigra* subsp. *pallasiana*, the result of this CDA can be explained by the fact that only *P. nigra* subsp. *pallasiana* populations from the Scardo-Pindic mountain system were distinguished from other Balkan black pine populations.

Hence, considering the result of all multivariate analyses (Figure 3), HS needle volatiles suggested differentiation of three phytochemical groups of *P. nigra* within the studied area in the same way as morpho-anatomical markers: 1. Dalmatian coast, distinguished by the highest concentration of α-pinene and the lowest concentration of β-pinene and germacrene D; 2. Greece (Scardo-Pindic mountain system), characterized by the highest concentration of (*E*)-caryophyllene; and 3. the remaining part of the Balkans with the Southern Carpathians, characterized by the highest concentration of β-pinene and the lowest concentration of (*E*)-caryophyllene.

Once again, no concordance was found after comparing the three obtained phytochemical groups of *P. nigra* with habitat type or sampling time of plant material (Table S1).

4. Discussion

4.1. Variability of Morpho-Anatomical Characteristics of Needles

The mean values obtained for the needle length of the studied *P. nigra* taxa are within the range of this characteristic shown in the key of Flora Europaea [10]. Thus, *P. nigra* subsp. *dalmatica* was characterized by the shortest and *P. nigra* subsp. *pallasiana* by the longest needles (Table 1). However, both taxa had wider and thicker needles with a more developed endodermis tube compared to *P. nigra* subsp. *nigra* and *P. nigra* subsp. *banatica*. These results support the conclusion reached by Vidaković [19], who described subspecies *dalmatica* and *pallasiana* as the most xerophytic Balkan *P. nigra* taxa. According to Vidaković [19], the more developed hypodermis and sclerenchyma (above, below, and between the vascular bundles) represent important protective layers against excessive insolation in these two taxa. On the contrary, subspecies *nigra* and *banatica* with narrower and thinner needles, a less developed hypodermis, and sclerenchyma (only above the vascular bundles) probably suffer much less from excessive insolation. However, in terms of the thickness of the epidermis with cuticle, the most xerophytic Balkan *P. nigra* taxa have shown considerable differences (Table 1): *P. nigra* subsp. *dalmatica* was characterized by the thickest epidermis with cuticle, while in *P. nigra* subsp. *pallasiana*, this layer was considerably thinner and similar to the other studied subspecies (Table 1). In agreement with our data, Vidaković [19] distinguished two *P. nigra* groups based on epidermis thickness in this area: subspecies *pallasiana*, *gocensis*, and *illyrica* (the last two were treated as *nigra* in the present study) with an epidermis thickness of less than 30 µm vs. subspecies *dalmatica* and *austriaca* (the last not considered in our study) with an epidermis thickness between 30 and 40 µm. Perhaps the biggest discrepancy between the present study and that by Vidaković [19] was related to the number of resin ducts. Namely, Vidaković [19] suggested that the number of resin ducts in *P. nigra* needles increases going from the east towards the west of the Balkan Peninsula. The results of the present study failed to fully uphold such a hypothesis since the smallest number of resin ducts was found in *P. nigra* subsp. *banatica* and *P. nigra* subsp. *nigra*. Therefore, our results are more supportive of a hypothesis that a greater number of resin ducts was a characteristic of more xerophytic southern *P. nigra* taxa (*P. nigra* subsp. *dalmatica* and *P. nigra* subsp. *pallasiana*) than a geographical trend of increase from east to west of the Balkans.

4.2. Variability of HS Needle Volatiles

To the best of our knowledge, this study is the very first report on the variability of needle terpenes of *P. nigra* isolated by the static HS procedure. Nevertheless, several authors studied the variability of needle terpenes of *P. nigra* in many parts of its natural range, whereby terpenes were isolated by extraction in nonpolar solvents [32–35]. In most of these studies, α-pinene represented the major compound of the needle extracts, just as in the present research, while the second dominant compound was either germacrene D (7 populations from Serbia) [35] or β-pinene (9 populations from Bulgaria) [34]. Furthermore, Rafii et al. [32] and Bojovic et al. [33], who presented the composition of the needle extracts separately for monoterpenes and sesquiterpenes, showed α- and β-pinene as the dominant monoterpenes and germacrene D and (*E*)-caryophyllene as the dominant sesquiterpenes in many populations across Europe. Additionally, these four terpenes have also been the most common dominant compounds in the needle essential oils of different *P. nigra* taxa [41–44]. Perhaps the most distinctive terpene profile was recorded for the needle essential oil of *P. nigra* subsp. *laricio* from Corsica, which was dominated by oxygenated diterpene manool oxide [45].

4.3. Taxonomic Consideration

The combination of the results of all multivariate statistical analyses with the applied set of morpho-anatomical and phytochemical markers revealed that *P. nigra* is differentiated into three groups within the Balkans and Southern Carpathians:

(1) The first group included the population from an island of Dalmatia, which corresponds to *P. nigra* subsp. *dalmatica* (Dalmatian black pine). This taxon is endemic to the Dalmatian coast and islands in Croatia, but with doubtful data on its precise distribution [21,22]. The distinctiveness of its populations was already suggested based on the morpho-anatomical characteristics of the needles [21], but they showed a lack of significant differentiation from populations of *P. nigra* subsp. *nigra* at the level of cuticular wax compounds [20]. If we consider three comprehensive molecular studies of *P. nigra*, we can notice that the situation is similar to that of phenotypic markers. Namely, some markers indicated that *P. nigra* subsp. *dalmatica* represents a distinct genetic lineage of black pine [16], while others failed to support such a hypothesis [15,17].

(2) The second group consisted of populations from Greece. These populations (together with neighboring populations from southernmost North Macedonia) have also shown a very sharp differentiation from other Balkan populations based on cuticular wax compounds [20]. Moreover, according to Naydenov et al. [17], populations from western Greece present one of five genetic groups of *P. nigra* (1. Morocco-Spain; 2. France-Corsica; 3. Western Greece; 4. Northern Turkey; and 5. the remaining part of the black pine natural range) based on mtDNA. In older literature sources [4,10], Greek populations were usually attributed to *P. nigra* subsp. *pallasiana* (Crimean black pine). This taxon is mainly distributed in Turkey and the Caucasus, but regarding the European distribution, Jalas and Suominen [4] showed that it appears across the eastern and central Balkans, Southern Carpathians, Crimea, and the European part of Turkey. On the other side, according to POWO [13], Greek populations (together with other Balkan and the Southern Carpathian populations) should be ascribed to *P. nigra* subsp. *nigra*, since *P. nigra* subsp. *pallasiana* has extremely small distribution range on the European continent (Crimea and the European part of Turkey). However, in the light of the most recent results of Naydenov et al. [17], who presented the populations from western Greece, northern Turkey, and the main area of *P. nigra* distribution as three distinct genetic groups, one can speculate that there is an additional subspecies of *P. nigra* within this region that corresponds to populations from Greece. This assumption is somewhat supported by the study of Scotti-Saintagne et al. [16], who, although they did not include any population from Greece, also identified three genetic lineages within the distribution region of

subspecies *nigra* and *pallasiana* (Central Europe, Crimea with Turkey, and Cyprus) using a set of different molecular markers.

(3) The third group included populations from the remaining part of the Balkans and the Southern Carpathians, corresponding to *P. nigra* subsp. *nigra*. Therefore, the combined results of all performed analyses have clearly shown that populations of *P. nigra* subsp. *banatica* (Banat black pine) belong to the same group as populations of *P. nigra* subsp. *nigra*, so the hypothesis about their separation at the rank of distinct subspecies was not upheld. Banat black pine is endemic to the southwestern Romania and northeastern Serbia (the Carpathian-Balkan mountain system), but with very controversial data on its taxonomic position [20]. For instance, populations of *P. nigra* from the Southern Carpathians are regarded as a separate subspecies (*P. nigra* subsp. *banatica*) in the Flora Romania [18], while in the Flora Europaea [10], they are ascribed to *P. nigra* subsp. *pallasiana* (without stating any distinct taxonomic position). Considering the point of view that *P. nigra* subsp. *pallasiana* appears in Europe only in Crimea and the European part of Turkey, current floristic databases [11–13] treat Banat black pine taxa as synonyms of *P. nigra* subsp. *nigra*. Anyway, based on the recent phytochemical [20] and molecular data [16,17,46], all populations of *P. nigra* from Serbia, Romania, Bulgaria, Bosnia, and Herzegovina and continental Croatia belong to a single genetic lineage that corresponds to *P. nigra* subsp. *nigra*, matching the results of the present study.

5. Conclusions

Differentiation of 19 native populations belonging to different *P. nigra* subspecies (*banatica*, *dalmatica*, *nigra*, and *pallasiana*) in the Balkans and Southern Carpathians was analyzed by using both morpho-anatomical characteristics and HS volatiles of needles. The combination of the results of all multivariate analyses with both types of markers revealed that *P. nigra* is differentiated into three groups within the studied area (the Dalmatian coast, Greece, and the rest of the Balkans with the Southern Carpathians). Thus, the first group included the population from the island of Dalmatia, which corresponds to *P. nigra* subsp. *dalmatica*. The third group consisted of populations from continental Croatia, Bosnia and Herzegovina, Serbia, Romania, and Bulgaria, which correspond to *P. nigra* subsp. *nigra*. In light of the recent molecular data that indicate that the Greek populations (the second group) represent a distinct genetic lineage of *P. nigra* placed between the populations from the principal area (*P. nigra* subsp. *nigra*) and Turkey (*P. nigra* subsp. *pallasiana*), one can speculate that there is an additional subspecies of *P. nigra* in this region that corresponds to populations from Greece. Extending our analyses to Asia Minor and Crimea will bring additional insight into the morpho-anatomical characteristics and volatile profile of needles, which might help clarify the issue of the phenotypic diversification of *P. nigra*, especially in the eastern part of its range.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/f15050739/s1. Table S1: Location and habitat description of the selected populations of four *P. nigra* taxa from the Balkan Peninsula and Southern Carpathians; Table S2: Morpho-anatomical characteristics of the needles of studied *P. nigra* populations classified according to mountain systems: descriptive statistics, results of ANOVA, and post hoc tests; Table S3: HS needle volatiles of studied *P. nigra* populations classified according to mountain systems: descriptive statistics, results of ANOVA, and post hoc tests.

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