Terpenes as Useful Markers in Differentiation of Natural Populations of Relict Pines *Pinus heldreichii*, *P. nigra*, and *P. peuce*

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Comparative analysis of terpene diversity and differentiation of relict pines *Pinus heldreichii*, *P. nigra*, and *P. peuce* from the central Balkans was performed at the population level. Multivariate statistical analyses showed that the composition of needle terpenes reflects clear divergence among the pine species from different subgenera: *P. peuce* (subgenus *Strobus*) *vs.* *P. nigra* and *P. heldreichii* (subgenus *Pinus*). In addition, despite the described morphological similarities and the fact that *P. nigra* and *P. heldreichii* may spontaneously hybridize, our results indicated differentiation of their populations naturally growing in the same area. In accordance with recently proposed concept of 'flavonic evolution' in the genus *Pinus*, we assumed that the terpene profile of soft pine *P. peuce*, defined by high amounts of six monoterpenes, is more basal than those of hard pines *P. nigra* and *P. heldreichii*, which were characterized by high content levels of mainly sesquiterpenes. In order to establish precise positions of *P. heldreichii*, *P. nigra* and *P. peuce* within the taxonomic and phylogenetic tree, as well as develop suitable conservation strategies and future breeding efforts, it is necessary to perform additional morphological, biochemical, and genetic studies.

Keywords: *Pinus heldreichii*, *Pinus nigra*, *Pinus peuce*, Terpenoids, Molecular diversity.

Introduction

Pinus L., with over 100 extant species, is the largest and the most widespread genus of conifers in the Northern Hemisphere.[1] A century ago, *Shaw*[2] proposed to split the genus into two lineages, Haploxylon (with only one fibrovascular bundle in the needle) and Diploxylon (with two fibrovascular bundles), considered two distinct subgenera in all later classifications.[3 – 6] The first subgenus - *Strobus* (soft pines) is characterized by non-decurrent pulvini at the cataphyll bases and deciduous fascicle sheaths, while the second subgenus - *Pinus* (hard pines) has decurrent pulvini at the cataphyll bases and usually persistent fascicle sheaths.[6]

The territory of central Balkans is inhabited by many conifer species, including three autochthonous pines of Tertiary origin: *i*) Bosnian pine (*Pinus heldreichii* CHRIST), a Balkan subendemic naturally occupying fragmented areas at high mountains in the Balkans and southern Italy, *ii*) Black pine (*P. nigra* ARNOLD), one of the most widespread pines in Europe, with a highly fragmented range that extends from North Africa through the Northern Mediterranean and eastwards to the Black Sea; both representatives of the subgenus *Pinus*, and *iii*) Macedonian pine (*P. peuce* GRISEB), a Balkan endemic of the high mountains of Bulgaria, Macedonia, Serbia, Montenegro, Albania, and Greece, which is a member of the subgenus *Strobus*.[7][8]

For a long time, the opinions on phylogenetic positions of these relict pine species have been contentious, particularly relationships between hard pines *P. heldreichii* and *P. nigra*. After *Shaw*,[2] who considered Bosnian pine only as a variety of *P. nigra*, *Mirov*[9] first revealed that *P. heldreichii* has a completely different terpene composition. Although the distinct taxonomic status of Bosnian pine has been confirmed in all subsequent studies, its position between the Eurasian and the 'true' Mediterranean hard pines remains debatable.[10]

Discoveries of spontaneous Bosnian pine hybrids with *P. nigra*[11] and *P. mugo* Turra[12] in Bosnia and Herzegovina, as well as several varieties and transitional forms of *P. heldreichii* have undoubtedly contributed to former perplexities.[13] However, the majority of recent molecular studies had indicated that *P. heldreichii* is more closely related to Mediterranean (subsection *Pinaster*) than to the Eurasian hard pine lineage (subsection *Pinus*) that includes *P. nigra*.[6][10][14] Hence, in modern infrageneric classifications of the genus, *P. nigra* and *P. heldreichii* belong to different subsections (*Pinus* and *Pinaster*, resp.) of section *Pinus* according to the chloroplast[6][10] and nuclear DNA sequences.[14]

The use of terpenes as chemotaxonomic markers has a very long tradition and is based on the fact that the terpene qualitative profile is under strong genetic control and not significantly influenced by environmental factors.[15][16] *Hanover*[17] discussed the efficacy of terpenes as genetic markers, pointing out their importance for researches of biodiversity, geographic variability, evolution and systematics, especially in studies of conifer order Pinales. On the other hand, there is certain variability in terpene composition, caused by various exogenous and endogenous factors: phases of plant's ontogenetic development, type of organ or tissue, ecological factors, procedure of processing the plant material, and particular terpene isolation procedure.[17] Therefore, the proper sampling and strict adherence to procedure in harvesting and storing plant material and terpene isolation are the necessary conditions that must be met in order to achieve valid interpretation of study results.

Chemodiversity of needle terpenes, obtained by pentane extraction, of *P. heldreichii*,[13][18] *P. nigra*,[19] and *P. peuce*[20] has been studied at the population level on the territory of Serbia and Montenegro. Therefore, with respect to the already published data, this study intends to use the terpene pattern in order to define the relationships among relict pines inhabiting the central part of the Balkan Peninsula. This opens many interesting questions, considering that *P. heldreichii* populations from southwestern Serbia and northern Montenegro, which are regarded as var. *pančići* Fukarek,[21][22] represent a transitional form towards the black pine.[8]

Differentiation between *P. heldreichii* and *P. peuce* populations from the central Balkans (with *Picea omorika* Pančić (Purk.) as outgroup), has already been approved by statistically selected terpenes[23] and *n*-alkanes.[24] However, in the present work accent was given on investigation of terpene differentiation between two morphologically similar hard pines: *P. heldreichii* and *P. nigra*, as well as to their individual

relationships with soft pine *P. peuce*. Although the differences in the terpene composition between *P. heldreichii* and *P. peuce* have already been investigated,[23] in this article we included one additional *P. heldreichii* population and used one more multivariate statistical analysis (canonical discriminate analysis). The aim of this study was to find, through several statistical analyses, a new set of terpene compounds which could help to elucidate diversity and differentiation of *P. nigra*, *P. heldreichii*, and *P. peuce*.

Bearing all this in mind, the terpene profiles were studied on 2-year-old needles from *ca.* 15 populations of *P. heldreichii*,[13][18] *P. nigra*,[19] and *P. peuce*[20] from naturally occurring stands in the central Balkans (*Fig. 1*,
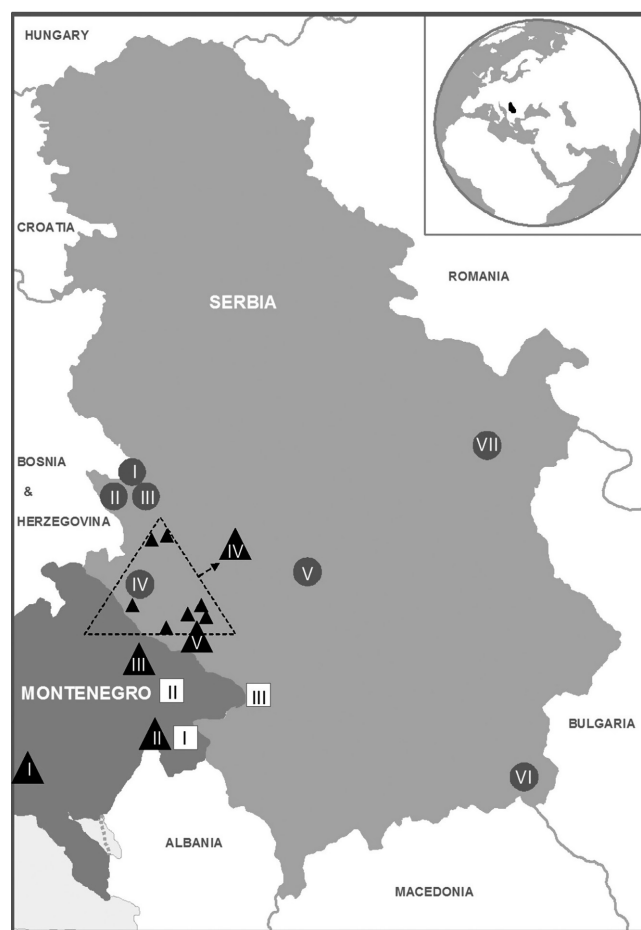

Figure 1. Location of analyzed populations. ● *Pinus nigra* (*P. nigra* subsp. *nigra*: I – Mt. Tara, Banjska stena, II – Mt. Tara, Omar, and III – Mt. Tara, Zmajevečki potok; *P. nigra* var. *gocensis*: IV – Priboj, Crni vrh, and V – Mt. Goč, Gvozdac; ▲ *P. nigra* subsp. *pallasiana*: VI – Mt. Dukat, Jarešnik; *P. nigra* var. *banatica*: VII – Lazareva Reka Canyon, Kovej); *P. heldreichii* (I – Mt. Lovćen, II – Mt. Zeletin, III – Mt. Bjelasica; *P. heldreichii* var. *pančići*: IV – small triangles represent scattered little groups and individual trees between Mt. Zlatibor and Pešter plateau considered as single population, and V – Mt. Revuša); □ *P. peuce* (I - Mt. Zeletin, II – Mt. Sjekirica, III – Mt. Mokra Gora).

Table 1. Location and habitat description of the selected populations of *P. nigra*, *P. heldreichii*, and *P. peuce*

Taxon	Locality	Population code	Latitude (N)	Longitude (E)	Altitude [m a.s.l.]	Substratum	Number of individuals analyzed	Date of collection
P. nigra subsp. *nigra*	Serbia: Mt. Tara, Banjska stena	I	43°57′04″	19°24′04″	1040	Limestone	30	August 2009
P. nigra subsp. *nigra*	Serbia: Mt. Tara, Omar	II	43°54′02″	19°23′18″	950	Limestone	30	August 2009
P. nigra subsp. *nigra*	Serbia: Mt. Tara, Zmajevečki potok	III	43°51′45″	19°25′05″	830	Serpentinite	30	August 2009
P. nigra var. *gocensis*	Serbia: Priboj, Crni vrh	IV	43°34′56″	19°35′06″	1100	Serpentinite	30	August 2009
P. nigra var. *gocensis*	Serbia: Mt. Goč, Gvozdac	V	43°33′32″	20°40′56″	790	Serpentinite	30	August 2009
P. nigra subsp. *pallasiana*	Serbia: Mt. Dukat, Jarešnik	VI	42°22′32″	22°24′10″	1300	Crystalline slate	30	August 2009
P. nigra var. *banatica*	Serbia: Lazareva Reka Canyon, Kovej	VII	44°01′02″	21°55′39″	750	Limestone	15	August 2009
P. heldreichii	Montenegro: Mt. Lovćen	I	42°25′	18°50′	1700 – 1800	Limestone	30	August 2003
P. heldreichii	Montenegro: Zeletin	II	42°37′	19°50′	1700 – 1900	Limestone, Serpentinite, Schist	30	August 2003
P. heldreichii	Montenegro: Bjelasica	III	42°53′	19°45′	1700	Limestone, Dolomite	30	August 2003
P. heldreichii var. *pančići*	Serbia: little groups and individual trees between Mt. Zlatibor and Pešter plateau considered as single population	IV	43°15′ – 43°30′	19°30′ – 19°55′	1100 – 1430	Limestone, Dolomite, Hornestone, Tuff, Neogene sediments	7	September 2003
P. heldreichii var. *pančići*	Serbia: Mt. Revuša	V	43°14′52″	19°51′39″	1525	Neogene sediments	30	August 2009
P. peuce	Montenegro: Mt. Zeletin	I	42°37′	19°50′	1700 – 1900	Limestone, Serpentinite, Schist	30	August 2003
P. peuce	Montenegro: Mt. Sjekirica	II	42°40′	19°50′	1700	Schist, Serpentinite	30	August 2003
P. peuce	Serbia: Mt. Mokra Gora	III	42°50′	20°23′	1750 – 1950	Limestone	30	August 2003

Table 1). The results could be important concerning chemotaxonomy, biogeography, phylogeny, and evolution of these three relict pine species.

Results and Discussion

Species Diversity with Respect to Abundance of Terpene Classes and Major Terpene Compounds

Mono- and sesquiterpenes were the main compound classes in needles of analyzed pines from the central Balkans, but in different ratios (*Fig. 2*). The total mono/sesquiterpene ratios were about 1.5:1 for both hard pines: *P. nigra* and *P. heldreichii*, and about 3:1 for the soft pine *P. peuce*. Namely, *P. peuce* has shown a slightly higher level of monoterpene hydrocarbons (60.5%) and particularly O-containing monoterpenes (9.5%) in addition to the lowest level of sesquiterpene hydrocarbons (24.6%). Monoterpene hydrocarbons also represented the main compound class in *P. nigra* and *P. heldreichii* (56.5 and 52.8%, resp.), but closer to the level of sesquiterpene hydrocarbons (41.6 and 37.6%, resp.) when compared to *P. peuce*. However, needles of *P. heldreichii* were also characterized by a slightly higher content of total diterpenes (2.4%), while in the other two pines diterpenes were detected mostly in traces.

Comparison of dominant terpenes among the studied pines has shown highly distinct profiles of the major terpene metabolites (*Table 2*; *Fig. 3*). The terpene profile of *P. peuce* was characterized by the highest number of compounds (six) detected in an average relative concentration over 5%. In addition to the main mono- and sesquiterpene hydrocarbons (α-pinene, germacrene D, camphene, β-pinene and (*E*)-caryophyllene), it also includes an oxygenated monoterpene (bornyl acetate), resulting in a considerably higher content of O-containing monoterpenes in this soft pine. On the other side, *P. heldreichii* and *P. nigra* were characterized by five and four, compounds detected in concentrations higher than 5%, respectively, and all of them were mono- and sesquiterpene hydrocarbons: limonene, α-pinene, germacrene D, (*E*)-caryophyllene, and β-pinene in *P. heldreichii*, while *P. nigra* had a similar profile but without limonene. Nevertheless, if only the terpenes found in high amounts (> 10%) are analyzed, there is a noticeable predominance of the same two volatiles (α-pinene and germacrene D) in *P. nigra* and *P. peuce*, while limonene, α-pinene, and germacrene D were volatiles with high contents recorded in *P. heldreichii*. Amounts of terpene compounds for each species were already reported.[13][18 – 20]

Variability and Differentiation of Studied Pines Based on Terpene Markers

Analysis of variance. The terpene profiles of all three pine species included 26 compounds recorded with concentrations higher than 0.5% (*Table 2*). Analysis of variance - ANOVA (with *Tukey* HSD for unequal N
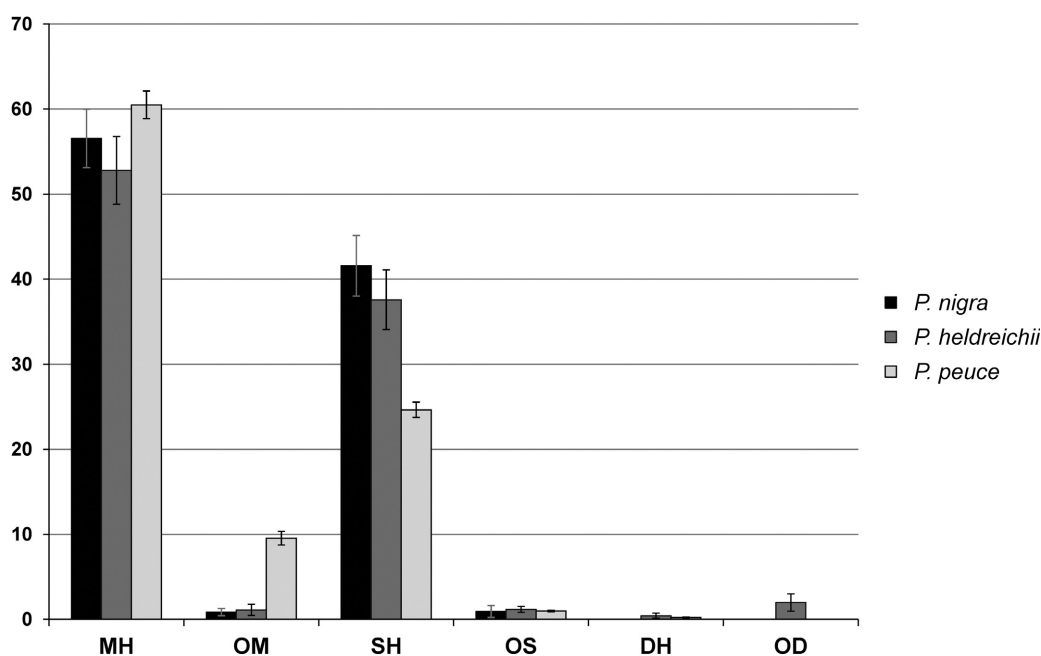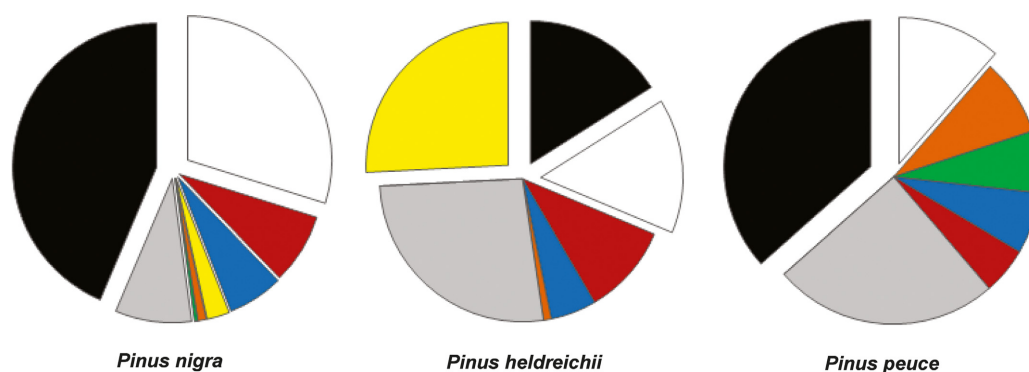

Figure 2. Differences between three pine species (in %) on the basis of main terpene classes: monoterpene hydrocarbons (MH), oxygenated monoterpenes (OM), sesquiterpene hydrocarbons (SH), oxygenated sesquiterpenes (OS), diterpene hydrocarbons (DH), and oxygenated diterpenes (OD). Error bars represent standard deviations of analyzed populations.

Table 2. Results of analysis of variance (ANOVA) for 26 needle terpenes detected in mean concentration greater than 0.5% in *P. nigra*, *P. heldreichii*, and *P. peuce*

Entry	Terpenes	Terpene class	$F^{[1]}$	$P^{[2]}$	Content [%][3]		
					P. nigra $n = 195$[4]	*P. heldreichii* $n = 127$	*P. peuce* $n = 90$
1	α-Thujene		77.70	***	0.6 ± 0.4[b]	0.2 ± 0.2[a]	0.6 ± 0.1[b]
2	α-Pinene		467.07	***	43.6 ± 9.1[c]	16.0 ± 8.2[a]	36.5 ± 3.6[b]
3	Camphene		1385.19	***	1.0 ± 1.2[a]	0.9 ± 0.4[a]	8.5 ± 1.9[b]
4	Sabinene		11.07	***	0.3 ± 0.2[a]	0.1 ± 0.2[a]	0.7 ± 2.0[b]
5	β-Pinene		3.14	*	6.2 ± 6.4[a]	5.2 ± 2.6[a]	6.8 ± 3.7[a]
6	Myrcene	Monoterpene hydrocarbons	419.94	***	0.8 ± 0.4[a]	2.2 ± 0.6[b]	1.0 ± 0.2[a]
7	α-Phellandrene		538.45	***	0.0 ± 0.0[a]	0.0 ± 0.1[a]	0.8 ± 0.4[b]
8	δ-3-Carene		12.59	***	0.1 ± 1.1[a]	2.1 ± 6.3[b]	0.0 ± 0.1[a]
9	Limonene		819.99	***	2.5 ± 2.8[b]	25.8 ± 9.3[c]	0.0 ± 0.0[a]
10	β-Phellandrene		3382.68	***	0.0 ± 0.0[a]	0.0 ± 0.0[a]	4.7 ± 1.0[b]
11	(E)-β-Ocimene		94.00	***	0.6 ± 0.5[c]	0.2 ± 0.3[b]	0.0 ± 0.0[a]
12	Terpinolene		2.14	ns	0.6 ± 0.5	0.5 ± 0.5	0.5 ± 0.1
13	Bornyl acetate		1508.44	***	0.4 ± 0.6[b]	0.1 ± 0.2[a]	6.8 ± 1.9[c]
14	Terpinen-4-yl acetate	Oxygenated monoterpenes	516.60	***	0.0 ± 0.0[a]	0.0 ± 0.1[a]	1.6 ± 0.8[b]
15	α-Terpinyl acetate		51.55	***	0.3 ± 0.6[a]	0.5 ± 0.3[b]	0.9 ± 0.4[c]
16	(E)-Caryophyllene		126.38	***	7.9 ± 2.6[b]	10.2 ± 2.6[c]	5.2 ± 0.8[a]
17	β-Gurjunene		300.76	***	0.0 ± 0.0[a]	1.1 ± 0.8[b]	1.1 ± 0.3[b]
18	Aromadendrene		341.61	***	0.0 ± 0.0[a]	0.7 ± 0.4[b]	0.6 ± 0.1[b]
19	α-Humulene		44.73	***	1.2 ± 0.4[b]	2.1 ± 1.9[c]	0.8 ± 0.5[a]
20	γ-Muurolene	Sesquiterpene hydrocarbons	32.69	***	0.5 ± 0.4[a]	0.9 ± 0.6[b]	0.6 ± 0.1[a]
21	Germacrene D		230.36	***	29.8 ± 9.1[c]	15.3 ± 7.5[b]	11.4 ± 3.0[a]
22	α-Muurolene		192.27	***	0.2 ± 0.2[a]	1.3 ± 0.9[b]	1.2 ± 0.4[b]
23	γ-Cadinene		49.96	***	0.4 ± 0.3[a]	0.8 ± 0.5[c]	0.5 ± 0.2[b]
24	δ-Cadinene		30.45	***	0.9 ± 0.6[a]	1.4 ± 0.9[b]	0.8 ± 0.3[a]
25	Germacrene D-4-ol	Oxygenated sesquiterpene	2.18	ns	0.7 ± 2.3	0.3 ± 0.2	0.5 ± 0.7
26	Isopimarol	Oxygenated diterpene	327.15	***	0.0 ± 0.0[a]	1.3 ± 0.9[b]	0.0 ± 0.0[a]

[1] *F*: ANOVA *F*-test. [2] *P*: Level of significance (*: $0.05 < P < 0.01$, ***: $P < 0.001$, ns: not significant). [3] Contents are given as percentages (mean ± standard deviation) of the total peak surface according to *Šarac et al.*,[19] *Bojović et al.*,[18] and *Nikolić et al.*;[13][20] means with different superscript letters within the same row (a,b,c) differ significantly (*Tukey* HSD for unequal N *post-hoc* test). [4] *n*: number of individuals studied for a given taxon.

Figure 3. Differences between three pine species (in %) on the basis of major terpene compounds. ■ α-Pinene; □ Germacrene D; ■ (E)-Caryophyllene; ■ β-Pinene; ■ Limonene; ■ Camphene; ■ Bornyl acetate; ■ Others.

post-hoc test) indicated statistically significant differences between means for all these volatiles except for terpinolene and germacrene D-4-ol. The most terpenes (α-pinene, limonene, (*E*)-β-ocimene, bornyl acetate, α-terpinyl acetate, (*E*)-caryophyllene, α-humulene, germacrene D, and γ-cadinene) have shown significant differences among all three pines, pointing to the existence of distinct terpene profiles for each of the species tested. Further, six other volatiles indicated divergence of *P. heldreichii vs. P. nigra* and *P. peuce*, five indicated separation of *P. peuce vs. P. nigra* and *P. heldreichii*, while the smallest number of volatiles (*i.e.*, three) indicated separation of *P. nigra* from *P. heldreichii* and *P. peuce*. Based on the number of compounds which indicate divergence, it was assumed that all three pines may be attributed to different terpene chemotypes, wherein chemotypes of *P. heldreichii* and *P. peuce* are the most distinctive.

Multivariate Analysis (PCA, CDA, and AHC)

Principal component analysis (PCA), based on a selected data set (412 individuals × 26 characters), was performed in order to determine the overall chemical variation and relationships among the individuals from analyzed populations of all three pine species. The first two principal component axes explain 49.4% of the total variation (with similar percentages of 25.2 and 24.3%, resp.). The scatter plot in the projection of the first two axes revealed good grouping of individuals within the species, and clear separation of the soft pine *P. peuce* from the hard pines *P. nigra* and *P. heldreichii* (*Fig. 4a*). Namely, samples from all populations of *P. peuce* form a group at the negative parts of both axes, while individuals of *P. nigra* and *P. heldreichii* were separated as two adjacent groups at the positive part of axis 2. However, individuals of two hard pines were additionally differentiated along the first axis: those of *P. heldreichii* have shown mainly positive, while those of *P. nigra* have shown negative values for axis 1. Within the *P. heldreichii* group, some separation of population V from Serbia was evident, but this trend along axis 1 was weaker than the main trends in the PCA already described. Several terpene characters were responsible for the explained variability (*Fig. 4b*). High contents of six monoterpene metabolites (camphene (*3*),[2] α-phellandrene (*7*), β-phellandrene (*10*), bornyl acetate (*13*), terpinen-4-yl acetate (*14*) and α-terpinyl acetate (*15*)) influenced separation of *P. peuce* individuals. Diversity

of *P. nigra* was mainly caused by high amounts of one monoterpene and one sequiterpene ((*E*)-β-ocimene (*11*) and germacrene D (*21*)). Finally, *P. heldreichii* individuals were characterized by high abundance of eight compounds, mostly sesquiterpenes (myrcene (*6*), limonene (*9*), (*E*)-caryophyllene (*16*), α-humulene (*19*), γ-muurolene (*20*), γ-cadinene (*23*), δ-cadinene (*24*) and isopimarol (*26*)).

Canonical discriminant analysis (CDA) was done in order to check the hypothesis that the analyzed sample was composed of discrete groups that are chemically differentiated from each other. The CDA based on 15 populations of *P. heldreichii*, *P. nigra*, and *P. peuce* has shown that the first two functions participated to 91.6% of the total discrimination, of which the first function was represented by 62.8% (*Table 3*). Five compounds, mostly dominant terpenes of these pines (α-pinene, β-pinene, limonene, germacrene D, and germacrene D-4-ol), had significant impact on both functions, while β-phellandrene and β-gurjunene considerably affected only the first, and δ-3-carene and aromadendrene only the second function. The scatter plot obtained by CDA suggested the existence of three chemically differentiated entities similar to those obtained by PCA (*Fig. 5*). All populations of *P. peuce* have shown positive values for the first axis, while *P. nigra* and *P. heldreichii* populations formed two groups at the negative part of axis 1, mostly separated along the second axis.

Agglomerative hierarchical cluster analysis (AHC) clearly separated *P. peuce* from *P. nigra* and *P. heldreichii* populations (*Fig. 6*), in agreement with the other two multivariate analyses. Therefore, all three analyses suggested that composition of needle terpenes reflects clear divergence between soft and hard pines (subgenera *Strobus* and *Pinus*, resp.). According to other phytochemical markers (phenolic data), the pines from the subgenus *Strobus* may be considered 'ancestral' in reference to the Laurasian origin of the genus.[25] Therefore, if we accept this concept, the terpene profile of a soft pine *P. peuce*, defined by high levels of six monoterpenes (camphene, α-phellandrene, β-phellandrene, bornyl acetate, terpinen-4-yl acetate, and α-terpinyl acetate) and, generally, the highest amount of total monoterpenes, is more basal than those of hard pines *P. nigra* and *P. heldreichii*, which were characterized by high contents of mainly sesquiterpene compounds (*Fig. 4b*). Hence, we may assume that 'terpene evolution' in these species went in the direction toward synthesis of a larger carbon skeleton of volatile molecules, and therefore, a greater structural diversity of the products. *Nikolić et al.*[23] reported somewhat different terpenes that influenced diversity of *P. heldreichii*, *P. peuce*

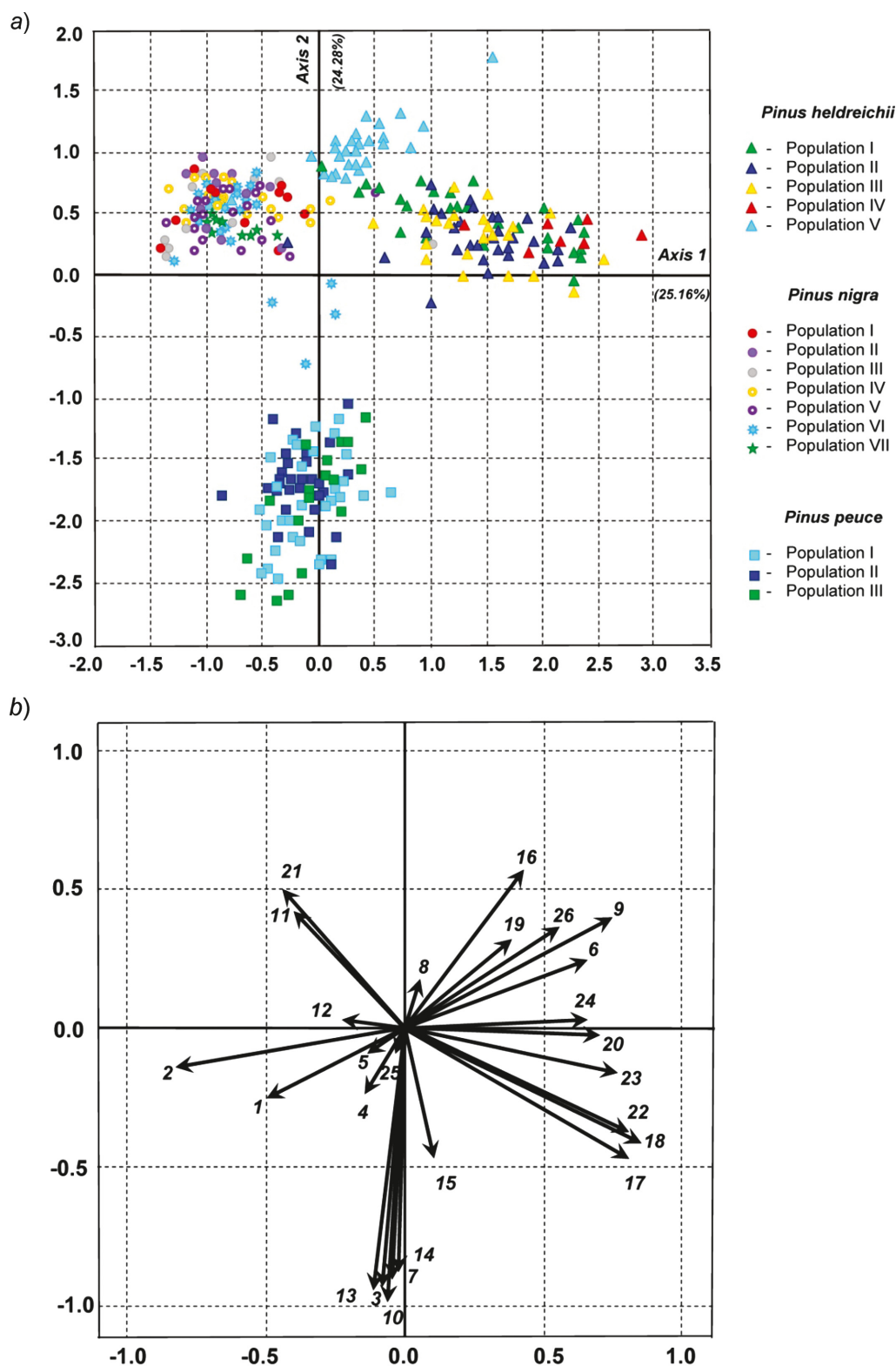[1]Numerals in italics refer to the entries in *Table 2*.

Figure 4. *a*) Principal component analysis (PCA) of 195 *P. nigra* individuals from seven populations, 127 *P. heldreichii* individuals from five populations and 90 *P. peuce* individuals from three populations. *b*) Representation of 26 selected variables (terpene compounds) on the plane of the first two axes. The italic numbers represent the entries of the components, *cf. Table 2*.

and one conifer species from different genus (*Picea omorika*). However, high content levels of several monoterpene compounds were mainly responsible for separation of *P. peuce* and high amounts of sesquiterpene volatiles for separation of *P. heldreichii*, in the same way as in results of our analysis.

Table 3. Standardized coefficients for the first three canonical axes (CA) of variation in 26 terpene compounds from the discriminant functional analysis of 15 *a priori* groups

Entry	Variable	CA1	CA2	CA3
1	α-Thujene	0.14	−0.09	−0.15
2	α-Pinene	**−1.09**	**−1.66**	−1.32
3	Camphene	0.21	−0.22	−0.13
4	Sabinene	−0.01	−0.16	−0.11
5	β-Pinene	**−0.62**	**−0.90**	−0.79
6	Myrcene	−0.04	0.25	0.17
7	α-Phellandrene	−0.34	0.23	−0.33
8	δ-3-Carene	−0.34	**−0.54**	−0.07
9	Limonene	**−0.79**	**−0.77**	−1.00
10	β-Phellandrene	**0.63**	−0.45	0.11
11	(E)-β-Ocimene	−0.24	−0.16	−0.19
12	Terpinolene	−0.33	−0.11	−0.20
13	Bornyl acetate	0.17	−0.30	−0.11
14	Terpinen-4-yl acetate	0.30	−0.30	0.11
15	α-Terpinyl acetate	0.01	−0.21	0.02
16	(E)-Caryophyllene	−0.37	−0.29	−0.30
17	β-Gurjunene	**1.01**	−0.08	−0.55
18	Aromadendrene	−0.41	**0.57**	−0.23
19	α-Humulene	−0.37	−0.40	−0.16
20	γ-Muurolene	−0.30	−0.28	−0.20
21	Germacrene D	**−1.27**	**−1.75**	−0.71
22	α-Muurolene	0.08	−0.05	0.27
23	γ-Cadinene	−0.09	−0.02	−0.12
24	δ-Cadinene	−0.12	0.04	−0.37
25	Germacrene D-4-ol	**−0.60**	**−0.55**	−0.30
26	Isopimarol	0.07	0.29	0.24
	Eigenvalue	43.98	20.20	2.68
	% explained variation	0.63	0.92	0.95

Significant coefficients are in boldface.

On the other hand, *P. nigra* and *P. heldreichii* represent two species of the subgenus *Pinus* with highly opposed and uncertain phylogenetic relationships. Interestingly, terpene metabolites were the first markers that suggested their demarcation,[9] as previously the Bosnian pine was considered only a variety of *P. nigra*.[2] In all subsequent studies, distinct taxonomic status of Bosnian pine has been confirmed; however its position between the Eurasian (subsect. *Pinus*) and the 'true' Mediterranean hard pines (subsect. *Pinaster*) remained unresolved.[10] In the older literature sources, *P. heldreichii* was either considered more closely related to *P. nigra*, *P. sylvestris*, and other Eurasian hard pines[26] or a species with the 'divider' position between these two groups.[27] In the analysis of genetic relationships between ten conifer species using RAPD markers,[28] *P. heldreichii* was found a place in subgenus *Pinus*, but it was, at the same time equally distant from *P. nigra* and *P. sylvestris* (species of subsect. *Pinus*). Furthermore, within the recent

infrageneric classifications of genus *Pinus*, *P. nigra* and *P. heldreichii* belong to different subsects. (*Pinus* and *Pinaster*, resp.) of section *Pinus* according to the DNA sequences from chloroplast[6][10] and nuclear genomes.[14]

Nevertheless, *P. heldreichii* from southwestern Serbia and northern Montenegro, assigned to var. *pančići*, is similar to *P. nigra* and to some extent to two natural hybrids (*P.* × *nigradermis* FUKAREK and VIDAKOVIĆ and *P.* × *mugodermis* FUKAREK) and one intermediate form (*P. nigra* f. *leucodermoides* FUKAREK and NIKOLIĆ) based on morphology of branches, needles and/or cones.[8] Despite the described morphological similarities and the fact that Bosnian and black pines can spontaneously hybridize, our results, presented here, showed terpene differentiation of their populations growing naturally in the same area. Recently, *Mitić et al.*[29] compared composition of essential oils obtained by hydrodistillation of needles of 27 taxa of section *Pinus* and confirmed positions of *P. nigra* and *P. heldreichii* within different subsects. (*Pinus* and *Pinaster*, resp.). Interestingly, percentages of occurrence of C-skeletons in the essential oils allowed identifying the preferential accumulation of different types of sesquiterpene C-skeletons between the examined subsects. *i.e.*, subsect. *Pinus* was characterized by a unique occurrence of the route germacrane → bicyclogermacrane as well as favoring the route germacrane → cadinane, while in subsect. *Pinaster*, the route germacrane → guaiane was unique. However, in the present study terpene profiles obtained by pentane extraction were analyzed and compounds such as bicyclogermacrene, α-cadinol, and guaiol (that showed significant differences between subsects. *Pinus* and *Pinaster*[29]) were detected in concentration lower than 0.5% or not even detected.[13][18 − 20]

Based on individual relationships of studied hard pines with a soft pine such as *P. peuce*, it may be assumed that the terpene profile of *P. nigra* (defined by high levels of one monoterpene and one sequiterpene) is more basal in comparison to *P. heldreichii* (characterized by the high amounts of eight compounds: two monoterpenes, five sesquiterpenes and even one diterpene). In this manner our results are largely consistent with the recently proposed concept of 'flavonic evolution' in genus *Pinus*, stating that pines growing under hot and dry climates (Mediterranean region) and containing very high levels of methylated flavonols are more evolved than those from cold and/or wet regions (Eurasia and North America).[25] However, any attempt to determine connections between extant species as well as their ancestors must consider that during the evolution of
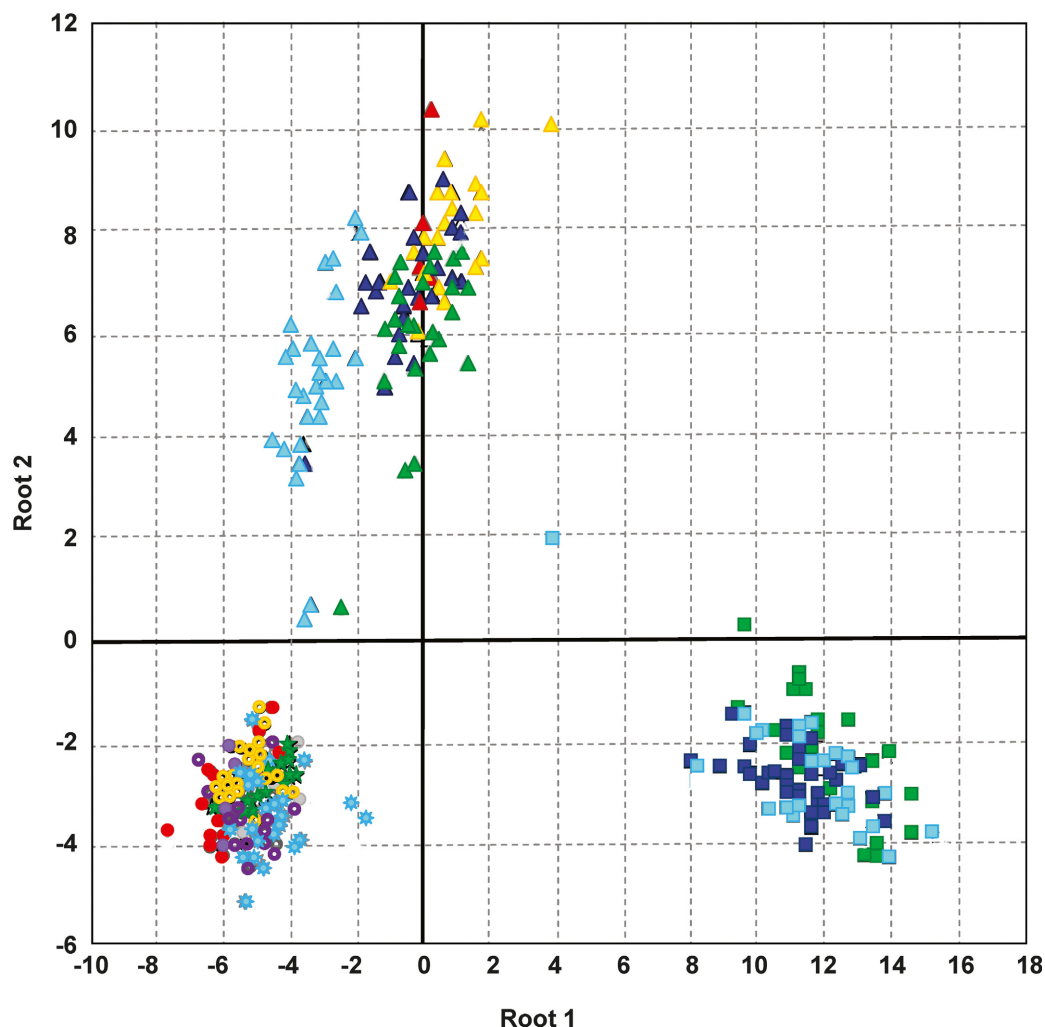
Figure 5. Canonical discriminant analysis (CDA) based on 26 selected terpenes of *P. nigra*, *P. heldreichii* and *P. peuce* with their populations as *a priori* groups. Symbols refer to populations as indicated in *Fig. 4a*.

species the function of terpenes and other secondary metabolites also evolved in line with environmental changes.[30]

As terpenoids constitute the largest class of plant specialized constituents, it should be pointed out that they play a wide range of roles in plant metabolism. In spite of the fact that many functions of terpenoids are known, overall knowledge regarding the roles of most constituents is completely unknown. Resolving taxonomic and phylogenetic relationships based on terpenoids is still a very difficult issue not only within the genus *Pinus*, but also in any plant group.

Evolution and diversification of terpenoids is obviously a very complex and long process. It is worth noting that in addition to mevalonate route for the synthesis of the C5 units (from which terpenoids are syntesized), a new, non-mevalonate (glyceraldehyde phosphate/pyruvate route) was discovered.[31] According to a recent report,[32] the second route is found in the plastids of all vascular plants (for the plastid-associated terpenoids such as monoterpenes, diterpenes, carotenoids, *etc.*), while the mevalonate pathway seems to be restricted to the cytosol/endoplasmic reticulum. The mevalonate route may be the main source of substrate for cytosolic terpenoids such as sesquiterpenes and triterpenes. This finding of 'alternative' route should be taken into account during further research of phytochemical, physiological and molecular aspects of synthesis and the role of terpenoids in plants and their taxonomic relevance.

Thus, possible explanation for sharp difference in contents in monoterpenes and sesquiterpenes between *P. heldreichii* and *P. nigra* from one, and *P. peuce* from the other side, might be found in further investigation of enzymes and genes involved in biosynthesis of terpenoids, which could better highlight the evolution of pines and, consequently, their taxonomic and phylogenetic relationships.
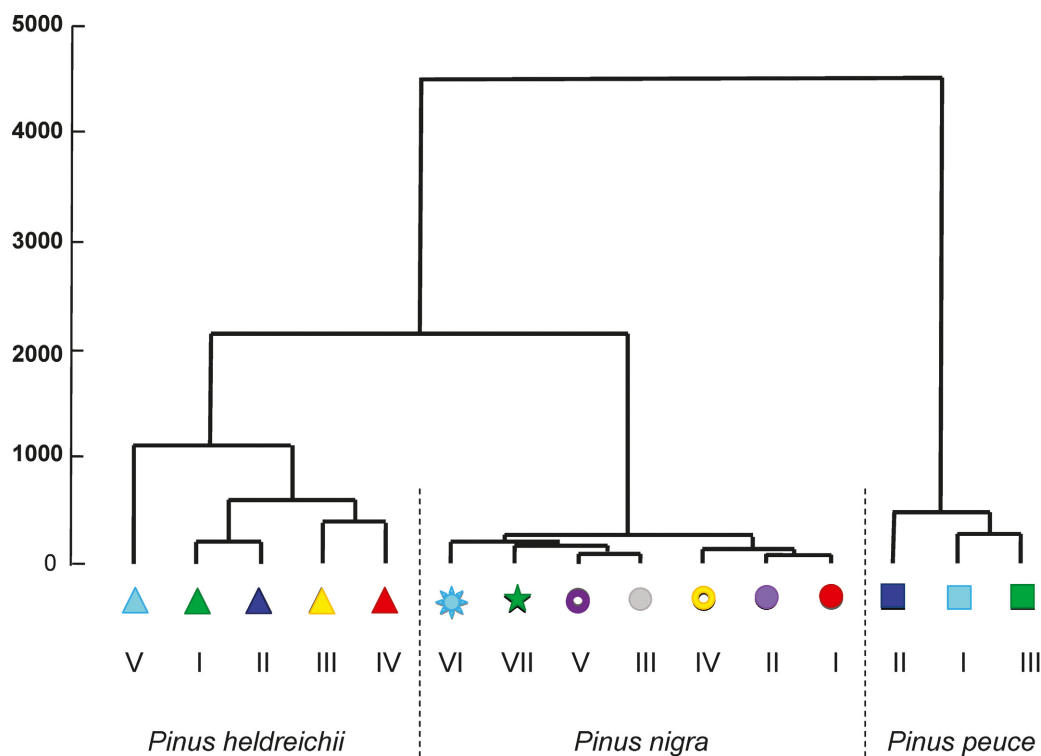
Figure 6. Dendrogram obtained by AHC clustering of *P. nigra*, *P. heldreichii* and *P. peuce* populations: percentages of selected terpenes as pattern, UPGMA as a criterion for the clusters development and *Euclidean* distances as diversity assessment criteria. Symbols refer to populations as indicated in *Fig. 4a*.

Terpene Diversity and Differentiation of Studied Pines at the Population Level

Pinus heldreichii is a Balkan subendemite with an extremely narrow range in the territory of Serbia, growing in the wild only in the southwest, in form of two enclaves that are described as var. *pančići* (*Fig. 1*, *Table 1*). The population from Mt. Revuša (population V) represents the largest natural population of this species in Serbia (over 200 trees), with number of individuals increasing over the last few decades, thanks primarily to the fact that it was spared from negative anthropogenic influences (discussed in *Bojović et al.*[18]). On the other hand, the second Serbian population (population IV) includes only some scattered little groups and individual trees between Mt. Zlatibor and Pešter plateau, which were almost destroyed by anthropogenic impact (the resinous wood was used for kindling, resin, and manufacturing of bowls). In the present study, terpene profiles of these two Serbian populations, together with three wild populations from Montenegro, were compared for the first time. According to the combined results of all multivariate analyses (*Figs. 4 – 6*), the largest Serbian population (population V) has shown a certain degree of separation from the rest of studied populations that largely overlapped with each other.

This result was somewhat unexpected, considering that the second Serbian population (population IV), described also as var. *pančići*, was grouped with Montenegro populations (I – III). The possible explanation is that plant material from these two Serbian populations was collected from late summer to early fall in 2 years with completely different climatic conditions (*Table 1*): for the largest Serbian population (population V) in 2009, while for all other studied populations (I – IV) in 2003, which was the year of the immense heat wave in Europe. European heat wave of 2003 led to the hottest summer on record in Europe since at least 1540, and the highest heat levels were recorded in July and August, mostly in Western Europe.[33] Therefore, certain separation of Serbian population V could be caused by different climatic conditions in 2009, which was generally a cooler and more humid year. However, results of comparison between the combined populations of *P. heldrechii* (collected in 2003 and 2009) and populations of *P. nigra* (collected in 2009) and *P. peuce* (collected in 2003) show presence of small differences in terpene composition under the influence of climatic factors, which may be detected on population level but do not influence their placement at species level. In the previous study that only included populations I – IV,[13] AHC

suggested the closest connection between the two spatially most distant populations I and IV.

The detected levels of terpene variability and differentiation within *P. nigra* populations from Serbia (determined as four infraspecific taxa: subsp. *nigra*, var. *gocensis* ĐorĐević, subsp. *pallasiana* (Lamb.) Holmboe), and var. *banatica* (Endl.) Georgescu et Ionescu; *Fig. 1*, *Table 1*) were lower than in populations of other two pines. Namely, in PCA and CDA scatter plots there was a significant overlap among the populations of *P. nigra* (*Figs. 4a* and *5*), while AHC also confirmed the highest degree of similarity within Serbian black pine populations (*Fig. 6*). As the territory of Serbia represents the contact zone for studied *P. nigra* taxa (including borders of their ranges) perhaps it is not surprising that all studied Serbian populations formed a single group. The observed population overlap may be explained by natural hybridization between the geographically close populations of different infraspecific *P. nigra* taxa, which are not reproductively isolated. Similar results were obtained by using epicuticular wax compounds as chemotaxonomic characters,[34] as well as molecular data (plastid and mitochondrial) that failed to support circumscription of *P. nigra* taxa within the territory of Serbia.[35] However, in our previous study of terpene markers[19] AHC had suggested the existence of three population groups where population VII (assigned as var. *banatica*) was the most distant. This arrangement of *P. nigra* populations is quite dissimilar to the results of the present study, as shown in the dendrogram.

In the case of *P. peuce*, only three natural populations were analyzed. Results of PCA and CDA have shown total overlapping of populations (*Figs. 4a* and *5*), while AHC (*Fig. 6*) detached the population II as the most distant. On the other hand, *Nikolić et al.*[20] reported a closer connection between populations II and III when compared to population I.

Conclusions

The composition of needle terpenes reflected clear divergence between the pine species from different subgenera: *P. peuce* (subgenus *Strobus*) *vs.* *P. nigra* and *P. heldreichii* (subgenus *Pinus*). In addition, despite the morphological similarities and the fact that hard pines *P. nigra* and *P. heldreichii* may spontaneously hybridize, our results showed differentiation of their populations naturally growing in the same area, based on terpene markers. In accordance with the recently proposed concept of 'flavonic evolution' in the genus *Pinus*, we assumed that the terpene profile of *P. peuce*, defined by high contents of six monoterpene metabolites and

generally the highest amount of total monoterpenes, is more basal than those of *P. nigra* and *P. heldreichii*, characterized by high abundance of mainly sesquiterpene compounds. Hence, 'terpene evolution' in these species might go toward synthesis of the larger carbon skeleton of molecules and, therefore, greater structural diversity of the volatile compounds. Additional morphological, phytochemical and genetic studies are necessary in order to establish precise positions of *P. heldreichii*, *P. nigra*, and *P. peuce* within the taxonomic and phylogenetic tree, and subsequently develop suitable conservation strategies and future breeding efforts.

Experimental Section

Plant Material

Plant material (fresh needles) from seven populations of *P. nigra* naturally grown in Serbia, five populations of *P. heldreichii* (three from Montenegro and two from Serbia), and three populations of *P. peuce* (two from Montenegro and one from Serbia) were analyzed. A map of the study area, indicating the locations of selected populations of studied pines, is presented in *Fig. 1*, and the corresponding geographic and geologic data as well as date of collection for every population are listed in *Table 1*. Two-year-old needles from lower third of crown of 30 randomly selected individuals in each of the populations (except *P. nigra* population VII with 15 and *P. heldreichii* population IV with seven individuals) were collected from late summer to early fall in 2003 and 2009. The entire plant material was deposited in polyethylene bags (labeled with data on sample plot, date of collection, locality), transferred to a freezer and stored at −20 °C prior to further analysis. In the present article, we merged and analyzed 195 *P. nigra*, 127 *P. heldreichii*, and 90 *P. peuce* individuals in total. Plant material was identified by Dr. *Srdjan R. Bojović* and Dr. *Biljana M. Nikolić*.

Isolation of Terpenes

Two-year-old needles of every individual tree, stored separately in a freezer at −20 °C until extraction, were cut into pieces of 2 − 3 mm length and extracted with pentane (1 g needles/ml solvent). The extracts were kept at 4 − 6 °C for 24 h, then filtered, and stored in chromatography vials with solid caps in a refrigerator until further analysis.[13][18 − 20]

GC-FID and GC/MS Analyses

The GC-FID and GC/MS analyses of terpenes of *P. heldreichii*[13][18] and *P. peuce*[20] were carried out with

Hewlett-Packard G1800C-GC and Hewlett-Packard G1800C-GCD apparatuses, resp. (*Hewlett-Packard*, Palo Alto, CA, USA), both equipped with an automatic liquid sampler (ALS), a flame ionization detector (FID), a mass selective detector (MSD), and a *HP-5 MS* fused-silica cap. column (30 m × 0.25 mm i.d., film thickness 0.25 μm). The oven temp. was programmed linearly rising from 40 to 280 °C at 4 °C/min; injector temp., 250 °C; detector temp., 280 °C; carrier gas, H$_2$ in GC-FID (1.0 ml/min) and He in GC/MS analysis.[13][18] [20] In the study of *P. nigra* terpenes,[19] *Agilent 7890A* apparatus (*Agilent Technologies*, Santa Clara, CA, USA) equipped with an auto-injection system (*Agilent 7683B Series*), a flame ionization detector (FID), a mass selective detector (MSD), and a *HP-5 MS* fused-silica cap. column (30 m × 0.25 mm i.d., film thickness 0.25 μm) was used. The oven temp. was programmed linearly rising from 60 to 300 °C at 3 °C/min and then isothermal at 300 °C for 10 min; injector temp., 250 °C; detector temp., 300 °C; source temp., 230 °C; quadrupole temp., 150 °C; carrier gas, He. Electron-impact mass spectra (EI-MS; 70 eV) were acquired over the *m/z* range 40 − 450[13][18][20] or 40 − 550.[19] For quantification purposes, area-percent values were determined by GC/FID.[13][18 − 20]

Compound Identification

The components were identified based on the comparison of their mass spectra with those reported by *Adams*[36] or with those compiled in the *Wiley-275* and the *NIST/NBS* libraries. The obtained results were correlated with the retention indices (*RI*).[13][18 − 20]

Statistical Analysis

Statistical data processing was carried out by STATISTICA 8 software (*Statsoft, Inc.*, Tulsa, OK, USA). Statistical matrices included the content (in percentage) of needle terpenes as original variables. In fact, to form a matrix, we had taken into account the terpenes that were present in more than 0.5%, while all terpenes found in traces (according to *Lieutier et al.,*[37] contents < 0.5%) were not considered. All selected components are listed in *Table 2*. The significance of differences between the studied species was determined by the univariate analysis of variance (ANOVA). A *Tukey* honest significant difference (HSD) for unequal N *post-hoc* test was used in order to evaluate statistical importance of difference between analyzed species, based on mean values of their chemical characteristics. Multivariate analysis included principal component analysis (PCA), canonical discriminant analysis (CDA), and agglomerative hierarchical cluster analysis (AHC). Unweighted pair group method with arithmetic mean (UPGMA) was used as a criterion for the cluster development, and *Euclidean* distances as diversity assessment criteria in AHC analysis.

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Author Contribution Statement

Zorica S. Mitić merged all final data, performed statistical analyses and their interpretation and drafted the first version of the manuscript. *Biljana M. Nikolić* gave substantial contribution to concept and design of the manuscript, critically redacted it until the final version and made general supervision of the research group. *Mihailo S. Ristić* and *Vele V. Tešević* analyzed chromatographic data up to the level of statistical processing and helped in determination of terpene classes and interpretation of obtained results. *Srdjan R. Bojović* revised statistical analyses, helped in their interpretation and revised the drafted version of the manuscript. *Petar D. Marin* participated to the general research design, critically redacted the manuscript and gave final approval of the version to be published. In addition, almost all listed authors, in accordance to their closer specialties, participated in the collecting of plant material, determination of species, isolation of terpenes, and/or biochemical analyses which had already been done in previous investigations, too.

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