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Chemotaxonomic Considerations of the *n*-Alkane Composition in *Pinus heldreichii*, *P. nigra* and *P. peuce*

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The *n*-alkane composition in the leaf cuticular waxes of natural populations of Bosnian pine (*Pinus heldreichii*), Austrian pine (*P. nigra*) and Macedonian pine (*P. peuce*) was compared for the first time. The range of *n*-alkanes was wider in *P. nigra* (C_{16}-C_{33}) than in *P. heldreichii* and *P. peuce* (C_{18}-C_{33}). Species also diverged in abundance and range of dominant *n*-alkanes (P. *heldreichii*: C_{23}, C_{27}, and C_{25}; *P. nigra*: C_{25}, C_{27}, C_{29}, and C_{23}; *P. peuce*: C_{29}, C_{25}, C_{27}, and C_{23}). Multivariate statistical analyses (PCA, DA and CA) generally pointed out separation of populations of *P. nigra* from populations of *P. heldreichii* and *P. peuce* (which were, to a greater or lesser extent, separated too). However, position of these species on the basis of *n*-alkane composition neither was in accordance with infrageneric classification nor with recent molecular and terpene investigations.

Keywords: *Pinus heldreichii* • *P. nigra* • *P. peuce* • Alkanes • Molecular diversity

Introduction

Pinus is the largest genus within family Pinaceae, consisting 113 species, which mainly inhabit Northern Hemisphere (with the exception of Sumatran pine, *Pinus merkusii*).[1] Classification of all pines to two subgenera: *Pinus* (Diploxylon, hard pines) and *Strobus* (Haploxylon, soft pines), which was founded a hundred years ago[2] and sometimes was disputed, nowadays have a final confirmation based on contemporary genetic studies ([3] and refs. cited therein). However, according to results of different molecular markers or gene segments some discrepancies in classification on the level of sections and/or subsections still exist[4] and need further investigations.

Two-needle pines, Bosnian pine (*P. heldreichii* Christ., syn. *P. leucodermis*) and Austrian pine (*P. nigra* Arn.), belong to subgenus *Pinus*, while five-needle pine, Macedonian pine (*P. peuce* Griseb.), endemite of Balkan Peninsula, belongs to subgenus *Strobus*.[3] Despite of morphological similarity and sometimes overlapped natural ranges with indication of natural hybridization,[5] *P. heldreichii* and *P. nigra* differ in morphological characters, in area of distribution (*P. heldreichii* is Balkan subendemite, while *P. nigra* is one of the most widespread pine species in Europe with fragmented distribution range that extends from North Africa through the northern Mediterranean and eastwards to the Black Sea), chemical composition,[6] cytogenetics,[7][8] and molecular genetics.[3] According to various systematists, *P. heldreichii* and *P. nigra* are classified into different subsections (*Pinaster* and *Pinus*, resp.) of section *Pinus*.[3][9][10] Recent researches also confirmed the displacement of *P. heldreichii* to Mediterannean pines (subsection *Pinaster*),[11-14] which are opposite of earlier statement[15] that classified *P. heldriechii* in intermediate position between hard and soft pines.

n-Alkanes in cuticular waxes of *P. heldreichii*, *P. nigra* and *P. peuce* have already been investigated at population level on the central Balkans.[16-19] Furthermore, recent investigation of taxonomic relations between *Picea omorika*, *Pinus heldreichii* and *P. peuce* populations in composition and abundance of *n*-alkanes gave interesting results.[20]

The aim of this work is to investigate for the first time relations between *P. heldreichii*, *P. nigra* and *P. peuce* natural populations (*Table 1* and Fig. 1) according to *n*-alkane composition previously published.[16,17,19]. Sets of *n*-alkanes data were merged, statistically processed and discussed with the aim to test their potential usefulness in understanding of chemotaxonomy, biogeography, phylogeny and evolution of these intriguing taxa.

Locality	Population code	Latitude (N)	Longitude (E)	Altitude (m a.s.l.)	Exposure	Terrain inclination[f]	Substratum	Number of individuals analyzed	Date of collection	Accesion number
					Pinus heldreichii					
Montenegro: Mt. Lovćen	I	42°25'	18°50'	1700-1800	S, SE	< 30	Limestone	30	August 2003*	PHE1
Montenegro: Mt. Zeletin	II	42°37'	19°50'	1700-1900	E, NE	< 30	Limestone, Serpentinite, Schist	30	August 2003*	PHE2
Montenegro: Mt. Bjelasica	III	42°53'	19°45'	1700	S, SE	< 35	Limestone, Dolomite	30	August 2003*	PHE3
Serbia: little groups and individual trees between Mt. Zlatibor and Pešter plateau considered as single population[a]	IV	43°15'-43°30'	19°30'-19°55'	1100-1430	S, SW, NW	< 30	Limestone, Dolomite, Hornestone, Tuff, Neogene sediments	7	September 2003*	PH4
					Pinus nigra					
Serbia: Mt. Tara, Banjska stena[b]	I	43°57'04"	19°24'04"	1040	NW	20 - 80	Limestone	30	August 2009**	PNI1
Serbia: Mt. Tara, Omar[b]	II	43°54'02"	19°23'18"	950	SW	40	Limestone	30	August 2009**	PNI2
Serbia: Mt. Tara, Zmajvečki potok[b]	III	43°51'45"	19°25'05"	830	W	30	Serpentinite	30	August 2009**	PNI3
Serbia: Priboj, Crni vrh[c]	IV	43°34'56"	19°35'06"	1100	N, NW	< 40	Serpentinite	30	August 2009**	PNI4
Serbia: Mt. Goč, Gvozdac[c]	V	43°33'32"	20°40'56"	790	S, SW	< 30	Serpentinite	30	August 2009**	PNI5
Serbia: Mt. Dukat, Jarešnik[d]	VI	42°22'32"	22°24'10"	1300	S, SW	< 30	Crystalline slate	30	August 2009**	PNI6
Serbia: Lazareva Reka Canyon, Kovej[e]	VII	44°01'02"	21°55'39"	750	N, NW	20 - 90	Limestone	15	August 2009**	PNI7
					Pinus peuce					
Montenegro: Mt. Zeletin	I	42°37'	19°50'	1700-1900	E, NE	< 30	Limestone, Serpentinite, Schist	30	August 2003*	PPE1
Montenegro: Mt. Sjekirica	II	42°40'	19'50'	1700	W, NW	< 15	Schist, Serpentinite	30	August 2003*	PPE2
Serbia: Mt. Mokra Gora	III	42°50'	20°23'	1750-1950	N	< 35	Limestone	30	August 2003*	PPE3

[a] var. *pančići*. [b] subsp. *nigra*. [c] var. *gočensis*. [d] subsp. *pallasiana*. [e] var. *banatica*. [f] in °. * collected by B. Nikolić. ** collected by S. Bojović.

Table 1. Geographic positions and habitat descriptions of examined populations of *P. heldreichii*, *P. nigra*, and *P. peuce*.

Figure 1.

Results and Discussion

Diversity of Species According to n-*Alkane Abundance and Main Profiles*

In the needle cuticular waxes of *P. nigra* range of *n*-alkanes was wider (C_{16} to C_{33}) than in *P. heldreichii* and *P. peuce* (C_{18} to C_{33}, compiled after previous results,[17][16][18] respectively). The most abundant were *n*-alkanes C_{25} and C_{27} (*P. nigra*), C_{23} (*P. heldreichii*), and C_{29} (*P. peuce*). Profiles of main *n*-alkanes (with abundance up to 10%) were as follows: C_{23}, C_{27}, and C_{25} in *P. heldreichii*, C_{25}, C_{27}, C_{29}, and C_{25} in *P. nigra*, and C_{29}, C_{25}, C_{27}, and C_{23} in *P. peuce*. Furthermore, *P. heldreichii* had the highest contents of C_{21}, C_{22} and C_{24}, and the lowest contents of C_{29} and C_{31}, while *P. peuce* had the highest contents of C_{31} and C_{28}. *Pinus nigra* also had the highest contents of C_{16}, C_{17}, C_{32} and C_{33}. Listed differences between species were statistically approved by ANOVA F-test (*Table 2*). However, in literature report,[21] among 21 pine species, the most dominant *n*-alkane were C_{31} (as in *P. heldreichii*), rarely C_{23} (as in *P. nigra*), or combination of two/three *n*-alkanes (as in *P. cembra*, etc.).

Table 2. Results of analysis of variance (ANOVA) for 18 *n*-alkanes in needle wax of *P. heldreichii*, *P. nigra*, and *P. peuce*.

Entry	*n*-Alkanes	F[1]	P[2]	Content (%)[3]		
				P. nigra N = 189[4]	*P. heldreichii* N = 97	*P. peuce* N = 90
1.	**C_{16}**	30.96	0.0000	0.91±1.41^{b}	0.00±0.00^{a}	0.00±0.00^{a}
2.	**C_{17}**	23.51	0.0000	0.86±1.53^{b}	0.00±0.00^{a}	0.00±0.00^{a}
3.	**C_{18}**	23.81	0.0000	3.49±2.63^{b}	3.62±3.24^{b}	1.20±2.36^{a}
4.	**C_{19}**	34.59	0.0000	1.38±1.98^{a}	4.32±3.09^{b}	1.78±2.90^{a}
5.	C_{20}	12.17	0.0000	4.89±3.18^{b}	4.61±2.84^{b}	2.96±2.82^{a}
6.	**C_{21}**	278.69	0.0000	1.90±1.60^{a}	9.18±3.50^{c}	5.71±2.23^{b}
7.	**C_{22}**	65.16	0.0000	3.82±2.32^{a}	8.43±3.65^{c}	5.99±3.33^{b}
8.	C_{23}	2.38	0.0939*	10.34±7.72^{a}	12.21±3.01^{b}	10.48±2.40^{ab}
9.	C_{24}	24.07	0.0000	5.72±2.50^{a}	8.06±2.88^{c}	6.57±1.37^{b}
10.	**C_{25}**	60.36	0.0000	16.52±5.92^{b}	10.79±1.51^{a}	11.07±2.31^{a}
11.	C_{26}	1.08	0.3395*	6.78±2.73^{a}	6.29±1.74^{a}	6.63±1.36^{a}
12.	**C_{27}**	45.54	0.0000	15.10±5.20^{b}	11.21±1.91^{a}	10.58±1.74^{a}
13.	C_{28}	12.58	0.0000	5.63±2.38^{a}	5.57±2.46^{a}	7.00±1.46^{b}
14.	**C_{29}**	65.41	0.0000	10.70±3.94^{b}	9.09±2.05^{a}	15.54±4.39^{c}
15.	C_{30}	6.36	0.0019*	3.82±2.06^{b}	3.04±2.81^{a}	4.40±2.38^{b}
16.	C_{31}	89.94	0.0000	4.42±2.79^{b}	3.05±2.88^{a}	9.24±3.99^{c}
17.	**C_{32}**	67.34	0.0000	2.44±1.76^{b}	0.21±0.58^{a}	0.43±1.97^{a}
18.	**C_{33}**	87.83	0.0000	1.28±0.53^{b}	0.30±0.81^{a}	0.17±1.01^{a}

[1] F: ANOVA F-test. [2] P: Level of significance (*not significant). [3] Contents are given as percentages (mean±standard deviation) of the total peak surface according to Bojović et al.,[17] and Nikolić et al.,[16][19] 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. Eleven *n*-alkanes (and their entries) which were chosen for multivariate analyses were given in bold.

For multivariate methods (PCA, DA and CA) eleven *n*-alkanes (C_{16}, C_17, C_{18}, C_{19}, C_{21}, C_{22}, C_{25}, C_{27}, C_{29}, C_{32}, and C_{33}) were selected, which had significant differences between species (0.01<P<0.05, *Table 2*), standard deviations up to 3.5 and/or coefficients of variation up to 70% (results were not presented).

Principal component analysis (PCA, Fig. 2) was performed based on a correlation matrix 382 x 11 (samples x variables) in order to determine chemical variability and relationships of listed three pine species. The first three principal component axes explain 66.9% of the total information (29.5%, 22.3% and 15.1%, respectively) (Fig.2). The greatest influence on the total variability or the formation of the axis 1 (29.5%) has C_{21} and C_{22}, on the formation of the axis 2 (22.3%) C_{16} and C_{18}, and on the formation the axis 3 (15.1%) C_{32} (*Table 3*). On the plain of axis 1 (29.5%) incomplete separation of widespread *P. nigra* from Balkan endemic and subendemic pines (*P. peuce* and *P. heldreichii*) was obtained (in 90% cases). Divergence of *P. nigra* was influenced by significantly high contents of *n*-alkanes C_{16}, C_{17}, C_{25}, C_{27}, C_{32} and C_{33} and significantly low contents of C_{21} and C_{22} (*Table 2*). Populations of *P. nigra* mainly overlapped, except of V (Goč, Gvozdac) (owing to high C_{16} - C_{19}) and IV (Priboj, Crni vrh) (owing to high C_{16}-C_{18} and C_{32}). There was no separation between populations of *P. heldreichii* and *P. peuce* on the plain of axis 2 (22.3%), although those of *P. heldreichii* had higher contents of C_{19}, C_{21} and C_{22}, while those of *P. peuce* had higher content of C_{29}. These results are not in accordance with PCA analysis of terpenes,[14] where *P. nigra* was closer to *P. heldreichii*, while *P. peuce* was separated. In *n*-alkane analysis, where *Picea omorika* was the third species,[20] *P. heldreichii* and *P. peuce* partially overlapped.

Table 3. Principal component analysis: factor loadings.

Independent variable	axis 1	axis 2	axis 3
C_{16}	-0.473	**0.729**	-0.128
C_{17}	-0.466	0.715	-0.167
C_{18}	-0.094	**0.731**	-0.119
C_{19}	0.340	0.477	-0.440
C_{21}	**0.804**	0.047	-0.201
C_{22}	**0.714**	0.257	0.266
C_{25}	-0.688	-0.256	-0.493
C_{27}	-0.660	-0.409	-0.454
C_{29}	0.042	-0.530	0.025
C_{32}	-0.436	0.254	**0.760**
C_{33}	-0.648	-0.078	0.519

Figure 2.

The same 11 *n*-alkanes were used for Discriminant analysis (DA) at the level of 14 populations of *P. heldreichii*, *P. nigra* and *P. peuce*. It showed that the axis 1 participated in 48.98% of the total discrimination, and the axis 2 with 17.32% (*Table 4A*). The axis 1 (48.98%) is determined by C_{21} and C_{17}, while the axis 2 (17.32%) is mostly defined by the C_{32} and C_{18}. DA shows that 14 populations were mostly overlapped (Fig. 3). Only three populations of *P. heldreichii* (Mt. Zeletin, Mt. Bjelasica i Mt. Lovćen) and three populations of *P. nigra* (Zmajevečki potok, Priboj, Crni vrh i Mt. Goč, Gvozdac) were clearly distinguished. In this analysis 50-100% of individuals were classified correctly. These results are in agreement with PCA (Fig. 2), but quite opposite of those based on terpene analysis of the same species,[14] where through DA analysis *P. nigra* populations were grouped together with those of *P. heldreichii*, while *P. peuce* populations were separated.

Table 4. Standardized coefficients for two discrimination axes of variation in eleven *n*-alkanes from the discriminant functional analysis (DA). Significant coefficients are given in boldface.

	A) Classification variable: Population		**B) Classification variable: Species**	
Independent variable	DF1	DF2	DF1	DF2
C_{16}	0.19	0.37	0.44	-0.17
C_{17}	**0.40**	-0.21	-0.20	0.15
C_{18}	0.20	**-0.65**	0.16	-0.28
C_{19}	-0.24	-0.44	-0.21	-0.22
C_{21}	**-0.48**	-0.09	**-0.58**	-0.42
C_{22}	-0.35	-0.14	-0.32	-0.26
C_{25}	0.33	-0.45	0.29	0.22
C_{27}	0.12	-0.31	0.16	**-0.70**
C_{29}	-0.23	0.05	-0.27	**0.72**
C_{32}	0.35	**0.66**	0.44	0.13
C_{33}	0.15	-0.40	0.16	-0.59
Eigenvalue	6.32	2.24	3.36	0.89
Relative %	48.99	17.32	79.13	20.87

Discriminant analysis (DA) at the species level (*P. heldreichii*, *P. nigra* and *P. peuce*) showed that the axis 1 participated in 79.13% of the total discrimination, and the axis 2 with 20.87% (Fig. 4). The axis 1 (79.13%) is determined by C_{21} while the axis 2 (20.87%) is mostly defined by the C_{29} and C_{27} (*Table 4B*). The first two discriminant axes separated individuals in three groups (Fig. 4). The axis 1 (79.13%) the most distinguished *P. nigra* from other species according to lower contents of C_{21}.The axis 2 (20.87%) extracted *P. peuce* from other species according to higher values of C_{29} and lower

values of C_{27}. With this analysis 84%, 98% and 91% individuals of *P. heldreichii*, *P. nigra* and *P. peuce*, resp. are correctly classified. The scatter plot obtained by the level of species (Fig. 4) more exactly explained existence of three differentiated entities (species) than scatter plot based at population level (Fig. 3). *n*-Alkane C_{21} mostly defined formation of axis 1 (79.13%), while C_{16}-C_{18} mostly define formation of axis 2 (20.87%) (on the basis of factor loadings (not presented). *n*-Alkanes present in higher content (C_{16}, C_{17}, C_{25}, C_{27}, C_{32} and C_{33}) are mostly poorly or moderately positive correlated ($P<0.05$). The exception is relationship of C_{16} and C_{17} as well as relationship of C_{25} and C_{27} which are highly positive correlated ($r>0.78$, $P<0.05$) (on the basis of correlation coefficient, not presented).

Figure 3.

Figure 4.

Cluster analysis (CA) (Fig.5) of all 14 populations, based on eleven n-alkanes, showed grouping in accordance with other multivariate methods, PCA (Fig. 2) and DA (Fig 3 and Fig. 4). Namely, according to obtained dendrogram (Fig. 5) all examined populations were classified into three main clusters. The first group composed of seven population of P. nigra, the second group constituted of three populations of P. peuce and the third group constituted of three populations of P. heldreichii. The only exception is the first group which contains one population of P. heldreichii (population IV identified as var. pančićі). Interestingly, P. heldreichii populations from southwestern Serbia, which are described as var. pančićі, represent a transitional form towards the black pine based on morphology features.[22] Namely, P. heldreichii var. pančićі is very similar to P. nigra and to some extent to two natural hybrids (P. x nigradermis Fukarek and Vidaković and P. x mugodermis Fukarek) and one intermediate form (P. nigra f. leucodermoides Fukarek and Nikolić) based on morphology of branches, needles and/or cones.[22] On the other hand, CA showed the formation of two separated clusters of P. nigra populations, which are separated almost at the same level as the ones for the other two species. In accordance with the geographic and ecological diversity of its habitat and its disjunctive distribution, P. nigra is an extremely variable pine species.[6] In various literature sources black pine was therefore described either as a collective species or a single species with more than 20 described subspecies.[22] Furthermore, the extremely high levels of chloroplast SSR (simple sequence repeats) differentiation of black pine populations in Western Europe were reported by Afzal-Rafii and Dodd.[23] These levels of genetic differentiation are among the highest reported for pines and approach the level determined for species differentiation between P. elderica and P. brutia,[24] suggesting deep divergence within P. nigra.

Figure 5.

Conclusions

On the basis of presented statistics it can be summarized that, according to contents of *n*-alkanes, differences between species were approved. PCA showed separation of *P. nigra* populations from partially overlapped populations of *P. heldreichii* and *P. peuce*. Similar results were obtained by DA on the level of populations as well as on the level of species: the existence of distinct *n*-alkane profiles for each of the species tested was confirmed pointing separation of *P. nigra* from *P. heldreichii* and *P. peuce* (which were, to a greater or lesser extent, separated too). However, CA showed the weakest grouping of populations within the species compared to other multivariate methods. It may be concluded that position of studied pines on the basis of *n*-alkane composition neither was in accordance with infrageneric classification[2] nor with recent molecular[3] and terpene investigations.[14]

For complete phylogenetic picture of conifers, further investigation of larger genomic data is necessary, as was already suggested in spruces.[23]

Experimental Section

Plant Material

Fresh plant material (needles) from four populations of *P. heldreichii*, seven populations of *P. nigra* and three populations of *P. peuce* were harvested from lower third part of trees and analyzed. Material was stored in deep freezer at -20 °C prior to further analysis. A map of studied area was presented in Fig. 1. Details of the each population were listed in *Table 1*. We merged data of 195 samples of *P.nigra*, 97 of *P. heldreichii* and 90 of *P. peuce* which were obtained, processed and published at population level in last 10 years. [16,17] [19,20] Plant species were identified by Dr *S. R. B.* and Dr *B. M. N.*

Extraction and Isolation of Needle Wax

The total wax of each sample was extracted by immersing 3 g of needles in 10 ml of *n*-hexane for 45 s. After extraction, the solvent was removed under vacuum at 60 °C. The concentrated extracts were chromatographed on a small-scale column using a Pasteur pipette filled with silica gel 60, previously activated at 120 °C. The wax was obtained by elution with 5 ml of *n*-hexane. The wax samples were stored at –20 °C until further analysis.

Chemicals and Reagents

n-Hexane (HPLC grade) and silica gel 60 (0.2–0.5 mm) were purchased from Merck (Darmstadt, Germany).

GC and GC–MS analysis

Gas chromatography (GC) and gas chromatography–mass spectrometric (GC–MS) analyses were performed using an Agilent 7890A GC equipped with an inert 5975C XL EI/CI mass spectrometer detector (MSD) and flame ionisation detector (FID) connected by capillary flow technology 2-way splitter with make-up. A HP-5MS capillary column (30 m×0.25 mm×0.25 µm) was used. The GC oven temperature was programmed from 60 to 300 °C at a rate of 3 °C min^{-1} and held for 10 min. Helium was used as the carrier gas at 16.255 psi (constant pressure mode). An auto-injection system (Agilent 7683B Series Injector) was employed to inject 1 µL of sample. The sample was analysed in the splitless mode. The injector temperature was 250 °C and the detector temperature 300 °C. MS data was acquired in the EI mode with scan range 30–550 m/z, source temperature 230 °C, and quadrupole temperature150 °C; the solvent delay was 3 min.

Compound Identification

The components were identified based on their retention index and comparison with reference spectra (Wiley and NIST databases) as well as by the retention time locking (RTL) method and the RTL Adams database.[25] The retention indices were experimentally determined using the standard method of Van Den Dool and Kratz[26] involving retention times of *n*-alkanes, injected after the sample under the same chromatographic conditions. The relative abundance of the *n*-alkanes was calculated from the signal intensities of the homologues in the GC-FID traces.

Statistical Treatment

Principal component analysis (PCA) was performed as *non a priori* multivariate analysis for easier comphrension of the structure of elements and characteristics in extensive tabular data. Discriminant analysis (DA) was employed to differentiate *a priori* defined groups and to assort the elements within the predefined groups. Cluster analysis (CA) was performed with the aim of identification of the relations among populations. The calculation of mean values (X) and standard deviations (S.D.) of the populations, test for normality (χ^2 test), one-way analyses of variance (ANOVA), Levene's test, principal component analyses (PCA) and cluster analysis (CA) were all carried out with the software Statgraphics Plus (version 5.0; Statistical Graphics Corporation, U.S.A.). For DA analysis software Statistica version 8 was used.

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Author Contribution Statement

B. M. N. merged all data, performed statistical analyses and their interpretation and drafted the first version of the manuscript together with graphic design. *Z. S. M.* gave contribution to concept and design of the manuscript and critically redacted it until the final version. *V. V. T., M. M .T.* and *I. Ž. Đ.* analyzed chromatographic data up to the level of statistical processing and helped in determination of terpene classes and interpretation of obtained results. *S. R. B.* revised statistical analyses, helped in their interpretation and revised the drafted version of the manuscript. *P. D. M.* and *V. V. T.* 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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Figure captions

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

Figure 2. Principal component analysis (PCA) of 195 *P. nigra* individuals from seven populations, 97 *P. heldreichii* individuals from four populations and 90 *P. peuce* individuals from three populations. The italic numbers represent the entries of *n*-alkanes, cf. *Table 2*.

Figure 3. Discriminant analysis (DA) based on eleven selected *n*-alkanes of *P. nigra*, *P. heldreichii* and *P. peuce* with 14 populations as *a priori* groups. Symbols refer to populations: 1= *P. heldreichii*, Mt. Zeletin; 2= *P. heldreichii*, Mt. Bjelasica; 3= *P. heldreichii*, Mt. Lovćen; 4= *P. heldreichii*, Mt. Zlatibor and Pešter; 5= *P. nigra* subsp. *nigra*, Mt. Tara, Banjska stena; 6= *P. nigra* subsp. *nigra*, Mt. Tara, Omar; 7= *P. nigra* subsp. *nigra*, Mt. Tara, Zmajevečki potok; 8= *P. nigra* var. *gocensis*, Priboj, Crni vrh; 9= *P. nigra* var. *gocensis*, Mt. Goč, Gvozdac; 10= *P. nigra* subsp. *pallasiana*, Mt. Dukat, Jarešnik; 11= *P. nigra* var. *banatica*, Lazareva Reka Canyon; 12= *P. peuce*, Mt. Mokra Gora; 13= *P. peuce*, Mt. Zeletin and 14= *P. peuce*, Mt. Sjekirica. Circles represent centroids with ellipses (95 percent confidence intervals).

Figure 4. Discriminant analysis (DA) based on eleven selected *n*-alkanes of *P. nigra*, *P. heldreichii* and *P. peuce* with species as *a priori* groups. Red, blue and green symbols indicated *P. nigra*, *P. heldreichii* and *P. peuce*, respectively.

Figure 5. Dendrogram based on a Unweighted pair-group average (distance: Pearson correlation coefficient similarity) of the studied eleven populations (mean values) (A) of three *Pinus* species (B). The numbers on the vertical axis refer to distance level, calculated on the basis of differences between population contents of selected components. For legend details see caption of Fig. 1.

Graphical Illustration