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Application of canonical discriminant analysis in differentiation of natural populations of *Pinus nigra* in Serbia based on terpene composition

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***Abstract*:**

Šarac, Z., Bojović, S., Nikolić, B., Zlatković, B., Marin, P.: ***Application of canonical discriminant analysis in differentiation of natural populations of Pinus nigra in Serbia based on terpene composition. Biologica Nyssana, 5 (1), September 2014: 11-15.***

The canonical discriminant analysis (CDA) was performed in order to check the hypothesis of chemical separation infraspecific taxa of *Pinus nigra* J.F. Arnold (ssp. *nigra*, var. *gocensis*, ssp. *pallasiana*, and var. *banatica*) in Serbia based on variability of the needle terpenes. The CDA, which maximizes variations between *a priori* groups, showed division of seven native *P. nigra* populations into three groups, which belong to three taxonomically recognized taxa (ssp. *nigra*, ssp. *pallasiana*, and var. *banatica*). The most important characters in discrimination were (*E*)-caryophyllene, α-humulene, terpinolene, germacrene D, α-pinene, and myrcene. The individuals of *pallasiana* group were poorer in (*E*)-caryophyllene and terpinolene and richer in α-humulene contrary to the individuals of *nigra* group. The individuals of *banatica* group had the highest content of α-pinene and myrcene. The obtained results were compared with recently published data on chemodiversity of *P. nigra* infraspecific taxa on the territory of Serbia.

Key words: canonical, chemotaxonomy, discriminant analysis, needle terpenes, *Pinus nigra*.

***Apstract*:**

Šarac, Z., Bojović, S., Nikolić, B., Zlatković, B., Marin, P.: ***Primena diskriminantne kanonijske analize u diferencijaciji autohtonih populacija Pinus nigra u Srbiji na osnovu sastava terpena. Biologica Nyssana, 5 (1), September 2014: 11-15.***

Diskriminatna kanonijska analiza (CDA) izvedena je u cilju provere hipoteze o fitohemijskom razdvajanju infraspecijskih taksona *Pinus nigra* J.F. Arnold (ssp. *nigra*, var. *gocensis*, ssp. *pallasiana* i var. *banatica*) u Srbiji na osnovu varijabilnosti terpena u četinama. CDA, koja naglašava varijabilnost između *a priori* definisanih grupa, pokazala je rezdvajanje sedam autohtonih *P. nigra* populacija u tri grupe (ssp. *nigra*, ssp.

pallasiana i var. *banatica*). Najvažniji karakteri u diskriminaciji bili su (*E*)-kariofilen, α-humulen, terpinolen, germakren D, α-pinen i mircen. Individue grupe *pallasiana* siromašnije su u sadržaju (*E*)-kariofilena i terpinolena, a bogatije u sadržaju α-humulena, suprotno od individua grupe *nigra*. Individue grupe *banatica* imaju veći sadržaj α-pinena i mircena. Dobijeni rezultati upoređeni su sa nedavno objavljenim podacima o hemodiverzitetu *P. nigra* infraspecijskih taksona na teritoriji Srbije.

Ključne reči: kanonijska, hemotaksonomija, diskriminantna analiza, terpeni u ;etinarima, *Pinus nigra*.

Introduction

Pinus nigra J.F. Arnold (Pinaceae) is a species of the tertiary origin belonging to the group of taxa typical for Mediterranean and submediterranean coniferous forests (Bogunic et al., 2007). It is one of the widespread, morphologically as well as taxonomically very variable pine species in Europe (Gaussen et al. 1993), with highly fragmented distribution range that extends from North Africa through the northern Mediterranean and eastwards to the Black Sea. In the flora of Serbia, Jovanović (1992) recognized two subspecies of black pine that can be geographically differentiated, each further divided into several varieties. Generally, in the western, southwestern and central parts of Serbia, *P. nigra* ssp. *nigra* is widespread, within which a distinct variety is recognized (var. *gocensis* Đorđević), while in eastern Serbia *P. nigra* ssp. *pallasiana* (D. Don) Holmboe has an extremely narrow distribution range. Namely, Crimean black pine (*P. nigra* ssp. *pallasiana*) was only recorded in southeastern Serbia (Rajevski, 1950) on the mountain Crnook (Jarešnik). Apart from this, in eastern and northeastern Serbia three additional populations of black pine are known, which Jovanović (1992) describes as var. *banatica* Georg. et Ion. within ssp. *pallasiana.*

The chemodiversity aspect of black pine on the territory of Serbia has been addressed concerning *n*-alkane (Bojović et al., 2012) and terpene (Šarac et al., 2013) variability. In the second study, 58 essential-oil components were identified in the needles of 195 trees from seven populations. Unlike principal component analyses (PCA), which showed an overlap of all populations, cluster analyses (CA) separated analysed samples into three basic groups: the first group consisted of populations from western (Banjska stena, Omar, Zmajevački potok), southwestern (Priboj) and central Serbia (Goč) (considered as ssp. *nigra* group), the second of population from southeastern Serbia (Jarešnik) (ssp. *pallasiana* group), and the third of population from northeastern Serbia (Lazareva reka), which had the most distinct oil composition (ssp. *banatica* group) (Šarac et al., 2013).

Bearing this in mind, the aim of the present study was to check the hypothesis of chemical separation of infraspecific taxa of *P. nigra* from Serbia by performing a canonical discriminant analysis (CDA). This method maximizes variations between *a priori* defined groups and thus characterize the degree of divergence among analyzed populations (James & McCulloch, 1990). Also, CDA was performed in order to determine the relative importance of terpenes as discriminators between *a priori* groups.

Material and methods

Plant material

The samples from seven typical populations of *P. nigra* infraspecific taxa - I - Banjska stena, II - Omar, III - Zmajevački potok (ssp. *nigra*), IV - Priboj-Crni vrh, V - Goč-Gvozdac (var. *gocensis*), VI - Jarešnik (ssp. *pallasiana*), and VII - Lazareva reka (var. *banatica*), growing wild in Serbia were analyzed. Details about sampling, locations and ecological conditions of the selected populations, numbering of populations, the extraction of the essential oils, the GC-FID and GC/MS analyses, and the identification of terpenes have been reported previously (Šarac et al., 2013).

Statistical Analysis

The CDA was carried out in order to check the hypothesis that the analyzed sample was composed of discrete groups, which are chemically differentiated one from other. From the total data set of 75 original compounds (for details see Šarac et al., 2013), 14 compounds were selected for CDA, *viz*., α-thujene, α-pinene, camphene, β-pinene, myrcene, limonene, (*E*)-β-ocimene, terpinolene, (*E*)-caryophyllene, α-humulene, γ-muurolene, germacrene D, δ-cadinene, and germacrene D-4-ol. The 61 compounds which were found in traces (content <0.5%, according to Rudloff et al., 1975; Lieutier et al., 1991) were excluded from further analysis. The CDA was computed on the selected data set (195 samples x 14 variables) with *a priori* defined groups. Statistical analyses were performed using the software Statistica 5.1 (StatSoft, 1997).

Results and Discussion

The CDA based on individuals from seven native *P. nigra* populations showed that the first two discriminant functions participated in 79.6% of the total discrimination, of which the first function with 64.7% (**Tab. 1**). The first discriminant function is approximately equal determined by the content of terpinolene, α-thujene, (*E*)-caryophyllene, α-pinene, and germacrene D. The second function is mostly defined by the content of germacrene D, α-pinene, and β-pinene (**Tab. 1**).

Table 1. Standardized coefficients for the first two canonical axes (CA) of variation in terpene variables from the discriminant functional analysis of 7 *a priori* groups.

Variables	CA1	CA2
α-Thujene	**-1.087**	0.404
α-Pinene	**1.017**	**-1.823**
Camphene	-0.408	-0.482
β-Pinene	0.829	**-1.618**
Myrcene	0.683	0.643
Limonene	-0.086	-0.408
(*E*)-β-Ocimene	0.498	-0.494
Terpinolene	**1.260**	-0.758
(*E*)-Caryophyllene	**1.053**	-0.140
α-Humulene	-0.880	-0.840
γ-Muurolene	0.016	-0.051
Germacrene D	**1.010**	**-2.305**
δ-Cadinene	0.729	-0.230
Germacrene D-4-ol	0.432	-0.959
Eigenvalue	1.493	0.344
% explained variation	64.7	79.6

The first discriminant function mainly separated population VI while the second function separated population VII from the other populations (**Tab. 2**, **Fig. 1**). The CDA with 7 populations was correctly classified only 53% of individuals on average (classification matrix values, data from the program). However, its results are sufficient to assume the existence of three groups of individuals: (1) populations I, II, III, IV, and V which can be designated as ssp. *nigra*, (2) population VI as ssp. *pallasiana*, and (3) population VII as ssp. *banatica*, in accordance with previous results of terpene analysis (Šarac et al., 2013). However, departing from the first group of individuals (ssp. *nigra*), population V was partially overlapped with the second group (ssp. *pallasina*) by axis 1. Differentiation suggestion obtained from seven populations was the inducement for performing the CDA with three *a priori* defined groups.

Table 2. Means of Canonical Variables.

Population	CA1	CA2
I	1.414	-0.241
II	0.481	-0.247
III	0.468	-0.149
IV	0.881	-0.106
V	-0.654	0.041
VI	**-2.366**	-0.273
VII	-0.053	**1.988**

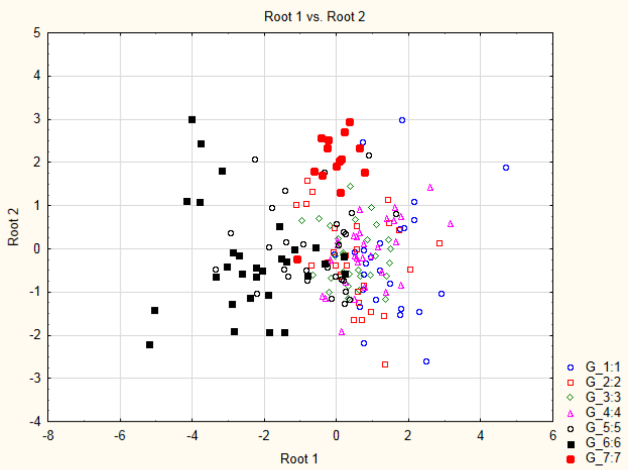

Fig. 1. Canonical discriminat analysis (CDA) based on the content of 14 terpenes isolated from 195 Pinus nigra samples of seven populations (I-VII).

The CDA based on individuals from three groups (taxa) showed that the first discriminant function explained 73.3% and the second 26.7% of the total discrimination (**Tab. 3**). The first function was mostly determined by the content of (*E*)-caryophyllene, α-humulene and terpinolene (Tab 3). The second function was mainly defined by the content of germacrene D, α-pinene, and myrcene. The first discriminant function separated *nigra* and *pallasiana* group, while the second function *banatica* group from the other groups (**Fig 2**). The individuals of *pallasiana* group had lower content of (*E*)-caryophyllene and terpinolene and higher content of α-humulene (considering that the majority of individuals was on the right side of the figure). The individuals of *nigra* group had higher content of (*E*)-caryophyllene and terpinolene, and lower content of α-humulene (the majority of individuals was on the left side of the figure). The individuals of *banactica* group had higher content of α-pinene and myrcene. The CDA with 3 *a priori* defined gropus was correctly classified 88% of individuals on average (classification matrix values, data from the program).

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

Variables	CA1	CA2
α-Thujene	0,741	-0,436
α-Pinene	-0.548	**1,682**
Camphene	0,496	0,411
β-Pinene	-0,464	1,523
Myrcene	-0,739	**-0,621**
Limonene	0,329	0,281
(*E*)-β-Ocimene	-0,348	0,464
Terpinolene	**-0,781**	0,785
(*E*)-Caryophyllene	**-1,069**	0,294
α-Humulene	**0,942**	0,670
γ-Muurolene	0,177	-0,102
Germacrene D	-0,477	**2,169**
δ-Cadinene	-0,719	0,345
Germacrene D-4-ol	-0,128	0,832
Eigenvalue	0,922	0,336
% explained variation	0,733	1,00

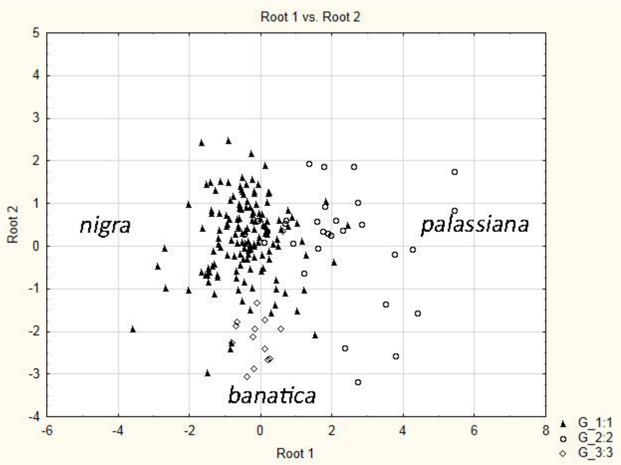

Fig. 2. Canonical discriminat analysis (CDA) based on the content of 14 terpenes isolated from 195 *Pinus nigra* samples of three groups (ssp. *nigra*, ssp. *pallasiana* and var. *banatica*).

In accordance with previously published results of CA (Šarac et al., 2013), CDA confirmed that based on the terpene variability in native populations of black pine in Serbia, three discrete groups can be distinguished. The first group (*nigra*) consisted of populations described as var. *nigra* (populations I-III) and var. *gocensis* (populations IV and V) within ssp. *nigra* according to existing morho-anatomical and phytocoenological studies (Jovanović, 1992). Also, our very recent (Šarac et al. (2013) and present study, showed that populations determined as var. *gocensis* exhibited a moderate tendency of separation from the first group (*nigra*) (population IV in CA and population V in CDA). Based on *n*-alkanes variability, the populations assigned as var. *gocensis* (IV and V) showed even grater tendency of splitting (Bojović et al., 2013). Population V has been classified into the first group (*nigra*), characterized as chemotype 1 by Bojović et al. (2012), while population IV belonged to the second group (*pallasiana*), assigned as chemotype 2. It was assumed that populations considered as var. *gocensis* could be transitional form between subspecies *nigra* in the west and subspecies *pallasiana* in the southest of Serbia.

Taxonomic position of var. *gocensis* within *P. nigra* complex is still not completely resolved. This variety was primarily described by Đorđević (1931) from the slopes of the mountain Goč in central Serbia. Later, Vidaković (1955), determined the wider distribution of this variety, and based on the leaf anatomy raised it to the subspecies level (ssp. *gocensis* Vid.). Comparing to the typical var. *nigra* it is different in some tree traits (bark of mature trees had prominent transverse and longitudinal furrows, resembling the bark of *Pinus heldreichii* H. Christ), as well as in needles structure. However, according to results of our analyses there is no such clear phytochemical distinction between var. *gocensis* and typical var. *nigra*.

The second group is composed mainly of trees of population VI from southeastern Serbia belonging to ssp. *pallasiana*. The presence of Crimean pine in Serbia was originally reported by Adamović (1909), but without precise data on its distribution. Rajevski (1950) was the first who established the position of this population within the territory of Serbia. The single enclava of Crimean pine is situated at the locality Jarešnik in the surroundings of Bosilegrad growing on the crystalline slates. The population in Serbia represents the northernmost disjunction of the Crimean black pine area spreading from the southern parts of Balkan Peninsula. Based on , *n*-alkane and terpene composition, ssp. *pallasiana* is clearly distinguished as separate group (taxon and chemotype) from ssp. *nigra* according to this and previous studies (Bojović et al., 2012; Šarac et al., 2013).

Finally, the third group consisted of trees from population Lazareva reka, determined as var. *banatica* within ssp. *pallasiana* (Jovanović, 1992). This taxon is previously described in Romania (Domogled - Valea Cernei) and according to the literature data the black pine populations from northwestern Romania have a very controversial taxonomic position (Boşcaiu & Boşcaiu, 1999). In the second edition of Flora Europea, Gaussen et al. (1993) treated these populations as an independent species, i.e., *P. banatica*. Analysis of the chemodiversity of the black pine populations from Serbia, based on both *n*-alkane and terpene composition, supports the view that var. *banatica* should be considered as distinct subspecies or even species (Bojović et al., 2012; Šarac et al., 2013).

Conclusion

The canonical discriminant analysis (CDA) of recently reported terpene composition of *P. nigra* in Serbia (Šarac et al., 2013), also showed division of populations into three discrete groups. The CDA confirmed the attitude that terpenes are good taxonomic markers at infraspecific level in the Pinales. Nevertheless, all these assumptions should be checked by further extending studies to the entire Balkan peninsula and the Mediterranean region, and especially including the classical locality (*locus classicus*) of *P. nigra* var. *banatica* from Romania (Domogled - Valea Cernei). Also, detail molecular analysis is needed to clarify relationships witin this extremely variable and complex taxon.

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