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Diversity of needle *n*-alkanes, primary alcohols and diterpenes in Balkan and Carpathian native populations of *Pinus nigra* J.F. Arnold

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ABSTRACT

This is the first report on population differentiation based on composition of three different compound classes (*n*-alkanes, primary alcohols and diterpenes) in the epicuticular waxes of European black pine (*Pinus nigra*) from Balkan Peninsula and Southern Carpathians. The *n*-hexane extracts of 263 samples, originating from 15 native populations of four *P. nigra* subspecies (*nigra*, *dalmatica*, *banatica* and *pallasiana*), were investigated by GC-MS and GC-FID analyses. In all studied populations *n*-alkanes represent the most abundant compound group, while the contents of diterpenes and alcohols were lower and variable. Multivariate statistical analyses (Canonical Discriminant Analysis and Agglomerative Hierarchical Clustering) suggest existence of two clearly differentiated chemical entities with the basic difference in the content of primary alcohols (especially *cis*-9-octadecen-1-ol and 1-hexadecanol). The first group (low alcohol content) included all populations of ssp. *nigra*, ssp. *dalmatica* and ssp. *banatica*, as well as the two northernmost populations of ssp. *pallasiana* from Serbia and Bulgaria, while the second group (high alcohol content) comprised individuals from Greek and Macedonian populations of ssp. *pallasiana*. According to the recently proposed concept of ancient split of major genetic lineages of *P. nigra*, both chemical entities detected in this study belong to the same (Balkan Peninsula) genetic group. Taxonomic implications of given results are briefly discussed in relation with previous data based on morphological and molecular characters.

1. Introduction

The European Black Pine (*Pinus nigra* J.F. Arnold) is a Tertiary relict (2.6–66.00 Ma) and one of the most economically and ecologically important forest species in the Mediterranean and Submediterranean regions (Mirov, 1967). It is present in all of the main European glacial refugia of biodiversity, including the Balkan, Iberian and Italian Peninsulas, Asia Minor and some Mediterranean islands (Gaussen et al., 1993). The long-term fragmentation and survival of individual populations caused pronounced adapting to the local environment, leading to high morphological variability among populations (Vidaković, 1991). In various literature sources black pine was therefore described either as a collective species (Villar, 1947; Svoboda, 1953; Fukarek, 1958) or a single species with more than 20 identified subspecies (reviewed in Vidaković, 1991). However, the first comprehensive study that included *P. nigra* individuals from across its entire natural range has revealed only three differentiated genetic groups, which corresponded to three wide geographical areas: Western Mediterranean, Balkan Peninsula, and Asia Minor (evidence from chloroplast DNA, Naydenov et al., 2016).

The most extensive forests of *P. nigra* are presently found in the Balkans and Turkey, while westwards from the Balkans and the Julian Alps the black pine forests become less frequent and fragmented. The territory of the Balkans and Southern Carpathians used to be characterized by a relatively great number of classification schemes of *P. nigra* (mostly based on morpho-anatomical and phytogeographical features), according to which we can distinguish from three (ssp. *nigra*, ssp. *dalmatica* (Vis.) Franco and ssp. *pallasiana* (Lamb.) Holmboe (Gaussen et al., 1964, 1993; Jalas and Suominen, 1973; The Plant List, 2013; Raab-Straube, 2014)) to six different subspecies (ssp. *nigra*, ssp. *dalmatica*, ssp. *illyrica* (Vid.) Fukarek, ssp. *gocensis* (Đorđ.) Vidaković, ssp. *pallasiana* and ssp. *banatica* (Borbás) Novák (Fukarek, 1958;

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Vidaković, 1991; Ciocârlan, 2000)) within this area.

Several authors suggested that compounds of epicuticular waxes are very informative as markers in studies of geographic variation and/or identification of infraspecific taxa of *Pinus* species (Bojović et al, 2012; Nikolić et al., 2010, 2012a; 2012b). Furthermore, Diefendorf et al. (2015) pointed out the significance of plant functional type and phylogeny as a driver of chain length distribution of wax compounds in terrestrial plants, indirectly controlled by long-term climate change on plant evolution. So far, the composition of epicuticular waxes of *P. nigra* has been addressed only regarding the contents of *n*-alkanes (Maffei et al., 2004; Bojović et al., 2012) or *n*-alkanes and primary alcohols (Mitić et al., 2016). The last study, which included 9 populations of three *P. nigra* subspecies (*nigra*, *banatica* and *pallasiana*) from Bosnia and Herzegovina, Serbia, Romania and Macedonia (the area of central Balkans and Southern Carpathians), has shown clinal variation of epicuticular waxes (in north-south direction) as a mechanism of chemical differentiation across the study area (Mitić et al., 2016).

In this article we presented new results for 6 populations of *P. nigra* from Serbia, Croatia, Bulgaria and Greece, identified as subspecies *nigra*, *dalmatica* and *pallasiana*, considering epicuticular wax *n*-alkanes, primary alcohols and diterpenes as well as for 9 populations, previously studied by Mitić et al. (2016), considering diterpenes. To the best of our knowledge, no study exists addressing the issue of diterpene distribution in epicuticular waxes of European black pine. Moreover, this is the first research of epicuticular waxes of endemic Dalmatian black pine (ssp. *dalmatica*) as well as of the populations of Crimean black pine (ssp. *pallasiana*) from the territory of Greece and Bulgaria. For multivariate statistical analyses (canonical discriminate analysis and agglomerative hierarchical clustering), in addition to the results obtained in this study, we used our previously published data (Mitić et al., 2016) relating to the content of *n*-alkanes and primary alcohols in smaller part of *P. nigra* range. In this way, epicuticular waxes of 15 populations of four *P. nigra* subspecies (*nigra*, *dalmatica*, *banatica* and *pallasiana*) from almost the entire territory of Balkan Peninsula and Southern Carpathians has been analyzed for the first time according to the contents of three different classes of wax compounds. Specifically, we addressed the following questions: 1) which type of characters (diterpenes, primary alcohols, *n*-alkanes) has the most important role in chemical differentiation of examined *P. nigra* populations? 2) is a correlation with geographical features stronger than correlation with lineages? 3) is there any correlation among chemical grouping and classic and molecular classification of *P. nigra*?

2. Materials and methods

2.1. Plant material

Plant material (2–3 year-old needles) from 15 native populations belonging to different infraspecific taxa of *P. nigra* (identified as ssp. *nigra*, ssp. *dalmatica*, ssp. *banatica* and ssp. *pallasiana*) growing wild in the Balkans and Southern Carpathians was selected. A map of the study area, indicating the locations of selected populations of studied taxa, is presented in Fig. 1, and the corresponding geographic and geologic data, date of collection, voucher information as well as the number of studied individuals for every population are listed in Table 1. Plant material deposited in labeled polyethylene bags (sample plot, date of collection, and locality) was immediately transferred to a freezer and stored at −20 °C prior to further analysis. Voucher specimens of each taxon were deposited in the Herbarium of the Department of Biology and Ecology, Faculty of Sciences and Mathematics, University of Niš (HMN) and the Herbarium of the Institute of Botany and Botanical Garden "Jevremovac", Faculty of Biology, University of Belgrade (BEOU). Identification of plant material was done by Dr. Zorica S. Mitić, Dr. Bojan K. Zlatković and Dr. Petar D. Marin.

2.2. Isolation of epicuticular waxes

The total wax of each sample was extracted by immersing 5 g of whole needles in 10 ml of hexane for 60 s. After extraction, the solvent was removed under vacuum at 50 °C. Clean-up of residues was done on a small-scale column filled with Florisil (60–100 mesh) using 5 ml of hexane as eluent. Eluates were concentrated to 0.5 ml under vacuum, and stored in amber-glass vials at 4 °C until further analysis.

2.3. GC-MS and GC-FID analyses

Chemical compositions of *n*-hexane extracts were determined by GC-MS (Gas Chromatography-Mass Spectrometry) and GC-FID (Gas Chromatography-Flame Ionization Detector) analyses. Samples were analyzed on a *7890*/7000B GC-MS/MS triple quadruple system (*Agilent Technologies*, Santa Clara, CA, USA, equipped with a *Combi PAL* auto sampler). The fused silica capillary column *HP-5MS* (5% phenylmethylsiloxane, 30 m × 0.25 mm, film thickness 0.25 μm) was used. The injector and interface operated at 250 and 300 °C respectively. The temperature program: held at 70 °C for 2.25 min and then from 70 °C to 300 °C at a heating rate of 5 °C min^{-1} than held at 300 °C for 10 min. The 5 μL of samples were injected 2:1 split ratio. Post run: back flash for 1.89 min, at 280 °C, with helium at 50 psi. MS conditions were as follows: ionization voltage of 70 eV, acquisition mass range 40–650, scan time 0.32 s. The GC-FID analyses were carried out under the same experimental conditions as described for the GC-MS analysis. The percentage composition was computed from the GC-FID peak areas without the use of correction factors. Constituents were tentatively identified by comparison of their linear retention indices (relative to C_8-C_{40} alkanes on the *HP-5MS* column) with literature values (Adams, 2007) and their MS with those from Wiley 6 and NIST02 by the application of the AMDIS software (the Automated Mass Spectral Deconvolution and Identification System, Ver. 2.1, DTRA/NIST, 2011).

2.4. Statistical analysis

Statistical data processing was carried out by STATISTICA 8 software (*Statsoft, Inc.*, Tulsa, OK, USA). Statistical matrices included the content of all identified wax compounds (in percentage) as original variables (Tables 2 and 3). The untransformed data were used in order to preserve their natural level of variability in multivariate analyses. The significance of differences between the studied characters was determined by univariate Analysis of Variance (ANOVA). Multivariate analysis included Canonical Discriminant Analysis (CDA) and Agglomerative Hierarchical Clustering (AHC). Unweighted pair group average (UPGMA) was used as a criterion for the clusters development, and Euclidean distances as diversity assessment criteria in AHC analysis.

3. Results and discussion

3.1. Variability of epicuticular wax composition of P. nigra

GC-MS and GC-FID analyses of *n*-hexane extracts of needle samples, collected from 263 individuals of *P. nigra* from 15 populations from Balkans and Southern Carpathians, have revealed presence of 21 components (Tables 2 and 3). The identified compounds include four diterpenes (two pimarane: pimaradiene and sandaracopimaradiene, and two abietane C-skeleton type: 18-norabieta-8,11,13-triene and abietatriene), four primary alcohols (1-hexadecanol, *cis*-9-octadecen-1-ol, *trans*-9-octadecen-1-ol and 1-octadecanol) and 13 *n*-alkane homologues with chain-lengths ranging from C_{21} to C_{33} (even if the relative contents of C_{32} and C_{33} were below 0.5% at the species level). *n*-Alkanes were the most frequent compound group in all studied populations and varied from 54.0 to 82.8% (73.4% on average), while contents of diterpenes and primary alcohols were much lower: 0.2–16.8% (4.5% on