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Diversity of nonacosan-10-ol and n-alkanes among twelve Pinus taxa

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Abstract

The content of nonacosan-10-ol and n-alkanes in needle waxes of twelve Pinus taxa (Pinus halepensis, P. heldreichii, P. mugo, P. nigra ssp. nigra, P. nigra ssp. dalmatica, P. peuce, P. pinaster, P. pinea, P. ponderosa, P. strobus, P. sylvestris, and P. wallichiana) were examined. In eight Pinus taxa (except of P. halepensis, P. pinaster, P. heldreichii and P. peuce) nonacosan-10-ol contents were examined for the first time. In all examined pines C29, C27 or C25 were the main n-alkane compounds. C range was mainly 18-33. In six Pinus taxa (P. mugo, P. nigra ssp. dalmatica, P. sylvestris, P. pinea, P. strobus and P. wallichiana) CPI and ACL values were examined for the first time. In the plane of Axis 1 (PCA), P. strobus, P. mugo, and P. wallichiana were strongly separated from P. heldreichii and P. pinea. In the plane of Axis 2, P. peuce, P. strobus, and P. wallichiana as well as P. heldreichii and P. pinea diverged from other examined species. In DA P. heldreichii, P. strobus, P. peuce, and P. wallichiana diversed. CA divided sections Pinus and Pinaster from section Strobi.

Keywords: Pinus, n-alkanes, nonacosan-10-ol, Carbon Preference Index (CPI), Average chain length (ACL), Principal component analysis (PCA), Discriminant analysis (DA)

Introduction

The genus *Pinus* (Pinaceae), with around 110 species, is the largest and the most widespread genus of conifers in the Northern Hemisphere (Farjon 2001). Pines are one of the most ecologically important tree species as a major, often dominant component of boreal, subalpine, temperate, and tropical forests as well as arid woodlands (Richardson and Rundel 1998). Economically, pines are an important source of timber, wood pulp, turpentine, charcoal, edible seeds (pine nuts) and ornamentals (Le Maitre 1998). Modern classification of *Pinus* recognizes two major lineages: subgenus *Pinus* (Diploxylon or hard pines, with two fibrovascular bundels in the needles) and subgenus *Strobus* (Haploxylon or soft pines, with one fibrovascular bundle in the needle). Both subgenera are further divided into two sections consisting of two or three subsections each (Gernandt et al. 2005).

The cuticular waxes are complex mixtures of very-long-chain (VLC > C_{20}) saturated aliphatic compounds, bearing no functionality or only one functional group at one end of the carbon chain (Racovita and Jetter 2016). Most commonly encountered are homologous series of even-numbered fatty acids, primary alcohols, alkyl esters and aldehydes, as well as odd-numbered alkanes, secondary alcohols and ketones (Kunst and Samuels 2003). In addition, detail population investigations based on the composition of secondary alcohol nonacosan-10-ol of the needle waxes of *P. halepensis* (Matas et al. 2003), *P. heldreichii* (Nikolić et al. 2012a), *P. peuce* (Nikolić et al. 2012b) and *Picea omorika* (Nikolić et al. 2013a) in different parts of their distribution were reported recently.

Among the compounds constituting the cuticular waxes, *n*-alkanes have been most commonly used as chemotaxonomic markers in different plant families (Maffei et al. 1994; 1996a; 1996b). In conifers, the efficacy of *n*-alkanes as chemotaxonomic markers have been discussed by Maffei et al. (2004), showing the differences in *n*-alkane composition at the familial and subfamilial level. *n*-Alkanes are usually examined in chemotaxonomic studies (Maffei et al. 2004 and refs. cited therein; Corrigan et al. 1978), phylogenetic studies (Bowman 1980), hybrid detection (Knight et al. 2004), air pollution studies (Lütz et al. 1990), etc. Variability of *n*-alkanes depends upon species, even within the same genus. In conifers, the most dominant is C31, less commonly C33 or C29 (Maffei et al. 2014), or even C25 and C27 (Oros et al. 1999)., which are also common in deciduous species, e.g. in the genus *Salix* (Teece et al. 2008) or *Populus* (Cameron et al. 2000).

It was already known that CPIs and ACLs could be use as chemotaxonomic markers (Herbin and Robins 1968), in environmental studies (Lockheart et al. 1998), in paleoenvironmental reconstructions (Corrigan et al. 1978), etc.

Furthermore, a number of studies suggested *n*-alkanes as very informative markers in the studies of geographic variation and/or identification of infraspecific taxa of *Pinus* (Nikolić et al. 2010, 2012a; 2012b; 2018a; Mitić et al. 2018a; 2018b), *Picea* (Nikolić et al. 2013a), *Juniperus* (Dodd and Poveda 2003; Rajčević et al. 2013), and other species (Günthardt – Goerg 1986). Furthermore, chemotaxonomic implications between several conifers were reported, too (Nikolić et al. 2013b, 2018b).

The aim of this investigation is to compare content of nonacosan-10-ol and *n*-alkanes in needle waxes of twelve *Pinus* taxa: 1. *Pinus halepensis* Mill., 2. *Pinus heldreichii* H. Christ., 3. *Pinus mugo* Turret, 4. *Pinus nigra* subsp. *nigra* J. F. Arnold, 5. *Pinus nigra* subsp. *dalmatica* J. F. Arnold, 6. *Pinus peuce* Griseb., 7. *Pinus pinaster* Aiton (syn. *P. maritima*), 8. *Pinus pinea* L., 9. *Pinus ponderosa* Douglas ex. C.Lawson, 10. *Pinus strobus* L., 11. *Pinus sylvestris* L. (syn. *P. silvestris*), and 12.*Pinus wallichiana* A.B.Jacks. (syn. *P. excelsa*). Significance of this research is also in the fact that, to our knowledge, nonacosan-10-ol content and CPI_s and ACL_s were not investigated in seven of selected *Pinus* taxa: *P. mugo, P. nigra* ssp.subsp. *dalmatica*, *P. pinea*, *P. ponderosa*, *P. strobus* L., *P. sylvestris*, and *P. wallichiana*.

Materials and Methods

Plant Material

Fresh leaf material of twelve *Pinus* taxa were harvested from lower third part of tree crowns in four repetitions (48 samples in total). Plant species from Belgrade parks (*Pinus mugo*, *P. nigra* subsp. *nigra* J. F. Arnold, *P. sylvestris*, *P. heldreichii*, *P. peuce*, *P. ponderosa*, *P. strobus* and *P. wallichiana*), were harvested and identified by Dr S. Bojović and Dr B. Nikolić. Those harvested from island Korčula in Croatia (*P. nigra* ssp.subsp. *dalmatica*, *P. halepensis*, *P. pinaster*, and *P. pinea*), were identified by Milan Vojinović, graduated forestry engineer. Material was stored in deep freezer at -20°C prior to further analysis. Voucher specimens were deposited in Institute of Forestry, Belgrade.

Extraction of Needle Wax for the Investigation of the Nonacosan-10-ol Content

A concentrated sample of epicuticular wax was collected from each tree by immersing 3 g of needles in 10 ml of *n*-hexane (HPLC grade; Merck, Darmstadt) for 45 s. The samples were then dried under vacuum at 60°C, and aliquots of 1 ml of these samples were used to determine the nonacosan-10-ol content by GC/MS analysis.

Extraction of Needle Wax for the Investigation of the n-Alkanes

The concentrated extracts, obtained as described above, were chromatographed on small-scale columns using a Pasteur pipette filled with silica gel 60 (SiO_2, 0.2 –0.5 mm; Merck) previously activated at 120°C (Mimura et al. 1998). The wax samples were obtained by elution with 5 ml of hexane and stored at -20°C until further analysis.

Organic solvents like benzene, chloroform, hexane, acetone, dichloromethane, methanol, and ethanol have been used on a large scale for surface wax extraction. Hexane is most commonly used in extraction nonpolar fraction of epicuticular waxes including *n*-alkanes (chain-length C21– C35) and primary alcohols (C22–C40). There are numerous previous reports efficiency of this method (Erosa et al. 2002; Medina et al. 2006; Nikolić et al. 2010; Kundu and Sinhababu 2013; Mitić et al. 2018b, etc.).

For this both nonacosan-10-ol and *n*-alkanes analyses one-, and two-year and sometimes three-year -old needles of trees, collected in spring and autumn separately (as a bulk) were used (ca. four to six repetitions of every taxon).

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

samples were 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 temperature 150°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 (Franich and Wells 1980). The retention indices were experimentally determined using the standard method of Van Den Dool and Kratz (Bargel et al. 2006) 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.

Calculation of CPI and ACL Values

The carbon preference index (CPI) of the total odd-numbered and even-numbered LNAs (CPI_{total}) was calculated using the equation of Mazurek and Simoneit (1997). The average chain length (ACL) of the total odd-numbered and even-numbered LNAs (ACL_{total}) was calculated by using the equation of Poynter and Eglington (1990). To compare the obtained results with those from literature sources, CPI25–33, CPI20–36, CPI15–21, and CPI25–31 were calculated by using the equation of Bray and Evans (1961) and ACL23–35 by using the equation of Poynter and Eglington (1990). The relative proportions of short-, mid-, and long-chain *n*-alkanes (*n*-C18–20, *n*-C21–24, and *n*-C25–33, resp.) were calculated according to Kuhn et al. (2010).

Statistical analyses

Statistical analyses were carried out using *Statgraphics Plus* (version 5.0; Statistical Graphics Corporation, U.S.A.) and *Statistica* (version 10, Stat. Soft. Inc. 2011).

Results and discussion

Variability of Nonacosan-10-ol Content

Nonacosan-10-ol contents varied among listed *Pinus* species from 60.82% (*P. nigra* subsp. *nigra*) to 83.42% (*P. strobus*) in average (Table 1; Fig. 1). Besides *P. nigra* subsp. *nigra*, *P. peuce* also had low content of nonacosan-10-ol (64.53%). On the other side, *P. strobus* had extremely high content (83.42%). Significant differences of *P. peuce* and *P. nigra* ssp.subsp. *nigra* from other analyzed species were confirmed by ANOVA test (results were not presented). From the highest to the lowest values subsections had as following: *Ponderosae* (i. e. *P. ponderosa*) – 77.30%, *Strobi* – 76.06%, *Pinaster* - 75.88%, and *Pinus* – 73.14% (Fig.1).

In previous results of *P. heldreichii* and *P. peuce* (Nikolić et al., 2012a, 2012b) average values of nonacosan-10-ol contents were not so high (55.5% and 55.9%, respectively), but in some trees they were also very abundant (73.2% and 72.3%, resp.). Differences of five-needle *P. peuce* and two-needle *P. nigra* subsp. *nigra* (with low nonacosan-10-ol content) from other analyzed species were confirmed statistically. However, these two pines belonged to different subsections (*Strobus* and *Pinus*, resp.).

Table 1, Fig. 1.

Variability of n-Alkanes

In all examined *Pinus* taxa C29, C27 or C25 were the main compounds. C range was mainly 18-35 (Table 2). In subsection *Pinus* C_{29} dominated. Domination of C_{29} in *P. mugo* was also shown in detail population investigation of Mitić et al. (2018b). In *P. nigra* ssp.subsp. *nigra* and *P. nigra* ssp.subsp. *dalmatica* C27 was also high. According to average values (Table 2) C29 and C27 were the most abundant. C range is mainly 18-35.

In previous results of *P. heldreichii* and *P. peuce* (Nikolić et al., 2012a, b) average values of nonacosan-10-ol contents were not so high (55.5% and 55.9%, respectively), but in some trees they were also very abundant (73.2% and 72.3%, resp.). Differences of five-needle *P. peuce* and two-needle *P. nigra* ssp.subsp. *nigra* (with low nonacosan-10-ol content) from other analysed species were confirmed statistically. These two pines belonged to different subsections (*Strobus* and *Pinus*, resp.). On the level of subsections, differences were not so great, and all were over 70%.

In subsection *Ponderosae (P. ponderosa)* C_{29} *n*-alkane was the main compound (Table 2). Previous results with *P. ponderosa* (Oros et al. 1999) had narrower C range (C23-C35) and C_{max}=35. In *P. peuce* and *P. wallichiana* C27, C29 and C31 dominated.

Literature reports signed that different environmental factors could changed composition (Kerfourn and Garrec 1992), biosynthesis (Franich and Wells 1980; Gordon and Percy 1999; etc.) as well as morphology and ultrastructure of wax (Gellini et al. 1985; Holmes and Keiller 2002; Sauter and Voβ 1986; and refs cited therein, etc). This is probably the reason for differences in PCA and Cluster analyses among presented and previous results of the same species (microsatellites, Nikolić et al 2018c).

Variability of CPI and ACL values

For all investigated taxa mean value of CPI_{total} is 2.8, and ACL_{total} was 15.0 (Table 2). Individual results of CPI_{total} ranged from 1.2 (*P. heldreichii*) to 5.1 (*P. nigra* ssp.subsp. *dalmatica*), and of ACL_{total} from 14.8 (*P. pinaster*) to 17.6 (*P. strobus* and *P. wallichiana*). Taxa of section *Pinaster* had the lowest CPI_{total} value (2.7), while pine of section *Ponderosae* had the highest one (4.0). ACL_{total} was the lowest in section *Ponderosae* (15.5) and the highest in sections *Strobi* and *Pinus* (17.4 and 17.2, resp.).

CPI25-33 values varied from 1.0 – 1.2 with average of 1.1 (Table 3). The lowest value had *P. ponderosa* (1.0) while the highest ones had *P. halepensis*, *P. heldreichii* and *P. peuce* (1.2). Mean values of all investigated taxa were up to 1.0, which exhibits odd/even predominance (OEP; because a CPI<1 indicates EOP, and CPI>1 indicates OEP (Kuhn et al. 2010).

CPI20-36 values were constant (1.0) in all analyzed taxa (Table 2).

CPI15-21 values varied from 0.0 – 4.0 with average of 1.5 (Table 2). The lowest value has *P. halepensis* (1.3) while the highest ones have *P. heldreichii* and *P. ponderosa* (2.1). Among all investigated sections the highest average value (1.8) was in the section *Pinus*. Mean values of all investigated taxa are up to 1.0, which exhibits odd/even predominance (OEP).

CPI25-31 values was predominantly 1.1, except in the case of *P. peuce*, *P. strobus* and *P. wallichiana* (section *Strobi*; 1.2).

Mean ACL23-35 had the lowest value in section *Ponderosa* (35.7) and the highest values in section *Strobi* (38.0 in average). Relative proportions of short-, mid- and long- chain *n*-alkanes were 1.4, 17.8 and 89.0 in average, respectively (Table 1). Section *Pinaster* had the highest value of *n*-C16-20. *n*-C21-24 range was the highest in section *Pinaster* and the lowest in the section *Ponderosae*. *n*-C25-35 range was the highest in section *Ponderosae*. In presented study CPI_s and

ACL_s of seven *Pinus* taxa (except of *P. halepensis*, *P. heldreichii*, *P. nigra* ssp.subsp. *nigra*, *P. peuce* and *P. pinaster*) were examined for the first time.

Mean ACL23-35 value was 36.9, with individual variation of 28.4 (*P. halepensis*) to 44.0 (*P. mugo*). The lowest value had section *Ponderosa* (35.7) and the highest section *Strobi* (38.0).

Relative proportions of short- mid- and long- chain *n*-alkanes were 1.4, 17.8 and 89.0 in average, respectively. Section *Pinaster* had the highest value of *n*-C16-20 (1.4), while section *Strobi* had the lowest value (0.9). Among *Pinus* taxa limited values had *P. sylvestris* (2.3) and *P. nigra* ssp.subsp. *dalmatica* (0.5). *n*-C21-24 range was the highest in section *Pinaster* (19.9) and the lowest in the section *Ponderosae* (14.4). Among *Pinus* taxa limited species were *P. halepensis* (4.3) and *P. heldreichii* (34.2). *n*-C25-35 range was the highest in section *Ponderosae* (84.4) and the lowest in the section *Pinus* (81.2). Among taxa limited species were *P. halepensis* (95.1) and *P. heldreichii* (63.7).

CPI_{total} of *P. ponderosa* reported in literature (Oros et al., 1999) was much higher (4.9) than in our investigations (4.0). The previous results were much higher in the case of *Pinus heldreichii* (Nikolić et al. 2012a), *P. peuce* (Nikolić et al. 2012b), and *P. nigra* (Bojović et al. 2012), (1.8, 2.3 and 2.7, resp.). ACL_{total} of *P. ponderosa* reported in literature (Oros et al. 1999) was much higher (29.5) than in presented investigations (15.5). Our previous results were much higher in the case of *P. heldreichii* (Nikolić et al. 2012a), *P. peuce* (Nikolić et al. 2012b), and *P. nigra* (Bojović et al. 2012), (26.1, 25.7 and 26.6, resp.).

Table 2.

PCA analysis

The first two axes of Principal component analysis (PCA) explained 60.4% of total information (Fig. 2). For this analysis nine *n*-alkanes, C_{23}-C_{31}, mostly abundant in all samples, were used. PCA showed the classification of elements into the two main groups. In the plane of Axis 1 *P. strobus*, *P. mugo*, and *P. wallichiana*, were strongly separated from *P. heldreichii* and *P. pinea*. In the plane of Axis 2, *P. peuce*, *P. strobus*, and *P. wallichiana* as well as *P. heldreichii* and *P. pinea* diverged from other examined species.

Fig. 2.

DA analysis

The DA (Discriminant analysis), based on nine *n*-alkanes, had shown that the first two axes explained 65% of the total information (Table 3). The first axis explained 48% of discrimination. The graetest influence on Root 1 was the distribution of C25, C30 and C31. The second discriminated (Root 2) was mainly determined by the values of C24, C25, C26, C27, C29 and C31. On the scatter plot (Fig. 3), first root showed that five needle pines (*P. peuce*, *P. stobus* and *P. wallichiana*) were separated from all two – and three- needle pines (except from *P. heldreichii*).

Fig. 3.

CA analysis

The CA (Cluster analysis, Fig. 4) based on the same nine *n*-alkanes as in previous multivariate methods, showed that section *Strobus* (five needle pines) were the most separated from other investigated *Pinus* taxa. Sections *Pinus* and *Pinaster* partially diversed, and *P. halepensis* was in dividing position between section *Strobus* and sections *Pinus* and *Pinaster*. According to

microsatellites analysis sections and subsections of the same 12 taxa of *Pinus* also diversed (Nikolić et al. 2018c and refs. cited therein).

Fig. 4.

Conclusions

Nonacosan-10-ol contents varied among listed *Pinus* species from 56.6% (*P. peuce*) 60.82% (*P. nigra* subsp. *nigra*) to 83.5% 83.42% (*P. strobus*). Contents of nonacosan-10-ol of eight *Pinus* taxa were examined in presented study for the first time. In all examined *Pinus* taxa *n*-alkanes C29, C27 or C25 were the main compounds. For all investigated taxa mean value of CPI_{total} was 2.8, and ACL_{total} was 15.0. CPI15-21 values varied from 0.0 – 4.0 with average of 1.5. CPI25-31 values was predominantly 1.1. Mean ACL_{23-35} value was 36.9, with individual variation of 28.4 (*P. halepensis*) to 44.0 (*P. mugo*). CPI_s and ACL_s of seven *Pinus* taxa were examined for the first time.

All three multivariate analyses showed that five needle pines were separated from two- and three- needle pines. *P. pinaster*, *P. heldreichii* and *P. pinea* were sometimes closer to section *Strobus* than to their own section (section *Pinaster*). This is not the case with the microsatellite analysis (of the same species) where analyzed pines of sections *Strobus*, *Pinus*, and *Pinaster* were clearly separated (Nikolić et al. 2018c). So, we could conclude that the taxonomic studies of *n*-alkanes are less significant than the genetic analysis.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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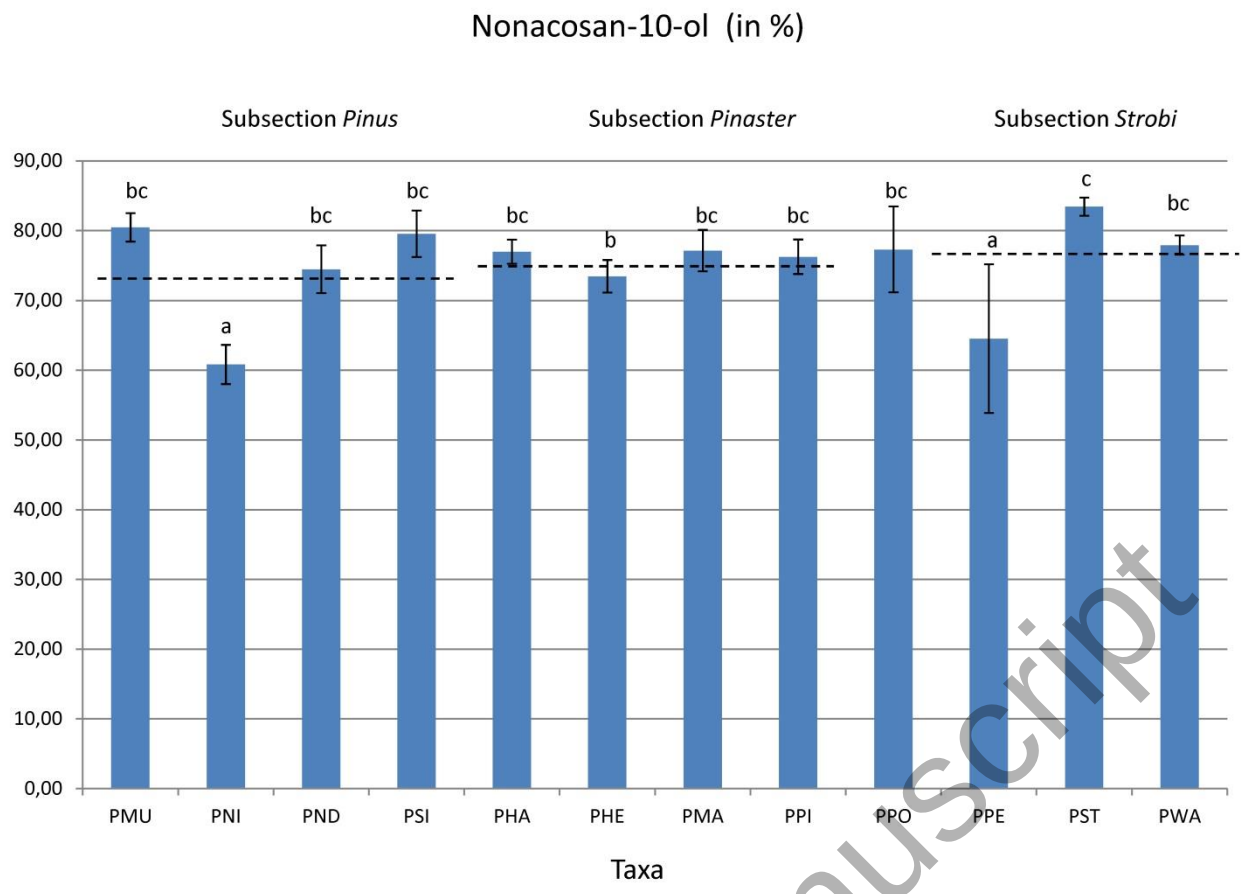

Fig. 1. Nonacosan-10-ol leaf contents of twelve Pinus taxa: 1. PMU (Pinus mugo), 2. PNI (P. nigra subsp. nigra), 3. PND (P. nigra subsp. dalmatica), 4. PSI (P. silvestris), 5. PHA (P. halepensis), 6. PHE (P. heldreichii), 7. PMA (P. pinaster), 8. PPI (P. pinea), 9. PPO (P. ponderosa), 10. PPE (P. peuce), 11. PST (P. strobus), and 12. PWA (P. wallichiana).

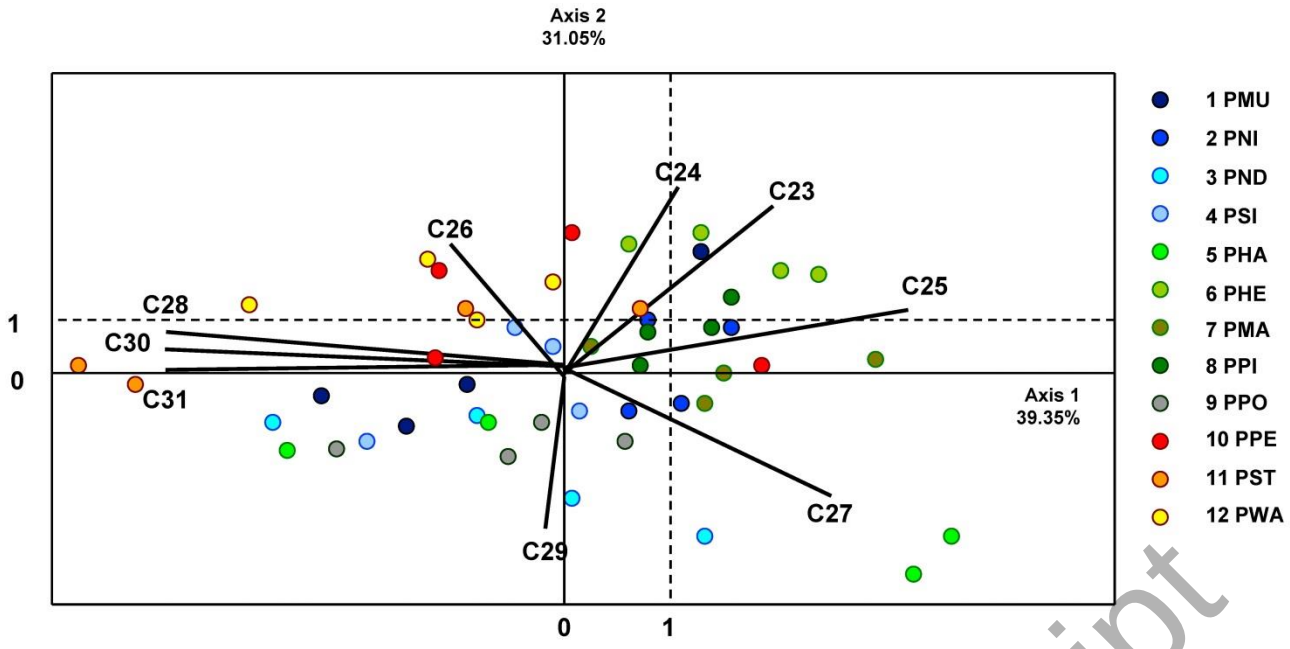

Fig. 2. Principle-component analysis (PCA) of nine selected n-alkanes (C23-C31) isolated from 48 pine-tree samples of twelve Pinus taxa. (for the names of species see caption of Fig.1).

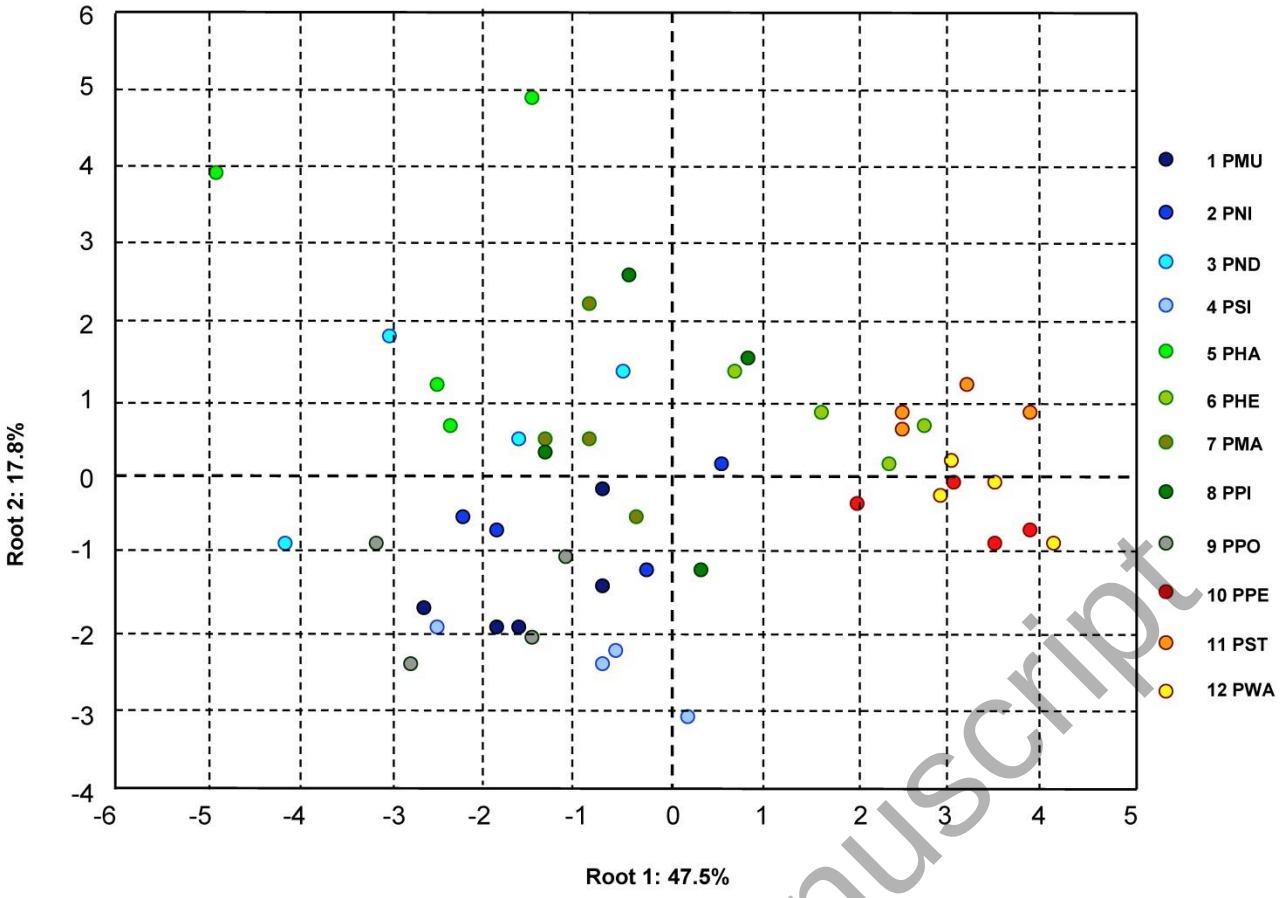

Fig. 3. Canonical discriminant analysis (CDA) based on the contents of nine selected n-alkanes (C23-C31) isolated from 48 samples of twelve Pinus taxa (for the names of species see caption of Fig.1).

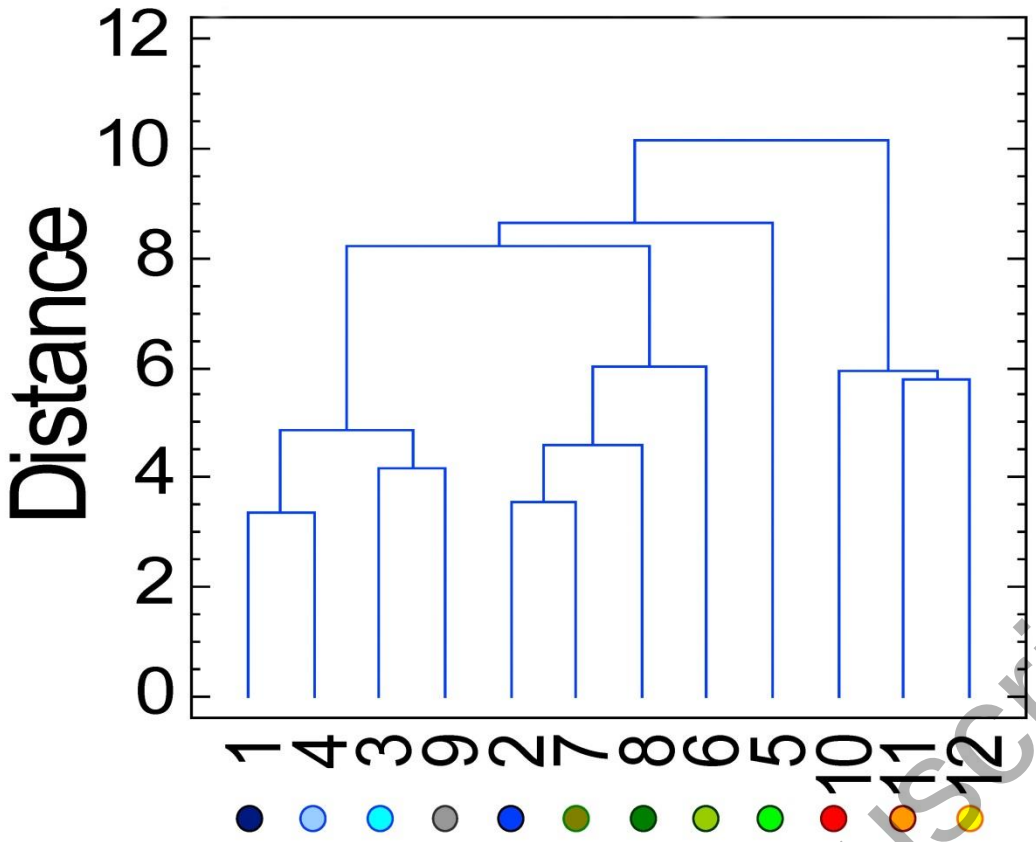

Fig. 4. Dendrogram based on a "nearest-neighbor method" (square Euclidean distance) of the studied nine n-alkanes (C23-C31) of twelve Pinus taxa (for the names of species see caption of Fig.1).

Table 1. Mean value (X) and standard deviation (SD) of nonacosan-10-ol of twelve Pinus taxa.

	TAXA		X ± SD
1.	*Pinus halepensis*	PHA	76.98 ± 3.44
2.	*Pinus heldreichii*	PHE	73.47 ± 4.67
3.	*Pinus mugo*	PMU	80.46 ± 4.08
4.	*Pinus nigra* subsp. *nigra*	PNI	60.82 ± 5.63
5.	*Pinus nigra* subsp. *dalmatica*	PND	74.48 ± 6.84
6.	*Pinus peuce*	PPE	64.53 ± 21.32
7.	*Pinus pinaster*	PMA	77.13 ± 5.91
8.	*Pinus pinea*	PPI	76.25 ± 4.91
9.	*Pinus ponderosa*	PPO	77.30 ± 12.29
10.	*Pinus strobus*	PST	83.42 ± 2.59
11.	*Pinus sylvestris*	PSI	79.54 ± 6.63
12.	*Pinus wallichiana*	PWA	77.92 ± 2.71

Table 2. Variability of the most abundant n-alkanes, Carbon preference indices (CPIs), Average chain length (ACLs), and Relative proportions of Short-, Mid- and Long-Chain n-alkanes in the needle wax of twelve Pinus taxa.

Taxon[a])		**C_{range}**	**C_{max}[b])**	**CPI_{total}[c])**	CPI25-33[d)]	CPI20-36[e)]	CPI15-21[f)]	CPI25-31[g)]	**ACL_{total}[h])**	ACL 23-35[i)]	***n*-C16-20** *Short-chain*	***n*-C21-24** *Mid-chain*	***n*-C25-35** *Long-chain*
											In % of total *n*-alkanes ($C18-35_{)}$		
P. mugo	Range	**18 – 35**	29	2.0 – 3.8	1.0 - 1.1	1.0	1.7 – 2.0	1.1	14.0 – 18.0	36.1 – 44.0	0.5 – 4.2	9.2 – 39.1	77.0 – 90.3
	Average		**29**	**3.0**	1.1	1.0	1.8	1.1	**17.2**	38.9	**1.8**	**18.9**	**79.3**
P. nigra subsp. *nigra*	Range	**18 – 35**	27, 29	2.7 – 5.5	1.1	1.0	1.3 – 1.9	1.1	16.0 – 18.0	33.7 – 38.8	1.7 – 4.7	10.8 – 29.0	69.0 – 86.5
	Average		**27, 29**	**3.6**	1.1	1.0	1.6	1.1	**17.0**	36.8	**3.0**	**22.5**	**74.5**
P. nigra subsp. *dal-matica*	Range	**18 – 35**	27, 29	2.8 – 8.2	1.1	1.0	0.8 – 2.6	1.1	17.0 – 18.0	31.1 – 40.6	0.4 – 0.6	3.1 – 12.8	86.8 – 96.4
	Average		**29**	**5.1**	1.1	1.0	1.6	1.1	**17.5**	35.1	**0.5**	**7.1**	**92.4**
P. syl-vestris	Range	**18 – 35**	25, 29	2.2 – 4.1	1.1	1.0	1.1 – 1.7	1.1	14.0 – 18.0	37.0 – 41.6	1.2 – 3.7	9.2 – 24.5	70.1 – 89.2
	Average		**29**	**3.0**	1.1	1.0	1.4	1.1	**17.2**	38.4	**2.3**	**19.3**	**78.4**
P. hale-pensis	Range	**18 – 35**	27, 29	3.2 – 24	1.1 – 1.2	1.0	0.0 – 2.2	1.1 – 1.2	14.0 – 18.0	28.4– 36.2	0.0 – 1.9	1.5 – 8.5	89.6 – 98.5
	Average		**29**	**2.4**	1.1	1.0	1.3	1.1	**17.2**	32.6	**0.6**	**4.3**	**95.1**
P. hel-dreichii	Range	**18 – 33**	27, 29	2.0 – 3.4	1.1 – 1.2	1.0	1.8 – 2.4	1.1 – 1.2	15.0 – 16.0	35.5 – 43.6	1.5 – 3.3	21.7 – 48.9	63.5 – 68.9
	Average		**27, 29**	**1.2**	1.1	1.0	2.1	1.1	**15.2**	40.7	**2.1**	**34.2**	**63.7**
P. pina-ster	Range	**18 – 35**	27, 29	2.6 – 5.6	1.1	1.0	0.9 – 2.1	1.1	14.0 – 15.0	31.6 – 38.0	0.5 – 1.4	13.5 – 25.5	73.6 – 85.2
	Average		**29**	**4.0**	1.1	1.0	1.5	1.1	**14.8**	34.8	**0.9**	**18.9**	**80.2**

P. pinea	Range	**18 – 35**	27, 29	2.7 – 3.5	1.1	1.0	1.0 – 1.9	1.1	16.0 – 18.0	35.0 – 38.1	0.7 – 5.3	14.1 – 27.3	70.4 – 85.2
	Average		**27, 29**	**3.2**	1.1	1.0	1.5	1.1	**17.0**	36.5	**2.1**	**22.5**	**75.4**
P. ponderosa	Range	**19 – 35**	29	3.6 – 4.6	1.0 - 1.1	1.0	1.0 – 4.0	1.1 - 1.2	14.0 – 17.0	34.6 – 36.7	0.5 – 1.9	11.2 – 20.5	79.0 – 87.2
	Average		**29**	**4.0**	1.1	1.0	2.1	1.1	**15.5**	35.7	**1.2**	**14.4**	**84.4**
P. peuce	Range	**19 – 35**	25, 29	2.5 – 5.9	1.1 – 1.2	1.0	1.2 – 1.9	1.1 – 1.3	16.0 – 18.0	32.1-34.2	0.6 – 1.5	17.9 – 24.2	75.2 – 81.3
	Average		**25, 29**	**3.6**	1.1	1.0	1.4	1.2	**17.0**	36.5	**1.0**	**21.3**	**77.7**
P. strobus	Range	**18 – 35**	27, 29	2.5 – 3.3	1.1	1.0	1.1 – 1.8	1.1 – 1.3	17.0 – 18.0	36.1 – 41.1	0.5 – 0.7	8.1 – 20.3	79.2 – 91.4
	Average		**29**	**2.9**	1.1	1.0	1.6	1.2	**17.6**	38.4	**0.6**	**13.9**	**85.5**
P. wallichiana	Range	**18 – 35**	25, 27, 29	2.1 – 3.3	1.1	1.0	1.1 – 1.8	1.1 – 1.2	17.0 – 18.0	36.2 – 42.3	0.7 – 1.5	12.4 – 24.1	74.4 – 86.4
	Average		**25, 29**	**2.7**	1.1	1.0	1.6	1.2	**17.6**	39.1	**1.0**	**17.4**	**81.6**
Section *Pinus*	Range	**18 – 35**	25, 27, 29	2.0 – 8.2	1.0 - 1.1	1.0	0.8 – 2.6	1.1	14.0 – 18.0	31.1 – 44.0	0.4 – 4.7	3.1 – 39.1	69.0 – 96.4
	Average		**27, 29**	**3.7**	1.1	1.0	1.8	1.1	**17.2**	37.3	**1.9**	**16.9**	**81.2**
Section *Pinaster*	Range	**18 – 35**	27, 29	2.0 – 24	1.1 – 1.2	1.0	0.0 – 2.4	1.1 – 1.2	14.0 – 18.0	31.6 – 43.6	0.0 – 5.3	1.5 – 48.9	63.5 – 98.5
	Average		**27, 29**	**2.7**	1.1	1.0	1.6	1.1	**16.0**	36.1	**1.4**	**19.9**	**83.8**
Section *Strobi*	Range	**18 – 35**	25, 27, 29	2.1 – 5.9	1.1 – 1.2	1.0	1.1 – 1.9	1.1 – 1.3	16.0 – 18.0	32.1 – 42.3	0.5 – 1.5	8.1 – 24.2	74.4 – 91.4
	Average		**25, 29**	**3.1**	1.1	1.0	1.5	1.2	**17.4**	38.0	**0.9**	**17.5**	**81.6**
1 – 12 [k])	Range	**18 – 35**	25, 27, 29	2.0–24.0	1.0 – 1.2	1.0	0.0 – 4.0	1.1 – 1.3	14.0 – 18.0	28.4 – 44.0	0.0 – 5.3	1.5 – 48.9	63.5 – 98.5
	Average		**29**	**2.8**	1.1	1.0	1.5	1.1	**15.0**	36.9	**1.4**	**17.8**	**89.0**

[a])Twelve species and subspecies and their sections are given. [b])C_{max}: The most abundant *n*-alkane is given in the row *Average* and two to three other dominant one in the row *Range*. [c]) CPItotal=∑odd C*n*/∑even C*n* (Mazurek and Simoneit, 1984, after Oros et al. 1999), C*n* is the concentration of *n*-alkane containing *n* carbon atoms; [d]) $CPI25_{-33}$=[∑(C_{25} – C_{33})odd/∑(C_{24} – C_{32})even+∑(C_{25} – C_{33})odd/∑(C_{26} – C_{34})even]/2 (Bray and Evans, 1961); [e]) CPI_{20-36}=[∑(C_{20} – C_{36})odd/∑(C_{19} – C_{35})even+∑(C_{20} – C_{36})odd/∑(C_{21} – C_{37})even]/2 (Bray and Evans, 1961); [f]) CPI_{15-21}=[∑(C_{15} – C_{21})odd/∑(C_{14} – C_{20})even+∑(C_{15} – C_{21})odd/∑(C_{16} – C_{22})even]/2 (Bray and Evans, 1961); [g]) CPI_{25-31}=[∑(C_{25} – C_{31})odd/∑(C_{24} – C_{30})even +∑ (C_{25} – C_{31})odd/∑(C_{26} – C_{32})even]/2 (Bray and Evans, 1961); [h]) ACL_{total}=(∑C*n* x *n*)/∑C*n* (Poynter and Eglington, 1990); [i]ACL_{23-35}=(23xC_{23}+25xC_{25} +27xC_{27}+29xC_{29} +31x C_{31} +33xC_{33}+35xC_{35})/(C_{23}+C_{25}+C_{27}+C_{29}+C_{31}+C_{33}+C_{35}) (Poynter and Eglington, 1990). Short-, mid- and long-chain *n*-alkanes are calculated according to Kuhn et al.(2010). [k]) Range and average of all *Pinus* 12 taxa.

Table 3. Standardized coefficients for the first two canonical axes (CA) of variation in 9 epicuticular wax compounds from the discriminant functional analysis of nine a priori groups.

	Root 1	Root 2
C23	0,559927	0,643121
C24	0,179590	**-0,942090**
C25	**1,177589**	**-0,786646**
C26	0,073834	**1,052820**
C27	-0,263850	**1,402519**
C28	-0,136557	-0,328993
C29	-0,217422	**-0,811722**
C30	**0,821572**	-0,565530
C31	**0,722108**	**0,772641**
Eigenvalue	6,169935	2,316560
Cum.Prop.	0,475022	0,653373

Significant coefficients are in boldface.