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Significance of diarylheptanoids for chemotaxonomical distinguishing between *Alnus glutinosa* and *Alnus incana*

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Abstract: Diarylheptanoids are a group of secondary metabolites widely distributed in the Betulaceae family and characteristic for *Alnus* species. In this study, the chemotaxonomic power of diarylheptanoids, such as hirsutanonol-5-*O*-*β*-D-glucopyranoside, rubranoside A, oregonin, platyphylloside, alnuside A and hirsutanonol, has been investigated in combination with principal component analysis (PCA) for differentiation of *Alnus* species. Concentrations of six diarylheptanoids in the bark extracts of two natural populations of *Alnus glutinosa* (black alder) and *Alnus incana* (gray alder) were determined by ultraperformance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS). PCA clearly shows the separation of three groups. Populations I (*A. glutinosa*) and II (*A. incana*) both consisted of individuals of the corresponding species. Four individuals from both sampled populations formed a separate group (population III), which possibly represents a hybrid group. Accordingly, diarylheptanoids may serve in combination with PCA as chemotaxonomic markers at the species level, which may also reveal hybrid species.

Keywords: *Alnus glutinosa*, *Alnus incana*, diarylheptanoid, hirsutanonol, linear discriminant analysis (LDA), oregonin, platyphylloside, principal component analysis (PCA), rubranoside A, UPLC-MS/MS

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Introduction

The genus *Alnus* Mill. (Betulaceae), commonly known as alder, is mostly distributed in the Northern Hemisphere, with the exception of a few South American species. The *Alnus* species occur as monoecious trees or shrubs that can be found in regions of floodplain forests or in mountains up to 2800 m a.s.l. (Krstinic et al. 2002). *Alnus* species are predominantly wind pollinated, and the seeds are dispersed mostly by waterways (Heuvel 2011). They enter into a symbiosis with *Frankia* (nitrogen-fixing actinobacteria), which enables them to grow on nitrogen-poor soil. Therefore, they play a significant role in ecosystem development because they improve the fertility of the soil and, as pioneer species help promote the growth of successional species by enriching the soil with nitrogen.

The genus *Alnus* includes between 20 and 35 species (Heuvel 2011; Bašić et al. 2014). Uncertainty about the number of *Alnus* species arises from the lack of clear morphological delimitations between taxa. Variations in leaf morphology, for example, show a continuum within and between taxa and this aggravates delimitation (Heuvel 2011). The taxonomy of *Alnus* is especially problematic for several species pairs or complexes, including *Alnus incana* (L.) Moench ssp. *incana* and *Alnus glutinosa* (L.) Gaertn., *Alnus trabeculosa* Hand.-Mazz. and *Alnus japonica* (Thunb.) Steud., and *Alnus formosana* (Burkill) Makino and *A. japonica* (Ren et al. 2010). Furthermore, species determination is complicated by hybridization and backcrossing events.

Three autochthonous alder species: *A. glutinosa* (L.) Gaertn., *A. incana* (L.) Moench, *Alnus viridis* (Chaix) DC., and one hybrid between *A. glutinosa* and *A. incana*, are distributed in Serbia (Vukićević 1996). *A. glutinosa* (black alder) is a tree up to 30 m in height and 40 cm in diameter, with dark brown cracked bark and glabrous leaves with a shape of obovate, round, emarginate or rounded-at-tip. *A. incana* (gray alder) is a coarse shrub or tree up to 20 m in

height and 50 cm in diameter, with smooth gray or brown bark and egg-shaped to elliptic leaves (Banaev and Bažant 2007). The most conspicuous differences between the two species are in bark appearance and in the infructescences, which have the pedunculate form in case of black alder in contrast to the sessile form of gray alder. Hybrids between black and gray alder cannot easily be recognized based solely on morphological characteristics because of the intermediate morphology between the parents, which may be overlapping or distinctly different (Parnell 1994; Banaev and Bažant 2007).

Populations of *Alnus* spp. included in this study were sampled on Golija Mountain (Golija-Studenica Biosphere Reserve, UNESCO), where *A. glutinosa* and *A. incana*, as well as the hybrid between these two species were previously recorded (Vukićević and Jovanović 1983). In the period succeeding the last glacial maximum, plant species expanded from refugia and populated other ecosystems. Adaptation to new habitats over time led to great changes within taxa and to hybridization between species. This may be the reason for the evolution of great intraspecies variability in black and gray alder on Golija mountain and the occurrence of hybrids (Vukićević and Jovanović 1983).

The approach to differentiate morphologically and anatomically similar species by means of analysis of secondary metabolism products is subsumed under the keyword chemotaxonomy. The performance of chemotaxonomy was demonstrated, for example, by Sandermann (1962) for the differentiation of various *Pinus* species based on terpenoid compounds in the extracts, or for chemotaxonomical identification of the similar woods of true mahogany, cedar, andiroba and curupixá (Pastore et al. 2011), or for *Dalbergia* species via FTIR analysis of ethanol-benzene extractives (Wang et al. 2016).

Diarylheptanoids are a class of secondary metabolites with a 1,7-diphenylheptane skeleton (Figure 1), which are candidates for chemotaxonomic studies. They can have a linear or cyclic structure, and they can be diversely substituted, which contributes to their manifold appearance. A pharmaceutical relevant diarylheptanoid is curcumin, a highly bioactive diarylheptanoid from *Curcuma longa* (Zingiberaceae), which induces cytotoxicity in tumor cells, and curcumin acts as a chemopreventive agent exhibiting synergistic effects with anticancer drugs and developing immunostimulating, anti-inflammatory, antioxidant, neuroprotective and numerous other activities (Allegra et al. 2017; Lopresti 2017; Redondo-Blanco et al. 2017). Over 400 diarylheptanoid compounds have been isolated from species of different genera and families (Lv and She 2012), and their appearance and variability is highly species specific. They are of chemotaxonomic significance for the genus *Alnus* and the Betulaceae family (Guz et al. 2002; Novaković et al. 2014a).

In the present study, the possibility of quantitative analysis of the secondary metabolites characteristic for *Alnus* species for differentiation between the *Alnus* taxa was investigated. The diarylheptanoid glycosides and the diarylheptanoid hirsutanonol examined herein have previously been reported as constituents of black and/or gray alder bark extracts (Martineau et al. 2010; Novaković et al.

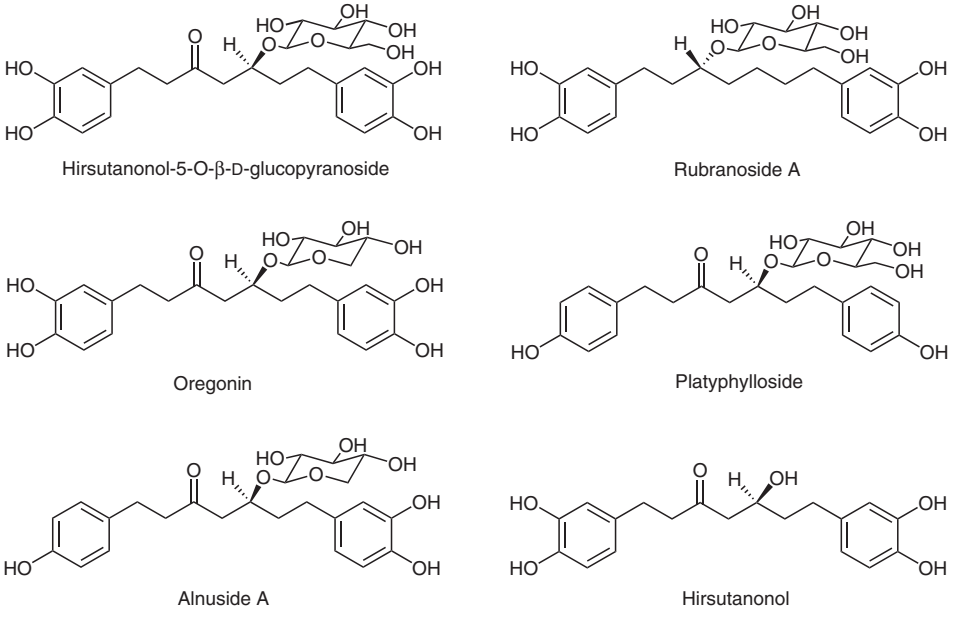

Figure 1: Chemical structures of diarylheptanoids investigated in this study.

2013). In focus was the question whether diarylheptanoids are reliable indicators for (1) chemotaxonomic identification and discrimination between the species *A. glutinosa* and *A. incana* and for (2) possible hybrids between these two species.

Materials and methods

Chemicals: Substances used as standards (hirsutanonol-5-*O*-β-D-glucopyranoside, rubranoside A, oregonin, platyphylloside, alnuside A and hirsutanonol; Figure 1) have previously been isolated from the bark of *A. glutinosa* (Novaković et al. 2013). Ethanol (96%) for extraction of plant material was freshly distilled. Solvents for ultraperformance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) analysis (chromatographic grade) were purchased from Sigma-Aldrich (St. Louis, USA).

Plant material: Field botanical determination of individuals was based on the species description, i.e. the appearance of the leaves, bark, habitus and habitat (Jovanović 2007). The bark from 42 trees of *Alnus* spp. was collected in the Golija-Studenica Biosphere Reserve (UNESCO–MAB) in June 2015 (Figure 2). The first population (*A. glutinosa*) was at the locality Rimski most (43°28′09.2″N, 20°14′05.9″E) within a *Querco-Carpinetum sensu lato* (sessile oak and hornbeam forest) community. The second population (*A. incana*) was at the locality Sastavci (43°27′38.5″N, 20°13′22.0″E) within a *Fagetum montanum sensu lato* (montane beech forest) community. The bark sampled from the populations of black and gray alder (both with $n=21$) were air-dried at room temperature for 2 weeks and then milled into powder by an electric grinder.

Preparation of bark extracts: Powdered bark (2 g) was extracted with 96% ethanol four times (4×20 ml, 24 h) at room temperature aided by an ultrasonic bath in the last hour of each extraction. For UPLC-ESI-MS/MS analyses, the extracts were dissolved in methanol to a final concentration of 1.0 mg ml^{-1} and then filtered through a 0.45-µm pore-size filter (Agilent Technologies).

UPLC Triple Quad MS-MS analysis: A stock solution of a mixture of diarylheptanoid standards (hirsutanonol-5-*O*-β-D-glucopyranoside, rubranoside A, oregonin, platyphylloside, alnuside A and hirsutanonol) was prepared in methanol (MeOH) at a concentration of 1000 mg l^{-1}. Calibration solutions with concentrations of 0.0001, 0.0010, 0.0100, 0.0500, 0.1000 and 0.1500 mg ml^{-1} were prepared by diluting the stock solution with MeOH. Calibration curves were constructed based on the peak areas.

Analyses were performed with a Waters Acquity Ultra Performance H Class system (Waters, Milford, MA, USA). UPLC separation was realized on an Acquity UPLC BEH C18 column (1.7 µm, 2.1 mm×150 mm) equipped with a VanGuard precolumn Acquity UPLC BEH C18 (1.7 µm, 2.1 mm×5 mm), also from Waters. The column was thermostated at 40°C, and the flow rate was 0.3 ml min^{-1}. The injection volume was 2.0 µl. The mobile phase consisted of 0.2% formic acid in water (solvent A) and acetonitrile (solvent B). The gradient elution program was as follows: 0–12.0 min 20–30% B, →12.0–12.1 min 30–100% B, →12.1–13.9 min 100% B, →13.9–14.0 min 100–20% B, →14.0–16.0 min 20% B. The compounds in focus were eluted within a 7-min run time.

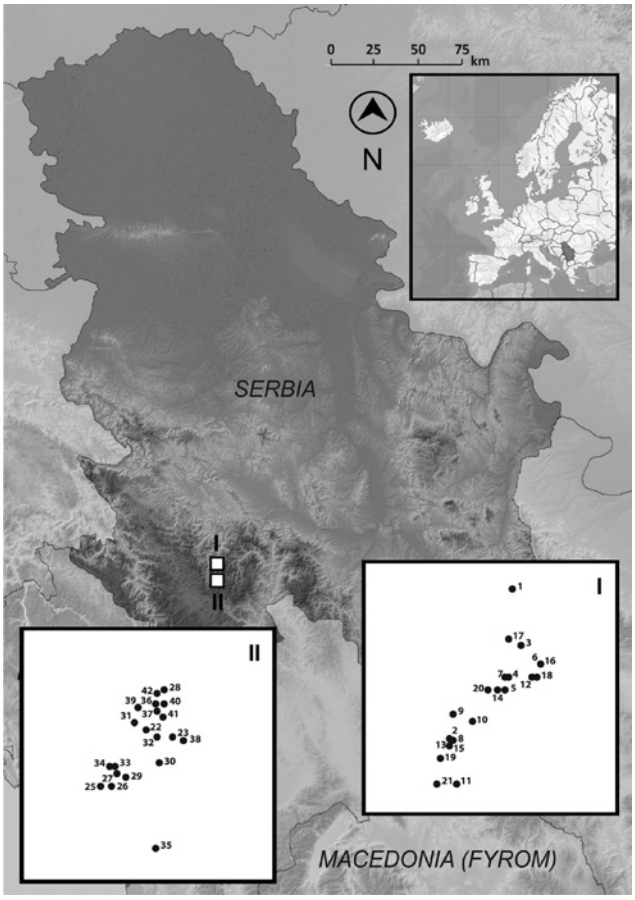

Figure 2: Locations of the studied populations: I – *A. glutinosa* (Rimski most: 43°28′09.2″N, 20°14′05.9″E; 640 m; $n=21$ trees); II – *A. incana* (Sastavci: 43°27′38.5″N, 20°13′22.0″E; 670 m; $n=21$ trees).

The UPLC was coupled to a triple quadrupole mass spectrometer Acquity TQD (Waters) equipped with the software MassLynx 4.1. The MS instrument was operated with an electrospray source in the negative ionization mode [M-H]$^-$. The ionization source conditions were as follows: capillary voltage 3.3 kV, source 150°C, desolvation gas 450°C, flow rate 500 l h^{-1}. Nitrogen and argon served as cone and collision gases, respectively. The diarylheptanoids were analyzed by multiple reaction monitoring (MRM) (Table 1).

Statistical analysis: The assumption of normality for each variable in a given population was tested with the Shapiro-Wilk test before statistical analysis. A parametric test (ANOVA) was applied for comparing the populations when the variables appeared to follow normal distribution ($P<0.05$). A non-parametric test (Kruskal-Wallis rank sum test) was used when the variables, even after the transformation, i.e. $y'=\log_{10}(y+1)$ did not appear to follow a normal distribution. Principal component analysis (PCA) and linear discriminant analysis (LDA) were carried out to visualize the data structure in clusters. Statistical analyses were performed based on the R statistical software (version 3.2.5: The R Foundation for Statistical Computing, https://www.r-project.org/) and Statgraphics Plus (version 5.0; Statistical Graphics Corporation, USA).

Table 1: Optimized MRM conditions for the analysis of the diarylheptanoids by UPLC-MS/MS method.

Compound	Precursor ion (m/z)	Product ion (m/z)	Cone voltage (V)	Collision energy (V)
Hirsutanonol-5-*O*-*β*-D-glucopyranoside	507.35	327.2	42	20
Rubranoside A	493.37	331.23	72	36
Oregonin	477.34	327.15	44	22
Platyphylloside	475.36	295.17	32	10
Alnuside A	461.35	311.13	40	16
Hirsutanonol	345.17	165.02	44	22

Results and discussion

Six diarylheptanoids, namely, hirsutanonol-5-*O*-*β*-D-glucopyranoside, rubranoside A, oregonin, platyphylloside, alnuside A and hirsutanonol, were quantified in *Alnus* spp. bark ethanol extracts. The sum of alnuside A and B was the input in further analyses, as they gave the same precursor and product ions for MRM, which could not be separated by UPLC.

PCA was performed for data evaluation and visualization (Figure 3). The first principal component (PC1) accounted for 49.7% of the total variance and the second principal component (PC2) for 17.8%. According to PCA, seven variables (i.e. the concentrations of the diarylheptanoids and the yields of the extracts) rendered possible to assign the individuals into two main groups, each corresponding to one of the two species (Figure 3): population I (19 individuals of *A. glutinosa*, left side of the graph) and population II (19 individuals of *A. incana*, right side of the graph). The basic statistical parameters for each variable and group, based on the PCA, are presented in Figure 4. In population I, the content of hirsutanonol was higher, whereas the extract yields and the contents of hirsutanonol-5-*O*-*β*-D-glucopyranoside and platyphylloside were lower in comparison with population II. Four individuals (4, 8, 38 and 39) formed a separate group (population III), which differs from the other groups by higher amounts of rubranoside A. Individuals 4 and 8 were identified within population I, whereas individuals 38 and 39 were located within population II. This can be interpreted that these four trees are hybrids between *A. glutinosa* and *A. incana*. Individuals 10, 21 and 36 formed the segregate position on the PCA graph, but they were eliminated as possible members of a group because of inconclusive representation on the discriminant axes. The differences between the three PCA groups were found to be statistically significant for each analyzed parameter (Figure 4). The differences were established by parametric or non-parametric tests, depending on the normality of the variable's distribution.

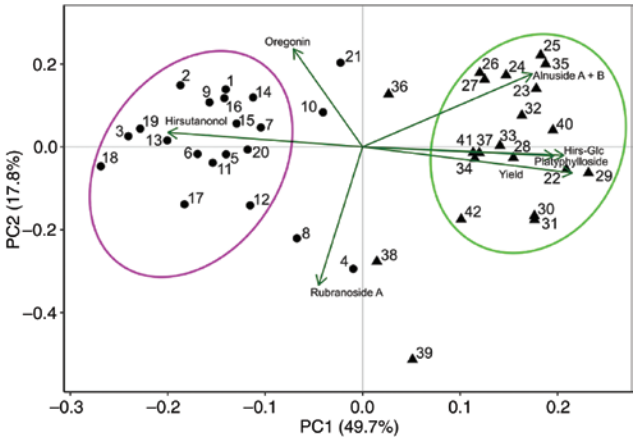

Figure 3: PCA of seven variables measured from 42 trees of *A. glutinosa* (●) and *A. incana* (▲) populations. ▬ Population I, comprised of *A. glutinosa* individuals; ▬ population II, comprised of *A. incana* trees.

Linear discriminant analysis (LDA) was performed on the set of the three predefined groups of individuals as implied by PCA (Figure 5). The first discriminant function explained over 85% of the discrimination. The first discriminant axis mainly distinguished population II from the other groups by the higher content of hirsutanonol-5-*O*-*β*-D-glucopyranoside (Figure 4). The second discriminant function mainly separated the group of four trees (population III), a possible hybrid between *A. glutinosa* and *A. incana*, from the other populations by its higher content of rubranoside A.

The occurrence of hybrids between black and gray alder is very common with sympatric species. Hybrid individuals are distributed with low frequency, around 10%, because of the phenological barriers between the species: *A. incana* begins to blossom earlier than *A. glutinosa*, although the flowering periods of the species may partially overlap (Banaev and Bažant 2007; Bašić et al. 2014), but there are certainly other mechanisms of their reproductive isolation. Nevertheless, natural hybridization between these species increases genetic variation and biodiversity within the genus. Genetic variability is an important precondition for the adaptive potential of forest tree species,

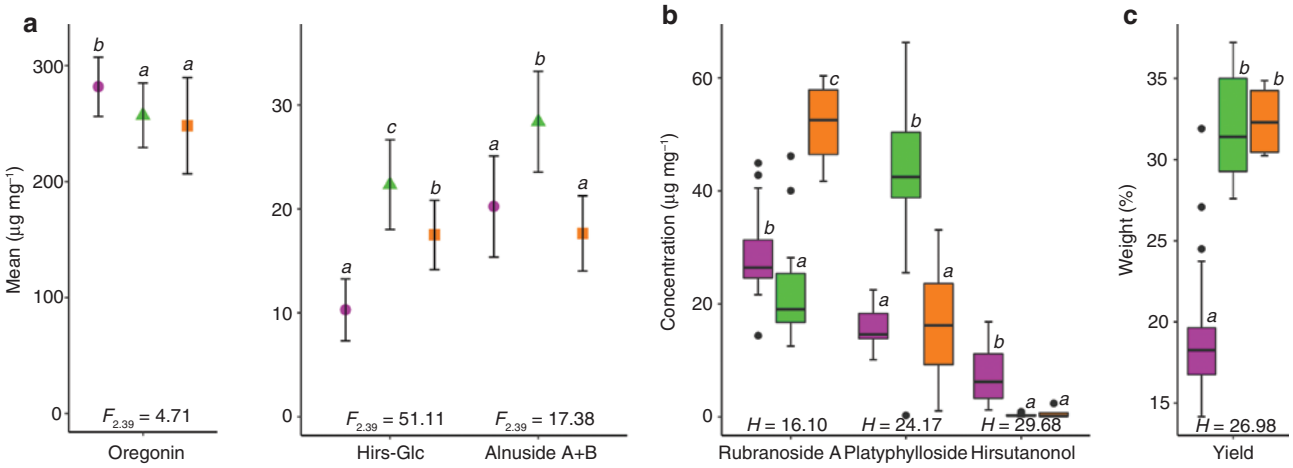

Figure 4: Comparison of three populations (population I, population II, population III) based on (a) mean values of concentrations of each compound (µg of diarylheptanoid mg of extract^{-1}), (b) median values of concentrations of each compound (µg of diarylheptanoid mg of extract^{-1}) and (c) median values of yields of the extracts (% w/w).
Means of the variables that followed normal distribution were compared by ANOVA ($P \leq 0.05$); *F* values are given at the bottom of the plots with degrees of freedom; Fisher's LSD post hoc test was applied. Medians of the variables that did not follow a normal distribution were compared by Kruskal-Wallis test ($P \leq 0.05$); *H* values are given at the bottom of the plots; to determine significant differences among the medians, features of Box-and-Whisker plots of median notch were visually examined. Error bars represent mean ± standard deviation (a) and interquartile ranges (b and c). Italic letters (*a*, *b*, *c*) above the error bars and boxes indicate statistically significant differences ($P < 0.05$).

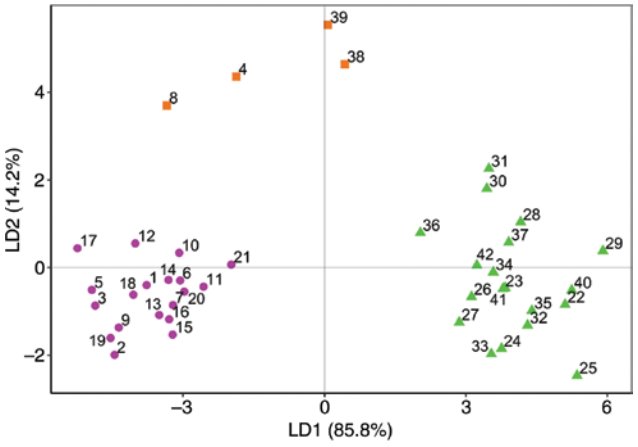

Figure 5: LDA of the three groups of *Alnus* spp., as indicated by PCA: (●) population I – *A. glutinosa* individuals; (▲) population II – *A. incana* individuals; (■) population III – miscellaneous group.

and at a long term for the preservation of species (Poljak et al. 2014). Besides, hybrid zones represent places of high ecological and evolutionary activity that provide spots of biodiversity forming new habitats for associate living organisms (Whitham et al. 1999; Tovar-Sánchez and Oyama 2006). Based on the chemical analysis only, it cannot be stated with certainty that the trees within population III are hybrids between *A. glutinosa* and *A. incana*. Isolation of trees 4, 8, 38 and 39 on the plane of the first two PC axes, as well as trees 10, 21 and 36, could also be attributed to individual differences due to phenotypic plasticity within respective populations. The plasticity of secondary metabolism plays an essential ecological role in the dynamic interactions between plants and their diverse and continuously changing environment (Hartmann 2007). Thus, the results of the present study should be understood as a hint to perform more detailed analyses in this regard, e.g. in terms of molecular markers in combination with morphometry studies. How powerful is in the meanwhile the DNA-marker method for species identification is demonstrated by a rapidly increasing literature (e.g. Höltken et al. 2012; Yu et al. 2016).

The content of bark metabolites depends on numerous biotic and abiotic factors (Lachaud et al. 1999; Plomion et al. 2001). For these reasons, the most reliable method for analyzing the bark would be to sample within particular growth cone, but in practice this could significantly damage the tree. In this study, the outer and inner bark was sampled, trying not to damage the cambium cell layer. This sampling method may have certain limitations regarding the variability of the contents of the analyzed compounds in different individuals. However, in order to increase the reliability of the results, we strived for uniformity during sampling by selecting the mature trees of similar age, and by sampling the material on the same day, from the same height and exposition.

Diarylheptanoids and their derivatives are distributed mainly in the families Betulaceae, Zingiberaceae

and Myricaceae, and within these families, in the genera *Alnus*, *Betula*, *Alpinia*, *Zingiber*, *Curcuma* and *Myrica* (Lv and She 2012). They can be considered as chemotaxonomic markers because oftentimes a certain genus or species is characterized by a specific type of diarylheptanoids. This is because related taxa share genes that encode enzymes, which are involved also in biosynthetic pathways of a characteristic set of secondary metabolites. On the other hand, not rarely, secondary metabolites of a substance group may also occur in unrelated taxa because of the convergent evolution of such substances or differential regulation of otherwise widely distributed genes that govern the biosynthesis of a particular secondary metabolite (Wink 2008). For example, oregonin was thus far identified only within *Alnus* and *Corylus* genera (Betulaceae) (Choi 2013; Riethmüller et al. 2016), with the exception of *Pinus flexilis* (Pinaceae) (Lee et al. 1998). The genera *Curcuma*, *Alpinia* and *Alnus* are represented mainly, but not exclusively, by the occurrence of linear diarylheptanoids (Claeson et al. 2002; Lv and She 2012), whereas cyclic diarylheptanoid derivatives are a characteristic of *Myrica* and *Morella* species (Silva et al. 2015). Substituents on the aromatic rings or on the heptane moiety usually point to distinct species. For instance, diarylheptanoids from the bark of *A. viridis* possess *p*-hydroxyphenyl aromatic groups, whereas *A. glutinosa* is represented by diarylheptanoids with *p*-hydroxyphenyl and/or catechol groups (Novaković et al. 2014a). Moreover, bark extracts from *A. glutinosa* and *A. incana* have similar diarylheptanoid contents (Martineau et al. 2010; Telysheva et al. 2011; Novaković et al. 2013), whereas *A. viridis* is in this respect closer to *Betula platyphylla* var. *japonica* (Novaković et al. 2014a), which may be significant for taxonomic considerations. Hydrophilic bark extractives of black and gray alder were found to exhibit antioxidant, cytotoxic, antimicrobial, antidiabetic and anti-adipogenic activities (Spoor et al. 2006; Frédérich et al. 2009; Martineau et al. 2010; Stević et al. 2010; Telysheva et al. 2011; Dahija et al. 2014). The main constituents of these extractives are diarylheptanoids and condensed tannins. It is assumed that diarylheptanoids play an ecological role as a part of the chemical defense system against herbivores; for example, diarylheptanoid glycosides isolated from birch phloem show an inhibitory effect on ruminant digestion (Sunnerheim and Bratt 2004). Members of this group of natural products are reported to have antiviral, antimicrobial, anti-adipogenic, anti-inflammatory, cytotoxic and many other activities (Mshvildadze et al. 2007; Tung et al. 2010; Lai et al. 2012; Lee et al. 2013; Novaković et al. 2014b, 2015).

Conclusions

Quantification of the most abundant alder diarylheptanoids in bark extracts was used for the first time for differentiation between the species *A. glutinosa* and *A. incana*. Diarylheptanoids proved to be good chemotaxonomic markers at the interspecific level. It was demonstrated that diarylheptanoids are reliable indicators for the identification and discrimination between the species *A. glutinosa* and *A. incana*. The analytical power of this approach for differentiation of hybrids needs to be verified by further research including genetic studies.

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