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Chemodiversity of two closely related tetraploid *Centaurium* species and their hexaploid hybrid: Metabolomic search for high-resolution taxonomic classifiers

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ABSTRACT

Species within the genus *Centaurium* readily hybridize and polyploid complexes are often seen in natural populations. We describe phytochemical profiles of newly discovered allohexaploid hybrid, here named *Centaurium pannonicum*, and its parental tetraploid species *C. erythraea* and rare *C. littorale* ssp. *compressum*. Our aim was to examine chemodiversity of these taxa in the area of Vojvodina (North Serbia) and to perform metabolomics search for chemical classifiers which would provide high resolution discrimination of parental and hybrid individuals. In sum, UHPLC-MS/MS Orbitrap metabolomics fingerprinting revealed seventy compounds in methanol extracts. Despite the lack of qualitative chemical novelty in hybrid plants, UHPLC-qqqMS targeted metabolomics approach, aimed at three secoiridoid compounds and seventeen phenolics, pointed to considerable differences in quantitative composition of these dominant compounds among the plant taxa studied. In addition to the difference in the ploidy levels, the hybrid taxon was well distinguished from both parental species based on metabolite profiles, and, for most individuals, positioned intermediately to the parental taxa in both PCA and hierarchical clustering. After optimizing and comparing several statistical learning methods, it was possible to narrow the number of taxonomic classifiers to five (three xanthones, one secoiridoid glycoside, and one phenolic acid), while increasing the differentiation resolution. The presented metabolomics approach will certainly, along with morphometrics and molecular genetics studies, have high impact on further elucidation of complex relationships among taxa within the genus *Centaurium*.

1. Introduction

Interspecific hybridization is described as the process by which new species originate through the sexual crossing between two different species (Rosenthal et al., 2008; Santos et al., 2012; Volker et al., 2007). As well as interspecific hybridization, polyploidization is a common phenomenon in the plant world, both of them having

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a great role in speciation. Speciation by polyploidization is well documented and represents a very common evolutionary mechanism in plants (Ainouche et al., 2004; Robertson et al., 2010; Soltis et al., 2004; Wood et al., 2009). When polyploidization follows interspecific hybridization, allopolyploids arise (Arnold, 1997; Soltis and Soltis, 2009). They can or may not become stabilized and reproductively isolated against the parental species when growing in sympatry. In the first scenario, newly originated polyploids are reproductively isolated from the parental taxa because of differences in the number of chromosome sets. Crossing the parental and hybrid taxa of different ploidy give offspring that have odd number of chromosome sets and therefore unbalanced genotype (Hegarty and Hiscock, 2005). Moreover, interspecific hybridization may lead to occurrence of so called hybrid swarms (extremely variable populations made of parental and hybrid individuals which mutually fertilize), emergence of new homoploid hybrid species, emergence of new allopolyploid species, or complete extinction of hybrid individuals (Soltis and Soltis, 2009). According to the literature (Banjanac et al., 2014; Brys et al., 2016; Çiçek et al., 2015; Guggisberg et al., 2006; Mansion et al., 2005; Ubsdell, 1979), genus *Centaurium* Hill (*Gentianaceae*) might represent a convenient model system to study both processes and their outcomes.

Common centaury (*Centaurium erythraea* Rafn) is one of the most famous and long-traditional pharmacological species from the *Gentianaceae* family (Grieve, 1971), distributed throughout almost all Europe, as far east as Afghanistan and south to Northern Africa (The Euro+Med Plantbase Project, 2016; U.S. National Plant Germplasm System, 2016). Its sister-species, *Centaurium littorale* (Turner) Gilmour is mainly found along the coastal dunes of Northern, Baltic, and Irish Seas (Hultén and Fries, 1986), but also in the continental areas of Central and East Europe (The Euro+Med Plantbase Project, 2016). It comprises two subspecies: *C. littorale* subsp. *littorale* and *C. littorale* subsp. *compressum* (Hayne) Kirschner (Marhold, 2011), both of which were reported to grow in France, Germany, Poland, Ukraine and western parts of Russian Federation. Since being published by various authors 40–50 years ago, some of these findings may raise doubts on subspecies determination validity. Also, different databases contain various taxonomical information considering the geographic ranges of the two subspecies, especially in East Europe (see The Euro+Med Plantbase Project, 2016; The Plant List, 2010; U.S. National Plant Germplasm System, 2016). Both *C. erythraea* and *C. littorale* are commonly found in tetraploid forms (Banjanac et al., 2014; Šiler, 2012; Ubsdell, 1976a). When growing sympatrically, they readily hybridize bearing morphologically intermediate hybrids, presumably stabilized F1 derivatives that sometimes produce backcrosses with *C. erythraea* (Mansion et al., 2005; Ubsdell, 1979). However, unstabilized derivatives of the F1 hybrids (with chromosome numbers ranging from $2n = 41$ to $2n = 59$), and also the hexaploid hybrid ("*C. intermedium*") are sometimes produced and have been described previously in detail (Mansion et al., 2005; Mansion, 2014; Ubsdell, 1976a, 1976b), the latter one being slated for a new species (Ubsdell, 1979). Mansion et al. (2005) also emphasized that it would be interesting to compare the histories of hexaploid taxa *C. turcicum* and *C. intermedium* since both may have involved similar progenitors (*C. erythraea* s.l. and *C. littorale* s.l.) in different areas. Recent findings have led to better understanding of the interspecific hybridization by studying the strength of the reproductive isolation mechanisms, differences in breeding systems, heterospecific pollen transfer patterns, autonomous selfing rate and efficiency of reproductive barriers between these two species (Brys et al., 2014, 2016), as well as molecular fingerprinting of hybrids and their parental lines (Banjanac et al., 2014).

Surprisingly little attention has been paid to the effect of hybridization on specialized metabolites' chemistry and survival of hybrids in natural populations (Fritz, 1999; Orians, 2000). However, modifications of metabolite profiles as a consequence of interspecific hybridization are readily studied (Caseys et al., 2015; Kirk et al., 2004, 2005). Progeny arisen from the interspecific hybridization often differ from their parents mainly in the quantitative content of the specialized metabolites; however qualitative differences are sometimes detectable (Banjanac et al., 2014; Harborne and Turner, 1984). These characteristics show complex patterns of inheritance and only rarely are the hybrids intermediate to the parental taxa (Rieseberg, 1995). However, closely related parental taxa are more likely to successfully hybridize producing similar types of compounds (Orians, 2000).

With the development of metabolomics, phytochemical profiling emerged as a powerful tool in functional genomics, chemotaxonomy, pharmacognosy, and biotechnology. In recent years, a handful of both experimental work and reviews underlined metabolomics approaches in screening for differences within and between plant taxa (Hadi et al., 2017; Messina et al., 2014; Mišić et al., 2015), introducing metabolomics as a bridge between genotype and phenotype (Saito and Matsuda, 2010). Aside *C. erythraea*, species belonging to the genus *Centaurium* have rarely been the subject of phytochemical studies and the majority of investigations have been targeted towards iridoids and xanthones, the most abundant specialized metabolites in this plant group (Jensen and Schripsema, 2002; Šiler et al., 2010, 2012, 2014). Nevertheless, previous phytochemical studies on *Centaurium* species revealed the presence of a variety of specialized metabolites, including terpenoids (monoterpenoids, sesquiterpenoids, diterpenoids, and triterpenoids), phenolics (phenolic acids, flavonoids, and xanthones), alkaloids and steroids (reviewed in Šiler and Mišić, 2016). Here we present a targeted metabolomics approach performed with the aim to investigate if quantitative profiles of twenty selected specialized metabolites would uphold the morphological differences among the two species: *Centaurium erythraea* and *C. littorale* ssp. *compressum*, and their interspecific hybrid offspring (here named "*Centaurium pannonicum*"). One of the major goals was to select a group of robust metabolic classifiers able to firmly differentiate the studied taxa.

2. Results and discussion

There is an evident lack of the scientific literature dealing with the distribution and genetic variations of species belonging to the genus *Centaurium* in the territory of the Balkan Peninsula and Southeast Europe (Šiler, 2012). On the other hand, there are several studies exploring the genetic basis and evolutionary relationships within the genus in the Northwest and Southwest Europe (Guggisberg et al., 2006; Mansion, 2004; Mansion et al., 2005; Ubsdell, 1979). Years of field research on *Centaurium* populations in the Subotica Sands in Vojvodina (Northern Serbia) brought insights into the evident existence of specific morphological forms of plants which did not suit the relevant keys for species descriptions. *Centaurium pulchellum, C. littorale* ssp. *compressum* and a potential interspecific hybrid, here named *C. pannonicum,* were detected around pounds and irrigation ditches, while *C. erythraea* individuals were not found in the studied area. Therefore, *C. erythraea* seeds collected from two geographically nearest populations, at Fruška Gora Mountain, were used for the purposes of this study (Table 4).

It is well known that exact delimitation of closely related species and subspecies of the genus *Centaurium* can often be very difficult based on the morphology only (Fig. 1). This is due to the fact that a high clinal variation in gross morphology is being frequently observed as well as natural hybridization between taxa, which tends to obscure taxon determination (Mansion, 2004; Melderis,