



High correlation of chemical composition with genotype in cryptic species of the liverwort *Aneura pinguis*

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ABSTRACT

Chemical constituents of cryptic species detected within the liverwort *Aneura pinguis* were identified using headspace solid-phase microextraction gas chromatography–mass spectrometry (HS-SPME-GC-MS). Fibre coating with divinylbenzene/carboxen/polydimethylsiloxane (DVB/CAR/PDMS) was used. A total of 48 samples of *A. pinguis* were analysed. The studied plants were identified genetically based on barcode DNA sequences and represented three cryptic species (A, B and F) of *A. pinguis*. Cryptic species A and B are genetically diverse; both represent three evolutionary lineages: A1, A2, A3 and B1, B2, B3, respectively. The cryptic species F that was recently detected is not diverse. The most characteristic compounds in analysed samples were sesquiterpene hydrocarbons (up to 17.7% for A1; 15.7% for A2; 20.6% for A3; 7.7% for B1; 2.0% for B2; 3.7% for B3; 10.2% for F), oxygenated sesquiterpenoids (up to 68.0% for A1; 54.7% for A2; 52.6% for A3; 63.5% for B1; 88.7% for B2; 82.7% for B3; 78.8% for F), and linear aliphatic hydrocarbons (up to 14.8% for A1; 1.1% for A2; 12.1% for A3; 6.9 for B1; 5.2% for B2; 1.1% for B3; 7.0% for F). The dominant compound in the studied samples was pinguisone. The second dominant compound present in the tested plant material was deoxopinguisone, except for lineage B2, where only a small relative concentration of this compound was found. A high content of deoxopinguisone in cryptic species A (lineages A1, A2 and A3) was accompanied by the presence of isopinguisone and methyl norpinguisone, whereas these two compounds were not detected in cryptic species B (lineages B1 and B3) and F. The chemical compounds detected in the studied samples of *A. pinguis* were subjected to multivariate statistical analysis. The results showed that the chemical composition depends mainly on the genotype of the plant and slightly on the habitat. However, there was no clear correlation between the volatile compounds and the date of collection of the studied plants.

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1. Introduction

Liverworts, mosses, and hornworts are the three divisions of plants known as bryophytes. Liverworts are small, herbaceous, and terrestrial plants and are estimated to have around 7000 species (Vanderpoorten and Goffinet, 2009). Fossil records, together with evidence from molecular phylogenetic studies, support the hypothesis that liverworts were among the first plants that colonized land and were present on land approximately 475 million years ago (Wellman et al., 2003; Cox et al., 2014). Phytochemical studies have

shown that liverworts contain a wide range of biologically active compounds, such as terpenoids and aromatic compounds, many of which are unique to this group of plants (Asakawa, 1995; Asakawa et al., 2013a). Liverworts, mosses and hornworts differ substantially from each other in terms of their chemical composition. These biologically active compounds are present predominantly in oil bodies; hence, they most often occur in liverworts, which are the only bryophytes having oil bodies (He et al., 2013).

Liverworts are extremely sensitive to changes in the environment, and therefore, they currently face extinction in many regions of the world (Stewart, 1995; Szweykowski and Buczkowska, 1996). Nevertheless, our knowledge of these plants is still rather limited compared to vascular plants; for example, the content of chemical compounds has been determined for approximately 6–10% of

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liverwort species (Asakawa et al., 2013a). The percentage of known species in terms of the composition of chemical compounds may be even less, since many of the liverwort species described on the basis of morphological features are genetically heterogeneous and consist of morphologically indistinguishable species known as cryptic species. Cryptic species have been discovered in many liverworts, e.g., *Conocephalum conicum* (L.) Dumort. (Szweykowski and Krzakowa, 1979), *Corsinia coriandrina* (Spreng.) Lindb. (Boisslier-Dubayle and Bischler, 1998), *Frullania tamarisci* (L.) Dumort. (Heinrichs et al., 2010), *Ptilidium ciliare* (L.) Hampe (Kreier et al., 2010), *Porella platyphylla* (L.) Pfeiff. (Heinrichs et al., 2011), and species of the genus *Herbertus* Gray (Feldberg et al., 2004).

Aneura pinguis (L.) Dumort. (Aneuraceae), a common and widely distributed thallose liverwort, also has a complex genetic structure. Genetic studies revealed that *A. pinguis* consists of several cryptic species (Wachowiak et al., 2007; Bączkiewicz et al., 2008; Wawrzyniak et al., 2014). The differences between three of these species measured by Nei's genetic distances (mean $D = 1.34$) are even greater than those found between taxonomically recognized species of higher plants (Bączkiewicz and Buczkowska, 2016). However, this genetic diversity is not sufficiently reflected in the morphology of the plant to allow for the recognition of distinct taxa. A reason for this may be the very simple morphological structure of the plant, providing only a limited number of diagnostic features.

The composition of chemical compounds is another source of markers helpful in species identification and in solving taxonomic problems in identifying plant species (Salimpour et al., 2011; Ethordević et al., 2014; Celiński et al., 2015). Ludwiczuk and Asakawa (2010) showed that sesquiterpenoids, diterpenoids and aromatic compounds can serve as chemosystematic markers in liverworts. Studies of the chemical variation of liverworts revealed the presence of specific compounds at the family, genus, and species level including cryptic species, such as in *C. conicum* (Ludwiczuk et al., 2013; Ludwiczuk and Asakawa, 2014). Chemotaxonomic studies of different liverwort species revealed that differences in the composition of chemical compounds between closely related species are very important not only for species identification but also for determining evolutionary relationships between species (Asakawa, 2004; Ludwiczuk et al., 2011; Ludwiczuk and Asakawa, 2014).

Interest in chemical research into *A. pinguis* dates back to the late 1960s. Benešová et al. (1969) reported, for the first time, the isolation of the pinguisane sesquiterpenoid pinguisone, and further studies identified several pinguisane derivatives, including a large quantity of methyl norpinguisonate (Asakawa et al., 1981) and three other derivatives isolated by Tazaki et al. (1996) from *in vitro* cultures of this liverwort. Pinguisanes have also been found in several other liverwort species (Asakawa, 1982; Asakawa et al., 2013b) and are important chemosystematic markers of several liverwort families, e.g., Ptilidiaceae, Trichocoleaceae and Porellaceae (Asakawa). To date, more than 40 pinguisanes have been described and some of them have been synthesized (Tazaki et al., 1995; Asakawa et al., 2013b).

The first chemotaxonomic studies on *A. pinguis* that took genetically distinct cryptic species into account, was reported by Wawrzyniak et al. (2014). The authors compared the chemical composition of four cryptic species of *A. pinguis* (A, B, C and E) and of *A. maxima*, and revealed the presence of compounds specific to each of the studied cryptic species. Further genetic studies of *A. pinguis* using DNA sequences revealed the presence of additional genetic diversity in cryptic species A and B (Bączkiewicz et al., 2017). Within cryptic species A, the authors distinguished three evolutionary lineages. Since the genetic differences between them in their DNA sequences are not sufficient to regard them as separate

cryptic species, they were treated as three genetic lineages, called A1, A2 and A3. Similarly, four evolutionary lineages were detected within cryptic species B. One of them was clearly distinct and was regarded as the new cryptic species F, while the other three differed to a lesser degree and consisted of three different genetic lineages, named B1, B2 and B3 (Bączkiewicz et al., 2017).

The aim of the present study was to determine chemical differences between and within cryptic species A and B. We examined the variation of volatile compounds in all genetically recognized lineages (A1, A2, A3, B1, B2, B3, and F). Analysed samples were collected from different places and at different times of the year to check whether the genetic variation within *A. pinguis* was also evident at the level of specialized metabolites.

2. Results and discussion

The volatile compounds were determined in 48 samples from seven genetic lineages (A1, A2, A3, B1, B2, B3, and F) that had been previously recognized (Bączkiewicz et al., 2017). Details on collection time and locality of the samples are shown in Table 1.

The volatile components detected in *A. pinguis* samples are presented in Tables 2a–2e in order of their elution from a Quadex 007-5MS column. Supplementary data related to this article are presented in Tables S2a–S2e. They include additional results of these measurements together with the values of relative standard deviation (RSD).

The total number of compounds detected were A1–51, A2–57, A3–54, B1–49, B2–49, B3–29, and F–37. The most characteristic compounds present in all analysed samples of *A. pinguis* were sesquiterpenoids, mainly oxygenated sesquiterpenoids, and linear aliphatic hydrocarbons. The structures of the most characteristic volatile components detected in the cryptic species of *A. pinguis* are presented in Fig. 1. The present structures were identified only by GC-MS, so that the absolute configuration of each compound has been tentatively drawn.

Hexanal (**4**) and 1-hexanol (**6**) are the major linear aliphatic compounds detected in the lineages A1, A3, B1 and F of the analysed liverwort species. The relative concentration of aliphatics among all volatiles detected in all samples ranged from 0.3 to 14.8%. The lowest relative concentration of these compounds was detected in lineages A2 (0.3–1.1%) and B3 (0.7–1.1%). In the samples belonging to the other genetic lineages, the percentage of these compounds ranged from 3.8 to 14.8%. Lineages A2 and B3 can be distinguished from one another by the content of oxygenated sesquiterpenoids. For lineage A2, the relative concentration of these compounds was not more than 55%, while for lineage B3 it was more than 79%. Additionally, lineage B3 was characterized by a relatively low relative concentration of the sesquiterpene hydrocarbons (1.9–3.7%) in comparison to all other analysed samples. The low content of sesquiterpenes (1.0–2.0%) was also characteristic of the B2 lineage.

The dominant components present in all liverwort samples were pinguisane sesquiterpenoids, and among them pinguisone (**50**) was the major compound. Its content was 31.5–43.9% of the total volatiles for lineage A1, and 25.6–29.1% for A2, while it was 10.9–17.1% for A3. In the case of lineages B1, B2, B3 and F, the content of this compound was much higher and was 55.2–58.4% for B1, 69.0–87.9% for B2, 71.0–73.5% for B3, and 72.1–76.3% for F. The second characteristic compound present in the analysed plant material was deoxopinguisone (**38**). The percentage of this compound in A1, A2, A3, and B3 ranged from 8.2 to 22.1%, while for B1, B2 and F the relative concentration of compound **38** was much lower and ranged from 0.3 to 3.4%. Among all analysed samples, the highest content of this compound was detected in A1 and A2. A high relative concentration of deoxopinguisone (**38**) in these

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