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Genetic diversity of three European *Veratrum* species revealed by Amplified Fragment Length Polymorphism

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Abstract. Chemical and genetic characterization of *Veratrum* species deposited in European collections is important for genepool preservation and identification of populations with desired metabolic properties. *Veratrum album, V. lobelianum* and *V. nigrum* are native to Europe, and in Poland are ranked as rare or threatened. Genetic variation of European *Veratrum* species was characterized by Amplified Fragment Length Polymorphism (AFLP) markers. The accumulation of jervine as a representative of steroidal alkaloids was measured in seeds. Distribution of 380 markers generated from eight primer combinations was useful for studying genetic relationships among and within species in the *Veratrum* genus and the most divergent populations were identified. Genetic variation between 12 populations of *Veratrum* species supports the classification of *V. lobelianum* as a subspecies of *V. album*. However, the results need further validation on extended material. A higher genetic diversity (22.3%) was observed between populations of *V. nigrum* as compared to *V. album* (14.5%). Contents of jervine allowed for discrimination of the studied *Veratrum* species and can be used as a potential chemotaxonomic marker. The highest jervine levels were found in *V. album. V. nigrum* seeds had only trace amounts and no jervine was detected in seeds of *V. lobelianum*.

Key words: genetic variation, hellebore, jervine, Melanthiaceae, molecular markers

1. Introduction

The *Veratrum* genus (Liliales, Melanthiaceae) comprises 17-45 species distributed over a wide range of habitats of the Northern Hemisphere (Zomlefer *et al.* 2003; Treier & Müller-Schärer 2011). *Veratrum album* L.*, Veratrum lobelianum* Bernh. and *Veratrum nigrum* L. are native to Europe. Only a few isolated populations of these species are present in Poland and ranked as rare or threatened (Allen *et al.* 2014). *Veratrum album* sensu lato is a complex of subspecies *V. album* ssp. *album* and *V. album* ssp. *lobelianum* (Schaffner *et al*. 2001) sometimes considered as separate species (Zomlefer *et al.* 2003). Plants of *Veratrum* are a rich source of unique bioactive steroidal alkaloids (Chandler & McDougal 2014). Quantitative and qualitative variations in the metabolic profile and pharmacological properties are affected by environmental factors and genetic diversity present in natural populations (Li *et al.* 2015).

There are three subtypes of *Veratrum*-type alkaloids including cevanine, veratramine and jervine that are divided according to structural features (Li *et al*. 2006). These steroidal alkaloids are well known for their pharmacological activities, including hypotensive, antithrombotic and antitumour functions (Tang *et al*. 2008a; Ivanova *et al*. 2011). In particular, cyclopamine showed antitumour activity and induced apoptosis using the mechanism of inhibition of the hedgehog pathway in a subset of the pancreatic cancer cell line (Thayer *et al*. 2003). Due to low cyclopamine concentration in *Veratrum* plants (about 0.01%), we focussed our attention on jervine that was present in higher concentrations (0.1%) and can be transformed to cyclopamine by Wolff-Kishner reduction with Huang-Minlon modification (Tang *et al*. 2008b). Jervine displays strong antifungal activities against the phytopathogenic *Phytophthora capsici* (Li *et al*. 2006).

Medical plants from Melanthiaceae are interesting specimens for genetic diversity studies (Li *et al*. 2011; Treier & Müller-Schärer 2011). For genetic characteristics targeting taxonomic questions, variation in cpDNA and the internal transcribed spacer (ITS)

region of rDNA are standard (Zomlefer *et al.* 2003; Griffin & Barrett 2004; Liao *et al*. 2007). Systems targeting conservative regions of DNA were ideal for separating evolutionary different populations of *Trillium grandiflorum* and *V. album* ssp. *oxysepalum* (Griffin & Barrett 2004; Kikuchi *et al*. 2010). However, to study genetic diversity within and between natural populations, amplified fragment length polymorphism (AFLP) system is a method of choice (Guthridge *et al.* 2001; Quagliaro *et al.* 2001; Tang *et al.* 2003; Llanes *et al.* 2011). This DNA fingerprinting technique targets multiple loci, generates dominant markers for whole-genome screening and has higher reproducibility, resolution, and sensitivity compared to random amplified polymorphic DNA (RAPD) and inter simple sequence repeats (ISSRs) methods (Vos *et al*. 1995; Blears *et al*. 1998).

Various genetic marker systems have been used to characterize variation in the *Veratrum* genus, including RAPD (Kleijn & Steinger 2002), AFLP (Treier & Müller-Schärer 2011) and simple sequence repeats (Kato *et al*. 2008; Kikuchi & Maki 2011). AFLP studies of 40 European natural populations of *V. album* revealed east-west direction in the genetic structure and suggested ancient migration from Asia (Treier & Müller-Schärer 2011).

So far, genetic diversity between and within European accessions of *V. nigrum*, *V. album* and *V. lobelianum* has not been investigated with AFLP markers. Chemical and genetic characterization of *Veratrum* species deposited in European collections generates new information useful both for genepool preservation and for identification of populations with desired metabolic properties. Jervine content was determined to preliminary test the suitability of this genera-specific steroid alkaloid as a potential chemotaxonomic marker.

2. Materials and methods

2.1. Plant materials

Plant collection consisted of six populations of *V. nigrum,* five populations of *V. album* and a single population of *V. lobelianum* maintained in botanical gardens across seven European countries (Table 1). Seeds from population N5 (*V. nigrum*) were germinated and plants were maintained *in vitro*, then lyophilized prior to DNA extraction. Seeds of the remaining families had reduced viability and, therefore, were used directly (3-7 random seeds) for DNA isolation. The names of genus and species follow Zomlefer *et al.* (2003).

2.2. DNA extraction and AFLP analysis

Total plant genomic DNA was extracted from seeds or lyophilized plants using a method developed by Milligan (1992). The DNA was quantified using agarose gel electrophoresis and diluted to a concentration of 200 ng·mL^{-1} prior to AFLP analysis. The AFLP analysis was carried out according to the methods of Thomas *et al*. (1995). Genomic DNA was digested with *MseI* and *PstI* restriction enzymes (5 U each) in Tango buffer (33 mM Tris-acetate, pH 7.9 at 37°C; 10 mM magnesium acetate; 66 mM potassium acetate; 0.1 mg/ml BSA) for 90 min at 65°C and 60 min at 37°C, respectively. The enzymes were subsequently heat inactivated at 80°C for 20 min and the products ligated with *Mse*I and *Pst*I adapters overnight at 37°C to generate template DNA for amplification. DNA was then purified by ethanol precipitation and dissolved in 20 µL of water. Non-selective (NS) amplification (30 cycles of 30 s at 94°C, 30 s at 56°C and 60 s at 72°C) was conducted using primers complementary to *MseI* and *PstI* adaptors without selective nucleotides (5'-GATGAGTCCTGAGTAA-3'

Table 1. Populations of *Veratrum* species used for genetic and chemical analyses

Acronym	Species	Sampling locality	Source of material	Number of plants
N1	*V. nigrum*	Nowy Sącz, POL	Private garden	3
N2	*V. nigrum*	Ljubljana, SVN	University Botanic Gardens	7
N3	*V. nigrum*	Bayreuth, DEU	Ecological Botanical Gardens	5
N4	*V. nigrum*	Romsey, GBR	Sir Harold Hillier Gardens	5
N5	*V. nigrum*	Brno, CZE	Masaryk University Botanic Garden	6
N6	*V. nigrum*	Yoskar-Ola, RUS	Botanical Garden of the Technological State University	5
L1	*V. lobelianum*	Ljubljana, SVN	University Botanic Gardens	6
A1	*V. album*	Bayreuth, DEU	Ecological Botanical Gardens	5
A2	*V. album*	Bolestraszyce, POL	Arboretum and Institute of Physiography	6
A3	*V. album*	Bonn, DEU	University Botanic Gardens	6
A4	*V. album*	Nancy, FRA	Conservatory and Botanical Gardens	4
A5	*V. album*	High Tatra Mts, POL	Collecting	6

and 5'-GACTGCGTACATGCAG-3', respectively). The diluted PCR products of the NS amplification were selectively amplified with eight combinations of *MseI* primers (M43, M47, M48, M50, M59) and *PstI* primers (P4, P12, P15, P16, P18, P26, P34), each containing three selective nucleotides at the 3'-end. Symbols of the selective primers are in accordance with the KeyGene standard list for AFLP primer nomenclature. Selective amplification was accomplished with a touchdown thermal cycle, as follows: 7 cycles at 94°C for 60 s, 65°C (-1°C per cycle) for 60 s, 72°C for 120 s, followed by 29 cycles at 94°C for 60 s, 56°C for 60 s and 72°C for 120 s and a final elongation at 72°C for 10 min. The PCR products were run on a 5% polyacrylamide sequencing gel and detected with silver staining (Fiust *et al*. 2015).

2.3. Extraction and quantitative analysis of jervine

Jervine was extracted from samples that were ultrasonicated with chloroform and ammonia hydroxide (20:3) for 40 min at room temperature. The extracts were filtered and evaporated to dryness under reduced pressure. The residue was dissolved in methanol (Wang *et al*. 2008). The amount of jervine in extracts was determined by ultra performance liquid chromatography (UPLC, Shimadzu) coupled with QTRAP 4500 mass spectrometry (AB Sciex) with triple quadrupole. Analytical separation was carried out using the UPLC system with a Kinetex XB – C18 LC column (50 mm x 2.1 mm I.D., 1.7 μm). The column temperature was set at 40°C. A binary gradient elution system consisted of acetonitrile (a) and water (0.01% formic acid) (b). Separation was achieved using the following gradient program: 0-5 min, linear gradient (0-100% a). The flow rate was adjusted to 0.5 mL·min^{-1} and an injection volume of 2 μL. Compounds were quantitated with an electrospray ion source in the positive ion mode (ESI-MS), under these conditions: temperature 600ºC, spray voltage: +5000 V. The fragmentation transitions were for multiple reaction monitoring (MRM). MRM was performed with 50 ms dwell time per channel. The following transitions were detected: m/z 425.89/313.10 Da and 425.89/114.0 Da for jervine (Tang *et al*. 2008b; Grobosch *et al*. 2008).

2.4. Data analysis

For the diversity analysis, the amplification products were scored for the presence (1) and absence (0) of bands to form a binary matrix. These data were used to calculate polymorphism information, including the number of polymorphic/species-specific fragments and PIC (polymorphism information content) according to Roldan-Ruiz *et al*. (2000). The AFLPsurv 1.0 (Vekemans *et al*. 2002) and Popgene ver. 1.32 (Yeh & Boyle 1997) were used to evaluate basic parameters for the population genetic structure, including Ht – total gene diversity, Hw – gene diversity within populations, Hb – genetic differentiation among populations and Fst – Wright's fixation index (Lynch & Milligan 1994). The relationships between individuals were visualised in TreeView 1.6.6 (Page 1996) as a dendrogram constructed using the unweighted pair-group method with arithmetic averages (UPGMA) method based on Nei's genetic distances (Nei 1972) computed in PHYLIP (Felsenstein 1989). PAST ver. 2.07 software (Hammer *et al*. 2001) and STRUCTURE v.2.3 (Pritchard *et al*. 2000) were used for principal coordinate analysis (PCoA) based on correlation matrix, and to discern populations. The package of STATISTICA v.10 was used for analysis of variance (ANOVA).

3. Results

3.1. Amplified fragment length polymorphism (AFLP) analysis

The genetic diversity among and within *V. nigrum, V. album,* and *V. lobelianum* represented respectively by 6, 5, and single populations from European collections was analysed using the AFLP method. Eight random combinations of selective primers resulted in 380 markers,

Table 2. Combinations of selective primers and obtained polymorphisms. Species-specific markers were identified for *Veratrum nigrum* (N) and *V. album* (A)

Primers	Number of fragments			PIC
	Total	Polymorphic	Species specific	
P16+M43	26	25	1(A)	0.329
P18+M48	39	37	0	0.278
P4+M50	58	53	2(A)	0.249
P12+M47	50	49	1(N)	0.274
P26+M59	51	50	0	0.235
P15+M59	69	67	1(N)	0.246
P16+M50	49	48	0	0.272
P34+M48	38	37	0	0.195

including 366 (96.32%) that were polymorphic (Table 2). The average number of markers per primer set was 47.5 and varied from 26 (P16+M43) to 69 (P15+M59). The average number of polymorphic fragments per primer combination was 45.8 and the percentage of polymorphic bands oscillated in the narrow range from 91.38% (P4+M50) to 98.04% (P26+M59). The polymorphism information content (PIC) parameters identified the most informative markers and showed the distribution of identified polymorphisms. The mean (PIC) values for primer combinations ranged from 0.195 to 0.329 (Table 2).

3.2. Genetic diversity

Genetic relationships among 12 populations representing three species were analysed using allele frequencies and Nei's distances. Clustering of the populations revealed two main genotypic groups (Fig. 1). The first group comprised the six populations of *V. nigrum*, while the second cluster comprised *V. album* and *V. lobelianum*. Due to the genetic structure of these populations and the resolution of two distinct clusters, the data were split into *V. album* and *V. nigrum* sets for further population analyses.

Out of the 380 polymorphic fragments identified in the whole set of genotypes, more polymorphic fragments were found in *V. album* genotypes (285; 75.0%) when compared to *V. nigrum* (253; 66.6%). Nei's gene diversity (He) in *V. album* populations (0.176) was higher than that for *V. nigrum* (0.161). We identified 127 markers present only in populations of *V. album* (including *V. lobelianum*), and three alleles were fixed in all these populations and absent in *V. nigrum.* 88 markers

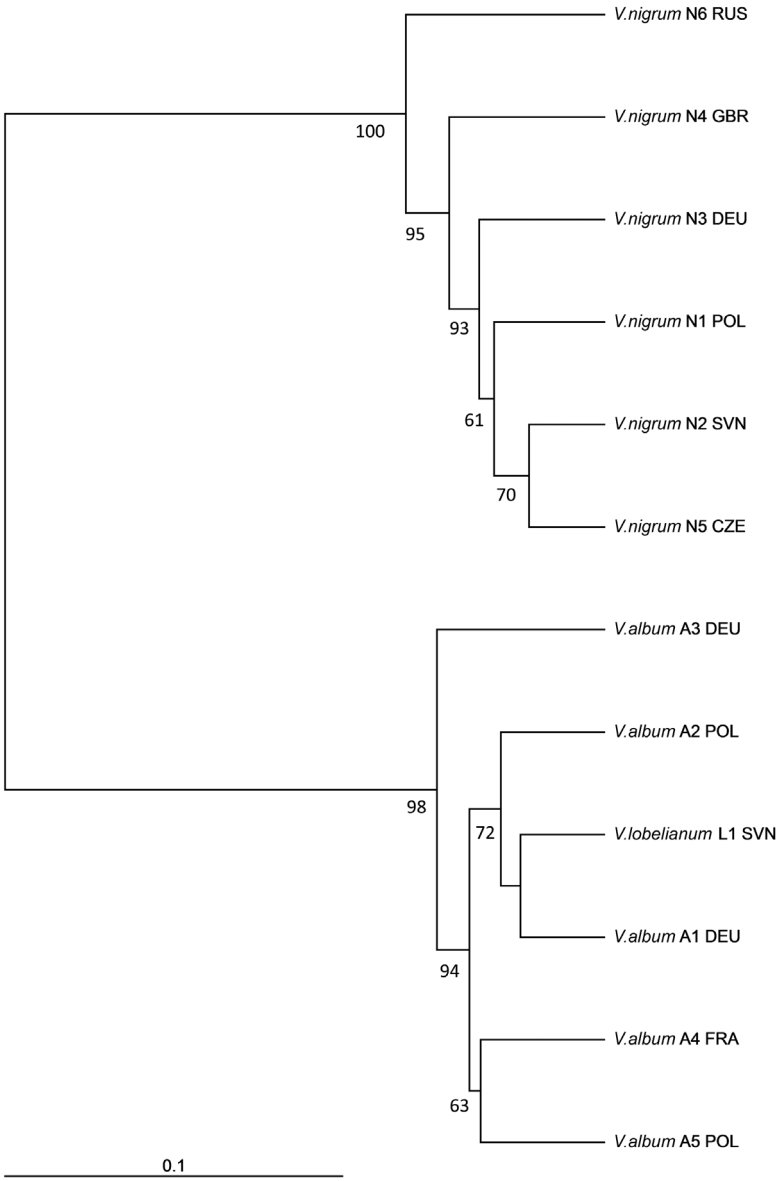

Fig. 1. Unweighted pair group method with arithmetic mean (UPGMA) dendrogram for 12 populations of *Veratrum* spp. based on Nei's genetic distances established for allele frequencies in 380 loci. Values on nodes represent the significance of the cluster for 100 bootstraps

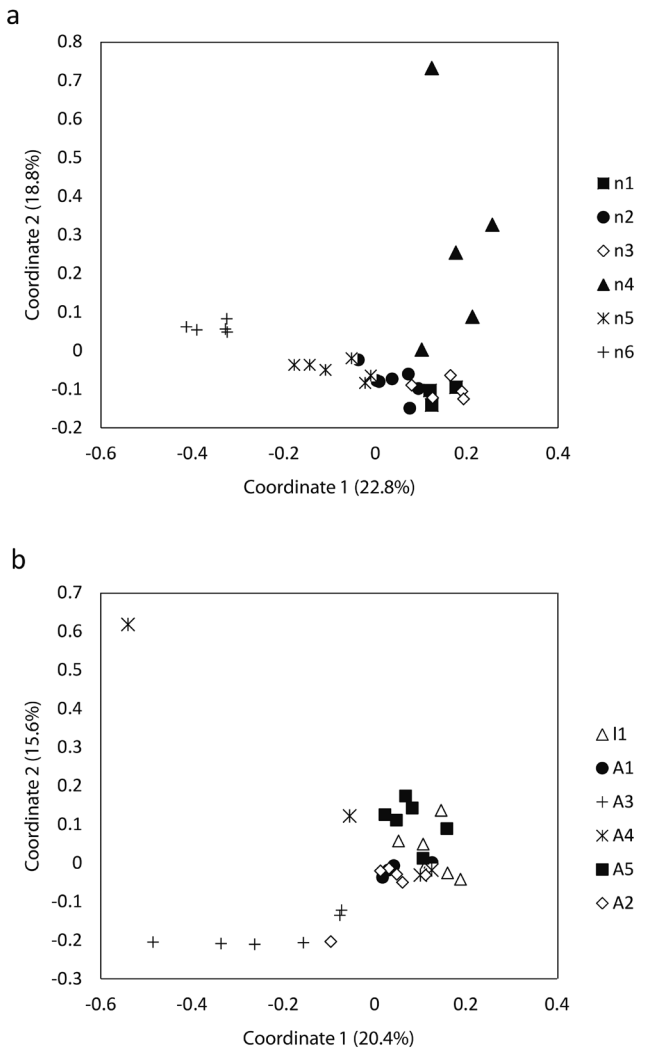

Fig. 2. Distribution of individuals in six populations of *V. nigrum* (a) and six populations of *V. album*, including *V. lobelianum* (b) across the two first principal coordinates

were detected only in *V. nigrum* and two markers were present in all genotypes of *V. nigrum* and absent in the *V. album* gene pool. High bootstrap values indicate that the resolution was sufficient to discern groups at a statistically significant level.

The genotypes of *V. album* and *V. nigrum* were spread across the two first principal components (Fig. 2) accounting for 36.0% and 41.6% of the overall variation, respectively. Populations of *V. nigrum* from the United Kingdom (N4) and Russia (N6) are separated from the remaining populations from central and eastern Europe on the second and first coordinates (Fig. 2a). Populations of *V. album* formed two groups, with a German population (A3) isolated from the main gene pool (Fig. 2b). Analysis with STRUCTURE software confirmed the significance of population structure within *V. album* and *V. nigrum*, with the two and three subpopulations discerned, respectively.

The total gene diversity of *V. nigrum* (Ht = 0.179) can be divided into variation deposited within (Hw = 0.139, 77.7%) and between populations (Hb = 0.040, 22.3%). Majority of the genetic diversity (Ht = 0.197) in *V. album* was assigned to variation within populations (Hw = 0.168; 85.5%). Wright's fixation index (Fst) was higher for *V. nigrum* (0.225) than for *V. album* (0.115), which confirmed that the total gene diversity in *V. nigrum* was shaped more by variation among populations.

3.3. Content of jervine

The mean content of jervine was determined in seeds of 10 populations by the UPLC-MS method. ANOVA revealed that the jervine content varied between species and populations ($p < 0.0001$). The highest average

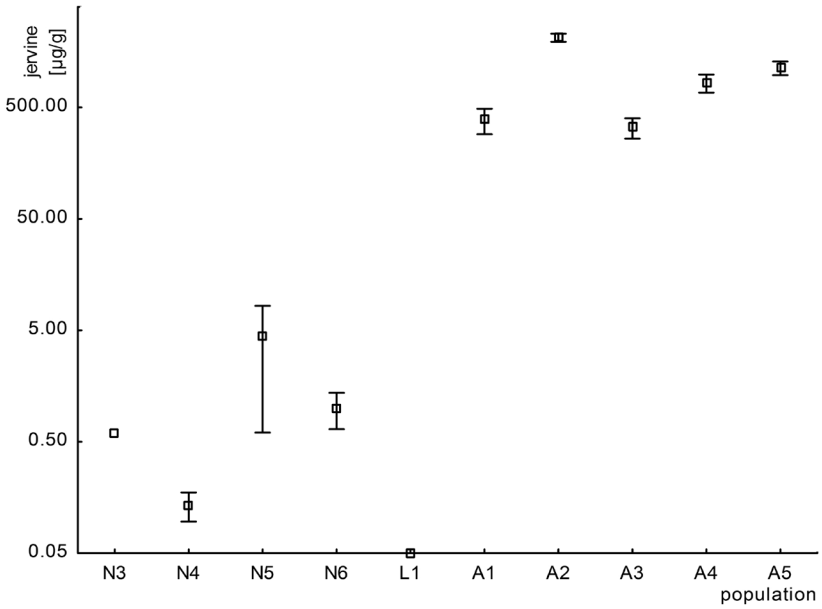

Fig. 3. Mean and standard error of jervine content in the seeds of *Veratrum nigrum* (N3-N6), *V. lobelianum* (L1) and *V. album* (A1-A5)

Table 3. Jervine content [μg·g^{-1}] in the studied populations of *Veratrum album, V. lobelianum, and V. nigrum*

Species	No. of populations	Jervine content [μg·g^{-1}]		
		mean±SD	Min	Max
V. nigrum	4	1.74±3.69	0.09	12.14
V. lobelianum	1	0.00±0.00	0.00	0.00
V.album	5	952.02±686.06	210.00	2416.00

concentration of jervine (952.02 μg·g^{-1}) was found in *V. album*, and its content varied by more than 10-fold between populations (Fig. 3, Table 3). More than 500 times less jervine was detected in seeds of *V. nigrum* (1.74 μg·g^{-1}). No jervine was detected in seeds of the single population of *V. lobelianum*.

4. Discussion

The AFLP technique provides an efficient and reproducible method for the simultaneous screening of multiple loci. In comparison with other fingerprinting techniques, it generates a large number of informative markers scattered across the genome (Magdy *et al*. 2016). In *Veratrum album* and other medicinal plants (*Ypsilandra thibetica*, *Tribulus terrestris*, *Fritillaria* sp.) the AFLP method generated from 69.9 to 147.8 bands per primer combination (Sarwat *et al.* 2008; Li *et al.* 2011; Treier & Müller-Schärer 2011; Metin *et al.* 2013). In our studies, on average, 47.5 markers per randomly selected primer set were scored. The efficiency of AFLP measured as the number of bands per primers combination may be compromised by several factors, such as quality control of the AFLP procedures (contamination), properties of primers (preselection, number of selective bases), combination of restriction enzymes, method of detection, and genetic variation deposited in analyzed samples (Pompanon *et al.* 2005).

According to taxonomic classification, the *Veratrum* genus in Eurasia is divided into two sections *Alboveratrum* (e.g. *V. album*) and *Fuscoveratrum* (e.g. *Veratrum nigrum* L.). *Veratrum album* is separated into two subspecies, *V. album* ssp. *album* (central- and south-European distribution) and *V. album* ssp. *lobelianum* (Euro-Siberian distribution) but this distinction is often ignored (Schaffner *et al.* 2001). Furthermore, variation of chloroplast DNA (cpDNA) and ITS sequences supports the above division, with *V. lobelianum* as separate species (Liao *et al.* 2007; Kikuchi *et al.* 2010). Results of the AFLP analysis (Fig. 1) are consistent with discerning of sections, but variation in random DNA fragments indicates that *V. lobelianum* is not significantly different from *V. album*.

Recent AFLP studies revealed that 77-85% and 91-94% of genetic diversity was allocated within populations of *V. album* or *V. album* ssp. *oxysepalum*, respectively (Kikuchi *et al.* 2010; Treier & Müller-Schärer 2011; Kikuchi *et al.* 2013). In the six European populations of *V. album*, we found a similar level of variation (85.5%), and we identified population A3 that represents unique genetic characteristics. Compared to *V. album*, the variation within populations of *V. nigrum* was lower (77.7%). This was confirmed by identification of the two subpopulations (N6 and N4) and higher Wright's fixation index. Our results are also consistent with high intraspecific variation previously reported for ITS sequences of *V. nigrum* (Liao *et al.* 2007). Genetically well diversified populations representing high variation may be preferred for characterizing genomes and transcriptomes.

The *Veratrum* genus is a source of steroidal alkaloids that have high anticancer activity and show health-beneficial effects (Li *et al.* 2012). The *Veratrum* species characterized in this study displayed great variation in the content of jervine. The concentration of jervine varied significantly between populations of *V. nigrum* and *V. album*. Only trace amounts of jervine were found in seeds of *V. nigrum*, and no jervine was detected in seeds of *V. lobelianum*; however, this finding needs confirmation on extended materials. Although metabolic profiles are often affected by environmental factors and vary during plant development (Kleijn & Steinger 2002), jervine content seems to be a promising chemotaxonomic marker. In spite of high interspecific variation, the concentration of this metabolite provides distinct ranges characteristic for the three tested species of *Veratrum*. Additionally, chemical profiling can be used to identify genotypes with target metabolic potential, and further genetic testing leading to mapping of loci involved in the biosynthesis of valuable metabolites.

5. Conclusions

Genetic variation of European *Veratrum* species was characterized by AFLP markers and the contents of genera-specific steroid alkaloid (jervine) was determined. The AFLP method was useful for studying the genetic relationships among and within species in the *Veratrum* genus and the most divergent populations

were identified. Genetic variation between 12 populations of *Veratrum* species supports the classification of *Veratrum lobelianum* as a subspecies of *V. album*. However, the obtained results need further validation on extended material. Content of jervine used as a potential chemotaxonomic marker allowed for discrimination the studied *Veratrum* species.

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