

## ADENOPHORA LILIIFOLIA: CONDITION OF ITS POPULATIONS IN CENTRAL EUROPE

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This study deals with populations of the European-South-Siberian geoelement *Adenophora liliifolia* (L.) A. DC. in the Czech Republic, Slovakia, Hungary, Romania, and Poland, where this species has its European periphery distribution. We studied the population size, genetic variability, site conditions, and vegetation units in which *A. liliifolia* grows. Recent and historical localities of *A. liliifolia* were ranked into six vegetation units of both forest and non-forest character. A phytosociological survey showed differences in the species composition among localities. Only a weak pattern of population structure was observed (only 22% of total genetic variation present at the interpopulation level, AMOVA analysis), with moderate values for gene diversity ( $H_j = 0.141$ ) and polymorphism ( $P = 27.6\%$ ). Neighbor-joining and Bayesian clusterings suggest a similar genetic background for most of the populations from Slovakia, the Czech Republic, and Poland, contrary to the populations from Hungary, Romania, as well as two populations from Central and South Slovakia. This might be explained by a relatively recent fragmentation of the *A. liliifolia* populations in Central Europe. Nevertheless, it seems that several populations in Romania, South Hungary, and Slovakia were isolated for a longer period of time and their genetic differentiation is more evident.

**Keywords:** AFLP, Campanulaceae, European periphery distribution, declining population, European-South-Siberian geoelement, genetic variability, vegetation

### INTRODUCTION

The present-day flora of Central Europe reflects its geographic position, varied geology and topography, as well as climate and vegetation history, and it is influenced by glacial cycles during the Quaternary Period (Grulich, 2012; Kaplan, 2012). Numerous species that are extinct in Central Europe survived in Eastern Asia (e.g., *Platykladus orientalis* (L.) Franco (Farjon and Filer, 2013)), in Transcaucasia (e.g., *Pterocarya pterocarpa*

(Michx.) Kunth ex Iljinsk. (Denk et al., 2001)), or in the Balkan Peninsula (e.g., *Picea omorika* (Pančić) Purk. (Ravazzi, 2002)). Some species, such as *Ligularia sibirica* (L.) Cass. (Šmídová et al., 2011) or *Pedicularis sudetica* Willd. (Hendrych and Hendrychová, 1988), became glacial or postglacial relicts, but also new local endemics appeared, e.g., *Galium sudeticum* Tausch, *G. cracoviense* Ehrend., *Cochlearia polonica* E. Froelich (Cieślak et al., 2007, 2010, 2015; Cieślak and Szelag, 2009, 2010; Kolář et al., 2013, 2015) or *Sorbus sudetica* (Tausch) Bluff et al. (Kaplan, 2012). Climatic

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changes during the Quaternary Period strongly affected the species composition and the species distribution in Europe (Szafer, 1946–47, 1954; Kaplan, 2012). Since the Neolithic Period, humans have become another important factor affecting the regional floras (Szymura, 2012; Hejcman et al., 2013; Roleček et al., 2014; Plieninger et al., 2015).

Many species, such as *A. liliifolia* (L.) A. DC, changed their distribution and their recent presence in floras is highly influenced by human activity. *A. liliifolia* is considered to represent the European-South-Siberian goeement, which tolerates extreme continental climate with a short growing season, warm but short summers, and long winters with severe frosts (Kucharczyk, 2007; Kaplan, 2012; Kucharczyk et al., 2014). The centre of *A. liliifolia* distribution is in Western Asia-Southern Siberia, and from there it extends to Mongolia and Western China in the East, and to the North-West of Turkey, and to South-, Eastern-, Central- Europe up to Western Europe in the West (Tacik, 1971; Fedorov, 1978; Deyuan et al., 2011; Urgamal, 2014). Although Smelansky et al. (2004) reported *A. liliifolia* as a common species in the steppes and forest-steppes in Southern Siberia, Boronnikova (2009) reported a 25% decrease of populations in the Perm region (Ural, Russia) during the last 15 years due to agricultural activities in the territory. Also, in the whole Central European region, *A. liliifolia* populations are declining not only in the number of localities, but also in the number of plants representing a single population.

*A. liliifolia* is scattered across Europe and forms isolated populations in Germany (Meusel and Jäger, 1992; Castroviejo et al., 2010), Austria, Switzerland (Moser, 1999), Italy, Czechia (Martinovský, 1967; Kovanda, 2000), Poland (Witkowski et al., 2003; Korzeniak and Nobis, 2004; Ciosek, 2006; Kapler et al., 2015), Slovakia (Goliášová and Šipošová, 2008), Hungary (Farkas and Vojtkó, 2012, 2013; Vojtkó, 2013), Croatia, Bosnia and Herzegovina, Montenegro, Serbia (Vladimirov et al., 2009; Vukojičić et al., 2011), Romania (Jones et al., 2010; Indreica, 2011), and Slovenia (Babij, 2004; Acetto, 2007). In Belarus, the species was thought to be extinct (Kozlovskaja, 1978), but one population at Sporowski Zakaznik was restored with plants multiplied *in vitro* and cultivated in the Minsk Botanical Garden of the Belarussian Academy of Sciences (Williams and Gotin, 2012). The information about *A. liliifolia* from France (Schnittler and Günther, 1999) and Bulgaria (Dimitrov, 2002) is uncertain, as no herbarium records from France and the current Bulgarian territory exist.

*A. liliifolia* is protected in Europe according to the Directive on the conservation of natural habitats and of wild fauna and flora (92/43/EEC); it is

considered as a species of least concern (Bilz et al., 2011); and it is threatened by vigorous shrubby vegetation and by inappropriate forest management (Anonymous, 2009). *A. liliifolia* is considered a plant species of European Community interest, whose conservation requires designation of special areas of conservation. Moreover, it is an indicator species of thermophilous forest hotspots, signaling remnant pools of biodiversity (Kiedrzyński et al., 2015). A typical habitat of *A. liliifolia* is the coppice, which is a formerly widespread way of forest management. However, changes in landscape management during the last two centuries caused the extinction of this species because of the shady and more eutrophicated high forests (often with conifers) that replaced the coppices (Szymura, 2012; Müllerová et al., 2015). Today *A. liliifolia* grows in lowlands in small populations in remnants of former light oak forests, their ecotones, and adjacent meadows. At higher altitudes it grows on the rocky outcrops in beech forests (Moser, 1999; Dražil, 2002), and in the portions of riparian forests receiving large quantities of sunlight (Siklósi, 1984; Farkas and Vojtkó, 2012, 2013). These ecological demands make *A. liliifolia* a suitable model species for studying the changes and the impact of human activities on populations of species with similar characteristics.

In spite of the critical conservation status of *A. liliifolia* in Europe, no large-scale population genetic studies have been done so far. Only two studies have investigated the population structure of *A. liliifolia*: Boronnikova (2009) analyzed four populations from the Ural region (Perm, Russia), and Manole et al. (2015) described the genetic diversity of one *A. liliifolia* population from Romania. The present study aimed to provide overall information on the current condition of *A. liliifolia* populations in Central Europe, and thus to better know the factors threatening this species and suggest appropriate management for the current populations. We performed: 1) a survey and comparison of *A. liliifolia* populations in Czechia, Slovakia, Hungary, Romania, and Poland; as well as 2) a screening of the genetic variability and relationship among the studied populations by means of Amplified Fragment Length Polymorphism (AFLP).

## MATERIALS AND METHODS

### STUDY SPECIES

The lilyleaf ladybells *Adenophora liliifolia* is a herbaceous perennial diploid ( $2n = 34$ ) plant from the Campanulaceae family with erect, leafed and branched stems. The root is spindle-shaped or branched. The plant usually grows to a height of

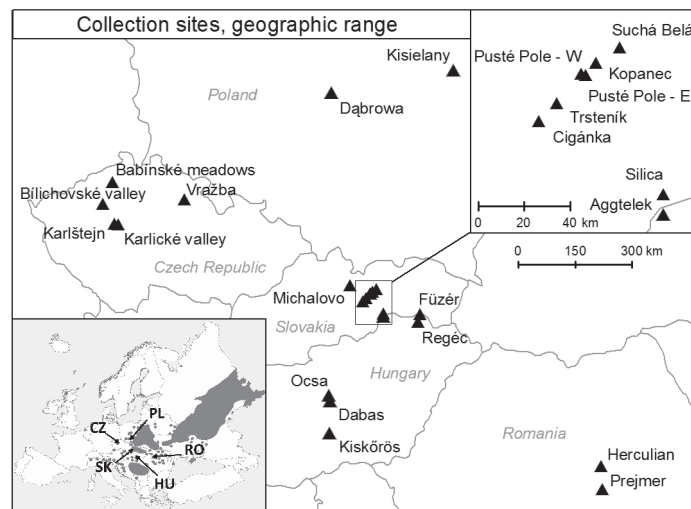
40–90 cm (Kovanda, 2000), although plants with heights of 205 cm have been observed in Poland (Ciosek, 2006). Basal leaves and leaves of young plants are long petiolate, with cordate to rounded and coarsely serrate blades. Stem leaves are sessile, alternating with an elliptical to lanceolate, serrate to entire blade with a wedge-shaped base. Inflorescences are panicles or racemes, flowers are fragrant. Calyx lobes are triangular, pointed, 3–4 mm long and finely serrate. The corolla is bell-shaped, 12–20 mm long, pale blue, rarely white. The pistil is twice as long when compared to the corolla. The species flowers from late June to August and is pollinated by insects. The fruits are pear-shaped, curved, 8–12 mm long capsules, opening with three holes at the base. The seeds are flattened, reddish brown, from 2.0–2.5 mm long and are spread by wind (Kovanda, 2000; Kucharczyk et al., 2014). The precise ecological demands of *A. liliifolia* require further studies. According to Ellenberg et al. (1992), the ecological demands are as follows: light (L) = 7; temperature (T) = 6; continentality (C) = 6; moisture (F) = 6; soil reaction (R) = 8; nutrients (N) = 2.

#### CHARACTERISTICS OF *A. LILIIFOLIA* POPULATIONS AND THEIR LOCALITIES

Monitoring in Czechia, Slovakia, Hungary, and Romania was performed according to the *Natura 2000* methodology (Marhoul and Turoňová, 2008) during July and August in 2012 or 2013. The number of tufts and number of fertile and sterile stems in each tuft were determined at each locality

in all the countries. The condition of the locality and its changes were observed during the monitoring of populations in 2012–2013. The danger of possible damage such as grazing, drying, grubbing out, damage from human activities, etc., was qualitatively recorded. Morphological differences including stem height, number of leaves per stem, length and width of 3 leaves at the central part of the stem, and number of branches and flowers in an inflorescence were observed too. Information about the Polish localities of Kisielany and Dąbrowa originates from papers by Ciosek (2006) and Rapa (2012). In total we studied 23 localities (all current localities in Czechia, Slovakia; chosen representative and accessible localities in Romania, Hungary, and Poland (Fig. 1). The characteristics of the studied localities are shown in Tab. 1 and Fig. 2, and were summarized from published data (Comitetul de Stat al Geologiei – Institutul Geologic CSG-IG, 1968; Mihai, 1975; Miklós, 2002; AOPK ČR, 2005; Káčer et al., 2005; Cháb et al., 2007; Tolasz, 2007; European Soil Data Center, 2008–2015; Dövényi, 2010; Climate Change Knowledge Portal, 2015; IUSS Working Group WRB, 2015; One Geology – Europe, 2015).

The localities were ordinated with Principal Component Analysis (PCA). The climatic characteristics (annual mean temperature, annual precipitation), the altitude, affiliation with particular biogeographic regions in Europe, and forest/non-forest character of vegetation were used as supplementary data to assist with data interpretation. The calculations were done in the CANOCO 4.5 program (ter Braak and Šmilauer, 2002).

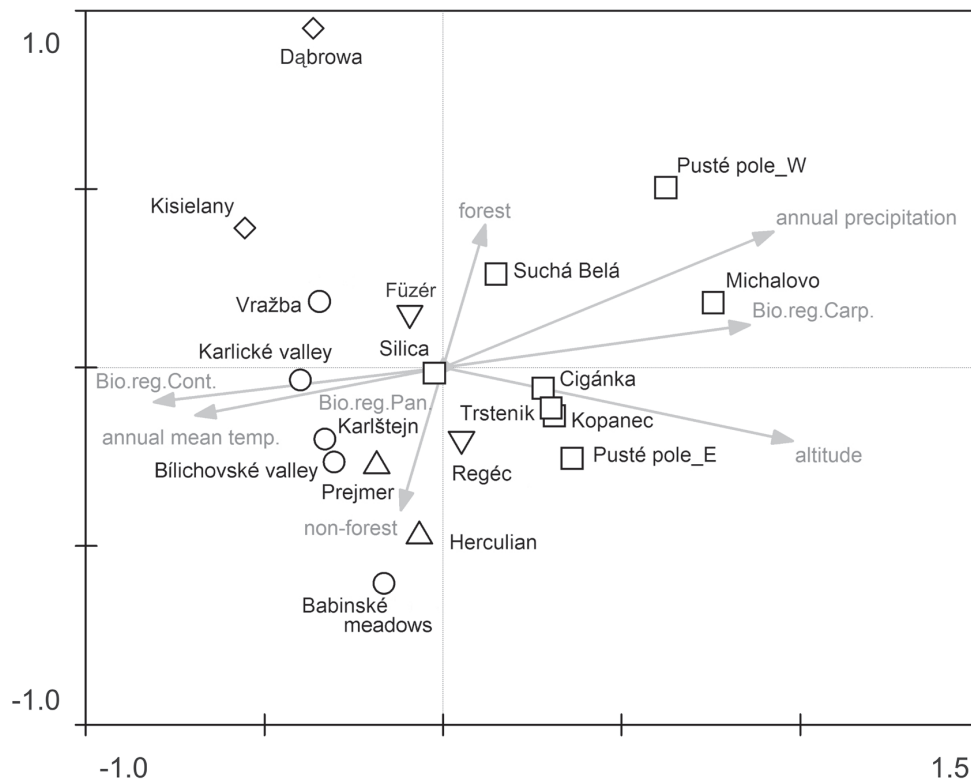


**Fig. 1.** Map of the studied localities of *A. liliifolia* in Czechia, Slovakia, Poland, Hungary and Romania and its geographic range (made by J. Gamráč in ArcGIS 10 program).

TABLE 1. Site conditions of the studied localities (GPS coordinates indicate the approximate center of the locality)

Site	Latitude Longitude	Alt. (m)	Bedrock	Soil (acc. to WRB 2014)	Habitat acc. o EH Habitat Directive
<b>Czechia</b>					
Babínské meadows	50°35'52" 14°07'36"	538	mesozoic marl, claystone	eutrophic cambisol, planosol, stagnosol	Intermittently wet <i>Molinia</i> meadows
Bílchovské valley	50°15'51" 13°53'57"	429	mesozoic marl, claystone	cambisol	Oak-hornbeam forest
Karlické valley	49°57'07" 14°15'24"	325	paleozoic limestone	cambisol	Oak-hornbeam forest
Karlštejn	49°57'35" 14°10'24"	400	paleozoic limestone	cambisol, phaeozems	Central European basiphilous thermophilous oak forest
Vražba	50°20'05" 15°49'19"	330	mesozoic marl, claystone	cambisol	Oak-hornbeam forest
<b>Hungary</b>					
Aggtelek	48°31'14" 20°33'08"	495	light steinalm limestones	modal cambisol, chernozem, kastanozem	Mountain hay meadows
Dabas	47°10'04" 19°16'03"	100	organic rich sediment, lacustrine and paludal clay, silt, calcareous mud, peat	histosol, planosol, stagnosol	Riparian mixed gallery forests
Füzér	48°33'42" 21°25'13"	520	rhyolite, andesite	stagnosol, fluvisol, podsol, retisol, phaeozem	Intermittently wet <i>Molinia</i> meadows
Kiskőrös	46°39'11" 19°16'29"	104	organic rich sediment, lacustrine and paludal clay, silt, calcareous mud, peat	histosol, planosol, stagnosol	Riparian mixed gallery forests
Ocsa	47°15'42" 19°15'35"	247	organic rich sediment, lacustrine and paludal clay, silt, calcareous mud, peat	histosol, planosol, stagnosol	Riparian mixed gallery forests
Regéc	48°26'19" 21°21'56"	680	andesite	histosol, fluvisol, podsol, andosol	Intermittently wet <i>Molinia</i> meadows
<b>Poland</b>					
Dąbrowa	50°45'55" 22°09'02"	200	outwash sands and gravels, Lithotamnium limestone	haplic arenosol, haplic luvisol	Thermophilous oak forests
Kisielany	52°15'12" 22°12'26"	146	outwash sands and gravels, clays	stagnic retisol	Thermophilous oak forests
<b>Romania</b>					
Herculian	46°07'05" 25°42'38"	635	volcanic sediments	andosols, chernozem, kastanozem	Central Europ. basiphilous thermophilous oak forest
Prejmer	45°43'59" 25°44'20"	518	quaternary sediments	histosol, stagnosol, fluvisol	Intermittently wet <i>Molinia</i> meadows
<b>Slovakia</b>					
Cigánka	48°45'49" 20°03'43"	825	dolomitical rocks	podzolic cambisol	Limestone beech forest

Site	Latitude Longitude	Alt. (m)	Bedrock	Soil (acc. to WRB 2014)	Habitat acc. o EH Habitat Directive
Kopanec	48°54'54" 20°17'16"	850	sandy and gravelly sediments	cambisol, litosol rubble rendzina	Limestone beech forest
Michalovo	49°00'43" 19°45'05"	1136	dolomitical rocks	cambisol, carbonate litosol	Limestone beech forest, relict pine forest on limestone
Pusté pole – E	48°53'05" 20°14'50"	914	sandy and gravelly sediments	cambisol, litosol rubble rendzina	Limestone beech forest
Pusté pole – W	48°53'16" 20°13'44"	990	sandy and gravelly sediments	cambisol, litosol rubble rendzina	Limestone beech forest
Silica	48°34'27" 20°33'12"	596	light steinalm limestones	histosol, stagnosol, fluvisol	Intermittently wet <i>Molinia</i> meadows
Suchá Belá	48°57'18" 20°22'46"	680	sandy and gravelly sediments	cambisol, litosol rubble rendzina	Limestone beech forest
Trsteník	48°48'36" 20°07'53"	860	sandy and gravelly sediments	histosol, stagnosol, fluvisol	Montane <i>Alnus incana</i> galleries, Alder swamp wood



**Fig. 2.** Ordination diagram of the studied *A. liliifolia* localities based on PCA analysis. Czech Republic – circle, Slovakia – square, Romania – up-triangle, Poland – diamond and Hungary – down-triangle. The first two axes explained 92% of the total variability. Bio. Reg.: Cont., Carp., Pan – biogeographical region in Europe: Continental, Carpathian, Panonian.



CHARACTERISTICS OF *A. LILIIFOLIA* HABITATS

The vegetation type in which *A. liliifolia* occurred was assessed using 43 phytosociological relevés from the surveyed population in Czechia (19 rel.), Poland (6 rel.), Slovakia (10 rel.), and Romania (8 rel.), 29 published relevés from Poland (7 rel.) by Ciosek (2006), from Hungary (20 rel.) by Farkas and Vojtkó (2012), and from Romania (2 rel.) by Indreica (2011), and 32 relevés from the TURBOVEG database for Czechia (Working Group for Vegetation Science, 2011; 3 rel.) and Slovakia (Working Group on Vegetation Research, 2012; 29 rel.). The nomenclature of the plant communities corresponds to that used in Chytrý (2007, 2013). The cover and the abundance of species was evaluated in the 9-grade Braun-Blanquet scale (Braun-Blanquet, 1964; Working Group for Vegetation Science, 2011). A synoptic table was made in JUICE 7 (Tichý, 2011) using the frequency percentage of a particular species. Only diagnostic species with a fidelity  $\geq 75\%$ , constant species with a frequency  $\geq 70\%$ , and dominant species with a cover threshold  $\geq 25\%$  are shown in Tab. 4. For each relevé, Ellenberg values for continentality (C), light (L), moisture (F), nutrient (N), soil reaction (R), and temperature (T) were excerpted using JUICE 7 (Tichý, 2011). Ellenberg indicator values were used to characterize the site conditions of 6 determined vegetation units in the STATISTICA 12 program (StatSoft, 2015).

## GENETIC ANALYSES

**Plant material and DNA extraction.** A total of 84 samples collected from 23 localities from five European countries (Tab. 5) were used for the genetic analyses. Each sample was represented by two leaves taken from one stem of a randomly selected tuft at each locality, and immediately preserved in plastic bags with silica gel until DNA extraction could be performed. Genomic DNA was extracted using a modified CTAB protocol (Doyle and Doyle, 1987). The integrity and quality of the extracted DNA was estimated using 1.5% agarose gel. The DNA concentrations were determined using a NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies, Delaware, USA).

**AFLP analysis.** AFLP analysis was carried out according to the procedure of Vos et al. (1995), with modifications according to Kitner et al. (2008). In total, eight selective primer combinations were chosen to generate the AFLP profiles (Tab. 2). The amplification products were separated on 6%, 0.4 mm-thick denaturing polyacrylamide gels (0.5×TBE buffer) using a T-REX sequencing gel electrophoresis apparatus (Thermo Scientific Owl Separation Systems, Rochester, NY, USA). As a size standard, 30-330-bp AFLP® DNA Ladder (Thermo Fischer Scientific) was used. Silver staining was used to detect the AFLP fragments after electrophoretic separation.

TABLE 2. Primers and primer sets for preamplification and amplification reactions with the total number of scored (NB) and polymorphic bands (NPB).

Preamplification Primers Sequence			
EcoRI 5' – G ACT GCG TAC CAA TTC A – 3'			
MseI 5' – G ATG AGT CCT GAG TAA C – 3'			
Amplification Primer Sets Sequences		N <sub>B</sub>	N <sub>PB</sub>
Set A	EcoRI primer E-GG / MseI primer M-AAC	41	31
Set B	EcoRI primer E-CC / MseI primer M-AAC	43	38
Set C	EcoRI primer E-CC / MseI primer M-AAT	49	44
Set D	EcoRI primer E-CG / MseI primer M-AAC	35	29
Set E	EcoRI primer E-CG / MseI primer M-AAT	42	36
Set F	EcoRI primer E-TCG / MseI primer M-AACG	22	17
Set G	EcoRI primer E-TCC / MseI primer M-AACG	26	20
Set H	EcoRI primer E-TC / MseI primer M-AACCG	33	27
total		291	242

## DATA ANALYSIS

To check the reliability of the AFLP analysis, the amplification for each primer combination with the whole sample set and, additionally, the amplification of randomly chosen samples (from two to three samples per each population) was repeated. The AFLP profiles were checked visually, and only clear and unambiguous bands were scored for their presence (1) or absence (0). In the last step, the results of scoring were compared and checked for the number of markers, intensity of the markers, and relative position of the markers. In the final binary matrix only verified markers (present in the original and repeated amplification) were used. The error rate was calculated as the difference in the total number and the number of fragments used in the final matrix. In order to determine whether genetic subpopulations can be detected in the analyzed sample set, the Bayesian approach was used as implemented in STRUCTURE 2.2 (Falush et al., 2007). Computation in STRUCTURE was set up for the recessive allele model and the admixture model with correlated allele frequencies. The  $K$  was set to 1–10 with 10 replicate runs for each  $K$  using the 1,000,000 MCMC iterations following the period of 100,000 burn-in iterations. For the graphical interpretation of clustering for the appropriate  $K$ , STRUCTURE HARVESTER (Earl and von Holdt, 2012), CLUMPP (Jacobsson and Rosenberg, 2007), and DISTRICT (Rosenberg, 2004) software were used. For the further visualization of the population genetic structure and relationships among individuals a Neighbor-joining (NJ) dendrogram was constructed [based on the Dice coefficient of similarity, 1,000 bootstrap replicates (Felsenstein, 1985)] using FREETREE software (Pavliček et al., 1999), and the resulting tree was visualised and arranged in FIGTREE v1.4.0 software (FIGTREE, 2015). The statistical indices for polymorphism ( $P\%$ ) and Shannon's Information Index ( $I$ ) were performed using GENALEX 6 software (Peakall and Smouse, 2006). The number of private bands ( $N_{PB}$ ; a band unique for a given population, but not for all individual), and the number of fixed private bands ( $N_{FPB}$ ; the number of bands common for all individuals within a single population) were calculated in FAMD 1.31 (Schlüter and Harris, 2006). The ARLEQUIN 3.5 (Excoffier and Lischer, 2010) was used for calculating the analysis of molecular variance (AMOVA) to inspect the partitioning and significance of the genetic variation distribution among and within the analyzed populations. AFLPdat (Ehrich, 2006) was used for the calculation of DW or "frequency-down-weighted marker" values according to Schönswetter and Tribsch (2005). DW values were used as a standardized measure of divergence and identification of long-term isolation. For the calcu-

lation of DW values no adjustment for the number of individuals was made, and DW values were calculated for all of the individuals within each population. AFLP-SURV 1.0 (Vekemans, 2002) (square root method) was used to assess the gene diversity under Hardy-Weinberg genotypic proportions ( $H_j$ ), also called Nei's gene diversity, the total gene diversity ( $H_t$ ), and fixation index ( $F_{ST}$ ). Correlation and regression analyses (to check the relationship between the obtained indices, population sizes, i.e., numbers of tufts and generative ramets), were computed in MS Excel add-in XLSTAT 2015 (Addinsoft, 2015), as well as the Mantel test to explore the hypothesis of isolation by distance (IBD) by examining the correlation between the matrices representing  $F_{ST}/(1-F_{ST})$  and the natural logarithm of geographic distance ( $\ln d$ ) for pairs of subpopulations (10,000 permutations). The regression analysis was also performed to provide the information about the linkage of the geographic position of localities (longitude) with polymorphism and gene diversity.

## RESULTS

### THE CURRENT CONDITION OF *A. LILIFOLIA* POPULATIONS

With respect to the population size in particular localities (number of tufts, number of fertile and sterile stems, and average number of stems in a tuft) the data are shown in Tab. 3. All of the Slovak localities (except for Michalovo and Silica) show a strong similarity based on a higher altitude, higher average annual precipitation, and *A. lilifolia* occurrence in forest vegetation units. Thus these localities represent a distinct group among other investigated localities (Fig. 2). The Romanian and the majority of the Czech localities are similar to one another. These localities represent non-forest habitats, or forest ecotones with higher than average annual temperatures. The Karlické valley and Vražba (CZ) are different, and their characteristics are close to the localities in Poland (Kisielany, Dąbrowa) and Hungary (Füzér, Regéc). Two localities – Babínské meadows (CZ) and Silica (SK) represent non-forest localities with higher average annual temperatures in comparison with the other localities.

### VEGETATION IN *A. LILIFOLIA* LOCALITIES

The linkage between *A. lilifolia* occurrence and the type of vegetation present in the locality can be seen in the synoptic table (Tab. 4). According to the analysis of all the recorded and published relevés (Ciosek, 2006; Farkas and Vojtkó, 2012; Indreica, 2011), six vegetation units in recent and historic

TABLE 3. Threat categories according to Red Lists of particular countries (CR – critically endangered, EN – endangered, VU – vulnerable), size of populations, morphological characteristics and number of species in phytosociological relevés in locality.

Locality	Threat in country	Size of population				Morphological characteristics					Number of species in a relevé
		Tufts	Stems	% fertile stems	Average number of stems/span in one tuft	Average height (cm)	Average number of leaves	Average ratio length/width leaf	Average number of flowers/infloresc.	Average number of branches/infloresc.	
<b>Czechia</b>											
Babínské meadows	CR	20	44	72.7	2.2/1–15	43	19.95	3.6	8.13	2.88	41–53
Bílchovské valley	CR	26	23	13	1.4/1–7	64.44	36.56	3.5	24.67	4.33	21–29
Karlícké valley	CR	22	26	23.1	1.2/1–3	33.5	19.44	2.7	5.33	0	58–60
Karlštejn	CR	22	63	84.1	2.9/1–6	91.78	36.37	3.2	46.89	9.68	38–81
Vražba	CR	83	200	96.5	2.4/1–14	98.04	32.71	3.5	48.18	9.34	38–64
<b>Hungary</b>											
Füzér	EN	18	22	31.8	1.2/1–2	34.42	19.7	2.6	16	3.33	30–42
Regéc	EN	38	62	17.7	1.6/1–4	26.08	15.97	2.3	4.9	0.5	16–49
<b>Poland</b>											
Dąbrowa	EN	53	76	73.7	1.4/1–8	missing data					39–58
Kisielany	EN	1000	1500	66.7	1.5/1–11	148.7	48	3.9	54	12	41–56
<b>Romania</b>											
Herculian	VU	9	18	22.2	2/1–5	48.83	24.05	4.3	10.75	4.29	31–42
Prejmer	VU	13	98	63.3	7.5/1–14	70.72	33.09	3.6	40.23	10.52	34–49
<b>Slovakia</b>											
Cigánka	EN	440	552	56.2	1.3/1–3	58.46	30.18	3.6	24.65	6.55	36–54
Kopanec	EN	8	15	100	1.8/1–3	85.2	33	3.1	18.5	3.1	49–54
Michalovo	EN	61	210	55.7	2.2/1–4	67.73	27.64	2.6	23.78	6.67	51–58
Pusté pole – E	EN	5	7	100	1.4/1–2	75.8	31	3.5	20	2.5	27–36
Pusté pole – W	EN	41	96	70.8	2.3/1–5	83.78	34.31	4.5	38.4	8.92	49–53
Silica	EN	38	60	11.7	1.6/1–5	44.83	21.34	3.4	14.75	4.8	36–43
Suchá Belá	EN	7	7	100	1/1–1	72.1	27	3.3	14.8	2.6	50–80
Trsteník	EN	343	474	49.8	1.4/1–6	93.37	27.93	3.6	31	7.64	47–57

localities of *A. liliifolia* were distinguished: 1) intermittently wet *Molinia* meadows (alliance *Molinion caeruleae* W. Koch 1926), 2) thermophilous oak forests (association *Potentillo albae-Quercetum* Libb. 1933), also Kiskőrös (HU), which is located in transition to alluvial forests, was ranked into

this unit, 3) Central European basiphilous thermophilous oak forests (alliance *Quercion pubescenti-petraeae* Klika 1933 corr. Moravec in Beg. et Theurill 1984), 4) oak-hornbeam forests (association *Tilio cordatae-Carpinetum betuli* Tracz. 1962), 5) limestone beech forests (association



TABLE 4. Synoptic table with 6 clusters using percentage frequency of species in the vegetation unit. Vegetation units: 1 – intermittently wet *Molinia* meadows, 2 – thermophilous oak forests (association *Potentillo albae-Quercetum*), 3 – Central European basiphilous thermophilous oak forests (alliance *Quercion pubescenti-petraeae*), 4 – oak-hornbeam forests (association *Tilio-Carpinetum*), 5 – limestone beech forests (association *Cephalanthero-Fagetum*), 6 – mosaic of montane *Alnus incana* galleries and alder swamp wood on basic and neutral substrate included in *Alnion incanae* alliance. Percentage of 70% and above in bold, except for unit 6 column, where percentage 100% in bold (only 2 relevés).

Vegetation unit	1	2	3	4	5	6
Number of relevés	24	22	8	19	30	2
<b>E3</b>						
<i>Quercus robur</i>		50		16		
<i>Carpinus betulus</i>		32		42		
<i>Quercus virgiliana</i>			63			
<i>Quercus petraea</i>				16		
<i>Fagus sylvatica</i>				26	50	
<i>Abies alba</i>				16	57	
<i>Alnus incana</i>						<b>100</b>
<i>Salix pentandra</i>						<b>100</b>
<b>E2</b>						
<i>Cornus mas</i>			<b>75</b>	5		
<i>Viburnum lantana</i>			<b>75</b>	11		
<i>Quercus petraea</i>	21	55	13	<b>74</b>	3	
<i>Corylus avellana</i>	4	45	13	<b>74</b>	13	
<i>Crataegus</i> sp.	8	64	13	26		
<i>Frangula alnus</i>	17	64		5	7	
<i>Alnus incana</i>						<b>100</b>
<i>Salix pentandra</i>						<b>100</b>
<b>E1</b>						
<i>Adenophora liliifolia</i>	<b>100</b>	<b>100</b>	<b>100</b>	<b>100</b>	<b>100</b>	<b>100</b>
<i>Betonica officinalis</i>	<b>83</b>	68	38	21		
<i>Molinia caerulea</i> s.l.	<b>83</b>	18				
<i>Convallaria majalis</i>	33	<b>86</b>		63	<b>80</b>	
<i>Lathyrus niger</i>	4	<b>77</b>	50	53		
<i>Carex montana</i>	25	<b>77</b>		5		
<i>Melittis melissophyllum</i>	4	<b>73</b>	13	58	13	
<i>Melica nutans</i>		64	25	<b>89</b>	57	50
<i>Brachypodium sylvaticum</i>	4	41	38	<b>74</b>		
<i>Carex digitata</i>	4	14		<b>74</b>	57	
<i>Aegopodium podagraria</i>	4	14		<b>74</b>	3	

TABLE 4.

Vegetation unit	1	2	3	4	5	6
Number of relevés	24	22	8	19	30	2
<i>Asarum europaeum</i>		5		<b>84</b>	23	
<i>Hepatica nobilis</i>		5		<b>89</b>		
<i>Pulmonaria obscura</i>		5		<b>79</b>		
<i>Calamagrostis varia</i>					<b>83</b>	<b>100</b>
<i>Galium schultesii</i>		41	25	16	<b>83</b>	
<i>Pimpinella major</i>	29			21	<b>70</b>	
<i>Laserpitium latifolium</i>		45			<b>80</b>	
<i>Rubus saxatilis</i>		14			<b>87</b>	
<i>Cirsium erisithales</i>					<b>77</b>	
<i>Carduus crassifolius</i> ssp. <i>glaucus</i>					<b>70</b>	
<i>Cruciata glabra</i>	54	9	38	5	7	50
<i>Ajuga reptans</i>	25	59		58	3	<b>100</b>
<i>Angelica sylvestris</i>	8	41		5	13	<b>100</b>
<i>Astrantia major</i>	54	55		37	40	<b>100</b>
<i>Lathyrus pratensis</i>	38	5		5	7	<b>100</b>
<i>Trollius europaeus</i>	17	14			3	<b>100</b>
<i>Colchicum autumnale</i>	38	5				50
<i>Succisa pratensis</i>	42	14			3	<b>100</b>
<i>Carex umbrosa</i>	13	9			3	50
<i>Deschampsia cespitosa</i>	4	5		21		<b>100</b>
<i>Leontodon hispidus</i>	33				17	50
<i>Thalictrum aquilegifolium</i>	4	27				<b>100</b>
<i>Listera ovata</i>		9			7	<b>100</b>
<i>Tanacetum clusii</i>					53	<b>100</b>
<i>Gentiana asclepiadea</i>					50	<b>100</b>
<i>Cirsium oleraceum</i>				37		<b>100</b>
<i>Carex paniculata</i>	17					<b>100</b>
<i>Carex panicea</i>	8					50
<i>Equisetum palustre</i>	8					<b>100</b>
<i>Gymnadenia conopsea</i>	8					<b>100</b>
<i>Filipendula ulmaria</i>	4					<b>100</b>
<i>Galium palustre</i>	4					50
<i>Knautia maxima</i>					10	<b>100</b>

TABLE 4.

Vegetation unit	1	2	3	4	5	6
Number of relevés	24	22	8	19	30	2
<i>Centaurea pseudophrygia</i>					3	100
<i>Carex davalliana</i>						50
<i>Carex flava</i>						100
<i>Valeriana simplicifolia</i>						100
<b>Juv.</b>						
<i>Acer campestre</i>		5	38	79		
<i>Alnus incana</i>						100
<b>E0</b>						
<i>Hylocomium splendens</i>	4				27	100
<i>Rhytidiadelphus</i> sp.					17	100
<i>Aulacomnium palustre</i>						100
<i>Climacium dendroides</i>						100

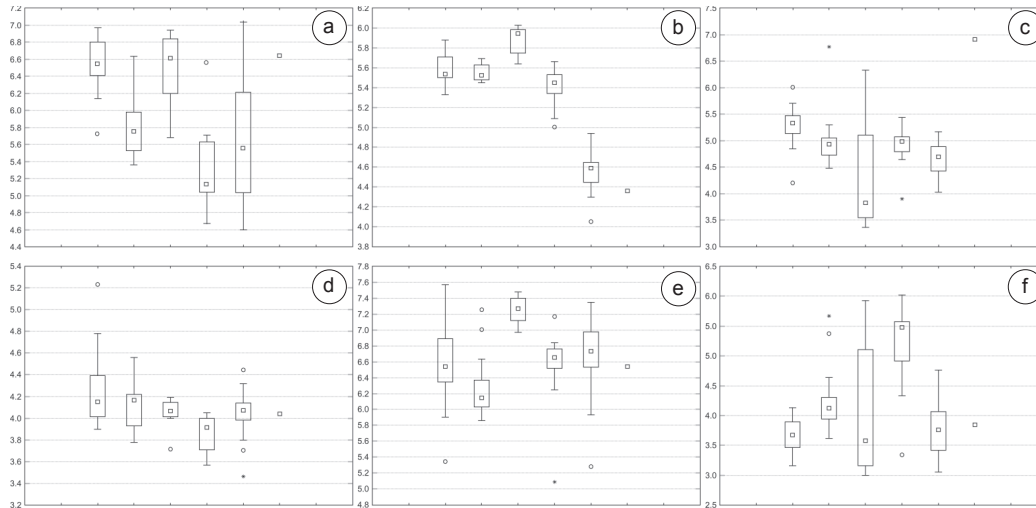
*Cephalanthero-Fagenion* R. Tx. 1955), 6) mosaic of montane *Alnus incana* galleries, montane fens with *Swertia perennis* from the *Caricion davallianae* Klika 1934 alliance and alder swamp wood on basic and neutral substrate included in the *Alnion glutinoso-incanae* Oberd. 1953 alliance (Tab. 4).

#### SITE CONDITIONS OF THE *A. LILIFOLIA* LOCALITIES

Site conditions of the six distinguished vegetation units with the presence of *A. lilifolia* were compared using Ellenberg's indicators (Ellenberg et al., 1992) for nutrients, soil reaction, temperature, light, moisture, and continentality (Fig. 3). When comparing the ecological demands of *A. lilifolia* according to Ellenberg et al. (1992) with the ecological conditions calculated from the phytosociological relevés using Ellenberg indicators (Fig. 3), we can say that the current optimal vegetation units for this species in Central Europe are Central European basiphilous thermophilous oak forests (alliance *Quercion pubescenti-petraeae*) and intermittently wet *Molinia* meadows. The light-, temperature-, moisture-, and soil reaction values calculated in these vegetation units are the most similar to the ecological demand of *A. lilifolia*. While intermittently wet *Molinia* meadows offer the most convenient conditions with respect to light, continentality, and moisture, Central European basiphilous thermo-

philous oak forests are most suitable for *A. lilifolia* with respect to temperature and soil reaction. Low moisture can be a limiting factor for this species in Central European basiphilous thermophilous oak forests. According to Ellenberg et al. (1992), the optimal value for nutrients is 2, but in all localities, this value was 3–6. Central European basiphilous thermophilous oak forests, which are comparatively the most convenient vegetation unit concerning nutrients, have the second widest amplitude with respect to this factor. The worst conditions for *A. lilifolia* were found in oak-hornbeam forests (association *Tilio cordatae-Carpinetum betuli*), limestone beech forests (association *Cephalanthero damassonii-Fagetum sylvaticae* Oberdorfer 1957), and mosaic of montane *Alnus incana* galleries and alder swamp wood, mainly because of low light intensity and temperature. In these habitats, *A. lilifolia* can only grow thanks to disturbances and management directed to an open forest.

*Adenophora lilifolia* shows a high morphological variability related to the geological bedrock, soil, moisture, and habitat in which it grows (Tab. 3). The investigated localities showed differences in species alpha-diversity. The most species-rich were two Czech localities (Vražba and Karlštejn) and three Slovak localities (Malý Sokol, Suchá Belá, and Michalovo). The fewest number of species were recorded in the Hungarian locality Regéc and in the Czech locality Bílichovské valley (Tab. 3).



**Fig. 3.** Comparison of 6 vegetation units (in this order: 1. *Molinion caeruleae*, 2. *Potentillo albae-Quercetum*, 3. *Quercion pubescenti-petraeae*, 4. *Carpinion betuli*, 5. *Cephalanthero damassonii-Fagetum sylvaticae*, 6. *Alnion glutinoso-incanae*) based on used relevés by means of Ellenberg indicator values (**a**) light, (**b**) temperature, (**c**) moisture, (**d**) continentality, (**e**) soil reaction, (**f**) nutrients).

#### MANAGEMENT IN *A. LILIIFOLIA* LOCALITIES

*Adenophora liliifolia* populations in Czechia are negatively influenced by many factors (e.g., overpopulated wild animals, inappropriate forest management, global eutrophication, expansive herbs, young trees, parasitic insects, and fungi). All Czech populations growing in forests, if not protected by fences against grazing, were browsed by overpopulated hoofed game. The level of grubbing out with respect to the underground organs was found to be increasing, especially in oak-hornbeam forests located at Karlštejn in Český kras PLA (Protected Landscape Area). The population in the Babinské meadows was negatively influenced not only by grazing and grubbing out, but also by expansion of *Calamagrostis epigejos* (L.) Roth. *Rubus* L. expansion also impacts *A. liliifolia* populations, mainly in oak-hornbeam forests. Not only the Czech localities, but also Trsteník in the Muránska planina NP (National park) in Slovakia and both of the Polish localities studied are affected by inappropriate forest management. Several Czech localities (Vražba, Bílichovské valley, and Karlštejn) have special management regulations protecting *A. liliifolia* against grazing by fences and against competitive vascular plants by cutting. Sheep pasturing occurs at the Silická planina in Slovenský kras NP (Silica, SK), and the *A. liliifolia* present at Silica is intensively grazed every year before it creates flowers and seeds. The most stable habitat for *A. liliifolia* populations is the limestone beech forest in the locality of Michalovo in Nízke Tatry NP. The plants grow there in slightly shaded parts of the forest, often

on rocky bedrock. They are rarely influenced by grazing, grubbing out, or by human activities, and their seeds have enough space for germination. The other localities (Slovenský raj NP, Muránska planina NP, Slovenský kras NP, Czech, Romanian, and Hungarian localities) are affected by grazing and grubbing out by wildlife.

#### GENETIC VARIABILITY

A total of 84 *A. liliifolia* plants from 23 populations were analyzed using eight AFLP primer combinations (Tab. 2), which generated 291 bands, of which 242 were polymorphic (83.2%). Replication of the analysis revealed high reliability of AFLP, with an error rate of 2.4%. Statistical indices (Tab. 5) were not computed for four localities which were represented by one or two samples. The highest values for Nei's gene diversity ( $H_j$ ) were observed for samples from Kopanec ( $H_j = 0.171$ ) and Pusté pole-E ( $H_j = 166$ ), both located in Slovakia. The lowest value was observed for the Czech population from Karlštejn ( $H_j = 105$ ). We recorded only a single fixed private band unique for populations located in Silica (SK) which was present among all of the sampled localities. The values for the DW index ranged from 1.7 to 4.2. The highest indices ( $DW = 4.2$ ) were observed for the Slovak populations located at Suchá Belá and Pusté pole E, followed by the Hungarian locality at Ocsa ( $DW = 3.9$ ). The lowest values were recorded for the Czech populations at Karlštejn ( $DW = 1.8$ ) and the Bílichovské valley ( $DW = 1.7$ ). The computation of Shannon's Information Index produced the lowest value for the

TABLE 5. List of analysed samples with population genetic statistics (n, number of samples;  $N_{PB}$ , number of private bands;  $N_{FPB}$ , number of fixed private bands; P%, polymorphism; I, Shannon's Information Index;  $H_j$ , Nei's gene diversity; DW, frequency down — weighed marker value; SE, sum of errors)

Site	ID of samples	n	$N_{PB}$	$N_{FPB}$	P%	I (SE)	$H_j$ (SE)	DW
<b>Czechia</b>		<b>18</b>	<b>2</b>	<b>0</b>	<b>56.1</b>	<b>0.194 (0.013)</b>	<b>0.128 (0.009)</b>	<b>2.3</b>
Babínské meadows	1–4	4	0	0	28.2	0.142 (0.014)	0.137 (0.010)	2.8
Bílichovské valley	5–8	4	0	0	25.9	0.131 (0.014)	0.127 (0.010)	1.7
Karlické valley	9–10	2	—	—	—	—	—	—
Karlštejn	11–13	3	0	0	18.5	0.087 (0.012)	0.105 (0.009)	1.8
Vražba	14–18	5	2	0	32.4	0.154 (0.014)	0.137 (0.010)	2.8
<b>Hungary</b>		<b>21</b>	<b>5</b>	<b>0</b>	<b>64.4</b>	<b>0.229 (0.013)</b>	<b>0.149 (0.009)</b>	<b>3.1</b>
Aggtelek	19	1	—	—	—	—	—	—
Dabas	20–21	2	—	—	—	—	—	—
Füzér	22–26	5	0	0	28.2	0.148 (0.015)	0.137 (0.011)	3.0
Kiskőrös	27–31	5	0	0	31.7	0.148 (0.014)	0.132 (0.009)	3.3
Ocsa	32–34	3	0	0	24.2	0.122 (0.013)	0.148 (0.010)	3.9
Regéc	35–39	5	0	0	37.2	0.177 (0.014)	0.152 (0.010)	3.0
<b>Poland</b>		<b>6</b>	<b>0</b>	<b>0</b>	<b>38.6</b>	<b>0.167 (0.014)</b>	<b>0.132 (0.009)</b>	<b>2.3</b>
Dąbrowa	40	1	—	—	—	—	—	—
Kisielany	41–45	5	0	0	36.2	0.168 (0.014)	0.141 (0.010)	2.6
<b>Romania</b>		<b>10</b>	<b>1</b>	<b>0</b>	<b>48.2</b>	<b>0.181 (0.013)</b>	<b>0.128 (0.009)</b>	<b>3.1</b>
Herculian	46–50	5	0	0	37.8	0.157 (0.014)	0.133 (0.010)	2.9
Prejmer	51–55	5	0	0	32.1	0.150 (0.014)	0.131 (0.009)	3.5
<b>Slovakia</b>		<b>29</b>	<b>14</b>	<b>0</b>	<b>68.8</b>	<b>0.229 (0.013)</b>	<b>0.144 (0.009)</b>	<b>3.1</b>
Cigánka	56–60	5	0	0	29.6	0.144 (0.014)	0.127 (0.010)	2.3
Kopanec	61–63	3	0	0	25.4	0.161 (0.015)	0.171 (0.011)	3.2
Michalovo	64–68	5	0	0	30.3	0.138 (0.013)	0.122 (0.009)	2.5
Pusté pole — E	69–71	3	0	0	26.5	0.146 (0.015)	0.166 (0.011)	4.2
Pusté pole — W	72–74	3	0	0	18.9	0.106 (0.013)	0.131 (0.010)	3.3
Silica	75–78	4	1	1	20.6	0.105 (0.013)	0.115 (0.010)	3.5
Suchá Belá	79–81	3	0	0	29.6	0.139 (0.014)	0.160 (0.011)	4.2
Trsteník	82–84	3	0	0	20.6	0.113 (0.013)	0.135 (0.011)	2.4
<b>total mean (populations separately)</b>					<b>27.6</b>	<b>0.139 (0.014)</b>	<b>0.144 (0.009)</b>	<b>3.0</b>



Czech locality at Karlštejn ( $I = 0.087$ ) and the highest value was computed for the Hungarian population from Regéc ( $I = 0.177$ ). The total gene diversity ( $H_t$ ) was 0.157.

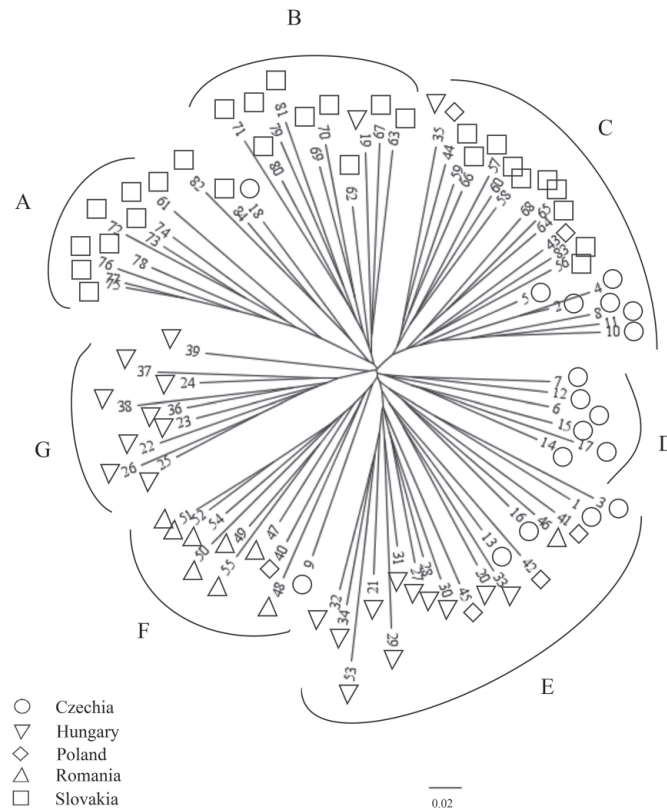
#### POPULATION GENETIC STRUCTURE

The Neighbor-Joining clustering analysis divided the *A. liliifolia* samples into seven main clades (Groups A–G; Fig. 4), where clustering of the samples was not strictly associated with their geographical origin and only a weak bootstrap (i.e., values below 15, not shown) support was recorded.

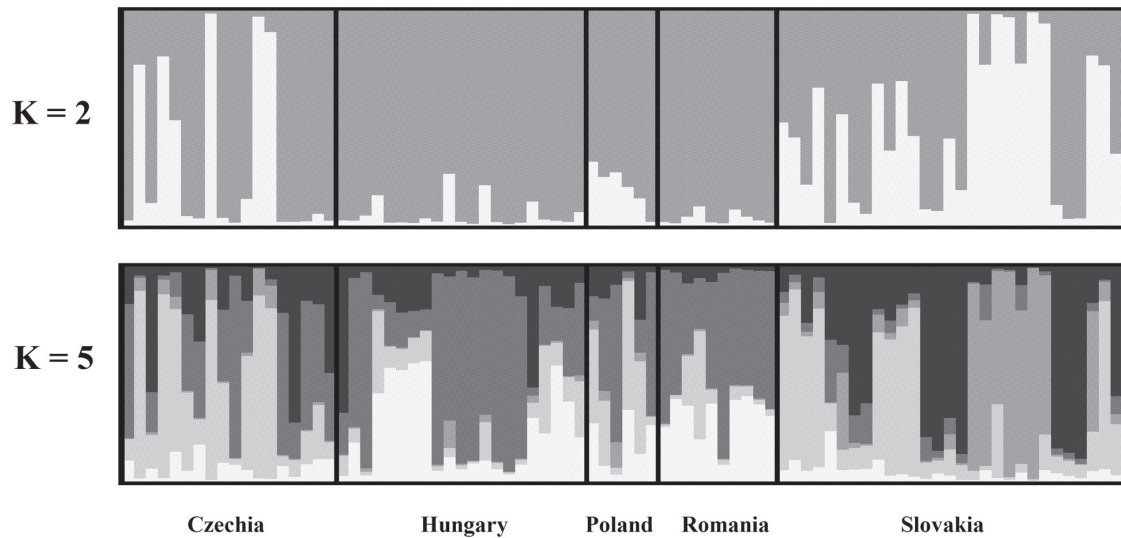
Nevertheless, a certain level of clustering which might be linked with the geographical origin can be observed on the NJ tree. All of the samples from Slovakia appeared in three groups (A–C). Group A represents samples solely from South (Silica) and Central Slovakia. Group B consists of samples from several Slovak localities and one outlying Hungarian sample (Aggtelek). Two samples from Trsteník (SK) together with one sample from Vražba (CZ) are located between Groups A and B. Two samples from Poland, one sample from Regéc (HU), and six samples from Czechia were

mixed with samples which originated mainly from Michalovo and Cigánka (SK) and formed Group C. Group D is separated from the previous groups and is represented only by six Czech samples, while the remaining 5 samples from Czechia are located in Group E (4 samples) and F (one sample). All of the samples from South Hungary, together with two Romanian and two Polish samples are present in Group E. Eight out of ten remaining Romanian samples were present in Group F. The samples from North Hungary (Füzér, Regéc) fell into a separate group, Group G (Fig. 4). The Polish samples were spread through the NJ tree in groups C (2 samples), E (3 samples), and F (one sample).

Further analysis of the population genetic structure by Bayesian clustering implemented in STRUCTURE suggested a subdivision into two or five clusters (maximum value  $\Delta K = 57.627$  for  $K = 2$  and  $\Delta K = 16.419$  for  $K = 5$ ) (Fig. 5). Bayesian clustering for  $K = 2$  stressed the genetic differences in the Slovakian populations from Slovenský kras NP (Slovakian karst, Silica) and Pusté pole (W), while all of the remaining samples were highly similar. Subdivision into five groups ( $K = 5$ ) basically reflects the results of the NJ clustering: i) a unique group of



**Fig. 4.** Unrooted Neighbour-joining dendrogram (based on 291 AFLP markers and Dice similarity matrix) of 84 *Adenophora liliifolia* samples from five European countries. Putative groups are designed by capital letters A–G.



**Fig. 5.** Results of the STRUCTURE analysis of 84 *A. lilifolia* samples showing results for  $K = 2$  and  $K = 5$ . Each vertical bar represents one individual with the color representing the probability of assignment to different clusters. The origin of the populations is displayed below the graphics.

Slovakian samples (roughly equal to Group A on the NJ tree:  $\sim$ NJ<sup>Group A</sup>); ii) the remaining Slovakian samples sharing a similar genetic background with some of the Czech and Polish samples ( $\sim$ NJ<sup>Groups B,C,D</sup>); iii) separation of samples from North Hungary ( $\sim$ NJ<sup>Group G</sup>); iiiii) similarity of the South Hungarian and Romanian samples with some samples from Poland and Czechia ( $\sim$ NJ<sup>Groups F,E</sup>) (Fig. 5).

The AMOVA computations revealed that 22% of the total genetic variation represents differences among the populations, while 78% is related to the genetic variation among plants within populations, with  $F_{ST} = 0.125$ . The Mantel test revealed no significant correlation between geographical distance and genetic distance or pairwise  $F_{ST}$  (i.e., lack of genetic isolation by distance;  $r = -0.141$ ,  $P = 0.1580$ ), except slightly positive correlations (not significant) of the geographic position of localities (longitude) with polymorphism ( $r = 0.466$ ,  $P = 0.217$ ) or genetic diversity ( $r = 0.426$ ,  $P = 0.182$ ).

## DISCUSSION

### POPULATIONS, SITE CONDITIONS, AND VEGETATION

Our survey of *A. lilifolia* populations in Central Europe revealed the decline in number of its localities in all of the investigated countries. Comparison of the present survey with historical data from the 19th and early 20th century showed that in the past *A. lilifolia* had occurred in 20 locali-

ties within the phytogeographical district of Czech Thermophyticum, and in 6 localities within the Mesophyticum. At present, the species occurs very rarely in 5 localities of the Czech Thermophyticum (Kovanda, 2000; Prausová and Truhlářová, 2009). In Slovakia, the species is still present in both of the phytogeographical districts of Pannonicum and Carpathicum (Goliášová and Šípošová, 2008). Currently, *A. lilifolia* grows only in about 10 localities in the Carpathians and their foothills, while historical data describe about 30 former populations in this territory. In Pannonicum *A. lilifolia* occurs in the Slovenský kras NP (only one verified locality near the Silica village). In Poland, this species was previously found in circa 100 localities within all of the phytogeographical provinces, but nowadays it is only known to occur in approximately 21–22 of them. Their location in the central and the north-eastern part of the country represents the current northern distribution border of *A. lilifolia* in Europe (Pawłowska, 1972; Ciosek, 2006; Kucharczyk, 2007; Piękoś-Mirkowa, 2008; Rapa, 2012; Kucharczyk et al., 2014; Kapler et al., 2015). Similarly, in Romania, *Adenophora* is only recorded in 6 out of 34 former localities and in two newly found localities in Transylvania (Indreica, 2011). In Hungary, it is recorded in 7 out of 30 former localities (Farkas and Vojtkó, 2012, 2013). In the Balkan Peninsula, in the former Socialist Federal Republic of Yugoslavia, the species remains common in river valleys, but is supposedly extinct in many localities (Acetto, 2007).

The highest number of *A. lilifolia* tufts per population were observed in the Slovak localities in

Muránska planina NP (Cigánka, Trsteník), followed by the Czech locality of Vražba (Tab. 3). This is probably related to the remoteness of these Slovak localities from populated areas and to appropriate management at the locality Vražba (CZ). *A. liliifolia* shows a high morphological variability related to the site conditions and habitat in which it grows. The tallest individuals were found in oak-hornbeam and beech forests, the smallest individuals were found in meadows (Regéc, Babinské meadows) and also in the Karlické valley which is influenced by inappropriate forest management, where young plants of *A. liliifolia* have been overgrown by juvenile trees and shrubs. The number of stems in a tuft varied from 1 (common at several localities) to 20 (Prejmer, RO). A single stem occurrence was most common in the Karlické valley (CZ), Fűzér (HU), and at Suchá Belá (SK), where most of the stems were sterile. The most species rich biotopes were observed at the Czech localities Vražba and Karlštejn, and the Slovak localities at Suchá Belá and Michalovo.

According to the Ellenberg indicators (Ellenberg et al., 1992), *A. liliifolia* is classified as a heliophilous, thermophilous, and basiphilous species requiring enough moisture and with little to no demand for nitrogen. On the contrary, many current localities (mainly Czech, Polish, and several historical Slovak localities) have a higher content of nutrients that support the development and spread of nitrophilous species including *Aegopodium podagraria* L., *Urtica dioica* L., and *Stachys sylvatica* L. which have become important competitors of *A. liliifolia*. It is assumed that basiphilous and mesotrophic thermophilous oak forests previously grew in these localities, but that they changed into mesophilous oak-hornbeam forests due to eutrophication (Müllerová et al., 2015) and missing disturbances like pasturage. The soils in the localities of Herculan (RO), Silica (SK), and the Babinské meadows (CZ) were found to have the highest pH of all of the studied localities because of both calcareous substrate and the greatest nutrient content resulting from a rapid humification process. Our finding of high soil pH in most of the current localities corresponds with other data about the occurrence of *A. liliifolia* on calcareous rocks in beech and pine forests, and also in subalpine grasslands in Slovenia (Babij, 2004; Acetto, 2007). In Switzerland (Moser, 1999) and Slovakia (Dražil, 2002), the species grows in calciphilous beech forests (association *Cephalanthero damassonii-Fagetum sylvaticae*). In Hungary (Siklósi, 1984; Farkas and Vojtkó, 2012, 2013), *A. liliifolia* was found in riparian forests (association *Fraxino pannonicae-Ulmetum glabrae* Aszód 1935 corr. Soó 1963).

Roleček (2007) states that *A. liliifolia* belongs to heliophilous species of subcontinental oak for-

ests which grow in climatically non-extreme sites. It has a limited ability for long distance dispersal and successional changes from subcontinental oak forests to oak-hornbeam forest or shady mixed oak forests do not facilitate its spreading. It is thought that *A. liliifolia* could survive in light oak coppices and grazed forests of lower elevations, and also in light forests at higher altitudes, mainly on rocky outcrops, in erosion-prone sites, areas influenced by the grazing of wild animals, and thus generally in various forest ecotones. The distribution of this species followed continually changing light conditions in forests. Válek (Válek in Rohlena and Dostál 1936) provided information about hundreds of *A. liliifolia* individuals that had reappeared in the locality of Vražba (Czechia) at clearings in mixed forests containing spruce after an attack of the moth *Lymantria monacha* L. The previously shady forest was then replaced temporarily by a non-forest or open forest area. Roleček (2007) states that the best conditions for this species are in the S Ural and in SW Siberia, where the continental climate prevents broadleaf, mesophilous trees and shrubs from extension and where *A. liliifolia* grows in the hemiboreal forests of *Brachypodio pinnati-Betuletea pendulae* Ermakov, Korolyuk & Latchinsky 1991 (Ermakov et al., 1991; Ermakov and Maltseva, 1999; Chytrý et al., 2012). Central European forest habitats have changed a lot since the Preboreal and Boreal period. Once light Preboreal and Boreal forests were later massively replaced by deciduous forests with more closed canopies in Central Europe (Kaplan, 2012). Open forests containing heliophilous trees like *Pinus sylvestris* L., *Betula pendula* Roth., and *Larix decidua* Mill. supposedly occurred in Central Europe and SW Siberia about 9,500 years BC; they disappeared from Central Europe due to climate changes and human activities. According to Roleček (2007), there are only fragments of relict hemiboreal forests surviving in Central Europe today. Martynenko (2009) designates the S Ural region as the easternmost part of the distribution range of thermophilous oak forests of class *Quercetea pubescentis* Doing Kraft ex Scamoni et Passarge 1959, namely *Lathyro-Quercion roboris* Solomeshch et al. 1989 nom. inval. alliance. According to Roleček et al. (2015), similar vegetation to that present in the S Ural region appeared in Central Europe after the expansion of oak during the Boreal period, and in suitable places it could resist, although the degree of climate oceanicity increased and highly competitive wood species expanded (*Fagus sylvatica* L., *Carpinus betulus* L.). This relict vegetation shelters rare species with disjunct distribution, such as *A. liliifolia*, *Veratrum nigrum* L., and *Dracocephalum ruyschiana* L. Patches of light oak or oak-pine forests and forest-meadow eco-



tones, which are suitable secondary habitats for light-demanding, often basiphilous species, were established by human activities in the Middle Ages. Therefore, *A. liliifolia* survives in Central Europe mainly in the light edges of forests and in intermittently wet *Molinia* meadows (Roleček, 2007). The current meadow vegetation of the *A. liliifolia* locality in the Central Bohemian Uplands PLA is considered as a relict of forest-steppe vegetation which prevailed in this area in the past and was later influenced by prehistoric settlement, agriculture, gradual overgrowing, and eutrophication. Despite these changes, a high level of biodiversity and species of the relict mesic sites vegetation remain here (e.g., *A. liliifolia*, *Potentilla alba* L., *Serratula tinctoria* L.). *Adenophora liliifolia* was also observed in the shrub association of alliances *Berberidion* Br.-Bl. 1950 and *Prunetalia spinosae* R.Tx. 1952, in *Peucedano cervariae-Coryletum* Kozł. 1925 em. Medw.-Korn. 1952 scrub (Ciosek, 2006; Kovanda, 2000; Kapler et al., 2015), in sunny patches at forest edges of *Tilio cordatae-Carpinetum betuli* (Kapler et al., 2015), and in mesic meadows (association *Anthyllido-Festucetum rubrae* Soó, 1971) (Farkas and Vojtkó, 2012, 2013). Hungarian relevés from Regéc and Fűzér were identified as the association of *Nardo-Molinietum hungaricae* (Kovács 1962) Borhidi 2001 (Farkas and Vojtkó, 2013). In Poland, *A. liliifolia* at its north-easternmost localities (Czarna Białostocka and Dobry Lasek) also grows in a mosaic of ruderal and segetal communities, often with ecotones of thermophilous scrubs and light spruce-pine-oakwoods (Kapler et al., 2015).

#### GENETIC VARIABILITY AND POPULATION GENETIC STRUCTURE

To compare the results of our genetic analysis we searched for studies dedicated to plants with an Euro-Siberian distribution similar to *A. liliifolia*. Two different species of *Stipa* L. (Poaceae) were studied by the AFLP method, and populations from their periphery in Central Europe were compared with populations within their main distribution area in Russia (Wagner et al., 2011, 2012). In both cases, the authors did not find any relationship between the size of the population (represented by the number of plants) and the observed genetic diversity, similar to the results of the present study. Nevertheless, this statement has to be proved in the future, due to the low number of analyzed individuals used in our study. In populations of *Stipa pennata* L., the genetic diversity declined significantly from the distribution's center to its periphery (Wagner et al., 2012). The same trend seems also to be present for *A. liliifolia*, as we observed only slight correlations between the geographi-

cal position of populations (increasing longitude), polymorphism, and heterozygosity (although not significant). For populations of *Stipa capillata* L., low values of polymorphism were found both in its distributional center (21.9%) and on its periphery (20.0%), values that are close to the value of polymorphism detected within the present study (average  $P = 27.6\%$  for the analyzed populations, Tab. 5). Similar results (low polymorphism both in the peripheral and the central populations) were observed in the relict steppe species *Iris aphylla* L. (Wróblewska, 2008). Another example of a species with a similar Euro-Siberian distribution is *Ligularia sibirica* L., whose populations from the Czechia and Slovakia were analyzed by Šmídová et al. (2011) using allozyme analysis. Similar to our study, the results of the investigated populations of *L. sibirica* showed high genetic diversity within populations (80.8%) and a lower level of genetic differentiation between populations ( $F_{ST} = 0.179$ ). Contrary to the results of our study, the genetic distance between populations correlated significantly with the geographic distance, and there was also a significant positive correlation between genetic diversity and population size. However, Šmídová et al. (2011) used codominant allozyme markers (tracing variation in proteins), which generally detect a lower level of genetic variation, contrary to the dominant and highly variable AFLP markers utilized in this study, which allow the direct examination of DNA sequence variation.

There are only a few studies which investigated the genetic diversity of populations of *Adenophora* spp., including two isozyme based studies by Ge et al. (1999) and Chung and Epperson (1999); and two more recent studies based on ISSR markers by Boronnikova (2009) and Manole et al. (2015). Boronnikova (2009) analyzed four *A. liliifolia* populations from the Perm region in Russia using ISSR markers, and detected (similarly to our study) a weak population genetic structure and high intrapopulation variation (nearly 84.5% of the total variation). The expected heterozygosity values ranged from 0.159 to 0.275, with a mean  $H_E = 0.228$ . These values are twice as great as the gene diversity values detected in the present study. This can be influenced by a) the greater number of sampled plants per population in the study by Boronnikova (2009); and b) the fact that the investigated populations were geographically closer to the species' center of distribution, thus possessing a greater degree of genetic variability. A recent study by Manole et al. (2015) investigated 12 mature specimens of *A. liliifolia* from one Romanian population using ISSR markers. Also in this study, a relatively high intrapopulation genetic variation was observed as measured by Shannon's index of genotypic diversity (0.812), contrary to our calculations (mean

value of  $I = 0.139$ , which may be caused by the different type of molecular markers used and/or the different sample size). Ge et al. (1999) investigated two *Adenophora* species in China – the endangered *A. lobophylla* D.Y. Hong and its widespread relative *A. potaninii* Korsh. For these two species, the differentiation among the investigated populations was higher among *A. potaninii* populations ( $F_{ST} = 0.155$ ) than among *A. lobophylla* populations ( $F_{ST} = 0.071$ ). The  $F_{ST}$  value obtained for *A. liliifolia* was 0.125, which is quite close to the results for *A. potaninii*. This result is probably due to the similar geographical distances between the most remote localities of *A. potaninii* and *A. liliifolia* (ca 850 and 1000 km respectively), contrary to the weak population genetic structure detected among the *A. lobophylla* populations, which were located at a maximum distance of 25 km from each other. In Korea, with respect to the endemic *Hanabusaya asiatica* Nakai (a genus closely related to *Adenophora*), there is an apparent pattern of isolation by distance among the assessed populations. Despite the fact that the allozyme differentiation among the populations is low ( $C_{ST} = 0.132$ ), the species maintains a high allozyme diversity ( $H_{ES} = 0.217$ ) (Chung et al., 2001). In *A. liliifolia* populations, we failed to find a significant correlation between geographical distance and genetic distance or pairwise  $F_{ST}$ .

*Adenophora liliifolia* is a species with prevailing sexual reproduction and regular generation of viable seeds (personal observation), which has a scattered occurrence in Europe and inhabits different habitats (in terms of abiotic conditions, see text above). The longest distance between the studied localities was more than 1000 km [between the Babínské meadows (CZ) and Prejmer (RO)] and our field observations showed morphological differences between the localities (e.g., shape of the leaves, presence or absence and different lengths of the leaf petioles, color of the corolla). Based on these observations, we initially expected to observe a clearly resolved inter-population genetic structure; however, this is not what was found.

The relatively high genetic diversity value obtained and the results of the AMOVA analysis showed that the majority of the genetic variation is present within populations. This pattern of genetic variability distribution may be due to vigorous sexual reproduction, which dominates over vegetative spreading within *A. liliifolia* populations (Manole et al., 2015). Furthermore, the low number of unique markers accompanied by the almost absolute absence of fixed-private markers, low  $F_{ST}$  and DW values, and weak inter-population genetic structure suggest that the separation of the analyzed populations took place only sub-recently, because of the short time for population differentiation by genetic

drift, which would result in the detection of a clear population genetic structure. On the contrary, we detected high overall variation and high similarity of the sampled populations, suggesting frequent gene-flow among populations. This, however, seems unlikely, due to the considerable geographical distance between the sampled localities utilized in this study. Our results rather suggest that there was a large meta-population of *A. liliifolia* in the Central European area, which has fragmented relatively recently into the isolated populations present today.

Nevertheless, some populations in the Pannonian Biogeographic Region (in Romania, South Hungary, and Slovak populations in Silica and Pusté pole (W) are genetically more different from the other remaining populations, thus these might have been isolated for a longer period of time.

#### CONSERVATION OF *A. LILIIFOLIA*

The conservation of *A. liliifolia* strongly depends on the specific management supporting its seedlings, which are not vigorous enough to survive without protection (e.g., by removing invasive or nitrophilous species; see e.g., Ciosek, 2006; Manole et al., 2015). Such management may be difficult because of the presence of other protected species, thus it should be planned with respect to the whole locality, not only to a single species.

Recently a conservation program for *A. liliifolia* was started in Czechia with the aim to find the most successful and efficient way of management for each of the present localities. It aims not only at preserving the natural populations *in situ* but also at developing appropriate techniques for *ex situ* preservation (creation of a sterile tissue culture, appropriate storage of seeds in seed banks, experimental germination tests and cultivation in order to identify the critical factors for seedling growth). The other countries of Central Europe suggested a similar approach to protect this species, although without the official funded conservation programs (e.g., Kucharczyk, 2007; Puchalski et al., 2014; Manole et al., 2015).

#### CONCLUSION

This study provides overall information about the present condition of *Adenophora liliifolia* populations in several countries located in Central Europe, combining molecular data with the results of a phytosociological survey. *A. liliifolia* was found in 6 vegetation units, where it prefers sunny places with moist alkaline soil. The richest populations are in the Polish locality of Kieselany and two Slovak localities – Trsteník and Cigánka (both in Muránska



planina NP). The greatest numbers of species in the phytosociological relevés were recorded in the Czech localities of Vražba and Karlštejn and in three Slovak localities – Malý Sokol, Suchá Belá, and Michalovo. Light forests and their edges are optimal biotopes for *A. lilifolia*. Eutrophication, shading, overpopulated wild animals, and expansive broad-leaved herbs are the main factors causing the decline of *A. lilifolia* populations in Central Europe. Despite the fact that the majority of the investigated populations (except for Slovakia) are rather isolated and geographically distant from each other, our results indicate high interpopulation homogeneity, typical for populations with extensive gene flow. The lack of stronger interpopulation differentiation can be explained by the relatively recent fragmentation of a larger population due to shrinking of suitable habitats, their disappearance, or overall changes in landscape management. The findings of the present study show that *A. lilifolia* populations are not primarily threatened by loss of genetic diversity, but are endangered by loss of suitable habitats. Therefore, a specific management strategy is necessary in most of the localities.

#### AUTHORS' CONTRIBUTIONS

Prausová – phytosociological research and analysis in Juice, monitoring of the Czech and Slovak populations; Marečková – monitoring of the Czech and Slovak populations, genetic analysis; Kapler – phytosociological research and monitoring of the Polish populations; Majeský – genetic analysis; Farkas – phytosociological research and monitoring of the Hungarian populations; Indreica – phytosociological research and monitoring of the Romanian populations; Šafářová – ecological statistical analysis in Canoco; Kitner – genetic analysis.

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