Palacký University Olomouc

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Adenophora liliifolia - present, past and future of the critically endangered species in Europe

Ph.D. Thesis

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Abstract:

Adenophora liliifolia (L.) A. DC is the only representative of the numerous Asian genus *Adenophora* in Europe, where it represents the European-South Siberian floristic element and a probable relic of the late glacial - early Holocene. The distribution of the species extends from southwestern Siberia and the Ural Mountains in the east to Western Europe, where the populations are highly fragmented. The species is still relatively abundant in the Carpathians (Slovakia), but population numbers in Europe are gradually declining. At present, *A. liliifolia* has the status of an endangered or critically endangered species in most countries of its occurrence.

In my dissertation thesis, I dealt mainly with the study of genetic variability and the interrelationships of European populations. My goal was also to try to answer how and when a species migrated from its Asian center to Europe and whether it could survive the cold Pleistocene in refugia located in Central Europe. I used two types of molecular markers to answer these questions - AFLP, and sequencing of selected regions of chloroplast and nuclear DNA. As an auxiliary method for interpreting genetic data, I used the prediction of suitable habitats in different periods using the MaxEnt algorithm. The presented work also includes information on the populations' size, habitat conditions, and vegetation units in which the species grows in Central Europe, with detailed information on localities in the Czech Republic.

The results of my work support the previous expectation that *A. liliifolia* most probably comes from the area of southwestern Siberia and the Urals, from where it later spread to Europe. However, a closer determination of the period in which this process occurred was not possible due to the overall low diversity of sequenced loci. The identified centers of increased haplotype diversity in the Western Carpathians, the Southeastern Carpathians, the Alps, and the Dinaric Mountains may have served as glacial refugia of the species. Other European populations are characterised by a significant predominance of a single haplotype lineage, which probably represents the lineage best adapted to the early Holocene Central European landscape conditions. The genetic diversity of European populations is rather at a medium level; a more pronounced population genetic structure can be observed among geographically distant populations. This, together with the longevity of the plants, may indicate the fragmentation of populations that have been interconnected in the relatively recent past. The process of fragmentation is most likely related to human activity, leading to the loss of habitats suitable for the species survival. Thus, it can be stated that *A. liliifolia* is not primarily endangered by the loss of genetic diversity but rather, as field observations suggest, the loss of suitable habitats. The results of this work also identify several areas in Europe that should be given priority in the implementation of conservation measures in terms of preserving genetic diversity.

Keywords: *Adenophora liliifolia*, critically endangered species, NATURA 2000, glacial refugium, species distribution modeling
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Abstrakt:

Adenophora liliifolia (L.) A. DC (zvonovec liliolistý) je jediný zástupce početného asijského rodu *Adenophora* v Evropě, kde představuje evropsko-jihosibiřský floristický element a pravděpodobný relikt pozdního glaciálu – raného Holocénu. Celkový areál druhu sahá od jihozápadní Sibiře a pohoří Ural na východě po západní Evropu, kde jsou populace značně fragmentované. Druh je stále poměrně hojný v Karpatech (Slovensko), ale počty populací v Evropě klesají. V současnosti má zvonovec liliolistý ve většině zemí svého výskytu status ohroženého nebo kriticky ohroženého druhu.

Ve své disertační práci jsem se zabývala zejména studiem genetické variability a vzájemných vztahů evropských populací. Mým cílem bylo také pokusit se odpovědět na otázky jak a kdy druh migroval ze svého asijského centra do Evropy a zda mohl přežít chladné období Pleistocénu v refugiích lokalizovaných ve Střední Evropě. K zodpovězení těchto otázek jsem použila dva typy molekulárních markerů – metodu AFLP a sekvenování vybraných regionů chloroplastové a jaderné DNA. Jako pomocnou metodu pro interpretaci genetických dat jsem použila predikci vhodných stanovišť v různých časových obdobích pomocí algoritmu MaxEnt. Součástí předložené práce jsou také informace o velikostech populací, podmínkách stanovišt a vegetačních jednotkách, ve kterých zvonovec liliolistý roste ve střední Evropě, s detailními informacemi o lokalitách v České republice.

Výsledky mé práce podporují předchozí tvrzení, že zvonovec liliolistý nejpravděpodobněji pochází z oblasti jihozápadní Sibiře a Uralu, odkud se později rozšířil do Evropy. Bližší určení období, ve kterém k tomuto procesu došlo, však nebylo kvůli celkově nízké diverzitě sekvenovaných lokusů možné stanovit. Evropské populace se vyznačují výraznou převahou jediné haplotypové linie, která pravděpodobně představuje linii nejlépe adaptovanou na podmínky raně-holocénní středoevropské krajiny. Výjimku představují tři oblasti se zvýšenou haplotypovou diversitou (západní Karpaty, jihovýchodní Karpat, a oblast Alp a Dinárských hor), které mohly představovat glaciální refugia druhu. Genetická diverzita evropských populací je spíše na střední úrovni, výraznější populačně genetickou strukturu lze pozorovat mezi geograficky vzdálenějšími populacemi. To může poukazovat na započatou fragmentaci populací, které byly propojeny ještě v relativně nedávné minulosti. Rozdíly mezi populacemi jsou stírány i díky vnitropopulační genetické variabilitě, která je udržována a generována kvetoucími dlouhověkými rostlinami. Proces fragmentace tedy nejpravděpodobněji souvisí s činností člověka, která vede k úbytku stanovišť vhodných pro přežití druhu. Lze tedy konstatovat, že zvonovec liliolistý není primárně ohrožen ztrátou genetické diverzity, ale především ztrátou vhodných stanovišť. Výsledky této práce také identifikují několik oblastí v Evropě, které by z hlediska zachování genetické diverzity měly mít prioritu při zavádění ochranářských opatření.

Klíčová slova: *Adenophora liliifolia*, kriticky ohrožený druh, NATURA 2000, glaciální refugium, modelování distribuce druhu
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Jazyk práce: Anglický

Declaration

I hereby declare that this thesis has been formulated by myself together with listed co-authors. All literary sources cited in this thesis are listed in the References section.

In Olomouc, ……………………………….

Mgr. Lucie Vaculná

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Author Contributions

CHAPTER 1 – Introduction and aims of the thesis

LV wrote the text.

CHAPTER 2 – A comprehensive description of *Adenophora liliifolia* habitats and populations within the Czech Republic

LV wrote the text.

CHAPTER 3 – The use of *Adenophora liliifolia* seeds collected at Dabrowa near Zaklików (Rzeszów voievodship) to reinforce the declining population in Niedzieliski Forest (Lublin voievodship)

LV provided important information about the Czech populations and the realised conservation management in the Czech Republic. AK gathered the seeds, provided the information from Polish and foreign literature, provided the classified information from Polish authorities, finalised the text. MN tested the viability of the seeds, performed, and documented the microscopic observations. JP coordinated the works of PAS BG with the work of the Regional Directorate for Environmental Conservation in Lublin province. AR, the corresponding author, supplied the information from Polish and foreign literature, finalised the text. BR performed the fieldwork at Niedzieliski Forest, helped plant the seedlings, and provided unpublished details on the Niedzieliski Forest vegetation. TB performed the field work at Niedzieliski Forest, transferred the seedlings from Powsin Botanic Garden to the Niedzieliski Forest, planted them. MaK provided the relevees from the Niedzieliski Forest. All of the authors contributed to and approved the final manuscript.

CHAPTER 4– *Adenophora liliifolia*: Condition of its populations in Central Europe

LV and RP contributed equally. RP – phytosociological research and analysis in Juice, monitoring of the Czech and Slovak populations; LV – monitoring of the Czech and Slovak populations, genetic analysis; AK – phytosociological research and monitoring of the Polish populations; ĽM – genetic analysis; TF – phytosociological research and monitoring of the Hungarian populations; AI – phytosociological research and monitoring of the Romanian populations; LŠ – ecological statistical analysis in Canoco; MK – genetic analysis. All of the authors contributed to and approved the final manuscript.

CHAPTER 5 – Genetic structure of endangered species Adenophora liliifolia and footprints of postglacial recolonisation in Central Europe

MK, LV, and ĽM designed experiments, performed statistical analyses, interpreted results, wrote the manuscript; LV performed the main part of laboratory analyses (with the assistance of MK, TA, and MT); RP, AK, DI, APS, and MT participated in sampling and manuscript preparation. All of the authors contributed and approved the final version of the manuscript.

GENERAL DISCUSSION

LV wrote the text.

SUMMARY

LV wrote the text.

Authors' abbreviation

LV - Lucie Vaculná (née Marečková); AI - Adrian Indreica; AK - Adam Kapler; AR - Adam Rapa; BR - Bogusław Radliński; DI - Dmytro Iakushenko; JP - Jerzy Puchalski; ĽM - Ľuboš Majeský; LŠ - Lenka Šafářová; MaK - Marcin Kołodziej; MK - Miloslav Kitner; MN - Maciej Niemczyk; MT - Marco Thines; RP - Romana Prausová; TA - Tahir Ali; TB - Tomasz Bajdak; TF - Tunde Farkas

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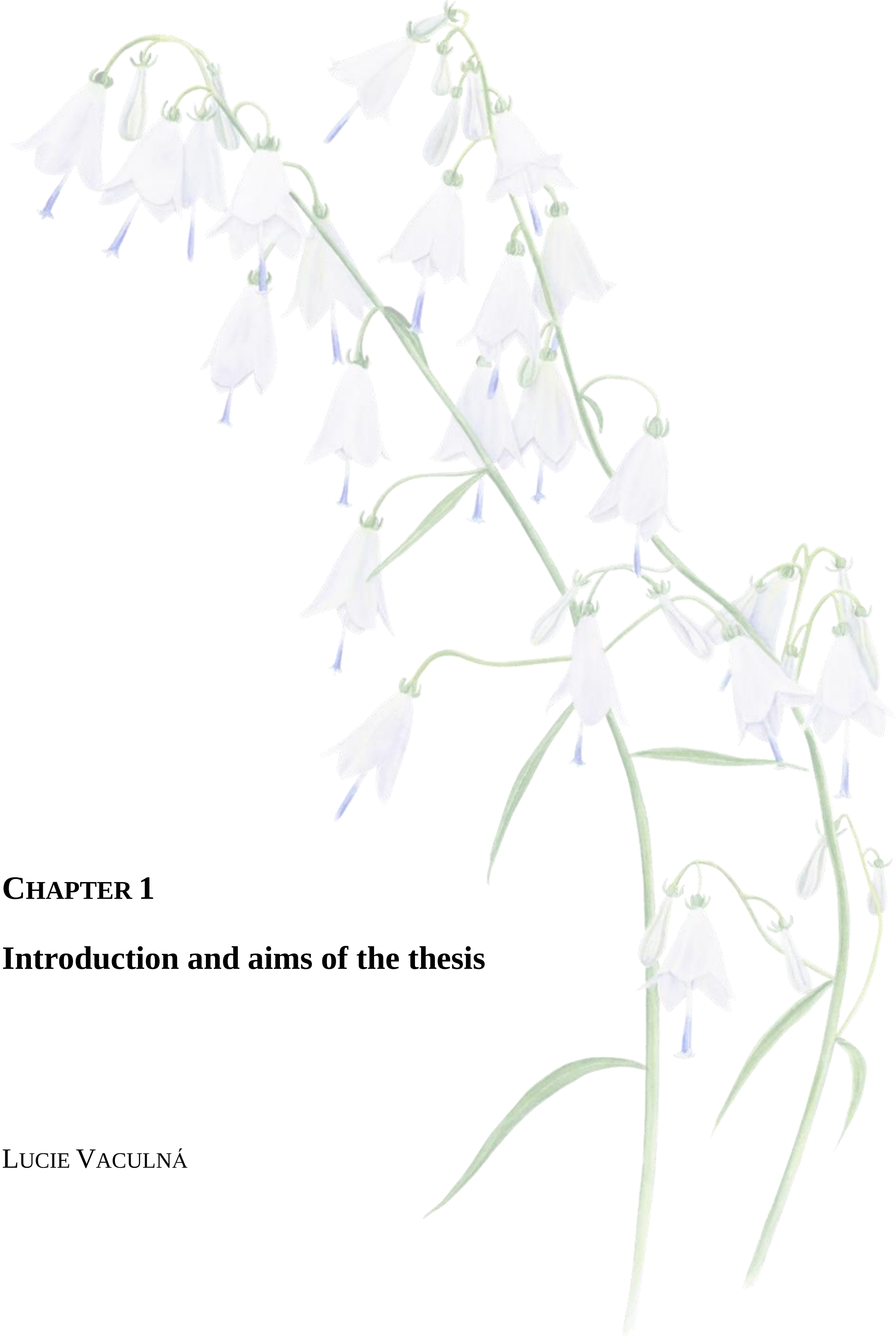


CHAPTER 1

Introduction and aims of the thesis

LUCIE VACULNÁ

Family Campanulaceae

The Campanulaceae Juss. represent a diverse clade of flowering plants encompassing nearly 2400 species in 84 genera, found on six continents, and growing in innumerable habitats from tropical rainforests to arctic tundra (Lammers 2007a; Crowl *et al.* 2016). The family includes five major lineages, variously treated as separate families or subfamilies (e.g., Lammers 1992, 2007a,b): (1) Cyphioideae Schönland include 65 perennial herbs of the genus *Cyphia* Bergius restricted to Africa; (2) Nemacladoideae M. H. G. Gustafsson is a group of 19 species of the genus *Nemacladus* Nutt. distributed in the southwestern United States and northern Mexico; (3) Cyphocarpoideae Gustafsson includes three poorly known species of the genus *Cyphocarpus* Miers, small annuals endemic to the Atacama Desert of Chile; (4) Lobelioideae Schönland, encompassing ca. 1200 species worldwide with the centre of diversity in the New World tropics, includes taxa with a diverse array of habits from tiny herbs to tree-like giant lobelias; and finally (5) Campanuloideae Burnett, a group encompassing ca. 1000 species distributed worldwide with a centre of diversity in the Holarctic, includes primarily small perennials and represents the only lineage in the Campanulaceae with radial floral symmetry.

The Campanuloideae is a highly diverse clade of angiosperms found mostly in the Northern Hemisphere, with the highest diversity in temperate areas of the Old World, namely in the Mediterranean and the Middle East. The representatives inhabit temperate to sub-tropical regions, where they occupy various habitats, from steppes to high elevation mountainous regions. Some species have wide distribution ranges, spanning entire continents, while others are narrow endemics (Crowl *et al.* 2014). Members of the Campanuloideae clade are primarily perennial herbs, with typical radial floral symmetry, also characterised by secondary pollen presentation (Crowl *et al.* 2016). The clade accommodates ca. 55 genera, with the most diverse genera *Wahlenbergia* Schrad. ex Roth (263 accepted species) and notably *Campanula* L. (440 species; The Plant List 2013). However, many genera, including *Campanula*, seem to be not monophyletic (e.g., Antonelli 2008; Cellinese *et al.* 2009; Mansion *et al.* 2012; Crowl *et al.* 2014, Crowl *et al.* 2016, Liveri *et al.* 2019).

The fossil records of the Campanulaceae are poor. Reliable records represent fossil seeds identified as *Campanula* sp. and †*Campanula paleopyramidalis* dated to Miocene [approximately 17–16 million years ago (Ma); Lancucka-Srodoniowa 1977, 1979]. Estimates from molecular dating suggested an origin for the Campanulaceae family in the Cretaceous to the Paleogene (67–40 Ma; Wikström *et al.* 2001; Bell *et al.* 2010; Knox 2014). The Campanuloideae subfamily was estimated to have originated in the late Palaeocene to early Eocene, ca. 56 Ma (Mansion *et al.* 2012; Crowl *et al.* 2016), and further diversification has been estimated to begin at 56–23 Ma (Cellinese *et al.* 2009; Mansion *et al.* 2012; Crowl *et al.* 2014).

Genus *Adenophora*

Lammers (2007a) characterises representatives of *Adenophora* genus as perennial herbs, often with large tuberous roots. Leaves are cauline, rarely opposite or 3–6-verticillate. Flowers are small to medium-sized, pendulous, short pedicellate, in loose racemes or panicles. Corollas are purple or blue (rarely white), campanulate, tubular, infundibular, or rarely urceolate; lobes are shorter than the tube. Stamens are commonly present; filaments dilated basally, forming a nectar chamber; anthers equalling or shorter than filaments; pollen 3–5-porate, echinate. Ovary 3-locular, crowned by a large, thick, annular cylindric or cupulate nectary; style exserted. Fruit capsular, laterally dehiscent by 3 pores or valves. Chromosome numbers differ within the genus, with 2n = 34, 36, 68, 72, 102. Although Lammers (2007b) states sixty-five species in *Adenophora* genus (with majority distributed in eastern Asia, one in western Europe – *Adenophora liliifolia* (L.) A. DC. and one in Crimea [nowadays accepted as *A. liliifolia* subsp. *taurica* Sukaczev; (Fig. 1)], there are ambiguities regarding the determination of some Asian taxa. Though many infrageneric taxa have been proposed, no modern classification attempt has resolved this large genus's taxonomy yet.

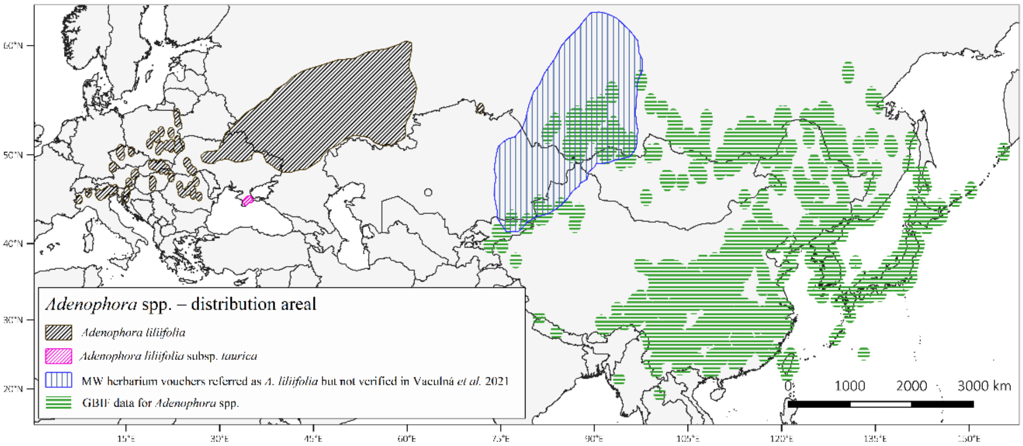


Fig. 1. Distribution range of *Adenophora* spp. Black dashed polygon – distribution of *Adenophora liliifolia* based on the literature and the study of Vaculná *et al.* 2021. Pink dashed polygon – distribution of *A. liliifolia* subsp. *taurica* on Crimea. Blue dashed polygon – samples from herbarium Moscow (MW) used for the above-mentioned study, deponed as *A. liliifolia*, but according to study Vaculná *et al.* 2021 representing another taxon of *Adenophora* genus. Green dashed polygon – distribution of other Asiatic *Adenophora* spp. based on GBIF occurrence data (GBIF Secretariat 2021).

Based on the phylogenetic and biogeographical study of Asian Campanuloideae (Yoo *et al.* 2018), the genus *Adenophora* split from its closest relative ca. 14.8 Ma, with further diversification during the late Miocene to Pliocene. The genus *Adenophora* forms monophyletic clade with *Hanabusaya asiatica* (Nakai) Nakai (Eddie 1997; Kim *et al.* 1999; Eddie *et. al.* 2003; Haberle *et al.* 2009). Most of the species of *Adenophora* have very similar ITS sequences and lack enough phylogenetic signal; therefore, phylogeny reconstruction based on ITS data solely is not appropriate (Eddie *et al.* 2003). Yoo *et al.* (2018) suggested that the ancestor of the genus *Adenophora* most probably originated in Europe/W. Asia, from where it has dispersed to its centre of diversification in East Asia. In tropical or temperate Asia, the genus further diversified and expanded its range. During the subsequent diversification, *A. liliifolia* dispersed back to Europe/W. Asia (Yoo *et al.* 2018).

Next to *Adenophora liliifolia*, species listed in numerous national Red Books and protected by law on several levels, the genus also includes other endangered or even endemic species. *Adenophora changaica* Gubanov & Kamelin, an endemic species of Mongolia (Urgamal and Oyuntsetseg 2017); *A. jacutica* Fed., endemic to Yakutia and listed in Red Book of Plants of Sakha Republic (Danilova 2017), or *A. lobophylla* D.Y.Hong, another endangered species confined to moist or damp valleys in the mountains of Jinchuan County in China (Zhang 1995). *Adenophora palustris* Kom. is widely distributed in East Asia, including eastern China and Korea, but with only six extant populations in Japan. Thus, the species is currently classified as "critically endangered" in the Japanese Red Data Book (Ministry of the Environment Japan 2015).

The root of *Adenophora* spp. has been widely used as a folk medicine in Eastern Asia (e.g., Korea, China, and Japan), believed to treat lung fever and enrich "Yin" in our body (Wiseman and Ellis 1996). *Adenophora triphylla* var. *japonica* (Regel.) Hara or *A. stricta* Miq. are prescribed as a crude drug "Sasam" in Korea (Shinilbooks Committee 2016), or "Nanshashen" in the Chinese Pharmacopoeia (Zhao *et al.* 2003). The species has also been reported to have anti-obesity, hepatoprotective, antioxidant, and anti-cancer effects (e.g., Lee *et al.* 2000; Gum *et al.* 2007; Kim *et al.* 2009; Choi *et al.* 2010; Kang *et al.* 2013).

Only a few population-genetic studies focused on the *Adenophora* genus. The spatial genetic structure of populations of *A. grandiflora* Nakai (Chung and Epperson 1999) was investigated by allozymes. Allozymes were also used to compare the genetic diversity of an endangered *A. lobophylla* and its widespread congener, *A. potaninii* Korsh. (Ge *et al.* 1999). Besides these studies, sets of microsatellite markers were developed for the genetic study of *A. palustris*, a critically endangered species in Japan (Masumoto *et al.* 2011; Otake *et al.* 2016). Inter Simple Sequence Repeats (ISSR) markers were used by Boronnikova (2009) and Manole (Manole *et al.* 2015) in the population genetic studies of *A. liliifolia*, however only on a very restricted geographical scale.

Adenophora liliifolia as the object of study and conservation interest

Adenophora liliifolia is an herbaceous perennial, long-lived (up to 40 years; personal observation) diploid (2n = 34) plant species. Stems are erect, leafed, and branched. The root is spindle-shaped or branched. The plants usually grow to a height of 40–90 cm (Kovanda 2000) but can reach up to 205 cm (Ciosek 2006). Basal leaves and leaves of young plants are long petiolate, with cordate to rounded and coarsely serrate blades. Stem leaves are sessile, alternate, with an elliptical to lanceolate, serrate to the 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 style is twice as long when compared to the corolla. The species flowers from late June to August and is pollinated by insects. The species is facultatively allogamous. 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 spread by wind (Kovanda 2000; Kucharczyk *et al.* 2014). The Ellenberg's species indicator values classified *A. liliifolia* as a heliophilous, thermophilous, and basiphilous species requiring enough moisture and with little to no demand for nitrogen (Ellenberg *et al.* 1992: light L = 7; temperature T = 6; continentality C = 6; moisture F = 6; soil reaction R = 8; nutrients N = 2; but see Pladias (2021), where these indicators are modified to Czech flora: L = 6, T = 6, F = 5, R = 7, N = 5, salinity S = 0).

Fig. 2. *Adenophora liliifolia* – detail of the flowering stem. Cigánka, National park Muránska planina, Slovakia, 2. 8. 2016. Author: Lucie Vaculná.

The taxon occurs mainly along forest edges and in forest openings within the Czech Republic, including forest roads and paths, windthrow sites, burnt sites, and forest clearings (Pladias 2021). It has a competitive-stress tolerant (CS) life strategy, a limited ability for long-distance dispersal and grows in climatically non-extreme habitats (Roleček 2007; Kaplan 2017). In the Central-European flora, the species represents an European-South-Siberian floristic element, with optimal conditions provided by the hemiboreal forests of Southern Ural and Southwestern Siberia (Roleček 2007). The centre of the species distribution is situated in Western Asia-Southern Siberia and disjointly reaches up to Western Europe (Germany, Switzerland; Fig. 1) (Tacik 1971; Fedorov 1978). In the middle Russian forest zone, it is a typical riparian species preferring light oak forests and their margins on major river flood plains (Seregin 2017).

Adenophora liliifolia is listed among the species of the European Community interest (Directive 1992), whose conservation requires the designation of special areas of conservation (SAC; NATURA 2000 sites). At the European level, the species conservation status was assessed as “unfavorable-bad” for finished reporting periods (2001–2006, 2007–2012, 2013–2018; EUNIS 2021). The species is nowadays endangered mainly through changes in habitat management and loss of suitable habitats, as described below. All European countries with the occurrence of the species listed the species as threatened. Next to the EU territory, the species is listed in 42 actual Red Books of several regions in Belarus, Ukraine, and numerous regions in Russia (Plantarium 2021).

Habitat management and population prosperity

A study subject of this thesis, *Adenophora liliifolia*, can be considered as “naturally rare,” light-demanding, relict species of the Late Glacial/Early Holocene forest-steppes. Such species adapted to the open landscape and the cold climate of the Late Pleistocene were formerly more widespread but became restricted during Holocene due to climate amelioration and forest expansion. These changes caused the retreat of light-demanding species to spatially restricted primary treeless habitats, open landscapes with steppe, tundra, or their mosaic (e.g., Bylebyl *et al.* 2008; Hensen *et al.* 2010). Some of these habitats were naturally stable, e.g., rocky outcrops; others were affected by stochastic events (fire, whirlwind), large herbivores’ pasturage, or later by human activity. Due to recent habitat alteration, fragmentation, and changes in habitat management resulting in the loss of suitable habitats, these species often become threatened (Hensen *et al.* 2010).

Human activity has been affecting the landscape since the Neolithic time. Historical forms of management such as coppicing, grazing, or litter raking shaped European forests and the composition of their biotic communities for centuries (Rackham 2003; Roleček *et al.* 2017). Meadows appeared in the landscape as a new habitat after the human-caused deforestation and were maintained by mowing, grazing, burning, or a combination of these activities (Petříček 1999). Their floristic composition depends on the intensity, frequency, and time when management measures occur (Willems 1988). Manual mowing, spread over the growing season, supports creating suitable areas for seed ripening and shelters for invertebrates. This method of meadow management has, however, either completely disappeared or receded.

Similarly, the traditional forest management forms (e.g., coppices) started to be abandoned across Europe since the 18th century (e.g., Müllerová *et al.* 2014, 2015). The transformation of most forest stands to high forests or plantations of economically productive fast-growing species has brought fundamental changes in site conditions and caused a decline of many, especially light-demanding species (Baeten *et al.* 2009; Naaf & Wulf 2010; Müllerová *et al.* 2015). Abandonment of low-productive and difficult accessible sites allowed them to undergo natural succession (Rybníček & Rybníčková 1974). Both the scenarios caused the alteration, fragmentation, or even disappearance of many habitats. The plant species populations confined to such habitats became strongly reduced and are today much more isolated from each other than in the past.

Some of the forests which were managed with traditional practice — particularly lowland oak forests — still harbour populations of otherwise vanishing species as a legacy of the long history of traditional management that kept the canopies open (Hédl *et al.* 2010; Jamrichová *et al.* 2013). These forests often represent biodiversity hotspots within the surrounding landscape and thus became the subject of conservation efforts in recent years. In such light forests, the highest floristic diversity is concentrated in forest patches with high light availability and high soil pH. Nowadays, light-demanding species are threatened with ongoing successional changes and by intensive competitive pressure of nitrophilous species. Active conservation measures are necessary, including opening up the canopies, early thinning of young stands, control of expansive and invasive species, and undergrowth grazing or mowing (Roleček *et al.* 2017).

Population genetics and its role in species conservation

Population genetics, and especially conservation genetics, has been substantially helping in species conservation attempts for the last two decades. From very first and nowadays obsolete techniques such as allozymes/isozymes diversity analyses to latest-fashion whole-genome genotyping, population genetics brings essential information about genetic diversity when dealing with vanishing species populations' support, repatriation, or reintroduction (e.g., Geffen *et al.* 2007; Amato *et al.* 2009).

Among the field's most inflected terms, genetic diversity takes the first place. Genetic diversity determines the potential population fitness and ultimately its long-term persistence because genes encode phenotypic information. Extinction risk has been associated with low genetic diversity, and the fact of reduced fitness in populations with low genetic diversity is generally accepted (e.g., Huenneke 1991; Ellstrand & Elam 1993; Bouzat 2010). Populations with higher genetic diversity tend to have a higher potential to store rare alleles useful in species adaptation to changing environmental conditions (Boulding 2008). The known relationship among populations allows researchers to determine priorities in conservation tasks or select which site is indispensable for the species' survival (Petit *et al.* 1998). In conservation actions aimed at population strengthening (e.g., sowing of seeds, planting of pre-grown plants, planting plants propagated *in vitro*), it is necessary to know the relationship between populations and choose from populations exhibiting high genetic similarity. These steps avoid introducing undesirable genotypes unsuitable for a particular site and prevent the risk of potential genetic erosion (irreversible loss of unique genotypes) (e.g., Lesica & Allendorf 1999).

Next to all of these, we can attempt to shed light on populations' history and outline factors that likely had the greatest impact on the current state of genetic variability and population structure. Phylogeography, another research discipline, deals with historical processes responsible for species' past and present geographic distributions. Current population status might be, among others, affected by, e.g., former bottleneck event, founder effect, or a restricted gene flow (Avise *et al.* 1987; Avise 2009). Phylogeographic analyses assume long-term natural evolutionary processes, and methods based on screening of both ancient and modern DNA are routinely considered in these analyses. Phylogeographic studies predominantly focus on the Quaternary period, particularly around the last glacial maximum (LGM). For example, analytical tools such as haplotype networks, genetic differentiation estimators, analyses of molecular variance (AMOVA), or dendrograms are assessed, and results are put in the context of general knowledge of the species biology. As a result, phylogeography has been considered one of the most integrative disciplines in biology. The number of phylogeographic studies has grown exponentially in recent years due to the increased accessibility of sequencing techniques (see the review of Rius & Turon 2020 and literature cited therein).

When assessing the genetic diversity of populations, one should be aware of species biology and life cycle (Loveless & Hamrick 1984; Hamrick & Godt 1996). Here is a paradox, when long-lived perennial or clonal species restricted to small and isolated populations may display a pattern of genetic diversity that is otherwise typical for large populations and indicates an extensive interpopulation gene flow. A possible explanation for this is that the isolation of populations and decreased population size are encountered only by a few most recent generations, and thus these characteristics cannot be reflected in the genetic population structure (Reisch *et al.* 2003; Segarra-Moragues & Catalán 2003; Šmídová *et al.* 2011). Moreover, "naturally rare" species, whose populations have always been more distant from each other, seem to be less sensitive to a reduction in genetic variation than species formerly distributed in large populations (so-called “new rare” species). It results from a fast restriction of “new rare” species distribution and population size reduction compared with “naturally rare” species occurring in small populations for a long time (Huenneke 1991; Lutz *et al.* 2000).

Species distribution modeling

Easy accessibility of environmental data, the possibility of the precise geographical location of studied individuals, increasing computational output of personal computers, grid computing, and the progress in machine-learning in general gave rise to the species distribution modeling, a new and powerful tool in species biological research.

Species distribution modeling (SDM), also known as environmental (or ecological) niche modeling, or range mapping, uses computer algorithms to predict the distribution of a species across geographical space and time using environmental data. These are most often represented by climate data (e.g., temperature, precipitation, and their distribution during the year) but can include other variables such as soil type, water availability, land cover, etc. The modeling approach is frequently used nowadays in conservation biology, ecology, and phylogeography (e.g., in the reconstruction of ancestral habitats). During the model calculation, the algorithm used in the process estimates the probability of habitat suitability, not, as often misinterpreted, the probability of species occurrence. The output is represented by the interval 0 - 1 for each square cell of investigated area (depending on data resolution) and is usually visualised in the map, where warmer colours represent higher probability.

Models outputs can be used to understand how environmental conditions influence the occurrence and abundance of a species and for predictive purposes (ecological forecasting). Predictions from SDM may be on 1) species’ future distribution under climate change, 2) species’ past distribution, 3) or potential future distribution of an, e.g., invasive species. Predictions of habitat suitability can be helpful in management decision-making (e.g., see the review of Pecchi *et al.* 2019 and literature cited therein). Typically, the SDM outputs are maps of suitable habitats. These must be inspected critically because the realised niche might be considerably narrower than the predicted one. There are often some variables, which might be essential or even crucial for species to prosper. However, it is hard or even impossible to convert them into data layers (e.g., ongoing habitat eutrophication, followed up by nitrophilous species spread resulting in canopy closing and total habitat change) or stochastic events.

The question frequently asked within the field of SDM is the question of thresholding, asking under which value the species can not occur. The simplest approach, which was also the most widely used in the past, consists in defining a threshold of environmental suitability (most frequently it was the value 0.6), below which conditions were believed to be unsuitable (so absence was attributed); and above which conditions were believed to be suitable (so presence was attributed). Nowadays, many authors warn against using this approach for several reasons: 1) the species are often absent from areas of high suitability because of factors acting at smaller spatial scales, such as biotic pressure (competition, predation), disturbances, stochastic events; 2) real species often occur in unsuitable areas, because of very particular conditions allowing their occurrence (microclimatic/microhabitat conditions, climatic refugia); 3) the threshold almost completely removes the previously defined relationship between the species and its environment. The gradual aspect is lost when the above-threshold part of the environmental gradient is always fully suitable, while the below-threshold part is wholly unsuitable (e.g., Leroy *et al.* 2015).

Even though the description of species distribution modeling sounds impressive and promising to answer many questions the scientists are looking for in their research, one must be aware of several snares before blindly adopting the outcomes. Several crucial points must be ensured, e.g., reliability of i) geographical reference points; ii) taxonomic determination; iii) relevance of chosen environmental data layers; otherwise, the results of modeling may easily lead to a wrong conclusion. One would assume that a higher number of reference points ensures more precise results, but this may not be necessarily true when using data from public databases without their precise verification. There might be a taxonomical problem, or in some world areas, the sampling is not as frequent, so discrepancies might occur within the resulted dataset (e.g., Duputié *et al.* 2014). Another problem arises with environmental data layers used. One should prefer to use data layers, which make ecological and biological sense for a given species in terms of previous findings and general knowledge of species' habitat demands (Jarnevich *et al.* 2015). The multicollinearity of the contributing environmental variables has to be tested, and as a prevention to misleading signals, highly correlated variables should be excluded (Dormann *et al.* 2013).

Aims of the thesis

During my research, I focused on the following questions:

1) What are the current conditions of *A. liliifolia* populations in Central Europe? Which factors threaten the species, and what type of appropriate management could be suggested for species' prosper?

2) What is the level of genetic diversity, and what is its spatial distribution within Europe? What are the relationships among extant *A. liliifolia* populations?

3) Is it possible to draw conclusions about the presence of potential glacial refugia and putative routes of post-glacial (re)colonisation of Europe based on the current distribution of genetic diversity and population structure?

4) Can species distribution modeling answer questions related to the history and future of *A. liliifolia* remnant populations?

The thesis consists of the following parts that aimed to answer the above-mentioned questions:

CHAPTER 2 – A comprehensive description of *Adenophora liliifolia* habitats and populations within the Czech Republic

The chapter brings information about documented *A. liliifolia* history within the Czech territory and describes in detail the habitat conditions of particular populations. The factors threatening the species and the implemented conservation measures are discussed in the chapter. The ongoing annual species monitoring results are displayed in graphs and tables.

CHAPTER 3 – The use of *Adenophora liliifolia* seeds collected at Dabrowa near Zaklików (Rzeszów voievodship) to reinforce the declining population in Niedzieliski Forest (Lublin voievodship)

This chapter describes the conditions of the declining *A. liliifolia* population in Niedzieliski Forest (Poland) in detail. It describes the on-site management activities to reinforce the population. The tentative results of the applied management are discussed and compared to the knowledge from the Czech population Vražba, and implications for the future are highlighted.

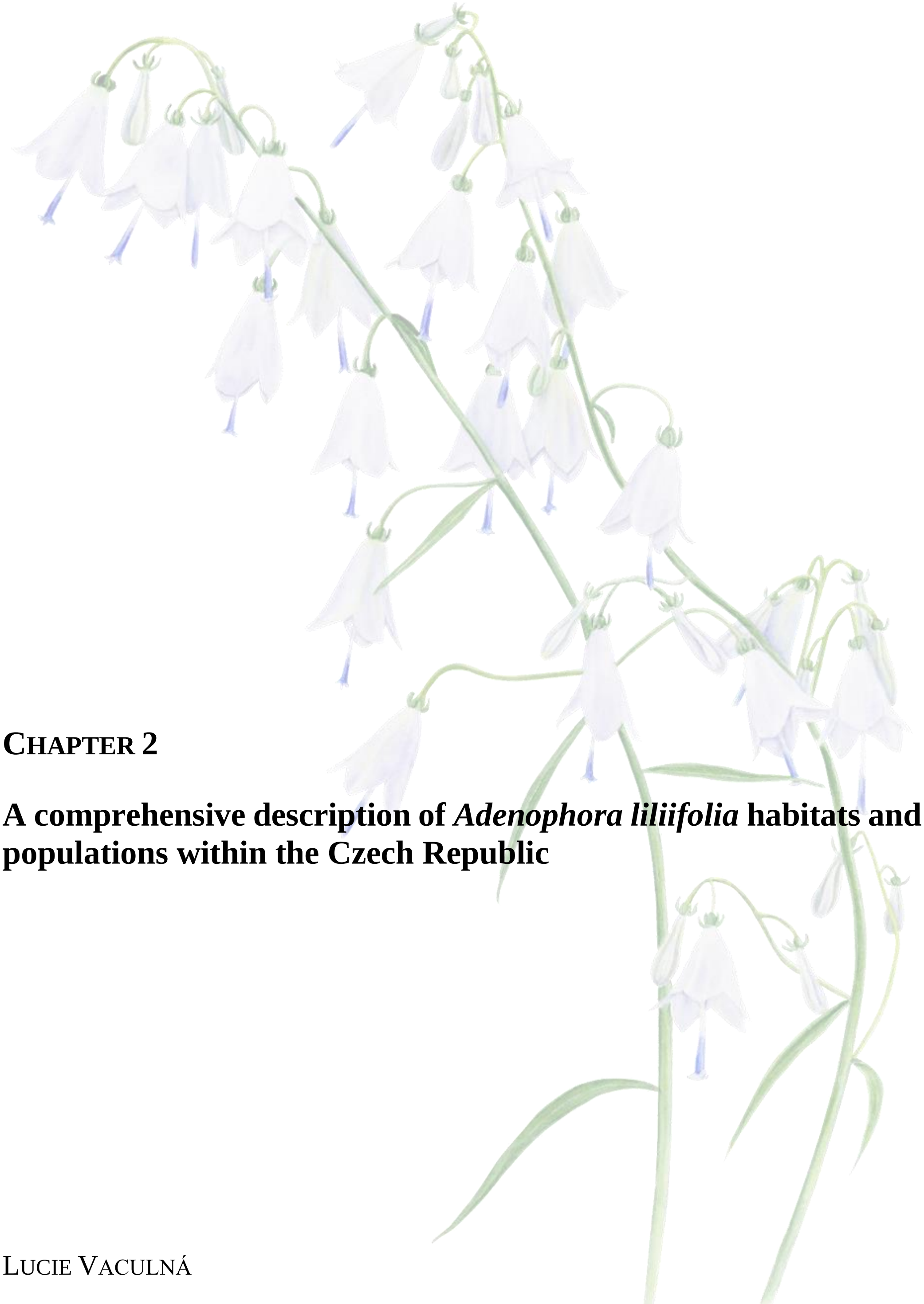
CHAPTER 4 – *Adenophora liliifolia*: Condition of its populations in Central Europe

This chapter summarises the current conditions of populations of *A. liliifolia* from the Czech Republic, Slovakia, Poland, Hungary, and Romania and describes habitat preferences and vegetation associations in which the species grow. The chapter brings the very first insight into the genetic relationship of European populations inferred by the amplified fragment length polymorphism (AFLP) method. It also reports the proportion of the sterile and fertile individuals, discusses the level of endangerment at particular localities, and suggests particular conservation interventions.

CHAPTER 5 – Genetic structure of endangered species *Adenophora liliifolia* and footprints of post-glacial recolonisation in Central Europe

This chapter brings insight into the biogeography of *Adenophora liliifolia* based on three chloroplast regions, fingerprinting data (AFLPs), and Species distribution modeling (SDM – Maxent). The current spatial distribution of genetic variability, possible ways of species migration during glacial/interglacial periods, and possible glacial refugia are discussed in the chapter. Results of distribution modeling predicted the possible scenarios for the species in the near future.

Note on nomenclature: Nomenclature is unified throughout this thesis using The Plant List, a working list of all known plant species. (The Plant List 2013).



CHAPTER 2

A comprehensive description of *Adenophora liliifolia* habitats and populations within the Czech Republic

LUCIE VACULNÁ

Chapter 2 - Motivation to the text

Even though my thesis's main topic is related to the population genetic diversity and phylogeography of *Adenophora liliifolia*, the conservation aspect of my research is inseparable and intertwines the whole study. I have been involved in *A. liliifolia* field research since my bachelor study, and therefore I feel there is a need to bring information about its genetics in a broader context. Moreover, the results of population genetic studies will be implemented in the ongoing Action Plan (of my co-authorship) for species protection in the Czech Republic. These circumstances led me to write this chapter, "Chapter 2". The chapter contains brief but comprehensive information about the current situation in the remaining Czech populations of *A. liliifolia*. Some of this information was already published (e.g., Prausová & Marečková 2017; Prausová *et al.* 2020), but a substantial part represents new and unpublished data from the annual field monitoring and recent findings from ongoing habitat management.

Introduction

Adenophora liliifolia could be considered a naturally rare species within the Czech Republic with about 20 localities (Kovanda 2005) comprising all historical and current distribution. Most of these localities fall under Bohemian Thermophyticum. However, the species was not verified in most of these localities for 50–120 years, notably at Vyškov and Opava regions (Prausová *et al.* 2020), and today the species persist in 6 main localities. According to Kovanda (2005), at least twelve populations have already extinct. The author further states that the main reason for the disappearance of the species is improper forests management, causing an undesirable succession. The habitats, he has been verifying at the turn of the 20th and 21st century, were overgrown with expansive species, e.g., *Rubus* spp., *Senecio ovatus* (G. Gaertn. & al.) Hoppe, or *Carex brizoides* L.

The oldest *A. liliifolia* herbarium voucher from the Czech territory is dated back to 1809 and comes from the Bohemian Karst, and is deponed at Masaryk University – BRNU. Other historical herbarium vouchers are from 1839–1983 from the locality "Babinské louky" in Central Bohemian Uplands (North Bohemia). The first record of the species from Eastern Bohemia in a forest between villages Velichovky and Habřina dates back to 1886. For the area of "Džbán" (or named alternatively by various authors, see below for details) in the Kladno region (Central Bohemia), the first documented *A. liliifolia* voucher is from 1888 and represents samples from the surroundings of Bílichov village. From the Kolín region (Central Bohemia), the species is documented between the years 1934–1943. From the Vyškov region (Moravian part of the Czech Republic), two vouchers exist from 1915 and 1919. The list of all the existing herbarium vouchers from the 19th and early 20th century is present as Appendix n.3 of the Action Plan for the species protection (Prausová *et al.* 2020).

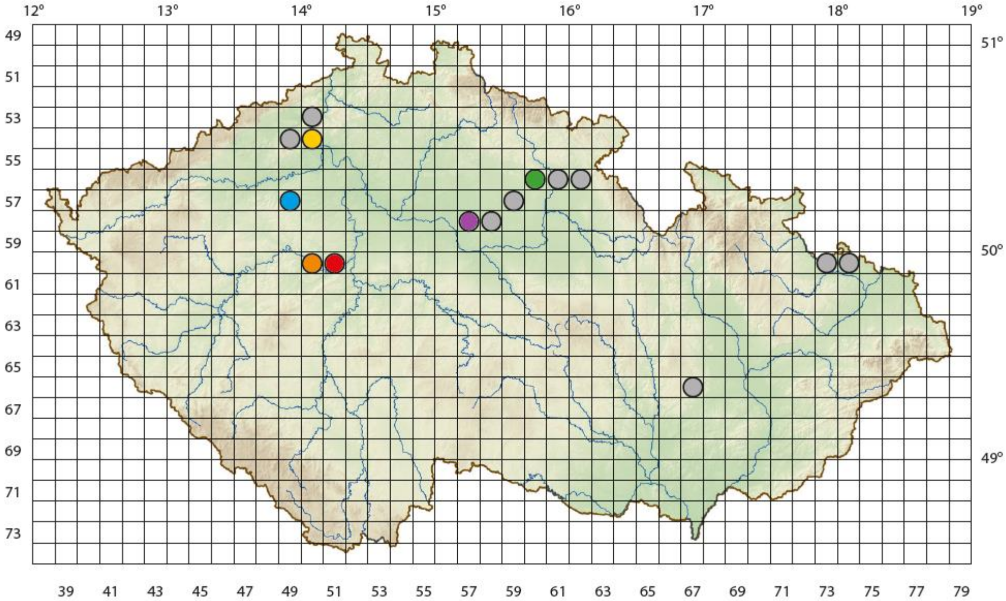


Fig. 1. Historical (grey points) and present (colour points) distribution of *Adenophora liliifolia* in the Czech Republic. Babinské louky – yellow, Bílichovské údolí/Džbán – blue, Český kras – red, new artificial site orange, Báňský les/Žehuň – purple, Vražba – green. (© AOPK ČR 2017, data layers © ČÚZK).

Several bachelor and diploma theses were dedicated to *A. liliifolia* in the Czech Republic, studying many aspects of species biology and ecology. Truhlářová (2008) studied habitat, size, and vitality of the Vražba population, with comparison to locality Babinské louky. The same author also studied environmental conditions in Czech populations (Truhlářová 2010). Marečková (2010) performed monitoring of the same population at Vražba locality in relation to its habitat' management. Comparison of the current state of *A. liliifolia* populations from the Czech, Slovak, and Hungarian populations was made by Řeháková (2013), and a comparison of phytocenoses with the occurrence of *A. liliifolia* within the Czech Republic and Slovakia was made by Vychytil (2013). Marečková (2013) monitored selected morphological features, habitat characteristics, and vegetation units of all Czech populations, and Bajerová (2015) focused on the description of the ecological demands of the species within Central Europe. Most recent studies are devoted to the importance of cryopreservation of *A. liliifolia* seeds for their germination (Špringrová 2018) and the life cycle and phenology of *A. liliifolia* (Kyselová 2019).

Methods

Since my bachelor studies, I have regularly participated in the annual field monitoring of the species within its Czech populations together with Dr. Romana Prausová (University of Hradec Králové, the monitoring' main coordinator), her students, and employees of the regional offices of the Nature Conservation Agency of the Czech Republic (AOPK ČR). The annual monitoring follows the methodology of AOPK ČR (Turoňová 2011; Action Plan Appendix n. 12 in Prausová *et al.* 2020), but more detailed observations are recorded for student theses, and in 6-year-interval the monitoring results are reported under the NATURA 2000 methodology (for details see below).

The annual monitoring takes part generally in the first half of August. This term is the most appropriate due to the species phenology – the plants are already flowering, making them better identifiable even within densely connected herbaceous vegetation. The number of individual tufts, number of stems present within the tuft, the proportion of fertile and sterile stems of particular tuft, and the occurrence of seedlings or juvenile plants are recorded every year for each locality. All the visible damage of the stems, e.g., caused by grazing of wild-game; flowers and ovaries nibbled by insects; digging out caused by rodents; stems wilting during seasons of limited precipitation; mildew or other plant-pathogen attacks are also recorded. The plants at each monitored locality are individually marked with a metal tag enabling their monitoring over time. This is a very convenient practice when elaborating bachelor and master theses studying individual plants' fitness and total population' prosper over time. During the annual monitoring, the ongoing management is evaluated at each locality, and its suitability or the necessity of adjustment is consulted.

For the purpose of NATURA 2000, reports are prepared in a 6-year interval. Results of monitoring reports for periods 2001–2006, 2007–2012, and 2013–2018 are available at Eionet Portal (Article 17 web tool 2021), along with regional reports from other member states hosting the species' populations. Within the reports, regions with the occurrence of *A. liliifolia* are divided into Alpine, Continental, and Pannonian. The main evaluation criteria are:

1. Range – square kilometers of the protected Special Area of Conservation (SAC) designed due to species occurrence and percentage of the species' actual coverage, along with the trend (increasing, decreasing, stable) and favourable reference range (equal, less, more, much more);
2. Population – individuals' counts (minimum, maximum, trend and method used for monitoring, i.e., individual counting/estimation);
3. Habitat – quality (insufficient/sufficient), status (favourable/unfavourable), and trend (increasing, decreasing, stable);
4. Future prospects – evaluated based on parameters Range, Population, and Habitat prospects (levels: unknown, good, poor, bad).

Applying these criteria, the system automatically evaluates the overall assessment.

Results & Discussion

National monument Vražba

Adenophora liliifolia was rediscovered at this locality in 2003, and since the year 2005, the locality has been regularly monitored. Thus, the population of *A. liliifolia* at the monument Vražba is for the longest-time continuously monitored population in the Czech Republic (Truhlářová 2008; Prausová & Truhlářová 2009). The occurrence of the species is in a forest complex in the Jaroměř region (Bohemian Thermophyticum, Jaroměřské Polabí, East Bohemia), about 1 km northwest of village Habřina and 13 km north of Hradec Králové city. The altitude is about 270–290 m a.s.l. The bedrock is formed by Cretaceous rocks from the Upper Turonian period, with calcareous claystone, which is locally present in powder form. There are also remnants of shallow overlaps of Pleistocene fluvial sandy gravels, originated from the Mindel glaciation, which are gradually washed away. On steeper slopes, calcareous claystone and siltstone are almost without overlap and reach the surface. Predominated soils are Cambrian pararendzina and leached cambisol soil. The average annual rainfall is about 600 mm, the average annual air temperature is 9 °C (Truhlářová 2008, 2010; Prausová & Truhlářová 2009; Marečková 2010, 2013; Prausová *et al.* 2020). The forest is classified as an oak-hornbeam forest, admixed with coniferous trees – mainly *Picea abies* (L.) H.Karst. and *Pinus sylvestris* L. (e.g., Marečková 2013). The area of the Natural monument Vražba is managed by the state enterprise Lesy ČR (State Forests of the Czech Republic).

The total area of *A. liliifolia* is approximately 0.46 ha. Due to the monitoring purposes, habitats with the occurrence of *A. liliifolia* are divided into four micro-habitats distant few dozen of meters (Truhlářová 2008; Prausová & Truhlářová 2009). The first micro-habitat (#1) is the most different due to the biggest slope inclination and southwest orientation. About 20-25 plants are growing in the area of ca. 100 m^2. At the time of rediscovery of the species, dominant tree species were spruce and larch, with a small admixture of broad-leaf trees and shrubs. In 2003, some of the trees were selectively felled, and the emerged gap was filled with planted oak seedlings. Thanks to these activities, micro-habitat #1 approached the most to the desirable light forest, the most likely habitat type with the presence of *A. liliifolia* in the Czech Republic in the past (Prausová & Truhlářová 2009). During seasons 2015 and 2016, the remaining coniferous trees were removed. The loosening of the habitat increased illumination, which led to an undesirable and rapid strengthening of herbaceous species prospering from available nutrients and calcareous bedrock. Dominant species during the subsequent years became *Vincetoxicum hirundinaria* Medik., *Brachypodium sylvaticum* (Huds.) P.Beauv., *Poa nemoralis* L., *Aegopodium podagraria* L., *Stachys sylvatica* L., *Rubus* spp. (Prausová 2015–2017). Soil analyses showed high saturation with basic cations [96.7%; mainly Ca^{2+} (29.94 $cmol^{(+)}$/kg), Mg^{2+} (1.52 $cmol^{(+)}$/kg), K^{+} (0.71 $cmol^{(+)}$/kg), and N^{+} (0.06 $cmol^{(+)}$/kg)]. Bedrock influence significantly more the properties of the substrate at this micro-habitat than in other parts of the Vražba area (Truhlářová 2008; Prausová & Truhlářová 2009). Each of the remaining micro-habitats #2, #3, and #4 occupies an area of 150–300 m^2, with 15–30 individual plants. The vegetation is represented by an oak-hornbeam forest. Micro-habitats #2 and #3 are a bit moister, with an admixture of *Fraxinus excelsior* (L.) and increased relative content of humus and nitrogen. The easily available nutrients result in overgrowing of *A. podagraria* and *Rubus* spp. In the micro-habitat #4, most plants grow in the light part of the micro-habitat along the forest road, slightly shaded by *Corylus avellana* L. (Fig. 7 - A, B).

Thanks to the fencing of micro-habitats, the population size and the proportion of fertile individuals remain relatively stable. The minimum number of counted plants was 66 (season 2009), and the maximum was 133 tufts (season 2010). The plants are protected against grazing from the overpopulated hoofed game, but the absence of grazing affects other plant species growing in the area, whose uncontrolled overgrowing oppresses *A. liliifolia*. The population responds positively to appropriate controlled interventions, particularly the habitats' enlightening done by reducing trees and shrubs and plucking of expanding *Rubus* spp. (Marečková 2013; Prausová 2015–2020; Prausová & Marečková 2017). The proportion of fertile stems increased in seasons 2010, 2013, 2016, and 2019–2021 following the manual plucking of blackberries (Fig. 2).

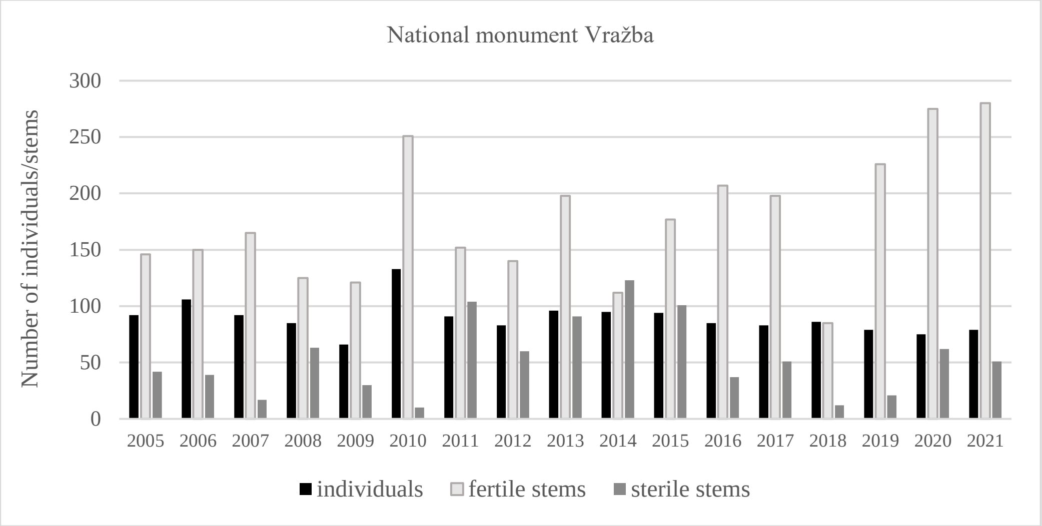


Fig. 2. Changes in population size (shown as the number of individuals, black colour) at Vražba during years 2005-2021. The increased proportion of fertile stems was recorded during seasons 2010, 2013, 2016, and 2019–2021. Such a trend follows when manual plucking of blackberries was done at the end of the previous vegetation season or early during the spring.

Natural monument Babinské louky

The locality Babinské louky is a part of an eponymous Natural monument in the district of Ústí nad Labem, cadastral area of Babiny I, and a part of the Protected Landscape Area (PLA) of České středohoří. It is the only natural non-forested location of the species in the Czech Republic. The locality is situated southeast of the village Čeřeniště and about 10 km north of city Litoměřice. *Adenophora liliifolia* grows here at the altitude of 540 m a.s.l. The bedrock is formed by upper Cretaceous marlstones and sandstones, and the primary soil type is eutrophic cambisol. Phytogeographically the area belongs to Bohemian-Moravian Mesophyticum. The average annual temperature is 7 °C, the average yearly rainfall is 600 mm. (Truhlářová 2010; Marečková 2013).

This locality was discovered in the late 80's of 20th century by Iva Machová and Karel Kubát (Machová and Kubát 2004). Previously documented herbarium collections from the period 1839–1983 originated from surrounding forest stands (Prausová *et al.* 2020). The locality is known for the high species diversity and the occurrence of rare plant species, e.g., *Pleurospermum austriacum* (L.) Hoffm., *Iris sibirica* L., *Parnassia palustris* L., *Trollius altissimus* Crantz, *Thesium bavarum* Schrank, and *Orchis mascula* (L.) L., whose populations are, however, shrinking. In comparison, populations of *Potentilla alba* L., *Betonica officinalis* L., *Salix rosmarinifolia* L., or *Serratula tinctoria* L. remain more abundant (Prausová & Marečková 2017).

Jan Otakar Martinovský (1967sec. in Prausová *et al.* 2020) assessed the area of Babinské louky as a slightly damp forest-meadow steppe in the submontane level, which is characterised by the presence of highland and mountain plant species, or even species of alpine level. Babinské louky, with its high species diversity, are often compared to species-rich meadows in the Bílé Karpaty (White Carpathians, representing the westernmost edge of West Carpathians) and to the current forest steppes in Russia, the presumed centre of *A. liliifolia*'s range.

The total species' area at Babinské louky is approximately 0.16 ha. The population size was highly variable and dependant on management (mowing) timing. During the years 2011 and 2012, only 3, respectively, 20 individuals were discovered at the site (Fig. 3). A high proportion of the plants were damaged by grazing (e.g., Marečková 2013; Prausová & Marečková 2017). The inappropriate mowing time also led to an increased competition pressure of *Calamagrostis epigejos* (L.) Roth, which oppresses *A. liliifolia* on-site. In recent years (2016, 2019), a semi-parasitic *Rhinanthus alectorolophus* (Scop.) Pollich was introduced to the locality to weaken the spreading of *C. epigejos,* and the fencing on species protection against the grazing was realised. The first results show the rapid answers to these interventions by an increase in the number of individuals (Prausová 2017, 2020). Thus, this management strategy looks to be appropriate.

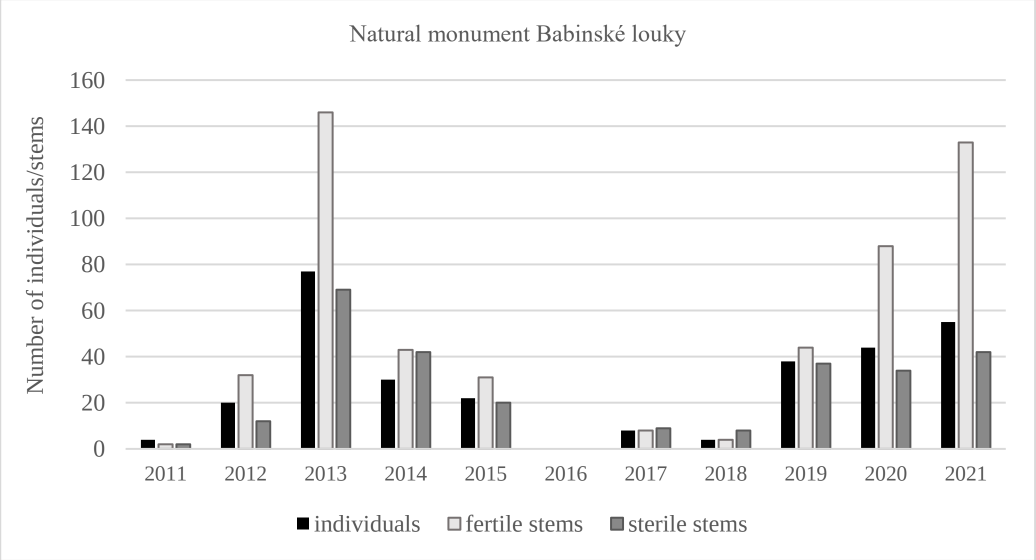


Fig. 3. Changes in population size (shown as the number of individuals) at Babinské louky during the years 2011–2021. The population size was in the past fluctuating and dependant on appropriate management (mowing) timing. Another problem represented grazing by hoofed game or cattle escaping from adjacent pasturages. The area has been fully fenced and protected from grazing since the season of 2019. Thanks to this measure, first from the population maximum in 2013, the trend of increasing population size started.

Natural park Džbán – Natural monuments Smradovna, Čertovka, Bor

The Natural park Džbán is situated on the border of Central Bohemia and Ústí nad Labem regions and includes parallel valleys of the Samotínský potok (Cikánský dolík) and the Zichovecký potok (Smradenské údolí) located northwest of Bílichov village. Phytogeographically the area belongs to Bohemian Thermophyticum with altitude varying from 340 to 420 m a.s.l. The average annual temperature is 8–9 °C, and the average yearly rainfall is 500 mm. The bedrock of the above-mentioned two valleys is formed mainly by Cretaceous sand-calcareous marlstones, glauconitic marlstones, and sandstones. Dominating soils are rendzina and decalcified cambisol (AOPK ČR 2005; Truhlářová 2010; Marečková 2013). For centuries, the whole area of Džbán has been conscientiously studied by Czech botanists, biologists in general, geologists, and other scientists, thanks to its proximity to Prague. Numerous studies mention its refugial character. From the botanical perspective, the locality hosts interesting plant species, e.g., *Veratrum nigrum* L., *Thesium rostratum* Mert. & W.D.J.Koch, *Schoenus nigricans* L., *Tofieldia calyculata* (L.) Wahlenb., *Carex dioica* L., *Utricularia minor* L., *Dictamnus albus* L., *Carex pediformis* C.A.Mey., *Arctostaphylos uva-ursi* (L.) Spreng. Also, malacozoological (Ložek 2006) and palynological (e.g., Rybníčková & Rybníček 1999) research were done in the neighbouring area.

At Smradenské údolí, *Adenophora liliifolia* grows along a forest road called "Hadovka" in-between the managed forest stands with a high proportion of conifers *Picea abies* and *Pinus sylvestris*. The total species area is ca. 0.84 ha, but the plants grow highly scattered (9–23 plants observed). Seedlings are regularly observed close to older specimens; however, the population's size remains stable and does not exceed 23 individuals. This site is referred to in the literature as "Smradovna." The species has been monitored, at this locality, since 1958 by Vladimír Bilinský, who in 1992 initiated management interventions (Truhlářová 2010). Since 2011 the population has been monitored under the NATURA 2000 and as a part of various bachelor and diploma thesis (e.g., Řeháková 2013).

Next to the above-mentioned population located in Bílichovské údolí, two other micro-habitats ("Bor" and "Čertovka") with the occurrence of *A. liliifolia* have been preserved in the forest near the road between villages Hříškov and Řevničov. On the site called "Bor," only a few (4–6) plants grow in an oak forest (less than 0.1 ha), whereas on "Čertovka," several (4–13) individuals are found in a well-lit pine-oak forest (ca. 0.25 ha). These two micro-populations were found in 2009 by Michal Severa, Jiří Brabec, and Václav Somola and are monitored only since the year 2015 (Prausová *et al.* 2020).

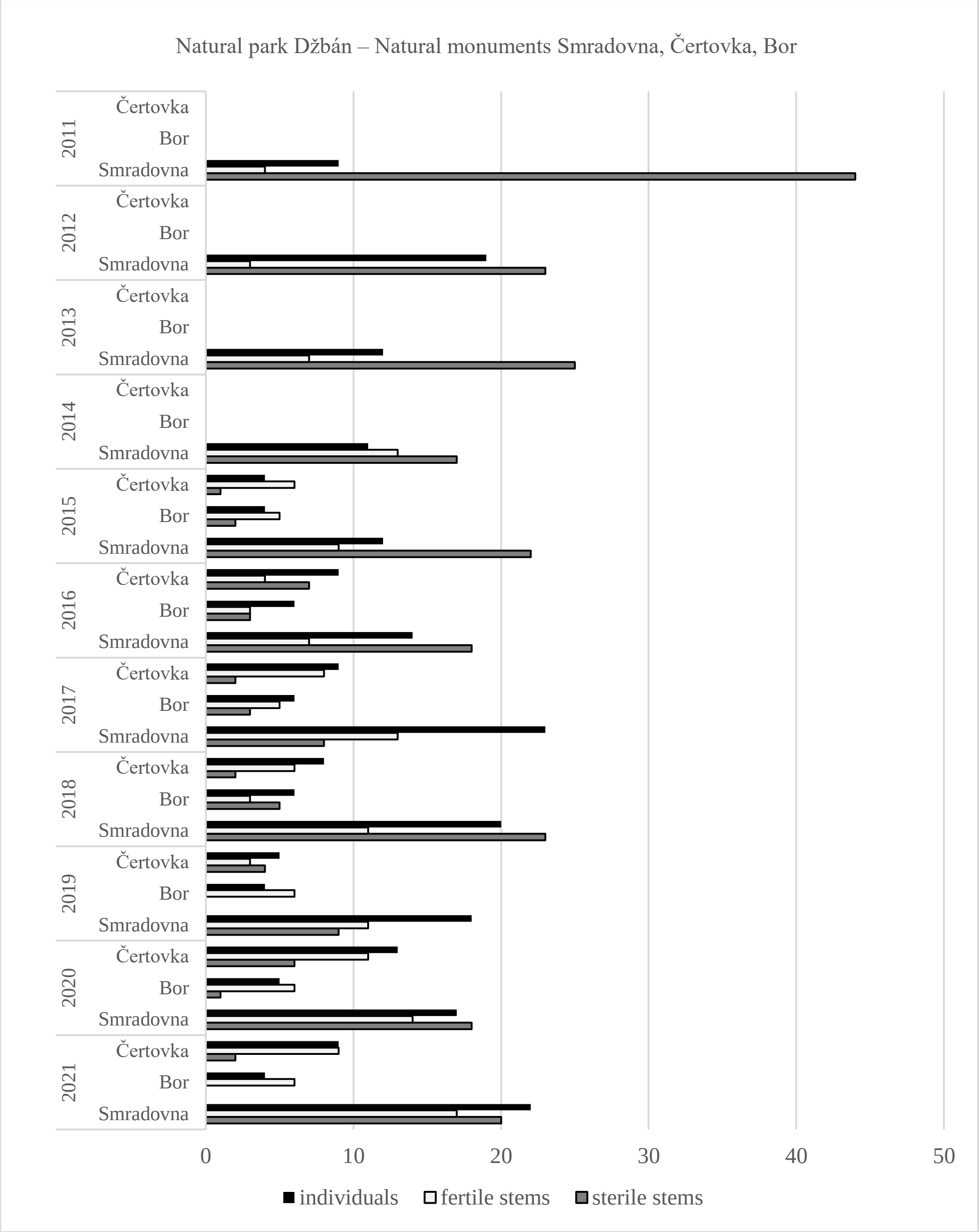


Fig. 4. Alterations of *Adenophora liliifolia* population size in Džbán (2011–2021), populations Bor and Čertovka have been monitored since 2015. Population Smradovna has been monitored since the 1950s, continuously supported by artificial seed dispersion along the forest road Hadovka.

National Nature Reserve Karlštejn

The National Nature Reserve Karlštejn protects the area in the core part of the Protected Landscape Area Český Kras. *Adenophora liliifolia* is present here in four micro-populations. The first of them is located in a large fenced area called "Na Mořině." This population is the most abundant. It is the only micro-population with the verified occurrence of *A. liliifolia* (2021 personal observation). The second micro-population "U Hájovny" (1 plant, unconfirmed in 2021); the third a small fenced area "U přírodňáku/Mokrý vrch" (last observation in 2007); and the fourth micro-habitat "V Kalhotách" (1–2 plants, unconfirmed in 2021). Dominant soil types are rendzina and pararendzina, predominantly developed on limestone. The altitude of the territory is about 390–410 meters a.s.l. Phytogeographically the area belongs to Bohemian Thermophyticum, with an average annual temperature of 8–9° C and average precipitation of 500–550 mm. The habitat with the species occurrence is represented by oak-hornbeam and central European oak forests (Truhlářová 2010; Marečková 2013; Prausová & Marečková 2017).

The first micro-population occupies an area of approximately 0.15 ha. The fence was created as a protection against grazing by hoofed game. The micro-population is located on the clearing site after spruce planting (during the early 1970s). Just before the felling of spruces, several hundred *A. liliifolia* plants were present on-site (according to Petr Moucha, a former worker of the state nature protection). In 1970, more than 100 flowering plants were recorded here. Regular monitoring has been carried out since 1971, but, e.g., in 1982, only two flowering plants were mentioned. Since the 1990s, the protection intervention has focused on eradicating small trees, tree seedlings, and shrubs (e.g., blackberries). During the 1990s, the micro-population served as a seed-source for plant propagation to strengthen other nearby populations, including the Karlické údolí. In recent decades, several dozen plants have been regularly documented here; from 2003 to 2007, about 30 plants were found at different stages of flowering and fruit formation (Prausová 2015–2020; Prausová *et al.* 2020). Since 2011, the population started to be regularly monitored, and the number of tufts ranged between 19 and 26 (Marečková 2013). The year 2015 left significant damage on the population due to severe drought. Many flowering plants were negatively affected by the long-lasting drought and raking by small animals (most likely rodents) (Bajerová 2015). In 2020, only one flowering stem was recorded, and the fitness of remaining sterile individuals was decreased (dried individuals or individuals with moldy leaves and dried terminal meristem). In 2021, eight plants developed strong stems; these were, however, grazed by wild animals, which got across the fencing (Prausová 2015–2020; Prausová & Vaculná, personal observation). A low number of seedlings and young plants were observed on-site, and all the remaining individuals are marked with metal tags and annually monitored for about ten years. A part of the fenced area is significantly overgrown with *Calamagrostis epigejos* and *Rubus* spp., which must be removed yearly from the site.

Natural monument Karlické údolí

The natural monument Karlické údolí located in the Protected Landscape Area Český kras (Central Bohemia), is surrounded by villages Karlík, Lety, Mořinka, Mořina, Roblín and Vonoklasy. Bedrock in this site is represented by Ordovician quartzite and sandy slates. A strip of basic igneous dolerite rocks and black slate is running through the centre of the area. This line separates the sedimentary quartz part in the southeast from the limestone and calcareous shale in the northwest. Dominant soil types are rendzina and cambisol. Population grows at an altitude of about 370–380 m a.s.l. Phytogeographically this locality belongs to Bohemian Thermophyticum, with an average annual temperature of 9°C, and average precipitation of 500 mm. *Adenophora liliifolia* grows at this locality in an oak-hornbeam forest with a higher proportion of rejuvenating *Fraxinus excelsior* (Truhlářová 2010; Marečková 2013; Prausová & Marečková 2017).

The occurrence of the species on this locality has been known since 1957, and the locality started to be monitored in the 1980s. In the past, the population was made by several hundred plants (Petr Moucha, pers. communication). However, the population size was rapidly declining. In 1996/1997, 140 cultivated individuals (raised from seeds originated from the micro-population "Na Mořině" (Karlštejn locality) were planted here to support and increase population size and vigor. In 2002, approximately 70 plants were counted on an area of 0.25 ha. Since 2005, the site's shading has been purposefully regulated by pruning trees, leaving the felled trees at the place, so the herb layer is worse accessible for grazing. In 2006, 48 non-flowering individuals were observed, most of them grazed; however, individuals between the fallen branches and trunks escaped grazing (Prausová *et al.* 2020). The metal marking of individual plants was repeatedly damaged, perhaps by persons searching for historical objects using a metal detector or by wild boar' digging (Prausová & Vaculná, personal observation). Since 2011, the number of individuals has ranged from 20 to 317. In the past years, under the undesirable light conditions, individuals of *A. liliifolia* used to be short in stature, usually consisting only of 1 or a few stems, often only in a rosette of juvenile leaves. They rarely bloomed, with only a few flowers. In 2010, about 10 plants bloomed for the first time since the 1990s, most likely due to better illumination (Prausová *et al.* 2020). In 2015, due to the severe drought and partly also grazing, no individuals bloomed. During the climatically favorable year 2020, with sufficient precipitation after several drier years, the population has been completely fenced, and 69 fertile stems were observed (Prausová 2017–2020). The highest number of 250 flowering stems was recorded during the last monitoring in 2021. It seems that the increase in the population size is a positive reaction to the management interventions (manual plucking of *Rubus* spp. and rejuvenating *Fraxinus excelsior*). The current population represents an area of approximately about 0.14 ha. It is planned to open the fence after the vegetation season to make it permeable to wildlife, which may help reduce expansive species.

Natural monument Kobyla

The natural monument Kobyla represents an abandoned limestone quarry. Here, an artificial population of *A. liliifolia* was established at the bottom of the quarry under the partial shade of trees. The altitude of the locality is about 450 m a.s.l. Bedrock consists mainly of limestone from the older Proterozoic. There is a Quaternary cover in the overburden of these layers. The topsoil layer consists primarily of various developmental stages of rendzina, brown soil, and loess clays (Prausová *et al.* 2020). The population area is approximately 0.17 ha. The quarry is overgrown by a mix of rocky vegetation with *Festuca pallens* Host and mesophilic meadow with *Arrhenatherum elatius* (L.) P.Beauv. ex J.Presl & C.Presl., together with shrubs. Shrubs in the quarry were in the past reduced. In the future, there may be a threat of *Calamagrostis epigejos*, rapidly expanding from another part of the quarry (Prausová & Marečková 2017; Prausová *et al.* 2020).

The experimental planting of *A. liliifolia* was established in 2002 when 12 individuals grown in the Botanical Garden of the Capital City of Prague were planted here. The source population of seeds was the micro-population “Na Mořině” (locality Karlštejn). Individuals were planted in two groups of six; one group was planted on the quarry bottom, the second on the opposite rock in the quarry wall. About 200 seeds were sown directly on the soil surface in eight different places within the quarry; some of them successfully germinated and developed into seedlings (Petr Moucha, and Josef Mottl, personal communication). This artificially established population is present here until today, i.e., for almost 20 years, but without further natural renevation and spread. During the period 2012–2021, 3–8 tufts were regularly found here (Fig. 5).

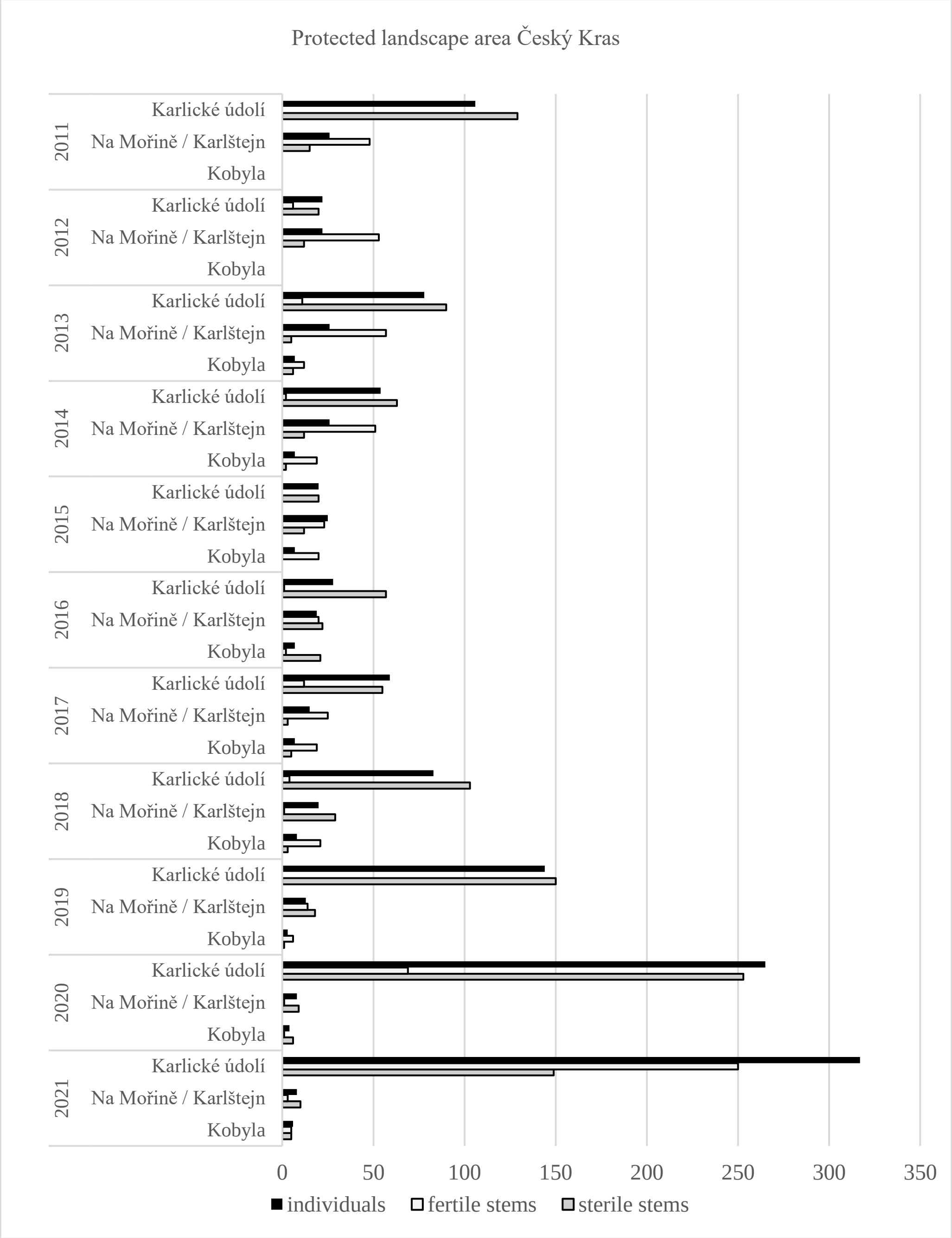


Fig. 5. The numbers of *Adenophora liliifolia* in PLA Český Kras (2011–2021), artificial population Kobyla has been monitored since 2013 only. Currently, the most prospering population in Karlické údolí is benefiting from the fencing of the area and the plucking of rejuvenating *Fraxinus excelsior* and *Rubus* spp. in the last four years. The number of individuals at artificial population Kobyla did not exceed the number of plants planted initially nearly 20 years ago; therefore, it seems no indigenous multiplication occurs on-site.

Natural monument Báňský les

Báňský les represents the westernmost part of a large forest complex, situated east of the town Poděbrady and between villages Žehuň, Hradčany, Dlouhopolsko and Lovčice. The largest part of this complex consists of the "Obora Forest," which is mostly enclosed and used for wild-game keeping (Obora Kněžičky, also called the Žehuň Game Reserve).

The basic relief features are determined by the distinctive asymmetrical ridge of Hradčanská cuesta. Its front slope falls steeply into the valley of the Cidlina river and has a south orientation. Steep slopes along 7 km create suitable habitats for the development of a wide range of xerothermal communities of plants and animals, which are protected in the Natural park Žehuňsko-Báň and NNR Kněžičky. The flat rear slope is slightly inclined to the north into the Mrlina river basin and is mainly wooded by deciduous forests with a predominance of oak. The steep south-facing slopes between Hradčany and Stará Báň, are covered by stands of flowery downy oak forests, transforming into non-forest vegetation of thermophilous shrubs, broad-leaved dry grasslands, and exposed white hillsides. However, most of the "Báňský les" area lies on a plateau that slowly bends to the village Dlouhopolsko. In addition to shady oak-hornbeams and plantings of spruce and pine, there are loose deciduous and mixed forest stands with oak, birch, pine, and in wet areas also with ash and alder trees, which are rich in the undergrowth with rare species. There are also three small meadow enclaves with *Molinia* sp. Only the southern slopes of the Nature monument Báň (10.5 ha) used to be protected, but since 2016 the protected area was enlarged to include part of forests on the plateau and renamed to the Natural monument Žehuňsko-Báň (Roleček & Šťastný 2020; Prausová *et al.* 2020).

Bedrock represents calcareous claystone and marlstones of the Cretaceous age. The average altitude is 240–272 m a.s.l. Phytogeographically the locality belongs to Bohemian Thermophyticum, with an average annual temperature of 9 °C, and average precipitation of about 500–550 mm (Roleček & Šťastný 2020; Prausová *et al.* 2020).

Surroundings of Žehuň along Labe river is one of the sites with the historical occurrence of *A. liliifolia* in the Czech Republic (Prausová *et al.* 2020). The species was repeatedly found here in the 1930s and 1940s by J. Klika, Z. Vulterin, and M. Deyl (Klika 1943; Kubát 1986). Although the localization of these findings in the Klika's article is somewhat confusing, the species probably occurred in the "Bažantnice" forest north of Dománovice; in the forest between Dománovice and Radovesnice II and perhaps in "Kozí hůra" south of Žehuň. Since then, despite repeated efforts (e.g., Roleček & Novák 2008), its occurrence has not been verified. Two individuals, both with two flowering stems) were found by Martin Šťastný in 2018 (Roleček & Šťastný 2020), and verified in 2019 (2 individuals, one flowering and one sterile), 2020 (2 individuals, one with 2 and one with 1 flowering stem), and in 2021 (2 individuals, one with 2 flowering and 1 sterile stem, second with 2 flowering stems). From the season 2019, plants are protected against grazing by individual fences.

Year of monitoring	**Monitored items**	**Vražba**	**Babinské louky**	**Na Mořině /Karlštejn**	**Karlické údolí**	**Kobyla**	**Smradovna**	**Bor**	**Čertovka**	**Báňský les**	**individuals total**
2005	**individuals**	**92**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**92**
	fertile stems	146	-	-	-	-	-	-	-	-	
	sterile stems	42	-	-	-	-	-	-	-	-	
2006	**individuals**	**106**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**106**
	fertile stems	150	-	-	-	-	-	-	-	-	
	sterile stems	39	-	-	-	-	-	-	-	-	
2007	**individuals**	**92**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**92**
	fertile stems	165	-	-	-	-	-	-	-	-	
	sterile stems	17	-	-	-	-	-	-	-	-	
2008	**individuals**	**85**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**85**
	fertile stems	125	-	-	-	-	-	-	-	-	
	sterile stems	63	-	-	-	-	-	-	-	-	
2009	**individuals**	**66**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**66**
	fertile stems	121	-	-	-	-	-	-	-	-	
	sterile stems	30	-	-	-	-	-	-	-	-	
2010	**individuals**	**133**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**133**
	fertile stems	251	-	-	-	-	-	-	-	-	
	sterile stems	10	-	-	-	-	-	-	-	-	
2011	**individuals**	**91**	**4**	**26**	**106**	**-**	**9**	**-**	**-**	**-**	**236**
	fertile stems	152	2	48	0	-	4	-	-	-	
	sterile stems	104	2	15	129	-	44	-	-	-	
2012	**individuals**	**83**	**20**	**22**	**22**	**-**	**19**	**-**	**-**	**-**	**166**
	fertile stems	140	32	53	6	-	3	-	-	-	
	sterile stems	60	12	12	20	-	23	-	-	-	
2013	**individuals**	**96**	**77**	**26**	**78**	**7**	**12**	**-**	**-**	**-**	**296**
	fertile stems	198	146	57	11	12	7	-	-	-	
	sterile stems	91	69	5	90	6	25	-	-	-	
2014	**individuals**	**95**	**30**	**26**	**54**	**7**	**11**	**-**	**-**	**-**	**223**
	fertile stems	112	43	51	2	19	13	-	-	-	
	sterile stems	123	42	12	63	2	17	-	-	-	
2015	**individuals**	**94**	**22**	**25**	**20**	**7**	**12**	**4**	**4**	**-**	**188**
	fertile stems	177	31	23	0	20	9	5	6	-	
	sterile stems	101	20	12	20	0	22	2	1	-	
2016	**individuals**	**85**	**0**	**19**	**28**	**7**	**14**	**6**	**9**	**-**	**168**
	fertile stems	207	0	20	1	2	7	3	4	-	
	sterile stems	37	0	22	57	21	18	3	7	-	
2017	**individuals**	**83**	**8**	**15**	**59**	**7**	**23**	**6**	**9**	**-**	**210**
	fertile stems	198	8	25	12	19	13	5	8	-	
	sterile stems	51	9	3	55	5	8	3	2	-	
2018	**individuals**	**86**	**4**	**20**	**83**	**8**	**20**	**6**	**8**	**2**	**237**
	fertile stems	85	4	1	4	21	11	3	6	4	
	sterile stems	12	8	29	103	3	23	5	2	0	
2019	**individuals**	**79**	**38**	**13**	**144**	**3**	**18**	**4**	**5**	**2**	**306**
	fertile stems	226	44	14	0	6	11	6	3	1	
	sterile stems	21	37	18	150	1	9	0	4	1	
2020	**individuals**	**75**	**44**	**8**	**265**	**4**	**17**	**5**	**13**	**2**	**433**
	fertile stems	275	88	1	69	1	14	6	11	3	
	sterile stems	62	34	9	253	6	18	1	6	0	
2021	**individuals**	**79**	**55**	**8**	**317**	**6**	**22**	**4**	**9**	**2**	**502**
	fertile stems	280	133	3	250	5	17	6	9	4	
	sterile stems	51	42	10	149	5	20	0	2	1	

Table 1. Table showing numbers of *Adenophora liliifolia* tufts (i.e., *A. liliifolia* individuals) per locality per year, representing also absolute numbers of individuals of the species growing within the Czech Republic from 2005 up to the year 2021. Green colour highlights the increase of individuals at particular locality compared to the previous year. The stagnation or decrease is reported for populations Vražba and Na Mořině (Karlštejn). Populations Babinské louky and Karlické údolí increased in the last four, respectively six years. Despite the nearly three-times higher numbers in 2021 than in 2016, natural renevation is lacking. The increase in the total numbers is instead represented by older plants, which were for years escaping counting due to insufficient light conditions in the rosette of juvenile leaves waiting for better conditions.

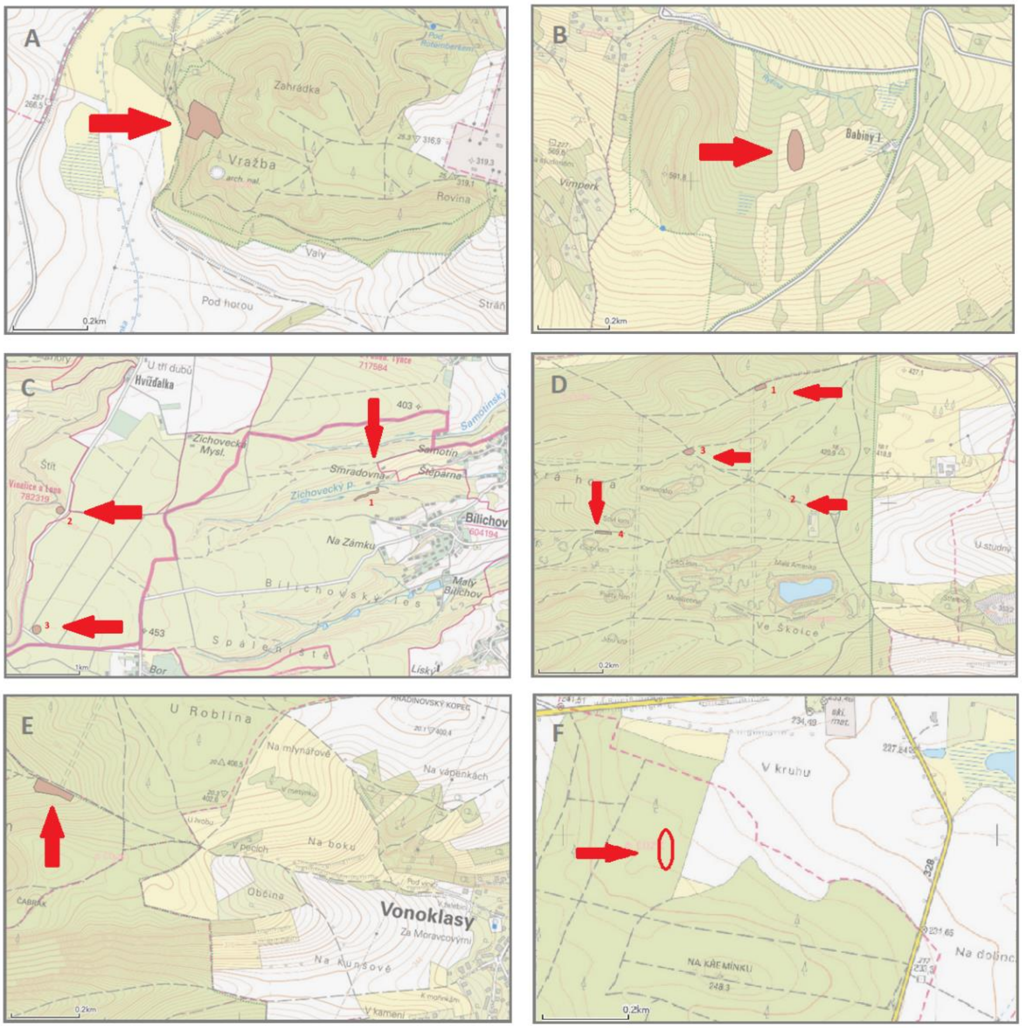


Fig. 6. Location of *Adenophora liliifolia* populations within particular localities. Populations (thus place with occurrence of *A. liliifolia*) are depicted by reddish-brown polygon. (A) Vražba, 79 plants in 2021. (B) Babinské louky, 55 plants in 2021. (C) Smradovna (1), 22 plants in 2021; Čertovka (2), 9 plants in 2021; and Bor (3), 4 plants in 2021; within Natural park Džbán. (D) Karlštejn National Nature Reserve: big fencing „Na Mořině” (1), 8 plants in 2021; „U Hájovny“ (2), no observed plant in 2021; small fencing „U přírodňáku/Mokrý vrch“ (3), no plant in 2021 and „V Kalhotách“ (4), no plant in 2021. (E) Karlické údolí, 317 plants in 2021. (F) Báňský les, 2 plants in 2021. Map original from AOPK ČR 2021 portal https://aopkcr.maps.arcgis.com/.

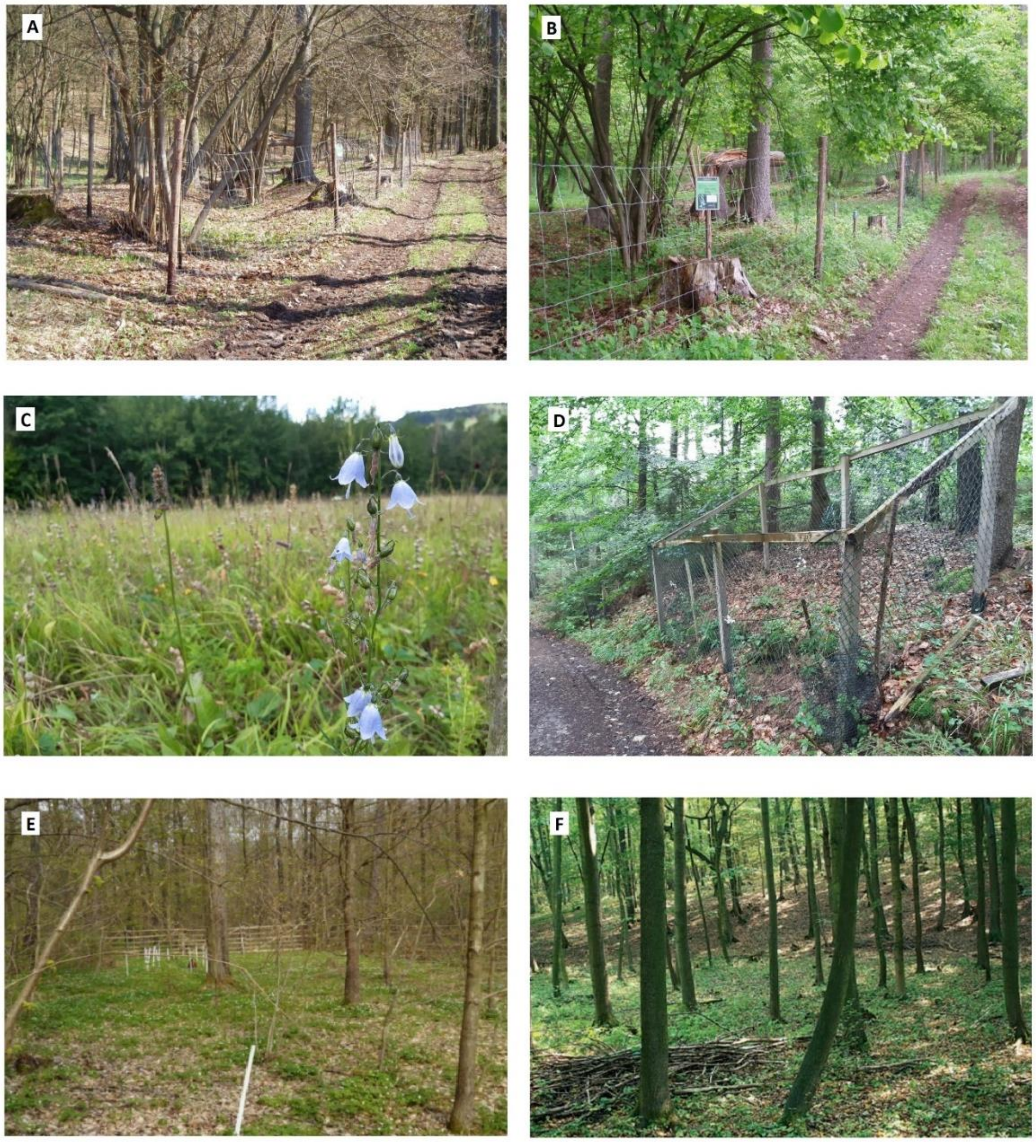


Fig. 7. Habitat types at particular localities with the occurrence of *Adenophora liliifolia* within the Czech Republic. (A) Vražba micro-habitat #4 (17. 4. 2010), (B) and the same place one month later (Vražba micro-habitat #4; 18. 5. 2010), Oak-hornbeam forest; (C) Babinské louky (5. 8. 2020), Intermittently wet *Molinia* meadow; (D) NM Smradovna along the "Hadovka" forest road (5. 8. 2021), Oak-hornbeam forest; (E) NNR Karlštejn fencing "Na Mořině" (24. 4. 2012), Central European basiphilous thermophilous oak forest; (F) Karlické údolí (August 2016), Oak-hornbeam forest. Authors of the photographs: Lucie Vaculná (A, B, C, D), Andrea Řeháková (E), Romana Prausová (F).

The situation of *Adenophora liliifolia* within its European territory

As mentioned earlier, *Adenophora liliifolia* is a species of EU Community interest subjected to a regular reporting process according to the NATURA 2000 methodology. Altogether, 70 NATURA 2000 Special Areas of Conservation (SAC) were designed because of the species occurrence in EU territory: Austria (2), Croatia (1), Czech Republic (5), Germany (2), Hungary (5), Italy (12), Poland (17), Romania (9), Slovakia (15), Slovenia (2) (EUNIS 2021). However, the comparison of historical data with present surveys showed a rapid decrease in the number of populations of *A. liliifolia* across its whole distribution, not only in the Czech Republic. In Slovakia, about 30 populations were known from the Carpathians, and only 10 are persisting; in Poland, the species was known from ca. 100 localities, but nowadays only 21–22 remain; about three populations remain in Austria; two in Germany, one in Switzerland, six in Hungary (Boronnikova 2009; Prausová *et al.* 2016; Valenti *et al.* 2018). The situation is unfavourable also in the eastern part of the species distribution – *A. liliifolia* is listed in 42 Red Books of Plants of Russian, Ukrainian, Belarus, and Baltic regions (Plantarium 2021).

To date, reporting data are available for periods 2001–2006, 2007–2012, and 2013–2018. During the first reporting period (2001–2006), the species was evaluated in Italy, Slovenia, Slovakia, Czech Republic, Germany, Poland, and Hungary. The favourable assessment was reported only for Italian and Slovenian Alpine bioregion and Slovakian Pannonian bioregion. Reports from Austria and Romania have been available since the second reporting period (2007–2012). Nevertheless, as favorable were re-evaluated only populations within the Alpine bioregions (Italy, Slovenia), while the situation for the population within Slovakian Pannonian bioregion was re-evaluated as Unfavourable. The last reporting period (2013–2018) assessed the status of the species within the EU territory as “Unfavourable-Inadequate” or “Unfavourable-Bad,” except the Alpine bioregion in Slovakia and Slovenia. It has to be noted that exact numbers of individuals for the Slovenian population are not available from reporting datasheet, and the population stays on the same level because of no change from the last report. Overall, there is no evidence of change leading to conditions more desirable for the species to prosper.

NATURA 2000 is only a legislative instrument designed to protect the rarest and most endangered species of animals, plants, and natural habitats within the European Union by the declaration of Special Areas of Conservation (SAC). This declaration by itself, however, changes nothing in the species’ habitat. The field experiences show the necessity of conservation management, adapted to the particular locality, continuously evaluated over time, and altered when necessary. Therefore, already implemented or recently prepared national action plans should aim to develop appropriate techniques for ex-situ preservation, e.g., creation of sterile tissue cultures, proper seed storage in seed banks, and cultivation to identify factors critical for seedlings growth; as well as to set management measures for a particular locality.

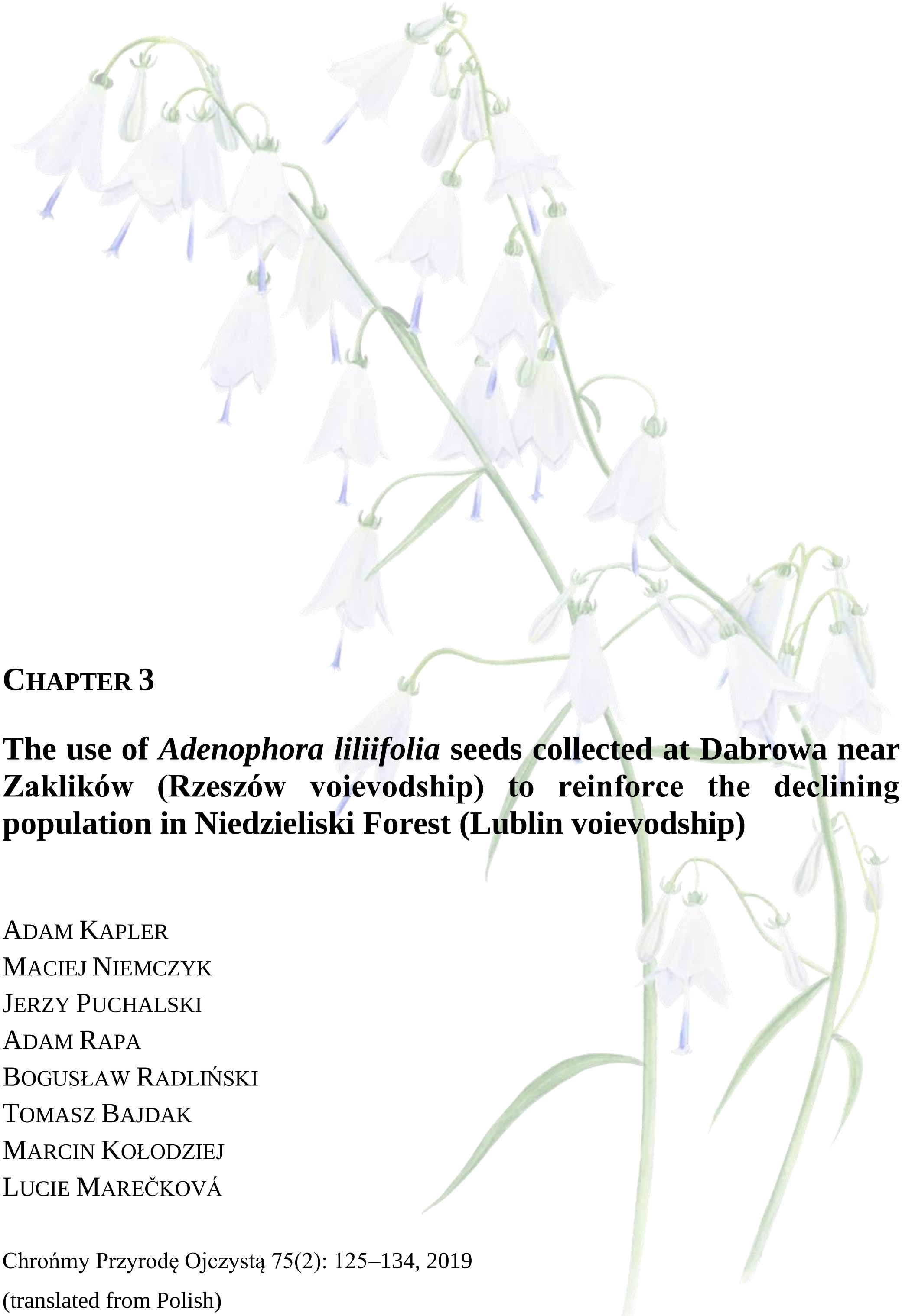
Conclusions

The seventeen-year-long (2005–2021) continuous and detailed monitoring of *A. liliifolia* at Vražba together with the ongoing conservation actions highlighted the critical factors threatening the species within the Czech Republic (Prausová & Truhlářová 2009; Prausová *et al.* 2016, 2020; Prausová & Marečková 2017). The same factors also threaten all the remaining Czech populations. In general, populations respond positively, by an increased proportion of fertile stems, to the opening of the canopy layer by selective removing of shrubs and trees. However, higher transmittance of sunlight causes fast overgrowing of competitively vigorous species (e.g., *Rubus* spp., *Aegopodium podagraria*, *Stachys sylvatica*), which oppresses *A. liliifolia*, and must be eradicated regularly. Another problem represents higher numbers of the hoofed game (roe-deers and fallow deers), which feed on-site and graze *A. liliifolia* stems. Therefore, the plants have to be protected by fencing. Even if all of these pitfalls are handled, only a limited number of new seedlings are observed, although the plants flower abundantly and produce numerous seeds.

Most of the annually observed plants are hefty, producing several robust and tall stems with dozens to hundreds of flowers (Prausová *et al.* 2020). Some of these individuals are monitored from the beginning of the monitoring and thus might be more than 20 years old (personal observation of Prausová & Samková). Adult and old individuals might persist unfavourable conditions thanks to the underground stock organ only with sterile leaves, waiting for the opportunity for flowering for years. A very limited number of seedlings is observed every year, and most of them do not persist to the next season. The lack of exposed soil surface might be the problem of low germination success, as most seedlings are observed in such places. Here they are, however, prone to wilting and other undesirable random events (Prausová *et al.* 2020). The seedlings' delicateness was proved when five healthy and strong seedlings resulted from the germination experiment were planted on-site in 2009 (Prausová & Truhlářová 2009); only two persisted to the next season 2010, but none of them survived till 2011 (Marečková 2010). The key to restoring suitable habitat is to keep a mosaic of opportunities, well-lit areas for species' flowering during the favorable season, and other more shaded parts for dry seasons, which seem to be probably more frequent in the future (Prausová *et al.* 2020; Prausová & Marečková 2017; and personal observation of Prausová & Vaculná).

The last five years (2017–2021) might be considered successful in species conservation within the Czech Republic. In 2020, the Action Plan for species protection was approved by the Ministry of the Environment. A significant increase of observed individuals from 168 in season 2016 to a total of 502 in the year 2021 was encountered. This was most of all thanks to the conservation measures finally implemented at Babinské louky and Karlické údolí localities. Both populations are now fully fenced. The meadow at Babinské louky is mowed at an appropriate time, and oppressing *Calamagrostis epigejos* is tempered by semi-parasitic *Rhinanthus alectorolophus*. Rejuvenating trees and seedlings of *Fraxinus excelsior* are manually plucked in Karlické údolí. The situation in the remaining populations is relatively stable. The most critical seems to be the state of the "Na Mořině" micro-population within the Karlštejn reserve. The fenced area was expanded; however, inappropriate fencing material was used. Trimming of *Corylus avellana* L. in the too-large area caused its uncontrolled rejuvenating. Omission of regular mowing caused the expansion of *Calamagrostis epigejos* and *Rubus* spp. Together with unresolved wilting of plants caused maybe by some pathogen or stochastic events such as rodents' consumption of plants' underground organs, the population size decreased from 26 individuals in 2014 to 8 miserably prospering in 2020, which were however grazed in 2021. It is also a question of whether the remaining individuals are not at the end of their life cycle as the population peak is reported from the late 1970s, and no juvenile plants were observed here (Prausová *et al.* 2020, and personal communication of Prausová & Vaculná).

Even when it looks like we know the most critical ecological requirements of *Adenophora liliifolia*, and the applied conservation management seems to be right-directed, there is obviously still some puzzle piece missing. The flowering plants produce numerous seeds, and even when their germination rate under laboratory conditions is not impressive, the majority of seeds are viable (successful rate about 36% after cold stratification, Bajerová 2015; but see Kapler *et al.* 2019 studying sites in Polish voivodeships Rzeszów and Lublin, Chapter 3). The desirable result of the Action Plan is to have populations able of independent propagation within the designated areas and adjacent surroundings. One of the findings of population genetic studies was that populations are not endangered by decreased genetic diversity, probably thanks to the longevity of individual plants (Prausová *et al.* 2016; Vaculná *et al.* 2021). Therefore, the problem seems to be caused by other factors, which must be proved by detailed population study and planting and sowing experiments.



CHAPTER 3

The use of *Adenophora liliifolia* seeds collected at Dabrowa near Zaklików (Rzeszów voievodship) to reinforce the declining population in Niedzieliski Forest (Lublin voievodship)

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Chrońmy Przyrodę Ojczystą 75(2): 125–134, 2019

(translated from Polish)

Abstract

One of the conservation measures included in the "Infrastructure & Environment Operational Programme – Implementation of protection tasks for Natura 2000 sites and nature reserves in the Lublin province" was to reinforce the declining population of *Adenophora liliifolia* at the Niedzieliski Forest Natura 2000 site, using local seeds. In 2013, seeds from populations located in the Lublin and Podkarpacie provinces were collected. The experiments realised in the Powsin Seed Bank Laboratory showed a lack of germination ability of seeds from the Lublin region. The only exception was one seedling obtained from the seeds collected at the Lasy Strzeleckie natural site. However, this single seedling soon died. On the other hand, seeds collected in the Dąbrowa near Zaklików Natura 2000 area (Podkarpacie province) showed a good germination rate of 42–65%. Therefore, authorities supervising the conservation project decided to use *A. liliifolia* plants obtained from the seeds collected in the Dąbrowa near Zaklików Natura 2000 area to reinforce the declining population Niedzieliski Forest.

Keywords: active protection, introduction, vitality of seeds, genetic rescue strategy.

Introduction

Adenophora liliifolia (L.) A. DC. is a rare and endangered species of Campanulaceae (Kucharczyk *et al.* 2014), under strict species protection (Rozporządzenie 2014) and included in Annex II of the European Union Habitats Directive (Prausová *et al.* 2016). *Adenophora liliifolia* is listed as a critically endangered species (CR) in both the Polish Red Book of Plants and the Polish Red List of Fern and Flower Plants (Kucharczyk *et al.* 2014; Kaźmierczakowa *et al.* 2016) as well as in the Red Book of Plants of the Podkarpackie Voivodeship and the Red List of Plants Lubelskie Voivodeship (Oklejewicz *et al.* 2015; Cwener *et al.* 2017). The populations of *Adenophora liliifolia* in Western and Central Europe, including Poland, have been decreasing in the last century, and its range border is moving eastwards (Tacik 1971; Durczak 1976; Kucharczyk 2007; Kucharczyk *et al.* 2014; Prausová *et al.* 2016). Particularly worrying is the disappearance of this plant due to secondary succession and the competitive pressure of herbivores in the Polesie, Kampinos, Ojców, and Wigry National Parks (Różycki 2013; Kębłowska 2016; Romański 2015; A. Sołtys-Lelek 2015 - personal communication). Based on the conclusions of the species monitoring from 2009-2011 (Rapa 2012a), and as a part of the National Conservation Plan for *Adenophora liliifolia* (Kucharczyk 2007), it was proposed to strengthen its highly vulnerable populations (Kapler *et al.* 2015). This study aims to present the effects of activities on the artificial strengthening of *A. liliifolia* population at the Niedzieliski Forest.

Research area

The Natura 2000 area Niedzieliski Forest (area: 267.2 ha), located southwest of Zamość, on the edge of Roztocze (Ukrainian: Розточчя, hilly geological and ethnographical region between Sandomierz Basin, Dniestr Basin, Lublin Upland, and Volhynian Upland), includes two forest complexes: the isolated forest Serwitut, preserved on a chalk hill surrounded by fallow land and intensively used fields; and the unnamed forest, from the north directly adjacent to the Roztoczański National Park (Gierczyk & Soboń 2008; Kołodziej 2017; Fig. 1). *Adenophora liliifolia* at Niedzieliski Forest was first found in 2007 (Gierczyk & Soboń 2008). In 2014–2017, the national monitoring of this plant, carried out by the Chief Inspectorate of Environmental Protection, was done on-site. In 2014, only six *A. liliifolia* individuals were confirmed (Rapa 2014; Kołodziej 2017). The species grows in the thermophilic variety of the subcontinental oak-hornbeam forest *Tilio cordatae* - *Carpinetum betuli*, now also called: *Carici pilosae* - *Carpinetum*, with rich undergrowth, with numerous occurrences of rare species, such as, for example, *Veratrum nigrum* and *Cypripedium calceolus* (Radliński 2010–2017 – personal observation; PZO 2011 project; Kucharczyk 2014; Rapa 2013 – personal observation; Raport 2014). The described site was considered when creating a List of Natura 2000 sites and included in the Niedzieliski Forest Special Protection Area.

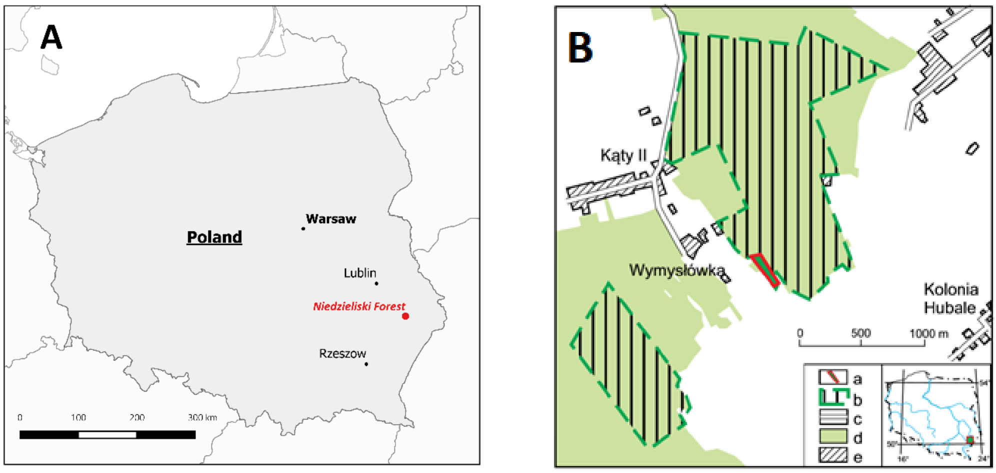


Fig. 1. (A) Location of Niedzieliski Forest in Poland. (B) Location of Niedzieliski Forest: a – reinforced population, b – PLH 060092 Special Area of Conservation Niedzieliski Forest, c – roads, d – forests, e – buildings.

Material and methods

Conservation measures of species' active protection

Conditions of the Niedzieliski Forest site

As part of the active protection of *Adenophora liliifolia*, two conservation measures were initially planned in Niedzieliski Forest. These were: 1) strengthening the population with individuals derived from seeds previously collected on-site and grown at the Polish Academy of Sciences Botanical Garden – Center for the Conservation of Biological Diversity (PAN OB – CZRB) in Warsaw-Powsin; 2) reduction of the undergrowth perennials and partial soil exposure in place of native occurrence of *A. liliifolia* and the place of the planned introduction of plants from cultivation, where other protected and rare species of light-demanding species, e.g., *Veratrum nigrum, Cypripedium calceolus,* and *Gladiolus imbricatus* grow. Unfortunately, seeds from the sites Skrzyniec, Wierzchowiska, and Strzeleckie Forests did not germinate. Therefore, to strengthen the Niedzieliski Forest population, *A. liliifolia* seeds from a natural position in the Lublin Upland, administratively belonging to the neighboring Podkarpackie Voivodeship in Ostoja Siedliskowa Natura 2000, Dąbrowa near Zaklików (Rapa 2012b) had to be used.

Methods of seeds' obtaining and testing

Seeds from natural stands in Lubelskie (Lublin province, Lublin voievodship) and Podkarpackie (Rzeszów voievodship or Subcarpathian voievodship) voivodships were obtained in 2013. As the first step to long-term storage of seeds, seed germination tests were carried out to assess seed viability using standard procedures (optimized during earlier work on this species). The obtained seeds were cleaned and then placed at 15 °C, in a chamber of stable relative humidity of 18%. After 30 days, some seeds were subjected to cool, 30-day stratification on a substrate saturated with distilled water in a refrigerator at 4 °C. During this time, the remaining part of the seeds was placed in the chamber to maintain constant humidity parameters. After completion of the stratification, germination tests were assumed in two variants: 1) seeds without stratification (control test), 2) seeds after cold stratification. Seeds germinated in Petri dishes (9 cm in diameter) in growth chambers. Variable temperatures 25/15 °C (day/night), photoperiod (16 hours light / 8 hours dark), and humidity 70% were set for germination. Due to the small number of available *A. liliifolia* seeds, other variants of their viability testing were not performed. The seeds were also subjected to the microscopic observation of embryo viability. The observations were made after the first unsuccessful seed sprouting attempts. Depending on the number of generative diasporas in the sample, the observations concerned either all seeds from a given sample or only non-germinating ones.

Locality	**Province/Municipality**	**Physico-geographical region**	**Number of seeds per variant**	**Germination rate (%)**	
				C	St
Skrzyniec	lubelskie/ Bełżyce	Lublin Upland	50	0	0
Wilderness of Strzelecki Forests	lubelskie/ Hrubieszów		50	2*	10*
Niedzieliski Forest	lubelskie/ Zamość		25	8*	**
Dąbrowa near Zaklików	podkarpackie/ Zaklików		50	42	65
Poleski National Park	lubelskie/ Urszulin	Polesie Lubelskie	-	the population extinct in the 20th century	
Wierzchowiska	lubelskie/ Bełżyce	Lublin Upland	-	lack of seed due to herbivore pressure	

Table 1. Results of viability tests for seeds collected from selected populations in SE Poland (C - control sample; St - after stratification; * dead seedlings; ** no variant due to too few seeds)

Improvement of subcontinental oak-hornbeam forest light conditions

During 12-16 September 2014, on the area of 0.05 ha, the light conditions were adjusted at locality Niedzieliski Forest. The overgrown shrubs were reduced by felling the most prominent individuals and pulling out smaller ones. The biomass was removed (Raport 2014). Before and after these activities, phytosociological relevées were recorded - in 2014 and 2017 (Rapa 2014; Kołodziej 2017).

Results

Seed viability

All seeds from locality Skrzyńca, 98% in the control sample and 90% in the sample after seed stratification from locality Strzelecki Forest were not viable (Table 1). Microscopic observations showed the complete absence or presence of diseased, poorly developed embryos in the seeds of *A. liliifolia*. Only seeds from Dąbrowa near Zaklików (Podkarpackie Voivodeship) could be used to enrich the target population. All seeds (50 pieces) from Zaklików turned out to be healthy and adequately developed (Fig. 2). Germination capacity was relatively high - 42% for the control sample and 65% for seeds after cold stratification (Table 1, Fig. 3). The obtained seedlings were developing very well (Fig. 4, 5).

Population dynamics

In 2014, there were six indigenous individuals in Niedzieliski Forest, of which one to two were in the generative stage (Table 2). In September 2014, a further five individuals were planted, grown from seeds taken from the Dąbrowa near Zaklików locality. In 2017, 12 individuals of *Adenophora liliifolia* were found on-site, four of which were from the introduction (Table 2). The share of newly introduced individuals was about 1/3 of the population in 2017 (Table 2).

Year	**Number of individuals**		**Total**	**The proportion of introduced individuals (%)**
	Native	**Introduced**		
2014	6 (1-2)	5 (0)	11 (1-2)	45.4 (0)
2017	8 (4)	4 (2)	12 (6)	33.3 (33.3)

Table 2. Structure of the *Adenophora liliifolia* population in the SAC Niedzieliski Forest in 2014 and 2017. The number of generative individuals is given in the brackets.

Fig 2. Seeds collected from the population in Dąbrowa near Zaklików. (9 October 2013; photo by Piotr Walerowski)

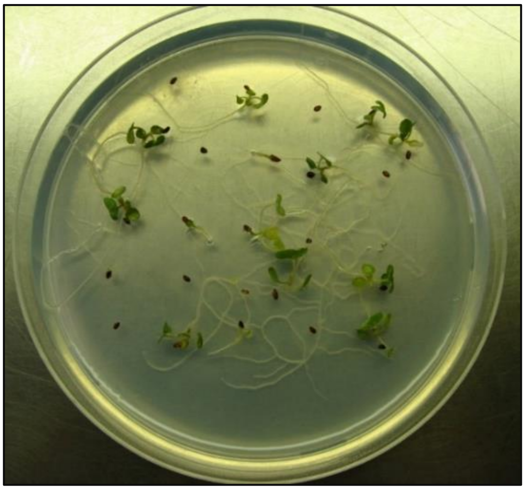


Fig. 3. Germination of *A. liliifolia* seeds. (12 November 2013; photo by Maciej Niemczyk)

Fig. 4. Seedlings of *A. liliifolia* (10 December 2013; photos by A. Kapler)

Fig 5. Juvenile *A. liliifolia*’s plants. (14 January 2014; photo by A. Kapler)

Discussion

Arguments for and against the use of seeds from geographically or genetically distant populations

The use of seeds taken from distant places to enrich the target population is a controversial method of active protection (Ren *et al.* 2012). Some scientists and field botanists argue that only a small proportion of plants from very genetically and geographically close populations may be used to strengthen a limited number of populations (Ricciardi & Simberloff 2009). On the contrary, others argue that it is beneficial to use different genetic lineages to increase genetic diversity, even if the population loses its genetic distinctness (Kamiński 2001; Vitt *et al.* 2009). In the light of the work of Prausová *et al.* (2016), the small relict populations of *A. liliifolia* located in the close geographical distance are characterised by high similarity. The transfer of plants/seeds between sites within the same physico-geographical region (Lublin-Lviv Upland) will likely not introduce different genotypes; therefore may be considered acceptable. The genetic rescue strategy used to protect populations of low genetic diversity (e.g., Ingvarsson 2001) is becoming increasingly popular in nature conservation.

Potential genetic differentiation of the south-east Poland populations

According to some opinions, strengthening the population in Niedzieliski Forest with plants from the same physical and geographical area crossed only by the voivodship border cannot be considered the introduction of a foreign material; therefore, it is entirely acceptable. However, the populations of the Lviv-Lublin Upland could constitute a genetic lineage separated from the populations from other regions of Poland. Therefore, the origin of the *Adenophora liliifolia* seedlings used to increase the number of plants in the Niedzieliski Forest is essential in maintaining the unique genetic character of this isolated population. For instance, Cieślak (2014) reported genetic separation for the closely located populations of xerothermic grassland species *Carlina onopordifolia*, *Cirsium pannonicum*, *Inula ensifolia* and *Linum hirsutum*. In the light of the research of Vaculná *et al.* (2021), the genetic distinctness of *Adenophora liliifolia* from south-eastern Poland to other native Polish populations does not seem to be particularly pronounced. However, it cannot be entirely ruled out. Differences might be discovered using more precise and advanced methods of analysis in the future.

Potential reasons for the undeveloped or poorly developed embryos

The exact determination of the reasons for the lack of embryos or their underdevelopment in seeds collected in the Lublin province requires further research. These may be 1) insufficient light at these sites during the flowering and fruiting of *A. liliifolia,* typical for habitats undergoing natural succession from bright oak to subcontinental oak-hornbeam forest (see Dąbrowski 1967; Kaźmierczakowa 1971, 1991; Głazek 1976; Jakubowska-Gabara 1996; Kucharczyk 2007; Rapa 2009, 2012b). Few publications are studying the effect of light intensity on the condition of pollen and pistil and its impact on seeds and seedlings development in Campanulaceae (Galloway 2001). 2) A small number of *A. liliifolia* individuals and their possible slight genetic variation in Lublin sites, typical for relict, highly isolated populations of rare vascular plant species (see Ellstrand and Elam 1993; Frankham 1998; Armbruster & Reed 2005; López-Pujol *et al.* 2009; Boronnikova & Nechaeva 2012; Prausová *et al.* 2016; Čepelová & Prausová 2017). It is worth noting that, in contrast to the poor-quality seeds collected from the small number of Lublin *A. liliifolia* populations, seeds obtained from extremely small populations in the province Łódź (Mierzyce, Stary Redzeń) and Podlasie (Puszcza Knyszyńska) germinated and gave seedlings capable of further development (Bednarek-Kolasińska 2018; Kucharski 2018; M. Kiedrzyński 2018 – personal observation; D. Wołkowycki 2016, 2017 - personal observation).

Effectiveness of active conservation measures

Removal of the shrubs and exposing the undergrowth at the *Adenophora liliifolia* locality Niedzieliski Forest resulted in faster conservation effects than planting individuals from a different site. An increasing proportion of native individuals in the generative stage and the emergence of native seedlings were reported soon after realizing the measures. The *Adenophora liliifolia* population reacted equally to active protection in the Czech Republic (Vražba locality, East Bohemia; L. Marečková - personal communication 2019), where the removal of shrubs and competitively strong perennials increased the number of generative shoots in native plants. Conclusions made based on the Czech experience on strengthening the population of *Adenophora liliifolia* in the Vražba locality show that it is necessary to continue removing shrubs and expansive perennials annually. After canopy opening and clearing of the undergrowth, a rapid expansion of competitively stronger perennials and shrubs (e.g., especially *Rubus* spp., *Calamagrostis* spp., *Aegopodium podagraria* L., *Chaerophyllum* spp.; Čepelová & Prausová 2017; L. Marečková - personal communication 2019) occurs. In the perspective of several years, intervention at Niedzieliski Forest proved to be more successful than analogous procedures at Vražba. Five juvenile plants germinated and grown at the University of Hradec Králové were planted in 2009 in Vražba locality. Three-year-old individuals were found in 2010. In 2011 neither one introduced plant was located. As the introduced plants were carefully marked with metal plates and not found in the following year, they could probably have been dug out, grazed, dried, or they died from other reasons or their combination. Attempts to sow seeds on the open soil gaps in the undergrowth conducted on the Vražba site did not have the effect of increasing the number of *Adenophora liliifolia* plants (L. Marečková – personal communication 2019).

Summary of results and conclusions

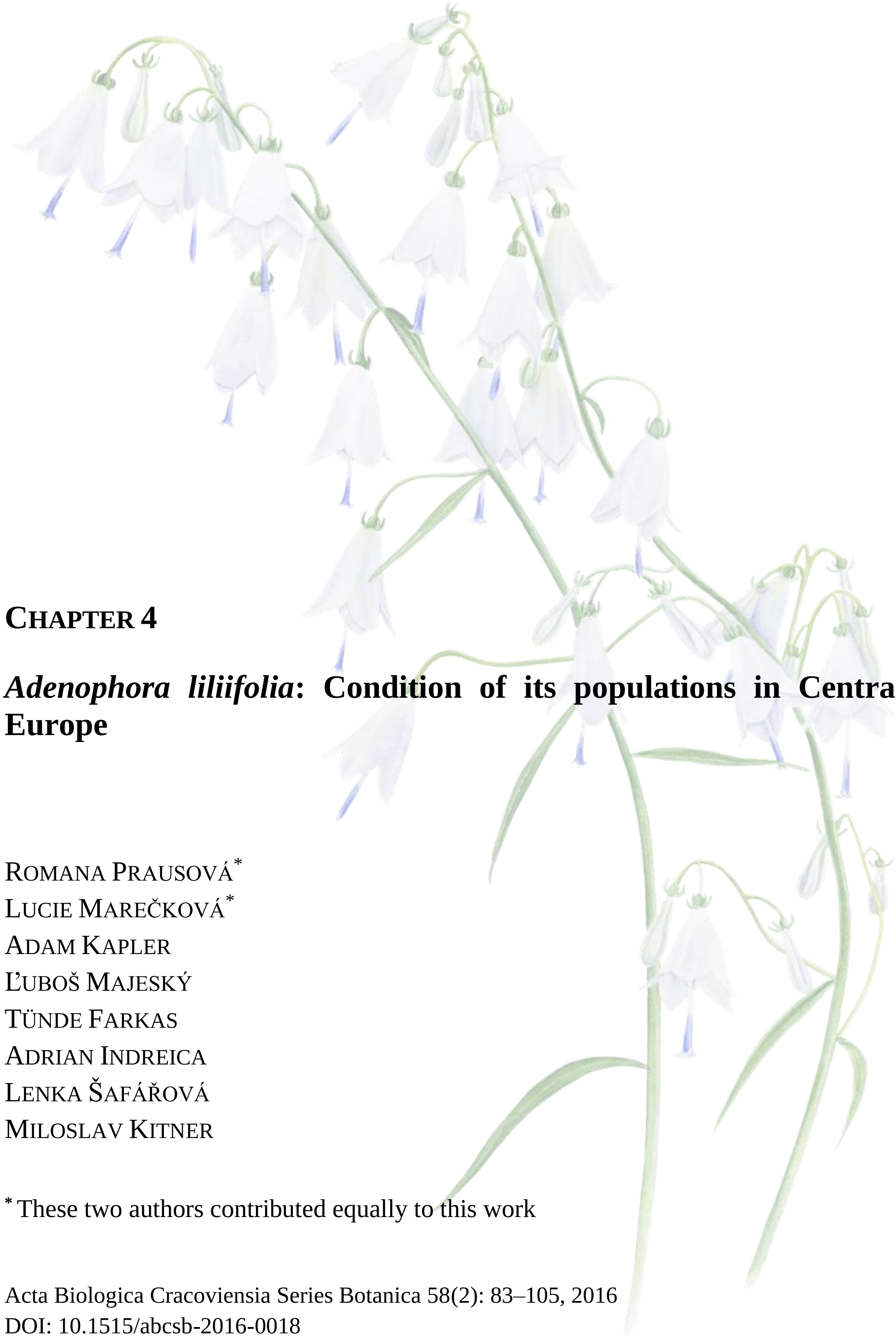
(1) Plants of *Adenophora liliifolia* from small, relict, isolated populations in the Lublin Province did not yield viable seeds in 2013.

(2) Seeds from a relatively large population of over 60 individuals in the Podkarpackie Voivodship from the Natura 2000 area “Dąbrowa near Zaklików” germinated well, giving healthy seedlings.

(3) Numerous populations of *Adenophora liliifolia* from south-east Poland are a better source of good quality seeds for effective conservative cultivation of seedlings than populations closer geographically but extremely poor in individuals.

(4) The shrubs’ removal at the *A. liliifolia* locality Niedzieliski Forest brought immediate conservation effects, increased the proportion of native individuals of the generative stage, and helped emerge native seedlings. This measure was more effective than reintroducing individuals grown from seedlings originating from another locality.

(5) Strengthening the population of *Adenophora liliifolia* in Niedzieliski Forest can be currently considered moderately successful (2017). However, the population remains extremely small. Of the five *A. liliifolia* plants introduced in 2014, four survived until 2017, with two individuals being in the generative stage in 2017. Only with a systematic and permanent increase in population size resulting from the natural *A. liliifolia* seedlings can the conservation measures be considered entirely successful. Active protective measures consisting in the removal of shrubs, young trees, and large perennials, however, proved to be beneficial also for other rare plant species found on-site, such as *Cypripedium calceolus*, *Cephalanthera rubra*, *Cephalanthera rubra*, *Cimicifuga europaea*, *Veratrum nigrum*, and *Gladiolus imbricatus*; which began to bloom and generate fruits more abundantly.



Chapter 4

Adenophora liliifolia: Condition of its populations in Central Europe

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Abstract

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 genetic structure was observed (22% 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, and 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.

Key Words: AFLP, *Campanulaceae*, the 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, and 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., *Platycladus orientalis* (L.) Franco (Farjon & 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 became glacial or postglacial relicts, such as *Ligularia sibirica* (L.) Cass. (Šmídová *et al.* 2011) or *Pedicularis sudetica* Willd. (Hendrych & Hendrychová 1988), 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 Szeląg 2009, 2010; Kolář *et al.* 2013, 2015) or *Sorbus sudetica* (Tausch) Bluff *et al.* (Kaplan 2012). Climatic 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 geoelement, 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; Hong *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 & Nobis 2004; Ciosek 2006; Kapler *et al.* 2015), Slovakia (Goliášová & Šípošová 2008), Hungary (Farkas & 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 (Wiliams & Gotin 2012). The information about *A. liliifolia* from France (Schnittler & 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 (Directive 1992); 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, signalling remnant pools of biodiversity (Kiedrzyński *et al.* 2015). The 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 & 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 genetics studies have been done so far. Only two studies have investigated the population structure of *A. liliifolia*: Boronikova (2009) analysed 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; and 2) a screening of the genetic variability and relationship among the studied populations by means of Amplified Fragment Length Polymorphism (AFLP).

Material and Methods

Study species

The Lilyleaf ladybells *Adenophora liliifolia* is an 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 the 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 & Turoňová 2008) during July and August in 2012 or 2013. The number of tufts and the number of fertile and sterile stems in each tuft were determined at each locality in all 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 (2012b). 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 Portal 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).

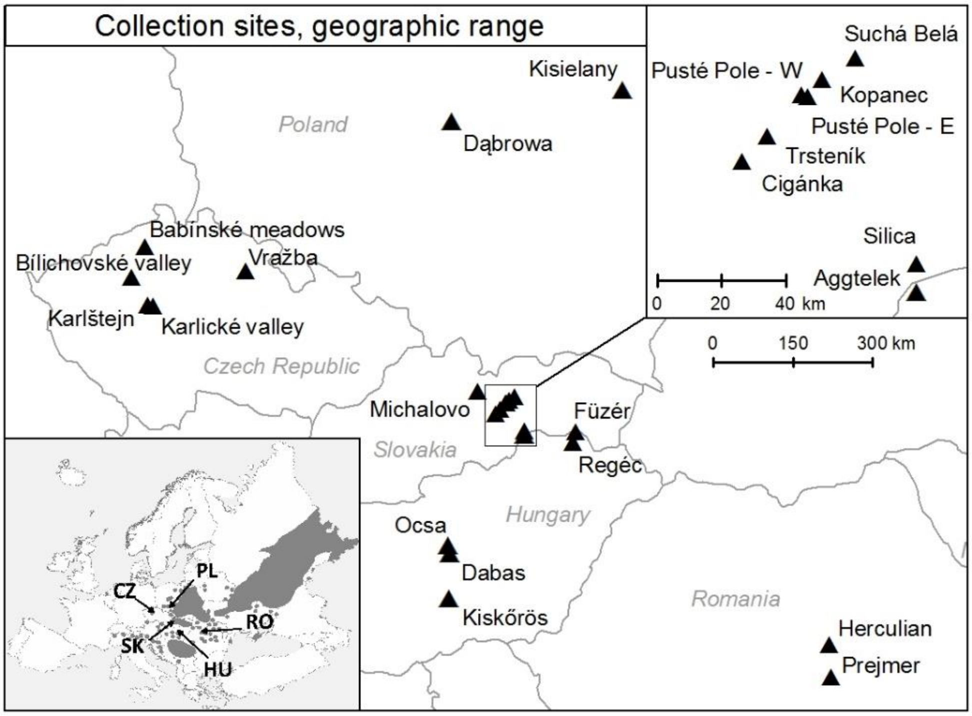

Fig. 1. Map of the studied localities of *A. liliifolia* in Czechia, Slovakia, Poland, Hungary, and Romania and its geographic range (made by J. Gamrát in ArcGIS 10 program).

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
Czechia					
Babinské meadows	50°35′52” 14°07′36”	538	mesozoic marl, claystone	eutrophic cambisol, planosol, stagnosol	Intermittently wet *Molinia* meadows
Bílichovské 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
Hungary					
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 *Molinia* 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 *Molinia* meadows
Poland					
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
Romania					
Herculian	46°07'05" 25°42'38"	635	volcanic sediments	andosols, chernozem, kastanozem	Central Europ. basiphilous thermophilous oak forest

Site	Latitude Longitude	Alt. (m)	Bedrock	Soil (acc. to WRB 2014)	Habitat acc. o EH Habitat Directive
Prejmer	45°43'59" 25°44'20"	518	quaternary sediments	histosol, stagnosol, fluvisol	Intermittently wet *Molinia* meadows
Slovakia					
Cigánka	48°45′49” 20°03′43”	825	dolomitical rocks	podzolic cambisol	Limestone beech forest
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 *Molinia* 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 *Alnus incan*a galeries, Alder swamp wood

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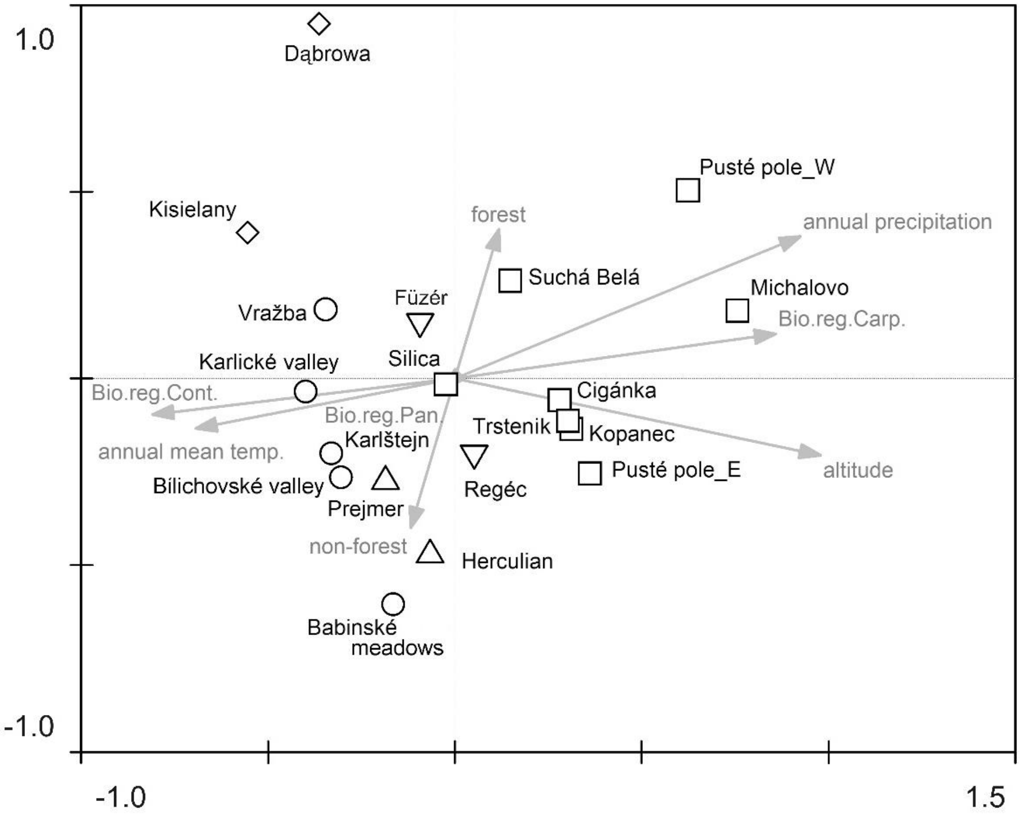


Fig. 2. Ordination diagram of the studied *A. liliifolia* localities based on PCA analysis. Czech Republic – circle, Slovakia – square, Romania – up-triangle, Poland – diamond, and Hungary – down-triangle. The first two axes explained 92% of the total variability. Bio. Reg.: Cont., Carp., Pan – a biogeographical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

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 Farskas & 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 were 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 ≥75%, constant species with a frequency ≥70%, and dominant species with a cover threshold ≥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 were estimated using 1.5% agarose gels. 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%, 0.4mm-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.

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_B**	**N_{PB}**
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

Table 2. Primers and primer sets for preamplification and amplification reactions with the total number of scored (N_B) and polymorphic bands (N_{PB}).

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 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, the intensity of the markers, and the 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 analysed 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 & von Holdt 2012), CLUMPP (Jakobsson & Rosenberg 2007), and DISTRUCT (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 (Pavlíček *et al.* 1999)], and the resulting tree was visualised and arranged in FIGTREE v1.4.0 software (Rambaut 2016). 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 individuals) and the number of fixed private bands (N_{FPB}; the number of bands bears by all individuals within a single population) were calculated in FAMD 1.31 (Schlüter & Harris 2006). The ARLEQUIN 3.5 (Excoffier & 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 analysed populations. AFLPdat 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 calculation 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 (square root method) was used to assess the gene diversity under Hardy-Weinberg genotypic proportions (Hj), also called Nei's gene diversity, the total gene diversity (Ht), 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 Fst/(1-Fst) and the natural logarithm of geographic distance (ln d) for pairs of subpopulations (10 000 permutations). The regression analysis was also performed to provide information about the linkage of the geographic position of localities (longitude) with polymorphism and gene diversity.

Results

The current condition of *A. liliifolia* 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. liliifolia* 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 one to 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 (Fűzér, 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. liliifolia* localities

The linkage between *A. liliifolia* 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 & Vojtkó 2012; Indreica 2011), six vegetation units in recent and historic localities of *A. liliifolia* were distinguished: 1) intermittently wet *Molinia* meadows (alliance of *Molinion caeruleae* W. Koch 1926), 2) thermophilous oak forests (association of *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 of *Quercion pubescenti-petraeae* Klika 1933 corr. Moravec in Beg. et Theurill 1984), 4) oak-hornbeam forests (association of *Tilio cordatae-Carpinetum betuli* Tracz. 1962), 5) limestone beech forests (association of *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 *Alnenion glutinoso-incanae* Oberd. 1953 alliance (Tab. 4).

Site conditions of the *A. liliifolia* localities

Site conditions of the six distinguished vegetation units with the presence of *A. liliifolia* 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. liliifolia* 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 (the alliance of *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. liliifolia*. While intermittently wet *Molinia* meadows offer the most convenient conditions with respect to light, continentality, and moisture, Central European basiphilous thermophilous oak forests are most suitable for *A. liliifolia* 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. liliifolia* were found in oak-hornbeam forests (association of *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. liliifolia* can only grow thanks to disturbances and management directed to an open forest.

Locality	Threat in country	Size of population				Morphological characteristics					Number of species in a relevée
		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.	
Czechia											
Babinské meadows	CR	20	44	72.7	2.2/1–15	43.00	19.95	3.6	8.13	2.88	41–53
Bílichovské valley	CR	26	23	13.0	1.4/1–7	64.44	36.56	3.5	24.67	4.33	21–29
Karlické valley	CR	22	26	23.1	1.2/1–3	33.50	19.44	2.7	5.33	0.00	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
Hungary											
Füzér	EN	18	22	31.8	1.2/1–2	34.42	19.70	2.6	16.00	3.33	30–42
Regéc	EN	38	62	17.7	1.6/1–4	26.08	15.97	2.3	4.90	0.50	16–49
Poland											
Dąbrowa	EN	53	76	73.7	1.4/1–8	missing data					39–58
Kisielany	EN	1000	1500	66.7	1.5/1–11	148.70	48.00	3.9	54.00	12.00	41–56
Romania											
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
Slovakia											
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	100.0	1.8/1–3	85.20	33.00	3.1	18.50	3.10	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	100.0	1.4/1–2	75.80	31.00	3.5	20.00	2.50	27–36
Pusté pole – W	EN	41	96	70.8	2.3/1–5	83.78	34.31	4.5	38.40	8.92	49–53
Silica	EN	38	60	11.7	1.6/1–5	44.83	21.34	3.4	14.75	4.80	36–43
Suchá Belá	EN	7	7	100.0	1.0/1–1	72.10	27.00	3.3	14.80	2.60	50–80
Trstenik	EN	343	474	49.8	1.4/1–6	93.37	27.93	3.6	31.00	7.64	47–57

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 the locality

Vegetation unit	**1**	**2**	**3**	**4**	**5**	**6**
Number of relevés	**24**	**22**	**8**	**19**	**30**	**2**
E3						
Quercus robur		50		16		
Carpinus betulus		32		42		
Quercus virgiliana			63			
Quercus petraea				16		
Fagus sylvatica				26	50	
Abies alba				16	57	
Alnus incana						**100**
Salix pentandra						**100**
E2						
Cornus mas			**75**	5		
Viburnum lantana			**75**	11		
Quercus petraea	21	55	13	**74**	3	
Corylus avellana	4	45	13	**74**	13	
Crataegus sp.	8	64	13	26		
Frangula alnus	17	64		5	7	
Alnus incana						**100**
Salix pentandra						**100**
E1						
Adenophora liliifolia	**100**	**100**	**100**	**100**	**100**	**100**
Betonica officinalis	**83**	68	38	21		
Molinia caerulea s.l.	**83**	18				
Convallaria majalis	33	**86**		63	**80**	
Lathyrus niger	4	**77**	50	53		
Carex montana	25	**77**		5		
Melittis melissophyllum	4	**73**	13	58	13	
Melica nutans		64	25	**89**	57	50
Brachypodium sylvaticum	4	41	38	**74**		
Carex digitata	4	14		**74**	57	
Aegopodium podagraria	4	14		**74**	3	
Asarum europaeum		5		**84**	23	
Hepatica nobilis		5		**89**		
Pulmonaria obscura		5		**79**		
Calamagrostis varia					**83**	**100**
Galium schultesii		41	25	16	**83**	
Pimpinella major	29			21	**70**	
Laserpitium latifolium		45			**80**	
Rubus saxatilis		14			**87**	
Cirsium erisithales					**77**	
Carduus crassifolius ssp. *glaucus*					**70**	
Cruciata glabra	54	9	38	5	7	50
Ajuga reptans	25	59		58	3	**100**
Angelica sylvestris	8	41		5	13	**100**
Astrantia major	54	55		37	40	**100**
Lathyrus pratensis	38	5		5	7	**100**
Trollius europaeus	17	14			3	**100**
Colchicum autumnale	38	5				50
Succisa pratensis	42	14			3	**100**
Carex umbrosa	13	9			3	50
Deschampsia cespitosa	4	5		21		**100**
Leontodon hispidus	33				17	50
Thalictrum aquilegiifolium	4	27				**100**

Vegetation unit	**1**	**2**	**3**	**4**	**5**	**6**
Number of relevés	**24**	**22**	**8**	**19**	**30**	**2**
Listera ovata		9			7	**100**
Tanacetum clusii					53	**100**
Gentiana asclepiadea					50	**100**
Cirsium oleraceum				37		**100**
Carex paniculata	17					**100**
Carex panicea	8					50
Equisetum palustre	8					**100**
Gymnadenia conopsea	8					**100**
Filipendula ulmaria	4					**100**
Galium palustre	4					50
Knautia maxima					10	**100**
Centaurea pseudophrygia					3	**100**
Carex davalliana						50
Carex flava						**100**
Valeriana simplicifolia						**100**
Juv.						
Acer campestre		5	38	**79**		
Alnus incana						**100**
E0						
Hylocomium splendens	4				27	**100**
Rhytidiadelphus sp.					17	**100**
Aulacomnium palustre						**100**
Climacium dendroides						**100**

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 of *Potentillo albae-Quercetum*), 3 – Central European basiphilous thermophilous oak forests (the alliance of *Quercion pubescenti-petraeae*), 4 – oak-hornbeam forests (association of *Tilio-Carpinetum*), 5 – limestone beech forests (association of *Cephalanthero–Fagetum*), 6 – a 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).

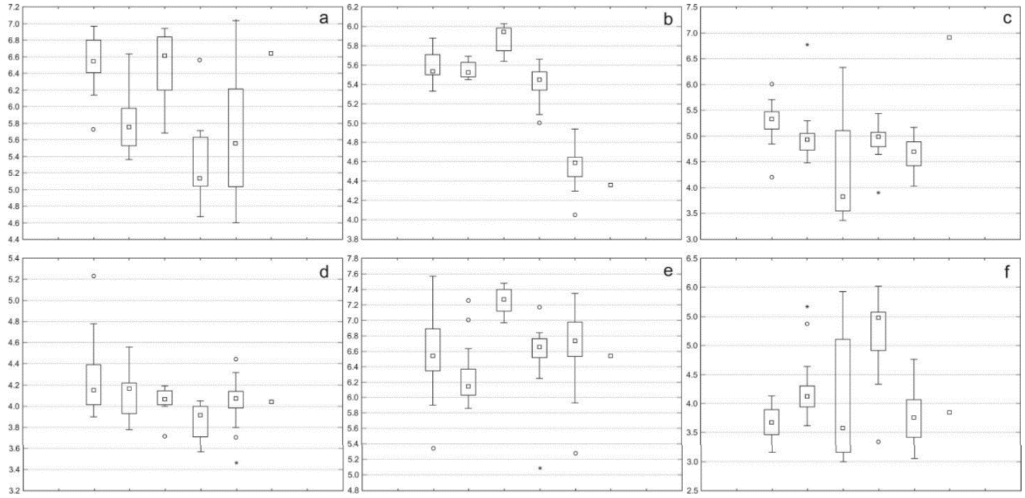


Fig. 3. Comparison of 6 vegetation units (in this order: 1. *Molinion caeruleae*, 2. *Potentillo albae-Quercetum*, 3. *Quercion pubescenti-petraeae*, 4. *Carpinion betuli*, 5. *Cephalanthero damassonii-Fagetum sylvaticae*, 6. *Alnenion glutinoso-incanae*) based on used relevées by means of Ellenberg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

A. liliifolia 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).

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 the 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 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 analysed using eight AFLP primer combinations (Tab. 2), which generated 291 bands, of which 242 were polymorphic (83.2%). Replication of the analysis revealed the 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 (Hj) were observed for samples from Kopanec (Hj = 0.171) and Pusté pole-E (Hj = 0.166), both located in Slovakia. The lowest value was observed for the Czech population from Karlštejn (Hj = 0.105). 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 Slovakian 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 Bílichovké valley (DW = 1.7). The computation of Shannon's Information Index produced the lowest value for the 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 (Ht) was 0.157.

Site	ID of samples	n	NPB	NFPB	P%	I (SE)	Hj (SE)	DW
Czechia		**18**	**2**	**0**	**56.1**	**0.194 (0.013)**	**0.128 (0.009)**	**2.3**
Babinské 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
Hungary		**21**	**5**	**0**	**64.4**	**0.229 (0.013)**	**0.149 (0.009)**	**3.2**
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
Poland		**6**	**0**	**0**	**38.6**	**0.167 (0.014)**	**0.132 (0.009)**	**2.3**
Dąbrowa	40	1	—	—	—	—	—	—
Kisielany	41 — 45	5	0	0	36.2	0.168 (0.014)	0.141 (0.010)	2.6
Romania		**10**	**1**	**0**	**48.2**	**0.181 (0.013)**	**0.128 (0.009)**	**3.1**
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
Slovakia		**29**	**14**	**0**	**68.8**	**0.229 (0.013)**	**0.144 (0.009)**	**3.1**
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
total mean (populations separately)					**27.6**	**0.139 (0.014)**	**0.144 (0.009)**	**3.0**

Table 5. List of analysed samples with population genetic statistics (n, number of samples; NPB, number of private bands; NFPB, number of fixed private bands; P%, polymorphism; I, Shannon's Information Index; Hj, Nei's gene diversity; DW, frequency down — weighed marker value; SE, the sum of errors).

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).

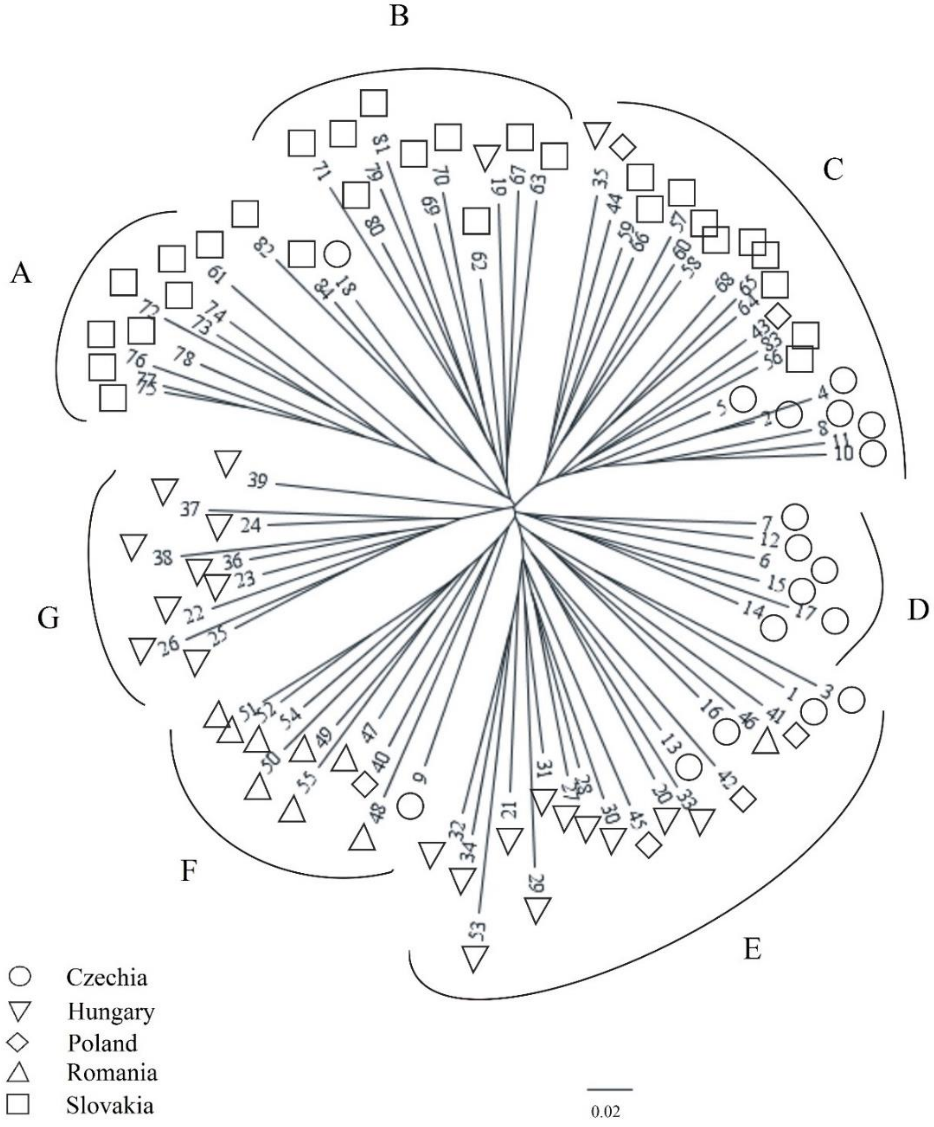


Fig. 4. Unrooted Neighbour-joining dendrogram (based on 291 AFLP markers and Dice similarity matrix) of 84 *Adenophora liliifolia* samples from five European countries. Putative groups are designed by capital letters A–G.

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

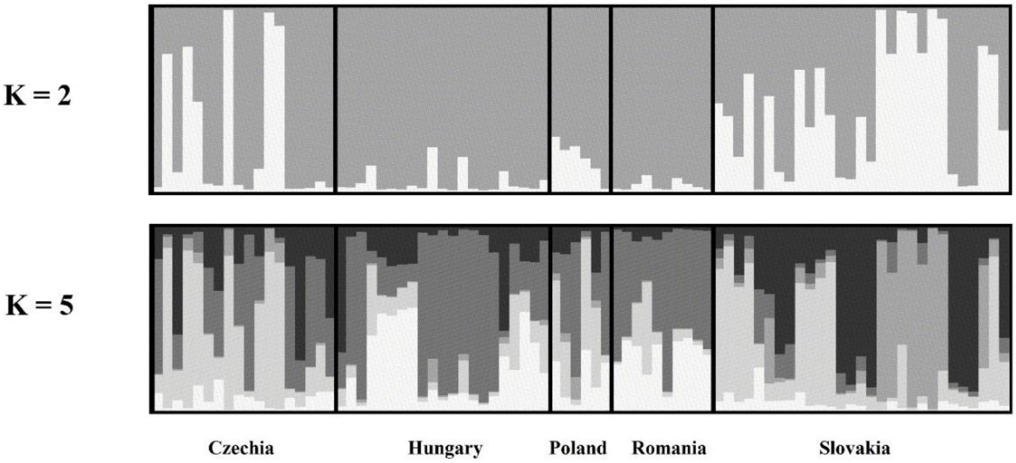


Fig. 5. Results of the STRUCTURE analysis of 84 *A. liliifolia* samples showing results for K = 2 and K = 5. Each vertical bar represents one individual, with the colour representing the probability of assignment to different clusters. The origin of the populations is displayed below the graphics.

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. liliifolia* populations in Central Europe revealed the decline in the 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. liliifolia* had occurred in 20 localities 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 & Truhlářová 2009). In Slovakia, the species is still present in both of the phytogeographical districts of Pannonicum and Carpaticum (Goliášová & Šípošová 2008). Currently, *A. liliifolia* grows only in about 10 localities in the Carpathians and their foothills, while historical data describes about 30 former populations in this territory. In Pannonicum, *A. liliifolia* 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. liliifolia* 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 & 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. liliifolia* tufts per population were observed in the Slovak localities in Muránska planina NP (Cigánka, Trstením), 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 at 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 Herculian (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 & 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 the heliophilous species of subcontinental oak forests, 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 forests 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 & 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 & Maltseva 1999; Chytrý *et al.* 2012). Central European forest habitats have changed a lot since the Preboreal and Boreal periods. Once light Preboreal and Boreal forests were later massively replaced by deciduous forests with more closed canopies in Central Europe (Kaplan 2012). Open forests, which contained 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 oceanity 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 ecotones, 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.). *A. 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) (Farskas & Vojtkó 2012, 2013). Hungarian relevés from Regéc and Fűzér were identified as the association of *Nardo-Molinietum hungaricae* (Kovács 1962) Borhidi 2001 (Farskas & 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 shrubs 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 a Euro-Sibirian 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 analysed individuals used in our study. In populations of *Stipa pennata* L., the genetic diversity declined significantly from the distribution center to its periphery (Wagner *et al.* 2012). The same trend also seems to be present for *A. liliifolia*, as we observed only slight correlations between the geographical position of populations (increscent 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 analysed 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 Czech and Slovak Republic were analysed 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 that 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) analysed four *A. liliifolia* populations from the Perm region in Russia using ISSR markers and detected (similar 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 influence 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 the 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 types 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* Nakaithere (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 $F_{ST.}$

A. 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., the shape of the leaves, presence or absence, and different lengths of the leaf petioles, the colour 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 suggests that the separation of the analysed 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 Slovakian 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.

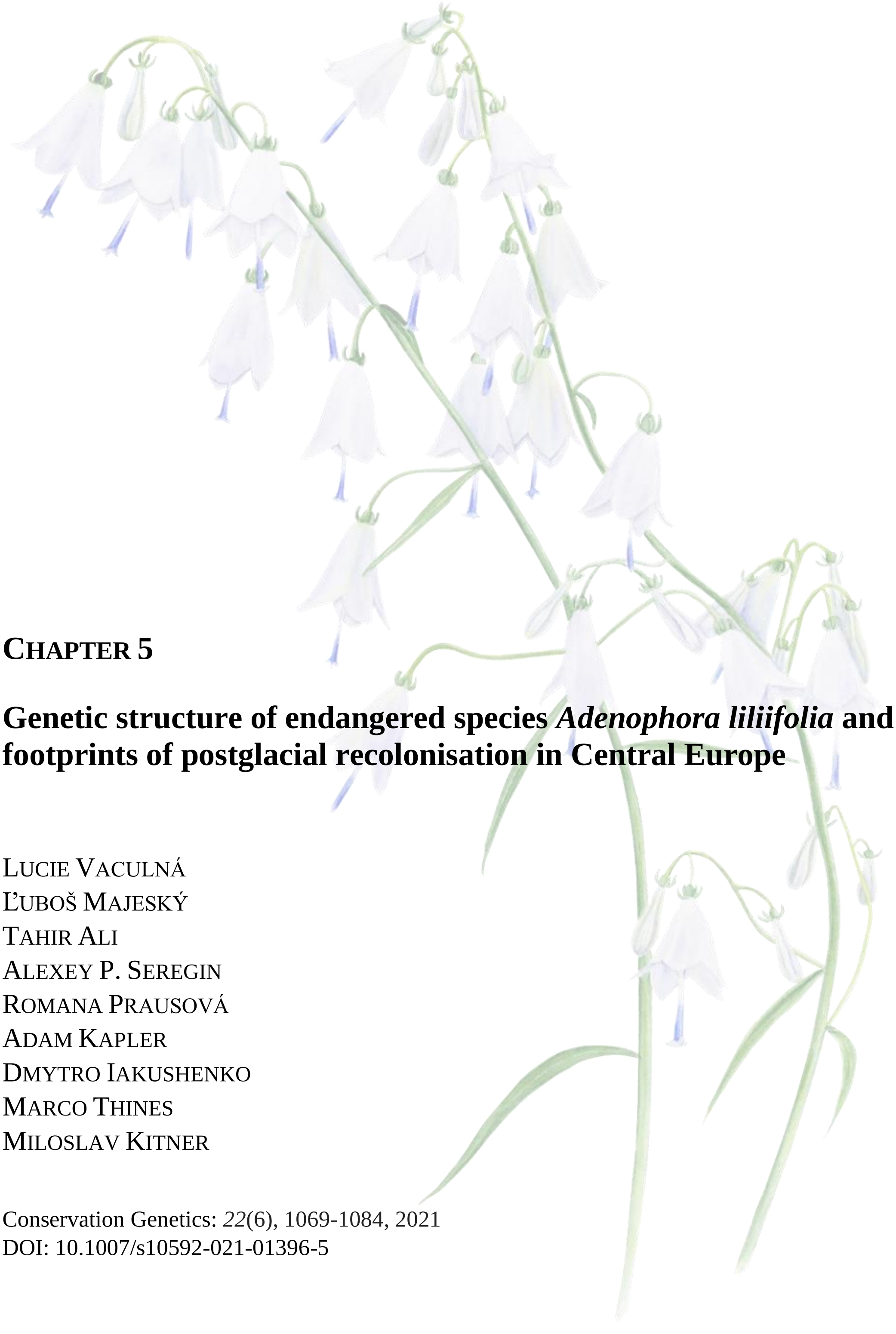
Conservation of *A. liliifolia*

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 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/government-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 *A*denophora *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 Kisielany 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. liliifolia*. Eutrophication, shading, overpopulated wild animals, and expansive broad-leaved herbs are the main factors causing the decline of *A. liliifolia* 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 the shrinking of suitable habitats, their disappearance, or overall changes in landscape management. The findings of the present study show that *A. liliifolia* 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 for most of the localities.



CHAPTER 5

Genetic structure of endangered species *Adenophora liliifolia* and footprints of postglacial recolonisation in Central Europe

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Abstract

Quaternary climatic fluctuations changed the floristic composition of large areas and forced biota to repeated movements following gradients of their ecological tolerance. Different and contrasting patterns of responses were reported for various species. In this study we focused on *Adenophora liliifolia*, a perennial herbaceous species representing a south Siberian floristic element in European flora, well adapted to a continental climate. We investigated the genetic diversity and phylogeography of the species within Europe. The results show the absence of stronger sequence differentiation across the investigated 3 600 km long transect, which reflects the young evolutionary origin of the species and/or repeated population contractions/expansions accompanied by bottleneck during the Quaternary climatic fluctuations. Along with this, the results suggest several regions located in Carpathians, Alps-Dinarides, and in non-glaciated parts of Central Russia with high haplotype diversity, which probably served as refugia. Low genetic diversity, the prevalence of a single cpDNA haplotype, and the genetic composition of the investigated European sites suggest re-colonisation of Central Europe from a refugium located in southern Europe. The distribution of genetic variation and moderate genetic differentiation of the investigated sites may indicate the process of fragmentation of the common genetic pool in Central Europe. Projections of ongoing climate change during the 21st century are not favorable for species persistence in Central Europe. In the case of *ex-situ* conservation actions, we recommend protection and proper management of rapidly vanishing populations and maintenance of genetic diversity, especially in regions with high haplotype diversity.

Keywords: AFLP – cpDNA sequencing – glacial refugia – NATURA 2000 – niche modeling – Quaternary history.

Introduction

To better understand the underlying factors of the current plant species distribution and predict their future in the rapidly changing Anthropocene environment, we can learn from species' responses to past environmental changes (Hampe & Petit 2005). The Quaternary represents an era of abrupt changes within a relatively short period, which caused enormous environmental alterations, especially during the Pleistocene (e.g., Maslin *et al.* 1996; Webb & Bartlein 1992), but also during the Holocene (e.g., Kalis *et al.* 2003; Parmesan & Yohe 2003). Climatic oscillations during the Pleistocene forced many species to cope with dramatic changes and led to local extinctions, genetic diversity reduction, but also to speciation (Comes & Kadereit 1998; Hewitt 1996; Willis & Niklas 2004). While the temperate and thermophilous species showed the paradigmatic north-to-south and/or east-to-west contraction-expansions as a response to glacial and interglacial periods (e.g., Taberlet *et al.* 1998), arctic-alpine species (to some extent also montane species) show the opposite response, expansions during cold periods, while contractions during warm periods and vertical rather than horizontal migration (e.g., György *et al.* 2016; Puşcaş *et al.* 2008; Schönswetter *et al.* 2005). Different patterns were observed for species of steppe and xeric grassland habitats (e.g., summarized in Kajtoch *et al.* 2016), which survived *in situ* within the Central Europe steppe habitats, which were widespread and supported by cold periods of Pleistocene (Frenzel & Troll 1952; Janská *et al.* 2017; Lang 1994; Willis & van Andel 2004). The observed patterns are various but somewhat similar to arctic-alpine species, i.e., expansion during glacial while contraction during interglacial periods (Kajtoch *et al.* 2016).

It was assumed earlier that the only option for many arboreal and thermophilous species had been the retreat to southern refugia (Hewitt 2004; Petit *et al.* 2002; Taberlet *et al.* 1998). However, recent decades brought new evidence about the Pleistocene landscape's appearance (e.g., Willis & van Andel 2004). Now, it is broadly accepted that boreal forest stands with cold-tolerant tree species like pine, spruce, birch (e.g., Juřičková *et al.* 2014; Willis *et al.* 2000), and even woodlands with beech (Magri *et al.* 2006; Magri 2008) were preserved much closer to the continental glacier's forehead in Central and Central Eastern Europe. The islets of the forested area served as refugia for herbaceous and mountain species, for instance, within Carpathian Mountains (e.g., Slovák *et al.* 2012; Stachurska-Swakoń *et al.* 2012, 2020; but see also Dítě *et al.* 2018). Unlike Alps, glaciers covered only the highest parts of Carpathian Mountains leaving lower massifs and deep valleys unglaciated (Mráz & Ronikier 2016; Ronikier 2011). Apart from the areas mentioned above, the primary vegetation type of Central Europe was represented by transitional forms between treeless vegetation of steppe-tundra, steppe, and forest-steppes (Frenzel & Troll 1952; Horsák *et al.* 2010; Jankovská & Pokorný 2008; Janská *et al.* 2017; Kuneš *et al.* 2008; Lang 1994).

Adenophora liliifolia (L.) Ledeb. ex DC. (Lilyleaf Ladybells) is a diploid (2n = 34), herbaceous, perennial, long-lived (up to 40 years; personal observation) and allogamous, insect-pollinated species, with competitive-stress tolerant (CS) life-strategy and a limited ability for long-distance dispersal, growing in climatically non-extreme habitats (Roleček 2007; Prausová *et al.* 2016; Kaplan 2017). The species represents a European-South-Siberian floristic element, with optimal conditions provided by the hemiboreal forests of Southern Ural and Southwestern Siberia (Roleček 2007). The center of the species distribution (Fig. 1) is situated in Western Asia-Southern Siberia and disjointly reaches up to Western Europe (Germany, Switzerland) (Tacik 1971; Fedorov 1978). *Adenophora liliifolia* is classified as a heliophilous, thermophilous, and basiphilous species requiring enough moisture (Prausová *et al.* 2016). The current best habitats for the species in Central Europe represent Peri-alpidic basiphilous thermophilous oak forests (the alliance *Quercion pubescenti-petraeae*, Chytrý *et al.* 2013) – concerning temperature and soil pH; and Intermittently wet nutrient-poor meadows (*Molinion caeruleae*, Chytrý 2007) – in respect to light, continentality, and moisture. In the middle Russian forest zone, it is a typical riparian species preferring light oak forests and their margins on major river floodplains (Seregin 2017). Lilyleaf Ladybells is listed among the species of the European Community interest (Directive 1992), whose conservation requires the designation of special areas of conservation (SAC; NATURA 2000 sites). The species conservation status at the European level was assessed as "unfavorable-bad" for finished reporting periods (2001-2006 and 2007-2012; EUNIS 2021). Currently, its European distribution is highly fragmented, restricted to small and isolated populations mainly in Central-Eastern Europe (Fig. 1; Prausová *et al.* 2016). A similar trend of population decrease is also reported for the European part of Russia (Boronnikova 2009). The most often highlighted factor causing the decline is the loss of suitable habitats.

In regard of *Adenophora liliifolia* distribution in Central Europe, it is considered an indicator species of Late Glacial-Early Holocene refugia of light and mesophilous habitats. These stands later mostly retreated with the spreading of dense forests during the middle Holocene (Dítě *et al.* 2018; Roleček 2007), and nowadays are persisting in limited space only thanks to the long history of traditional management that kept the canopies open. Such habitats still harbour populations of rare, declining species, and therefore, they become biodiversity hotspots and the subjects of conservation efforts in the changing landscape. *Adenophora liliifolia* might be used as a model species for co-occurring heliophilous, thermophilous, and basiphilous species with

European-South-Siberian distribution, whose prosperity is conditioned by appropriate site management.

Based on our previous research (Prausová *et al.* 2016), we hypothesized that *A. liliifolia* could survive cold periods of Pleistocene in refugia scattered across Central Europe within protected valleys of Carpathians, as well as within widespread steppe habitats. In the present study, we investigated species' genetic diversity and its spatial distribution with the aim to identify potential glacial/Early Holocene refugia and shed more light on the species' phylogeography. We also modeled the probability of species distribution within different periods in the past and under future climate change. Altogether, our results bring new perspectives on species' response to Quaternary climatic fluctuations, on the distribution of genetic diversity, and may be the very first step in the development of an efficient international conservation strategy for *A. liliifolia*.

Methods

Plant material and DNA extraction

Sampling was done under permission from corresponding government authorities, allowing collection of 1-2 leaves per individual plant, to prevent unnecessary harm to the populations composed frequently of 1-5 plants. Leaf material (silica-gel dried) was collected from 293 individuals representing 41 sampling sites within Europe (35 sites from NATURA 2000 SACs; one from Switzerland; four from Ukraine, and one from Russia; Tab. 1, Fig. 1), and deposited at the OL herbarium. These individuals were used for analyses based on amplified fragment length polymorphism (AFLP) and DNA sequencing. Additional 80 voucher specimens from the Moscow University Herbarium (Russia; MW) and eight from the Department of Botany and Zoology at Masaryk University in Brno (the Czech Republic; BRNU) were used for DNA sequencing, covering the easternmost part of the species distribution area. Genomic DNA was extracted using a modified CTAB protocol (Doyle and Doyle 1987). The integrity and quality of extracted DNA were assessed using 1.5% agarose gel electrophoresis, and concentration was determined using a NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies, Delaware, USA). For the control of repeatability and error rate calculation, 10% of samples were double extracted and represented replicates in all subsequent analyses.

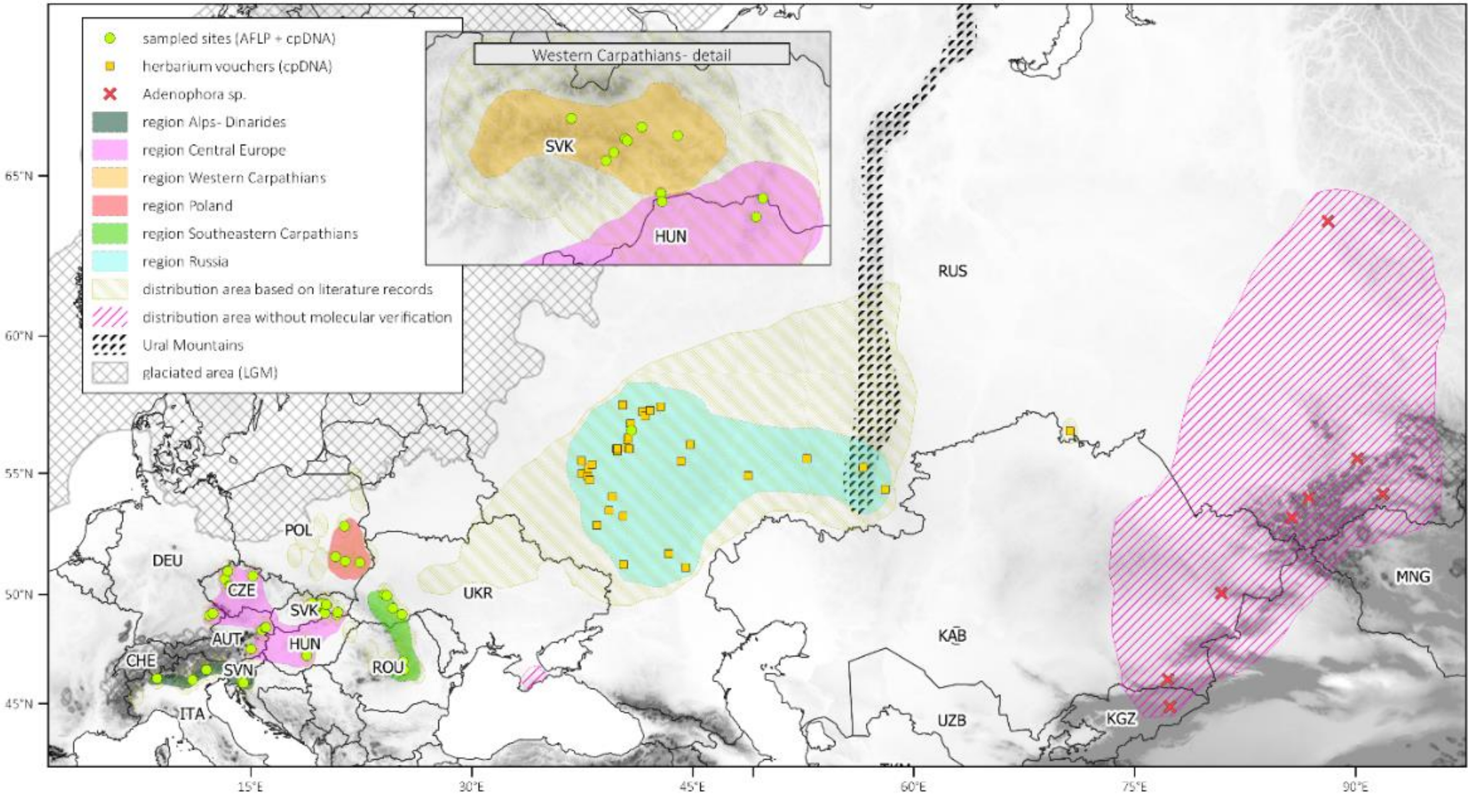


Fig 1. The distribution area of *Adenophora liliifolia*. Green circles represent sampled sites analysed by AFLP and cpDNA sequencing; orange squares represent sites gathered from the MW and BRNU herbaria used for cpDNA sequencing only. Red crosses indicate samples deponed in herbaria as *A. liliifolia*, but this determination was not confirmed within the present study. Coloured polygons show defined regions referred in the main text: Central Europe (magenta), Poland (red), Western Carpathians (orange), Alps - Dinarides (dark turquoise), Southeastern Carpathians (green), Russia (light blue). Dashed pink polygon reflects area historically mentioned as a part of *A. liliifolia*' distribution range, but without molecular verification. Grey dashed polygon shows ice sheet range during the LGM. Ural Mountains are highlighted by the black dashed line.

Table 1. List of the sampled sites of *A. liliifolia*. ID (identification code for populations); Seq (number of sequenced samples); AFLP (number of samples analysed by AFLP); geographic coordinates [Lon(E), Lat(N)]; NP (national park); OL ID (identification code of samples in OL herbarium); Habitat (simplified habitat categories).

Country	ID	Population	Seq	AFLP	Lat (N)	Lon (E)	OL ID	Habitat
Region Central Europe								
Germany	DE_A	Erlau	3	3	48.701	12.737	37884	*Molinia* meadow
	DE_B	Isar Mouth	2	2	48.783	12.967	37885	*Molinia* meadow
Austria	AT_A	Pischelsdorfer Wiese	17	9	48.027	16.521	37875	*Molinia* meadow
	AT_B	Sollenau	2	2	47.909	16.239	37876	*Molinia* meadow
	AT_C	Graz – Plabutsch	11	8	47.065	15.425	37877	beech forest
Czechia	CZ_A	Babinské louky	11	7	50.598	14.127	37878	*Molinia* meadow
	CZ_B	Bílichovské údolí	12	12	50.264	13.899	37879	oak forest
	CZ_C	Karlické údolí	7	5	49.952	14.257	37880	oak forest
	CZ_D	Karlštejn	4	1	49.960	14.173	37881	oak forest
	CZ_E	Vražba	12	12	50.335	15.822	37882	oak forest
	CZ_F	Žehuňsko – Báň	2	0	50.169	15.293	37883	oak forest
Hungary	HU_A	Aggtelek: Csiszár-Nyilas	1	1	48.516	20.564	37886	mountain meadow
	HU_B	Füzér: Drahos	10	8	48.542	21.455	37887	*Molinia* meadow
	HU_C	Regéc: Gyertyán-kút-rét	10	8	48.43	21.400	37888	*Molinia* meadow
	HU_D	Kiskörös: Szücsi erdö	10	8	46.632	19.204	37889	riparian mixed gallery forests
	HU_E	Dabas	2	0	47.168	19.268	37890	riparian mixed gallery forests
	HU_F	Ócsa	3	3	47.294	19.209	37891	riparian mixed gallery forests
Region Alps-Dinarides								
Italy	IT_A	Belluno, Soverzene - Gallina Valley	4	4	46.217	12.326	37892	beech forest
	IT_B	Passo Colletto Piccolo	3	3	45.771	11.350	37893	beech forest
Slovenia	SL_A	Rački potok	1	1	45.568	14.715	37908	riparian mixed gallery forests
	SL_B	Belica	6	3	45.495	14.805	37909	riparian mixed gallery forests
Switzerland	CH_A	Monte San Giorgio	1	1	45.925	8.949	37910	beech forest
Region Poland								
Poland	PL_A	Kisielany (Ciolkowo)	26	22	52.253	22.207	37894	oak forest
	PL_B	Dabrowa near Zaklikow	17	9	50.766	22.150	37895	oak forest

Country	ID	Population	Seq	AFLP	Lat (N)	Lon (E)	OL ID	Habitat
	PL_C	Niedzieliski Las	4	3	50.684	23.128	37896	oak forest
	PL_D	Krzemionki	10	7	50.975	21.498	37897	oak forest
Region Southeastern Carpathians								
Romania	RO_A	Prejmer	6	5	46.118	25.711	37898	*Molinia* meadow
	RO_B	Herculian	8	8	45.733	25.739	37899	oak forest
Ukraine	UA_A	Dziurkach	1	1	48.603	25.216	37911	subcontinental steppe meadow
	UA_B	Sinozhati	19	7	48.289	25.803	37912	subcontinental steppe meadow
	UA_C	Simlyn	1	0	49.168	24.842	n.a.	subcontinental steppe meadow
	UA_D	Kasova hora	1	0	49.226	24.696	n.a.	subcontinental steppe meadow
Region Western Carpathians								
Slovakia	SK_A	Silica NP Slovenský kras	8	3	48.574	20.553	37900	*Molinia* meadow
	SK_B	Pusté pole NP Nizké Tatry	7	5	48.888	20.229	37901	beech forest
	SK_C	Michalovo NP Nizké Tatry	7	7	49.012	19.751	37902	beech forest
	SK_D	Cigánka NP Muráňská planina	10	6	48.764	20.062	37903	beech forest
	SK_E	Trstěník NP Muránska planina	10	8	48.810	20.132	37904	montane *Alnus incana* galeries
	SK_F	Suchá Belá NP Slovenský raj	5	6	48.955	20.379	37905	beech forest
	SK_G	Puste pole NP Slovenský raj	7	4	48.881	20.253	37906	beech forest
	SK_H	Kopanec NP Slovenský raj	7	6	48.909	20.703	37907	beech forest
Region Russia								
Russia	RU_A	Zagryazhskoye	5	5	55.483	42.043	37913	riparian mixed gallery forests
		Summary:	**293**	**213**				

AFLP

AFLP analyses were performed according to Vos *et al.* (1995) with modifications described in Ali *et al.* (2017), using six primer combinations (Supplementary material 1a; [SM 1a]) selected by preliminary testing. Amplified fragments were separated by capillary electrophoresis (GenomeLab CEQ/GeXP DNA, Beckman Coulter, USA). Raw data were analysed with CEQ 8000 software using an incorporated dominant scoring algorithm (bin 1-2 bp, threshold 100), where peaks ranging from 70 to 550 bp were scored as either present (1) or absent (0).

DNA sequencing

Altogether, 13 genomic loci [12 chloroplast (cpDNA) and one nuclear (internal transcribed spacer, ITS); SM 1b] were screened for polymorphism in a 46-sample subset (i.e., at least one randomly selected sample per each sampled site). Based on observed variation, only three variable intergenic spacers (*trn*S-*trn*G, *trnS-trnfM, and psb*D-*trn*T) were used in further analyses. The PCR reaction consisted of template DNA (75 ng), 0.4 µM of each primer, 1× KAPA Taq buffer (containing 1.5 mM $MgCl_2$), 0.2 mM dNTPs, 0.4 U of KAPA *Taq* polymerase (Kapabiosystems, Boston, USA), and PCR-quality water in a 20 µl total volume. The reaction conditions were optimized for each of the three selected cpDNA loci separately (SM 1c). The PCR products were sequenced at the Biodiversity and Climate Research Centre (BiK-F) (Frankfurt am Main, Germany) or by Macrogene Europe (Amsterdam, Netherlands). Geneious 7.1.8 (Biomatters Ltd., Auckland, New Zealand) was used for contig assembly from partial reads, base call editing, sequence alignment, and sequence concatenation. Details of the observed variation are summarized in SM 2a together with haplotype characteristics for the variable loci for each analysed *A. liliifolia* sample. GenBank accessions: MN530067-93, MN519409-16 MT299777-8, MT304671-2, and MT308772-3. Haplotype/ribotype alignments are presented in SM3.

Data analysis

For data analysis and further interpretation, samples were grouped and analysed based on: i) population level (only for sampled sites with at least five samples), ii) defined geographic regions: 1) Central Europe; 2) Southeastern Carpathians (SE Carpathians); 3) Western Carpathians (W Carpathians); 4) Alps-Dinarides; 5) Poland; and 6) Russia (for details see Tab. 1). These regions were designed *ad hoc* to reflect genetically homogeneous regional groups recovered during analyses. These groups do not fully correspond to regional geographical division. Sampling sites HU_A/_B/_C geographically belong to W Carpathians; however, genetically, they are more similar to the sites included in the group Central Europe. Sampling sites from Ukraine lie on borderline in-between Eastern Carpathians and East European Plain (Pontic steppes) but were treated within SE Carpathians based on their genetic similarity with Romanian samples. Separation of samples from Poland relates to the premise that Poland's area was under the stronger influence of continental glaciers than the rest of the investigated area, with no or very restricted local refugia.

Genetic diversity inferred from AFLP

To avoid the loss of information by excluding rare bands, at sampled sites represented by less than five individuals, fragments present only once were disregarded from the final binary matrix (as discussed by Bonin *et al.* 2004). From the final AFLP binary matrix, the following statistics were calculated. The percentage of within-population polymorphic bands (PLP) and Nei's unbiased expected gene diversity (Hj) were computed using AFLP-SURV v.1.0 (Vekemans *et al.* 2002). Shannon Index (I) and the effective number of alleles (N_E) were calculated in GenAlEx 6.5 (Peakall & Smouse 2006, 2012). For assessing the possible relict status of the sampled sites, we computed the frequency-down-weighted marker (DW) (Schönswetter and Tribsch 2005) using the R-script AFLPdat (Ehrich 2006; R Core Team 2020). Greater DW values imply a greater amount of rare markers as expected for long-term isolated populations, whereas recently diverged populations should exhibit lower DW values. The private band number (nPB, bands present only in individuals of the given group), the fixed band number (nFB, monomorphic bands within the given group), and the fixed private band number (nFPB, monomorphic bands present only in individuals of the given group) were calculated in FAMD 1.31 (Schlüter & Harris 2006), as well as the matrix of genetic distances for the reconstruction of a Neighbor-joining (NJ) dendrogram (Dice similarity coefficient, 1,000 bootstrap replicates). The resulting dendrogram was visualized in FigTree v1.4.0 (Rambaut 2016).

To determine whether a genetic structure within/among sampled sites can be detected, the Bayesian clustering approach implemented in STRUCTURE 2.3.4 (Falush *et al.* 2007) was used. For this purpose, we used a binary matrix composed of AFLP and cpDNA data, where each multilocus cpDNA haplotype was binary coded as an additional character. Computation was set up for the recessive allele model and the admixture model with correlated allele frequencies (recommended for dominant markers) and without activated *locprior* option. The K was set to 1-37 with ten replicate runs for each K using 1,000,000 MCMC iterations following the period of 100,000 burn-in iterations. For the graphical clustering interpretation (based on the maximum ΔK value; Evanno *et al.* 2005), STRUCTURE HARVESTER (Earl & von Holdt 2012), CLUMPP (Jakobsson & Rosenberg 2007), and DISTRUCT (Rosenberg 2004) software were used. The CLUMPP output was also used for the admixture visualization in QGIS (Quantum GIS Development Team 2019).

To inspect the genetic variation distribution, AMOVA was performed in Arlequin 3.5.2.2 (Excoffier and Lischer 2010) as well as the calculation of pairwise F_{ST} and Slatkin's F_{ST} [$F_{ST}/(1-F_{ST})$] (significance of variance components tested using 10,000 nonparametric permutations). Isolation by distance (IBD) was examined by correlation of matrices of genetic distance [representing $F_{ST}/(1-F_{ST})$] and geographic distance with 10,000 permutations using Mantel test in GenAlEx 6.5. To reveal trends related to isolation by distance, correlation of Nei's gene diversity (Hj) and frequency-down-weighted marker (DW) versus latitude and longitude as linear regression was performed.

Chloroplast DNA diversity and haplotype network reconstruction

For estimation of sequence diversity, polymorphic, singleton, and parsimony-informative sites, number of haplotypes (N_H), haplotype diversity (H_D; Nei 1987), and nucleotide diversity (Pi; Tajima 1983) program DnaSP v.6 (Rozas *et al.* 2017) was used. The presence of bottleneck event and/or population contraction/expansion within defined geographic regions was tested by Tajima's D test. Values close to zero mean the observed variation is near to expected variation, and the population is evolving under mutation-drift equilibrium. Values below zero reflect the presence of abundant rare alleles, which may be explained as population expansion after a recent bottleneck, while values greater than zero reflect a deficiency of rare alleles, which may be caused by sudden population contraction. DnaSP v.6 was also used to test if subpopulations (corresponding to defined geographic regions) are genetically differentiated by calculating haplotype-based and nucleotide-based statistics with Chi-square test and permutation test, as implemented in the software.

The haplotype network was constructed by statistical parsimony method using R package Haplotypes (Aktas 2015). To retain maximum information in the concatenated cpDNA alignment and avoid indel exclusion, the minisatellite motif observed within the *trn*S-*trn*G spacer, and four other indels were coded as two additional characters using C for presence and A for the absence of a particular stretch/indel. Two variable polyA stretches were excluded from the alignment. The remaining indels with more than two characters per particular position (altogether four one nucleotide indels) were left in the alignment and were coded using a simple indel coding method (Simmons & Ochoterena 2000) possibility implemented in the package Haplotypes.

Niche modeling

Maxent (version 3.3.3; Phillips *et al.* 2006) was used for habitat suitability modeling analysing environmental data from a total of 78 cross-checked occurrence points (GBIF 2018; Plantarium 2018), which are listed in the supplementary material (SM 4a). The present-day climate data (1970 – 2000) as well as different Global Circulation Models (GCMs) for the Last Glacial Maximum (LGM; 22,000 BP), Mid-Holocene (6,000 BP), and models for the years 2050 and 2070 were extracted from the WorldClim database (Hijmans *et al.* 2005). Data resolution was 30s except for LGM (2.5m). For the ensemble models, we used the following GCMs: CCSM4, MIROC-ESM, and MPI-ESM-P for LGM projections; CCSM4, CNRM-CM5, HadGEM2-CC, HadGEM2-ES, and MPI-ESM-P for Mid-Holocene projections; and CNRM-CM5, GFDL-CM3, HadGEM2-ES, MIROC5, and MPI-ESM-LR for the future projections (SM 4b). Selected GCMs are evaluated as the best reproducing the historical behaviour of surface air temperature over Europe (Basharin *et al.* 2016; McSweeney *et al.* 2015) and are commonly used in species distribution modeling. Soil data were accessed from the ISRIC (Hengl *et al.* 2017) and GlobalChange (Shangguan *et al.* 2014) portals and vegetation cover data from the EarthEnv database (Tuanmu & Jetz 2014). Soil and vegetation data were used only for refining the present distribution assessment. We tested two possible emissions scenarios. Emissions in RCP 4.5 peak around 2040, then decline. Whereas RCP 8.5 emissions continue to rise throughout the 21st century (Meinshausen *et al.* 2011). The Maxent performance was evaluated by the threshold independent receiver-operating characteristic (ROC) analysis. Initially, Maxent was run under the default settings using all 19 climatic variables, selected soil variables, and layers representing vegetation cover. The multicollinearity of the contributing environmental variables was tested by the ENMTools (Warren *et al.* 2010). To prevent misleading signals, we excluded highly correlated variables with Pearson's $r > 0.8$. For the final modeling of the present distribution, nine ecologically meaningful variables were used (SM 4c). Maxent was run under the following settings: "random test percentage" = 25, "replicates" = 15, "replicated run type" = subsampling, "number of iterations" = 500 (default), "max number of background points" = 10 000 (default), "threshold rule" = equal training sensitivity and specificity. Models were assembled in the Dismo R package (Hijmans *et al.* 2017), and their predictions were visualized using QGIS.

Results

Genetic diversity and its distribution inferred from AFLP

Fingerprinting of 213 individuals produced 595 unambiguously scorable AFLP markers (4.9% error rate). More than half of the scored markers were polymorphic (350 polymorphic markers = 58.8%) and used for further analyses. Table 2 summarizes computed statistics at the regional scale. For population-based statistics, see SM 5a. The genetic diversity values both at the population but also at regional scale were low and similar across the sampled area (Hj = 0.093-0.162, median value 0.121; I = 0.119-0.224, median 0.165; PLP = 21.7-48, median 35.7 SM 5a). Spatial distribution of Nei's gene diversity (Hj) and frequency-down-weighted marker (DW) is given in SM 5b. We did not detect any fixed private bands (nFPB), and only a low number of private (nPB) at the regional level: eight for the region Central Europe and one for the region Russia (Tab. 2). DW values ranged from 1.38 (region Poland) to 2.28 (region Russia) with a median of 1.72.

Based on the greatest ΔK (SM 5c), two K values were further investigated: K = 3 and K = 7 (Fig. 2, SM 5d). The clustering for K = 3 differentiated mainly sampled sites from W Carpathians (1st cluster), Poland (2nd cluster), while all the remaining sites represented the 3rd cluster. Clustering for K = 7 further refined genetic groups' distribution defined by K = 3 and primarily showed further sub-separation of populations from Hungary and Russia. For both K values, a low to moderate degree of admixture is evident in all populations. The Neighbor-joining analysis grouped samples into eight clusters with weak bootstrap support (below 50%) (SM 5e, f), and samples' clustering was not strictly associated with their geographical origins.

The AMOVA results showed the greatest proportion of genetic variation (75.38%) present within sampling sites, 11.91% present among sampling sites within the six geographical regions, and 12.71% present among the six regions ($P < 0.001$). The overall differentiation among the sampled sites was $F_{ST} = 0.246$ ($P < 0.001$), while pairwise F_{ST} values ranged between 0.00075 (geographical distance; GGD = 50 km) and 0.428 (GGD = 540 km) (SM 6a). Mantel tests revealed the presence of a moderate to weak pattern of isolation by distance: 1) all sampling sites (r = 0.358, $P = 0.0001$); 2) Central European sites only (r = 0.470, $P = 0.0001$); 3) SE Carpathians (r = 0.519, $P = 0.335$); 4) W Carpathian sites (r = 0.067, $P = 0.429$). No correlation between Nei's gene diversity (Hj) or frequency-down-weighted marker (DW) and geographical location of sampling site was observed (Hj vs. Latitude r =0.118, P =0.50; Hj vs. Longitude r = 0.185, $P = 0.30$; DW vs. Latitude r = 0.06, $P = 0.72$; DW vs. Longitude r = 0.157; $P = 0.38$).

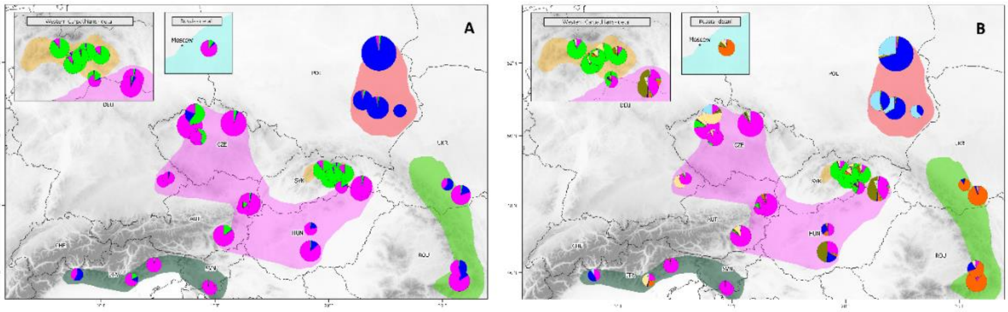


Fig. 2. Distribution of genetic diversity among *A. liliifolia* populations based on merged AFLP and cpDNA data. STRUCTURE clustering for K=3 (A), K=7 (B). Coloured polygons show defined regions referred in the main text (for details, see Figure 1 legend).

Chloroplast DNA diversity

From the 13 loci tested (SM 1b), only three (*trn*S-*trn*G, *trnS-trnfM,* and *psb*D-*trn*T) showed some variability (SM 2a), which was, however, low (e.g., gene diversity ranging from 0.053 for *psb*D-*trn*T to 0.596 for *trn*S-*trn*G; based on the whole dataset). The aligned *psb*D-*trn*T region was 908 bp with five SNPs, *trn*S-*trnf*M spacer was 878 bp long with three SNPs. The most variable region (*trn*S-*trn*G spacer, 963 bp long) had nine SNPs, a short 5 bp indel characteristic for two Romanian samples (sampled site RO_A), and a minisatellite (MS) region spanning four repetitions (i.e., four length variants of 0, 23, 46, and 69 bp long indel in alignment). All the three cpDNA loci were concatenated in a 2,767 bp long alignment. Nucleotide sequences were obtained for 50 of 80 MW (62.5%) and two of eight BRNU herbarium vouchers. Comparison of all the sequenced samples confirmed the taxonomic determination of 43 herbarium samples from MW as *A. liliifolia*, while eight samples represented different (not determined) *Adenophora* taxon (including KZ_MW-76; see below), and one sample represented taxon *A. coronopifolia* (RU_BRNU-01; SM 2b). Among 336 sequenced *A. liliifolia* individuals, 28 different cpDNA haplotypes (26 *A. liliifolia*: *hap_1* to *hap_26*; one *A. coronopifolia* – RU_BRNU-01/*hap_27*, and one *A.* sp. – KZ_MW-76/*hap*_28; EA3b) were distinguished (Tab. 2, Fig. 3, SM 2a, b). Concatenated alignment of only ingroup samples (i.e., without two outgroup lineages *hap_27* and *hap_28*) was 2,749 bp long with only 16 variable positions (excluding indels; 13 parsimony-informative + 3 singleton sites). A/C coding of the minisatellite and one indel slightly increased sequence polymorphism to 20 variable positions (17 parsimony-informative and 3 singleton sites).

Haplotype network (Fig. 3c) displays an interconnected topology reflecting low sequence divergence of identified lineages. Lineage *hap_14* seems to represent ancestral lineage to all the European lineages based on its geographical location (Poland, Russia, W and SE Carpathians; Tab. 2, SM 2a, b) as well as a direct connection with two outgroup lineages *hap_27* and *hap_28*. However, this interpretation should be taken with care due to low sequence divergence and possible homoplasies. The highest haplotype diversity (H_D) was recorded for W Carpathians (0.759), Alps-Dinarides (0.752), and Russia (0.650); the lowest for Poland (0.197; Tab. 2). The most common haplotype within the whole dataset was *hap_01* found nearly in all sampled sites except SE Carpathians and Russia (Tab. 2, SM 2a). Within the regions Central Europe and Poland, its frequency reached 0.87 and 0.89, respectively. This haplotype is only one mutational step apart from the suggested ancestral haplotype lineage *hap_14*.

On the contrary, several haplotypes show a narrower distribution (e.g., *hap_02* [AT_A] or *hap_04* [CZ_B] specific for the region Central Europe; *hap_03* [CH_A], *hap_07* [IT_A] present only locally within Alps-Dinarides). SE Carpathians were characterised by six haplotypes, two of which (*hap_16*, *hap_17*) were shared with the region Russia and one (*hap_18*) with Alps-Dinarides and W Carpathians. For the region Russia, 11 haplotypes were identified, three were shared with other regions, and eight were region-specific (see above). Within W Carpathians nine haplotypes were identified (Fig. 3b), what makes W Carpathians a hotspot of haplotype diversity of *A. liliifolia* in Europe outside Russia. Four haplotypes were unique for this region (*hap_23*, *hap_24*, *hap_25*, *hap_26*), while two haplotypes (*hap_06*, *hap_18*) were shared among W Carpathians, Alps-Dinarides, and Russia; *hap_14* was the most frequent lineage, and *hap_19* was shared with one Polish sampled site. Based on a subpopulation differentiation test, all the regions are significantly differentiated, $X^2 = 883.360$ (df = 115, $P < 0.001$). Tajima's D test revealed negative values for five out of the six defined regions pointing to population expansion following bottleneck. Only positive value pointing to population contraction was encountered for region Alps-Dinarides. However, none of the computed values was statistically significant (Tab. 2).

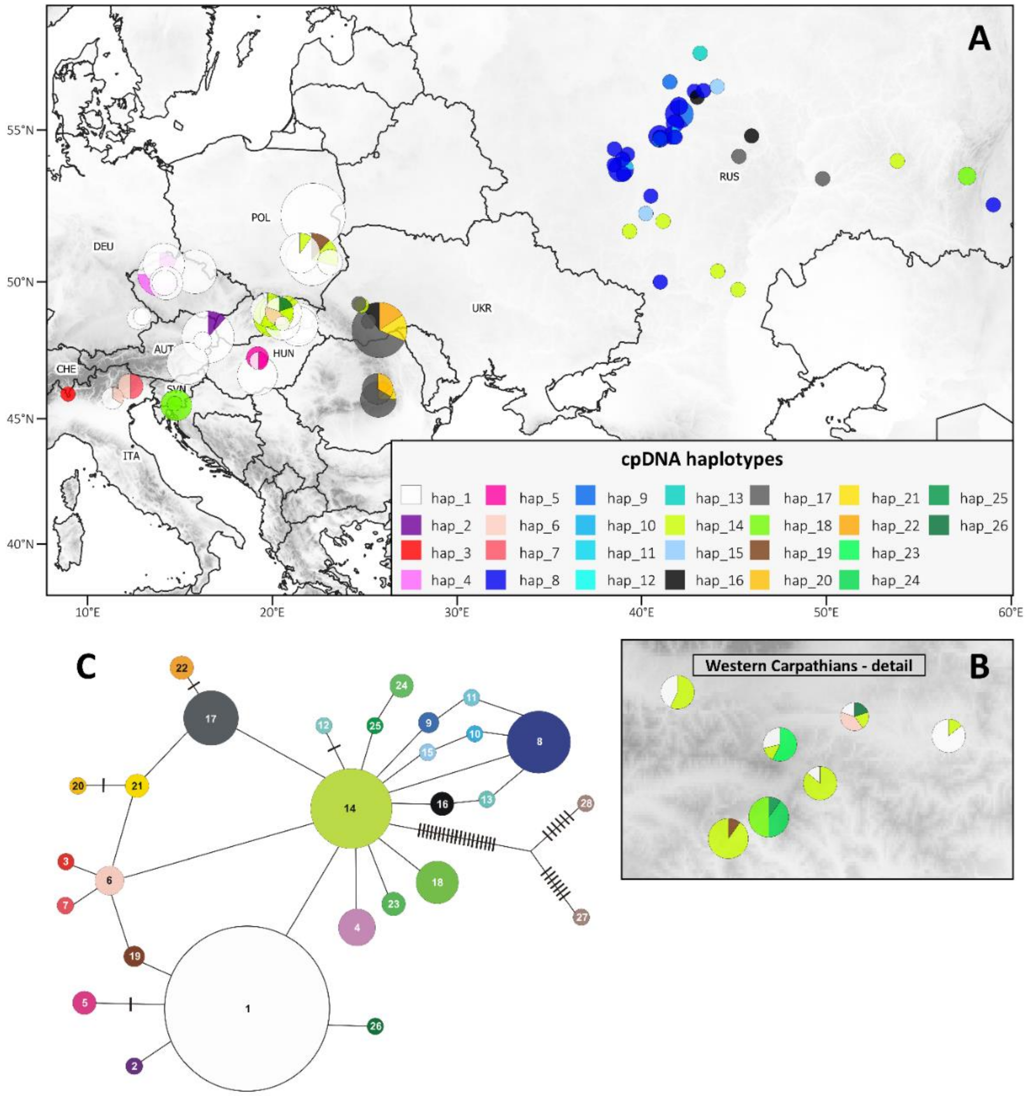


Fig. 3. Distribution of cpDNA haplotypes (A) with a detail on W Carpathians (Slovakia) (B). Figure (C) showing the parsimony network, based on three concatenated cpDNA loci and suggested relationships among the identified haplotype lineages. The circles' size corresponds to the number of sampled individuals (A, B), or samples bearing a particular haplotype (B); the small black lines represent unsampled lineages. Lineages *hap_27* (*A. coronopifolia;* RU-BRNU-1) and *hap_28* (*Adenophora* sp.; KZ_MW-76) represent the outgroup.

Table 2. Statistic based on AFLP and concatenated three cpDNA loci. nAFLP (number of samples used for AFLP analysis), PLP (percentage of polymorphic loci), nFB (fixed band number), nPB (private band number), nFPB (fixed private band number), Hj (Nei's Gene Diversity), I (Shannon's index), N_E (number of effective alleles), DW (frequency-down-weighted marker value [rare fragments]), nSeq (number of samples sequenced for three cpDNA loci), N_H (number of different haplotypes), H_D (haplotype diversity), Pi (nucleotide diversity), Tajima's $D^{[P]}$ (Tajima's D test, the significance of the test is shown as the superscript, ns = not significant), cpDNA (multilocus haplotype observed for the concatenated three intergenic spacers; + frequency of observed haplotype), SE (standard error).

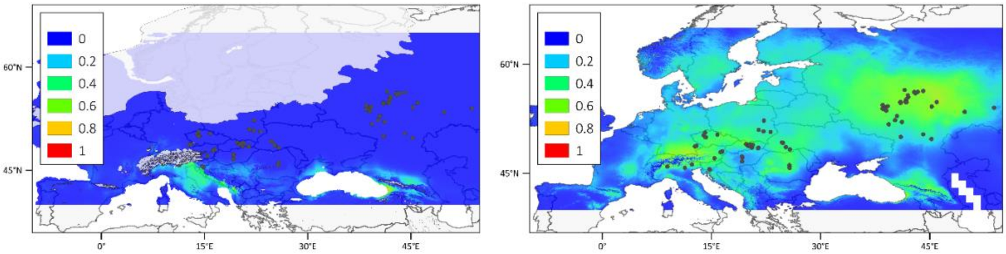
Region	nAFLP	PLP	nFB	nPB/ nFPB	Hj	I (SE)	N_E (SE)	DW	nSeq	N_H	H_D	Pi	Tajima's $D^{[P]}$	cpDNA (frequency)
Central Europe	89	48.6	3	8/ 0	0.159 (0.008)	0.263 (0.012)	1.241 (0.015)	1.71	119	4	0.231	0.00017	-1.081^{ns}	hap_01 (0.87), hap_02 (0.02), hap_04 (0.08), hap_05 (0.03)
Alps-Dinarides	12	35.7	31	0 / 0	0.134 (0.009)	0.200 (0.013)	1.194 (0.015)	2.02	15	5	0.752	0.00063	0.304^{ns}	hap_01 (0.13), hap_03 (0.07), hap_06 (0.2), hap_07 (0.13), hap_18 (0.47)
Poland	41	40.6	10	0 / 0	0.122 (0.008)	0.199 (0.012)	1.181 (0.014)	1.38	57	3	0.197	0.00008	-0.925^{ns}	hap_01 (0.89), hap_14 (0.07), hap_19 (0.04)
W Carpathians	45	37.4	22	0 / 0	0.120 (0.008)	0.196 (0.012)	1.177 (0.014)	1.54	61	9	0.746	0.00048	-0.347^{ns}	hap_01 (0.34), hap_06 (0.03), hap_14 (0.36), hap_18 (0.08), hap_19 (0.02), hap_23 (0.07), hap_24 (0.07), hap_25 (0.02), hap_26 (0.02)
SE Carpathians	21	34.3	21	0 / 0	0.129 (0.008)	0.205 (0.012)	1.189 (0.015)	1.73	36	6	0.576	0.00040	-0.721^{ns}	hap_14 (0.03), hap_16 (0.06), hap_17 (0.65), hap_20 (0.06), hap_21 (0.1), hap_22 (0.1)
Russia	5	32.3	37	1 / 0	0.126 (0.010)	0.158 (0.012)	1.169 (0.016)	2.28	48	11	0.650	0.00040	-0.862^{ns}	hap_08 (0.58), hap_09 (0.06), hap_10 (0.02), hap_11 (0.02), hap_12 (0.02), hap_13 (0.02), hap_14 (0.10), hap_15 (0.04), hap_16 (0.04), hap_17 (0.04), hap_18 (0.04)

Maxent modeling

The Maxent niche-modeling was evaluated with a receiver operating characteristic (ROC) curve, with an AUC value for the replicate runs equal to 0.914 (±0.019). Min Temperature of Coldest Month (Bio6) contributed most to the model (35.2%), followed by Warmest Quarter Precipitation (Bio18, 21.7%; SM 4c). This suggests climatic variables determining *A. liliifolia'* distribution are low temperature during winter (temperature in sampled sites varies from -14 °C to -4 °C) and amount of precipitation during the growing season (optimal amount of rainfall 330 mm; for details, see SM 4d). The environmental variable with the highest gain, when used in isolation, is Bio4 (Temperature Seasonality). The gain decreased the most when Bio6 was omitted. LGM prediction (Fig. 4A) showed a considerable reduction of climatically suitable habitats with optimal habitat restriction to an area south of Alps in Italy, Balkan Peninsula, and Caucasus (the region not covered by sampling and not considered). The model for the Mid-Holocene era (Fig. 4B) highlighted the area north from Alps and SE from Moscow as the most climatically suitable. The present-time prediction identifies a large area of suitable habitats in Central-Eastern Europe. This distribution is in concordance with actual species distribution in Europe (Fig. 4C). However, the actual area of suitable habitats is much narrower because it is influenced by many other factors that are difficult to consider in modeling (e.g., abandonment of traditional site management, canopy closing, ongoing succession, increasing pressure of nitrophilous species prospering on the environment eutrophication). The scenarios for years 2050 and 2070 (Fig. 4D, SM 4e) indicate habitat constrictions and shifting of climatically suitable habitats towards the north-east.

A) Last Glacial Maximum (22, 000 BP)

B) Mid-Holocene (6,000 BP)



C) Present (1970-2000)

D) Future projection under scenario rcp8.5 (2070)

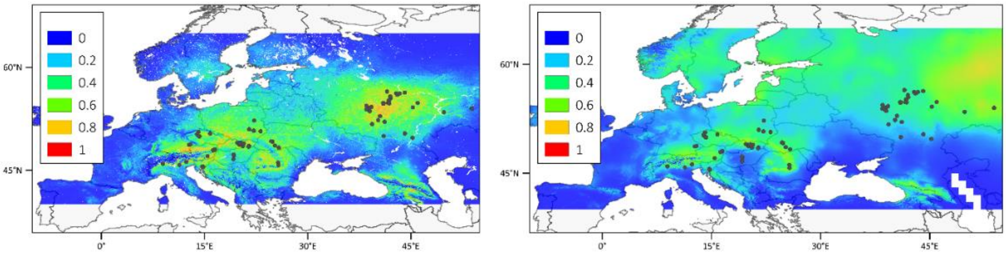


Fig. 4. Maxent prediction of habitat suitability for *A. liliifolia* populations: A) LGM (Last Glacial Maximum, ~22,000 BP); B) Mid Holocene (6,000 BP); C) present time (1970-2000); D) future projection under scenario rcp8.5 (2070). Log suitability is expressed between 0 and 1. Higher values (represented by warmer colours) indicate an increased probability of finding suitable habitats. Dots on the map represent sampled populations.

Discussion

The present study provides the first insight into the phylogeography of *Adenophora liliifolia*, a unique European representative of the predominantly Asiatic genus. One remarkable finding is the overall low sequence divergence of all the sequenced loci along a great geographical distance (3 600 km). The nuclear ITS marker, successfully used for disentangling phylogeography of several plant species (e.g., Comes & Abbot 2001; Eidesen *et al.* 2007; Volkov *et al.* 2010), was almost uniform across the sampled sites. The lack of sequence divergence may indicate the young evolutionary origin of *A. liliifolia* or a recent range expansion (Schönswetter *et al.* 2005; Krawczyk *et al.* 2018; Urbaniak *et al.* 2018). The distribution of observed cpDNA haplotype lineages points to several regions within Carpathians, Alps-Dinarides, and Russia with elevated haplotype diversity, which may represent refugial areas.

Phylogeography of *Adenophora liliifolia*

Based on the recent phylogeny of East Asian Campanuloidae (Yoo *et al.* 2018), the genus *Adenophora* split from its closest relatives ca. 14.8 Myr ago, while *A. liliifolia* further diverged from sister taxa between 4.64 and 3.56 Myr, just before Pleistocene's abrupt climatic changes (the divergence time estimation has a large confidence interval, thus the species divergence may be more recent). The absence of different ITS ribotypes across the investigated 3 600 km transect contradicts the estimated divergence time. Substitution rate of the ITS region varied considerably among different plant groups and life histories (0.38×10^{-9} to 8.34×10^{-9}; Kay *et al.* 2006), and although the mutation rate for *A. liliifolia* is unknown, we would expect more changes accumulated over ~ 4 Myr than observed. Together with low variability of sequenced cpDNA loci, it suggests *A. liliifolia* is evolutionarily younger taxon. Based on the observed haplotype diversity and its spatial distribution, we hypothesize *A. liliifolia* originated in the area between Ural Mountains and Southern Siberia, from where the species migrated westward. This migration probably took place only recently during Late Pleistocene and was enhanced by the spread of vegetation of boreal/hemiboreal forests and/or forest-steppes/steppes through Central Russia and the Pontic region. This is supported by the connection of the region of Central Russia with Central–eastern Europe by three haplotype lineages (*hap_14* – suggested ancestral lineage to all the European haplotype lineages; *hap_16*, *hap_18*; Fig. 3c). The observed spatial distribution of shared haplotype lineages (Fig. 3a,b), together with AFLP data (Fig. 2), however, suggests only a restricted gene flow among the Russian and the European populations, resulting rather from their earlier connection. We cannot also exclude the possibility of repeated and independent origin of the lineage *hap_14*, once in the Russian territory and the second time in Europe from *hap_01* lineage. Very low sequence divergence, observed within the present study, hampers reconstruction of a reliable phylogenetic tree but also dating of dispersal event of *A. liliifolia* out of Asia, which would help to identify the most ancestral lineage.

Contrary to the invariable ITS region, cpDNA data show some geographic pattern, which corresponds to defined geographical regions. A survey of cpDNA sequence data unveiled four spatially restricted haplotype diversity areas. Three of them are situated within Europe (W Carpathians, SE Carpathians, and Alps-Dinarides), and the fourth is lying on Russian territory. These areas may represent the refugia of *A. liliifolia* during Late Pleistocene.

Western and SE Carpathians were already recognized as glacial refugia of mountain flora (e.g., Fér *et al.* 2007; Mráz *et al.* 2007; Stachurska-Swakoń *et al.* 2012, 2020; Wąsowicz *et al.* 2016) or even steppe and grassland flora (e.g., Cieślak 2014; reviewed in Kajtoch *et al.* 2016), together with Alps and Dinarides which are considered important refugia for beech-forest associated herbaceous flora (Dvořáková *et al.* 2010; Slovák *et al.* 2012; Willner *et al.* 2009). On the other hand, the area of Central Russia south of Moscow was not covered by an ice sheet during LGM (Svendsen *et al.* 2004), and palynological data suggest general cooling in this period did not dramatically change available habitats (Tikhomirov & Kulikova 1987). Several habitats suitable for *A. liliifolia* were present here, including boreal forest, forest-steppe, or steppe (Hurka *et al.* 2019; Janská *et al.* 2017; Markova *et al.* 2002). Apart from these four putative refugial regions, haplotype diversity in the rest of the investigated European sites was probably reduced during multiple glacial-interglacial cycles. In this area, only a few rare spatially restricted lineages were left (e.g., *hap_02*, *hap_04,* and *hap_05*) along with the most widespread lineage *hap_01* (Fig. 3, Tab. 2). These rare lineages might have survived locally in climatically favorable places. Bílichovské valley (CZ_B) may represent such an exemplary habitat, where Early Holocene soil layers rich for malacofauna typical for open forest habitats were found (Ložek 2006); and relict species as *Veratrum nigrum* L., *Thesium rostratum* Mert. et Koch and *Carex pediformis* C. A. Mey are co-occurring with *A. liliifolia*. However, niche-modeling points only to the southern refugium (foothills of SE Alps-Dinarides; Fig. 6A) and failed to reveal W and SE Carpathian refugia. This can be explained by the limited resolution of data layers, failing to predict microclimatic/microhabitat conditions on such a fine scale.

Based on AFLP data, the majority of the investigated sites show a low level of admixture, especially within Poland and W Carpathians. These two regions are highly distinct and represent different genetic clusters (Fig. 2, SM 5d, e, f). Homogeneity of AFLP data and low admixture pattern of the investigated Polish sites can be explained by leading-edge re-colonisation accompanied by a substantial bottleneck (Hewitt 1996; Soltis *et al.*1997). The result of Tajima's D test suggests one of the strongest bottleneck event for this region, however without statistical significance (Tab. 2). The presence of particular haplotype lineages (Fig. 3) and pairwise genetic distances (SM 6a, b) points to possible sources of migrants from Central Europe. Though, the presence of lineage *hap_14* in Poland may point to the presence of cryptic refugium located in Southern Poland. The area of modern-day Poland was close to the continental ice sheet, however, the south-eastern part has not been glaciated since the Sanian/Elsterian glaciation (e.g., Marks *et al.* 2016), and several phylogeographic studies suggest that steppe biota found suitable habitats here and persisted from the Late Glacial in the area of South-Eastern Poland (e.g., Cieślak 2014; Cieślak & Drobniak 2019; see also review by Kajtoch *et al.* 2016).

Different genetic composition of the sampled W and SE Carpathian sites indicates that populations preserved within these regions were isolated and without gene flow, a common pattern observed for species with glacial refugia in Carpathians (e.g., Wąsowicz *et al.* 2016; Stachurska-Swakoń *et al.* 2012; Mráz *et al.* 2007). Low admixture pattern and homogeneity of the investigated W Carpathian sites suggests migrants only marginally enriched this area but also *vice versa*. Region of W Carpathians contributed only minorly to the species' postglacial movement out of the refugia. A similar trend was also observed for locally preserved stands of beech in W Carpathians, which did not significantly contribute to the re-colonisation of Central and Northern Europe during Holocene (Magri *et al.* 2006; Magri 2008). Limited contribution of W Carpathian genotypes to re-colonisation might be due to local adaptation or competitive disadvantage over faster spreading/or already present "general-purpose" lineage represented by *hap_01*. We hypothesize that re-colonisation of Central and Central-Eastern Europe was directed from the southern refugia (where *hap_01* is present), and *A. liliifolia* might have accompanied the spread of oak (Petit *et al.* 2002).

Neighbor-joining analysis revealed clusters that mix samples of different geographic origin (SM 5e). From these clusters, cluster VI (and partially also IV) might be considered as a result of directed re-colonisation or may mirror former frequent gene flow among regions. The prevalence of only one haplotype (*hap_01*) in the majority of the investigated sites (Fig. 3a), and its absence in the region Russia, suggests this lineage evolved in Europe west of Russia. Evolution of this haplotype lineage could be the response to newly appearing habitats in Europe. Identification of recolonisation direction is not straightforward, however, based on the admixture pattern revealed in STRUCTURE analysis we hypothesise, recolonisation was directed from an area situated close to presumed southern refugium (a scenario proposed above). An advantageous, fast-spreading haplotype lineage (*hap_01*) might have enriched populations in already persisting and scattered refugia. On the other hand, the observed pattern can also be explained as the continuous presence of stands of *A. liliifolia* within the Central European region throughout the Late Pleistocene. Persisted populations outside the mountain chains might have been selected for *hap_01* lineage representing a "general-purpose" lineage, being the most resistant and adapted to conditions of Late Pleistocene-Early Holocene glacial steppe/forest-steppe.

Genetic diversity of *Adenophora liliifolia*

The observed values of genetic diversity were low and similar across the investigated sites (Tab. 2, SM 5a), which may indicate several processes. Fast leading-edge re-colonisation of Central Europe during Late Pleistocene – Early Holocene might have influenced lower genetic differentiation of the sampled sites through founder event (Waters *et al.* 2013). However, a strong bottleneck within preserved populations through selection for a "general purpose" lineage *hap_01* may be the cause of lower variability as well.

Although no statistically significant relationship between Hj or DW and geographical location was revealed, pairwise F_{ST} values are higher among particular pairs of geographically distant sites. This suggests an ongoing differentiation through isolation. Recorded F_{ST} value along the investigated geographical transect ($F_{ST} = 0.246$) reflects a continual breakdown of a larger genetic pool and the beginning of gradual differentiation of fragmented populations. On the other hand, this may also mirror the differentiation of refugial sites. For the investigated W and SE Carpathian sites, AFLP data do not exhibit a typical pattern of refugia but rather show diversity evenly distributed across the sampled regions. Lower values of gene diversity and rare fragments in the two proposed refugia (W and SE Carpathians, Tab. 2, SM 5a) might result from lower effective population size caused by population shrinking (Ellstrand & Elam 1993; Szczecińska *et al.* 2016; Young *et al.* 1996), while admixture may have increased gene diversity of recolonized populations (e.g., Havrdová *et al.* 2015). This may also suggest the existence of a larger genetic pool within Central (Eastern) Europe, which was present for a longer time, and enabled gene flow which hinders more pronounced population differentiation.

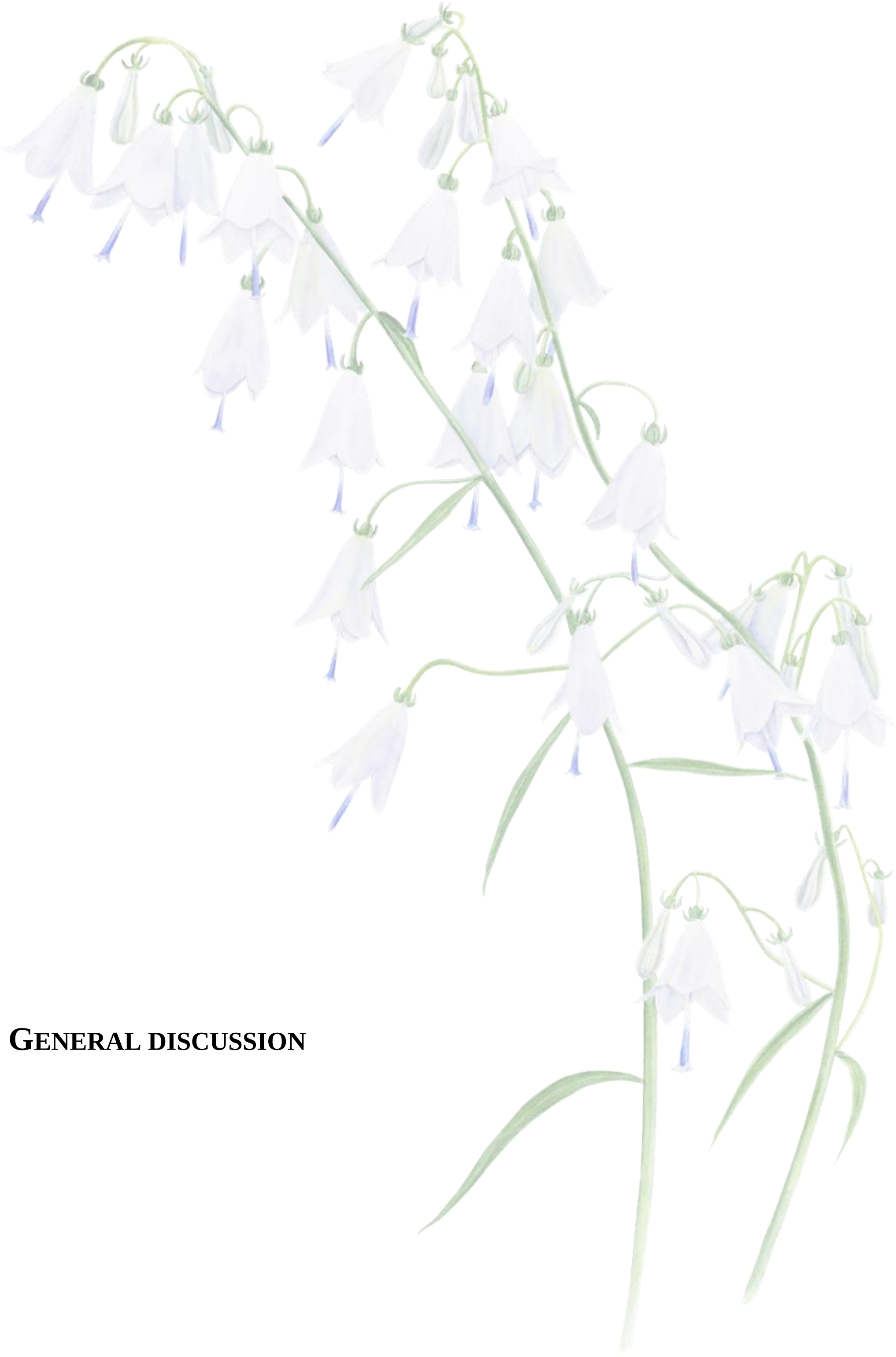
Similar level of genetic diversity, as reported here for *A. liliifolia,* was observed for four populations of *A. liliifolia* on Ural, He = 0.228 (ISSR markers, Boronnikova 2009). Different East-Asian *Adenophora* species show an analogous level of genetic differentiation based on allozymes, e.g. *A. grandiflora* Nakai: 2 populations 40 km apart, He = 0.259, G_{ST} = 0.027 (Chung & Epperson 1999). Study of another two species show also a similar level of genetic differentiation, endangered *A. lobophylla* D.Y. Hong: populations 25 km apart F_{ST} = 0.071, versus widespread *A. potaninii* Korsh.: populations on 850 km long transect, F_{ST} = 0.155 (Ge *et al.* 1999).

Concluding, the overall low genetic diversity may be equated with the young evolutionary origin of the species and/or repeated population contractions/expansions accompanied by bottleneck during Quaternary climatic fluctuations. All of this could lead to a reduction of variability of the genetic pool, which (re)colonized Europe and which was further depleted during the Pleistocene. Such conjoint effect of local persistence/recolonisation is reported for some tree species in Europe (Petit *et al.* 2003) and the Mediterranean (Fady & Conord 2011). On the other hand, the absence of a stronger genetic differentiation and overall similarity of the investigated sites could also point to the process of gradual fragmentation of a larger genetic pool once present within Central Europe.

Conservation recommendations and *A. liliifolia*' prospects

Our research shows that European populations are not primarily threatened by decreased genetic diversity but rather by fragmentation and habitat loss, which results in population shrinking and extinction (Prausová *et al.* 2016). Comparison of historical data with present surveys showed a rapid decrease in the number of populations of *A. liliifolia* across its whole distribution; e.g., from the Czech Republic, about 26 historical localities are known, but only 6 of them are still present; in Slovakia, about 30 populations were known from Carpathians, only 10 are persisting; or in Poland, the species was known from ca. 100 localities, but nowadays, only 21–22 remain (Boronnikova 2009; Prausová *et al.* 2016; Valenti *et al.* 2018). NATURA 2000 reports assess the species conservation status as “unfavorable-bad” since 2001, and there is no evidence of change in the future. Probably only due to the longevity of the species, the situation is not critical yet. We performed the first genetic study of the species, and we recognized several genetically unique areas, which deserve priority in protection. Some of these populations face acute threats. These regions should be addressed as a matter of priority and treated with special care in any *ex-situ* conservation attempts. The results of Maxent modeling suggest that ongoing climate change will even worsen the situation and influence the future distribution of *A. liliifolia* in Europe. Both scenarios for years 2050 and 2070 show gradual habitat constrictions. Under the most stringent course (rcp85, Fig. 4D, SM 4e), prediction shows a noticeable shift in habitats' suitability towards the north-east in Russia. The vastness of Russian territory seems to preserve much broader options for *A. liliifolia* survival than Central Europe, where populations face increasing pressure. Possible limitations of the presented predictions might be seen in a relatively low number of presence records used for modeling and omission of some hard-to-be predicted but essential variables (ongoing habitat eutrophication, followed up by nitrophilous species spread resulting in canopy closing and total habitat change).

The conservation efforts should focus on maintaining the present genetic diversity and the increase of populations' size. There is no need for a single conservation strategy, and the conservation should be adjusted in particular regions to main threats. However, when possible, population reinforcement should be done exclusively from local sources. Local lineages represent locally best-adapted genotypes and may show reduced fitness in any non-native habitat. Our results may be used as a guide for finding closely related populations within each region (e.g., pairwise F_{ST} values in SM 6a). Conservation of *A. liliifolia* strongly depends on specific management supporting its seedlings, which are not vigorous enough to survive without protection (e.g., by removing invasive and nitrophilous species). National action plans already implemented or recently prepared aim at developing appropriate techniques for *ex-situ* preservation, e.g., creation of sterile tissue cultures, appropriate storage of seeds in seed banks, experimental germination tests, and cultivation to identify factors critical for seedlings growth. Further studies on *A. liliifolia* should focus on demographic monitoring, determining exact influences causing habitat and individual loss, and test of fitness in transplantation experiments.



GENERAL DISCUSSION

The current situation of *Adenophora liliifolia* within Europe and evaluation of factors threatening the species

Seventeen-year monitoring of *A. liliifolia* populations in the Czech Republic highlighted the critical factors threatening the species. Most of the stands the species inhabits within Central-East Europe and the Czech Republic, in particular, represent habitats shaped in the past by traditional forest and meadow management. Such activities as, e.g., coppicing and mowing are nowadays abandoned in many sites. The species in these habitats is endangered by ongoing secondary succession, and its persistence is therefore conditioned by a human intervention which, however, needs to be coordinated. Populations respond positively to the opening of the canopy layer by selective removing of shrubs and trees by an increased proportion of fertile stems. However, higher transmittance of sunlight causes fast overgrowing of competitively vigorous species, which oppresses *A. liliifolia*, and must be regularly eradicated. Moreover, plants have to be also protected from grazing by fencing in most of the sites. However, the most critical fact in the species' future within the Czech Republic is the absence (or only minimal level) of natural regeneration. Although plants bloom and produce numerous seeds regularly, only a few seedlings are observed annually, and only a minority of them persist to the next season. A similar situation may be present in other countries, where, to my knowledge, not such detailed and repeated monitoring was done.

The only exception is the study of Shevchenko and Kraynyuk (2019), focused on supposedly closely relative species *Adenophora liliifolia* subsp. *taurica* Sukaczev, the endemic taxon of Crimea. The authors studied developmental biology, distribution, and the population age structure of two closely situated populations on the area of about 100 m^2 in pine forests of *Pinus kochiana* Klotzsch ex K.Koch (a synonym of *Pinus sylvestris* var. *hamata* Steven) at the altitude of 1 200 m a.s.l. The projective coverage of the species was 5–10%, with a population size of up to 100 individuals. The study is reporting 85% (respectively 78.4% on the second site) of observed individual plants being in "virginal" life-period, while only 15% (respectively 21.6%) individuals in the generative phase. This observation is highly different from the situation we are witnessing in the Czech Republic, where the majority of plants are flowering – thus are in the generative phase, while sterile individuals are found mainly on sites with inappropriate habitat conditions. However, the question may be the determination of the life phase because "virginal" plants, shown in the published picture, have only a rosette of juvenile leaves, which might, according to our knowledge, represent old individuals, persisting unfavourable conditions. The main threats and limiting factors listed by the authors are the global transformation of the climate and narrowing of the species' ecological niche, as well as the natural-historical rarity of the species, low competitive ability at the border of the range, and anthropogenic disturbance of the habitat.

The situation of *Adenophora liliifolia* during the last five years within the Czech Republic has brought a slightly positive turn. The species was rediscovered after 75 years in 2018 in Central Bohemia, close to the town of Poděbrady (Roleček & Šťastný 2020). The most recent herbarium vouchers from this area were collected during the 1930s and 1940s. The Action Plan for the species defining the goals in protection and bringing statutory and financial covering of necessary costs was approved in 2020 (Prausová *et al.* 2020, https://www.zachranneprogramy.cz/zvonovec-liliolisty/ke-stazeni/). A significant increase of observed individuals from 168 in season 2016 to 502 in 2021 was encountered, representing nearly three times higher absolute numbers of individual plants recorded in the country. However, it has to be noted that this overall increase is associated with the increased abundance in two populations (Karlické údolí and Babinské louky). In contrast, other populations stagnate or even encounter a decrease in the number of individuals (Prausová & Vaculná, personal observation).

Knowledge exchange and information sharing are essential for the successful conservation of threatened species. Close cooperation among Central- East Europe colleagues has been established, especially between the Czech Republic and Poland, where enormous efforts are being made on *A. liliifolia* rescue. A regional plan on species protection in the Lublin region was published in 2007 (Kucharczyk 2007). The strategy on seeds cryopreservation is developed, as well as germination tests and growing experiments were done. The seedlings obtained in growing experiments were used in transplantation experiments, e.g., in Niedzieliski Las (Kapler *et al.* 2019). However, despite the optimistic results announced, the situation of species' natural persistence is getting worse in the region as well as in the whole Poland. In Niedzieliski Las, there are only four (September 2021) healthy, flowering individuals left. The population used to be more abundant; however, part of the plants was grazed by wild game or possibly dug up by humans. In the Lublin province, there are a few other *A. liliifolia* populations with a decreasing number of surviving individuals (Bodaczowski Las, Skrzyniec, Strzeleckie Las), as well as in adjacent regions of Rzeszów province (the Zaklików population). In recent years, populations in Bodaczowski Las, Wierzchowiska and Adamowskie Las (Łysa Góra, Lublin province) were lost. Those populations could represent an important evolutionary line, which migrated from W Ukraine. Regarding the rest of the Polish populations investigated in Vaculná *et al.* (2021), the situation in the Krzemionki Opatowskie site is better than a few years ago. The population is healthy and abundant. Seeds from this population will be stored at Kostrzyca Forest Gene Bank. However, the site is potentially threatened by local investment interests to establish a new limestone quarry and power plant construction. The situation at the Kisielany site is worsening due to ongoing secondary succession. Moreover, part of the site was destroyed during the construction of new roads for firefighters. Similarly, the situation at the Zaklików site is also worsening due to ongoing succession. The main goal of the project "FlorIntegral," implemented in 2018, was the restoration of *A. liliifolia* population in Kampinos Primeval Forest in Kampinos National Park (NP) near Warsaw, destroyed by wild boar activity in 2017. The restoration of this population was done from seeds collected from the genetically closest populations – Kisielany and Krzemionki Opatowskie. Five replacement posts were established with a total area of 6 ha, where protective treatments as reduction of undergrowth vegetation and elimination of invasive species were carried out. On 51 plots representing various microhabitats altogether, 1 752 pre-grown *A. liliifolia* individuals were planted. The situation is monitored at various levels (individual, population, and habitat) (A. Kapler, personal communication).

At the European Union level, the situation of the species stays critical. The last report from period 2013–2018 assesses the species' status within the whole EU territory as "Unfavourable-Inadequate" or "Unfavourable-Bad." The only exceptions are the Alpine bioregions (denoted as "Alpine" in NATURA 2000 methodology to emphasize their non-lowland character) of Slovenia and notably Slovakia. In Slovakia, the species grows mainly in the mountains in places distant from human settlements, rocky outcrops, and well-lit edges of beech or relict pine forests. Such habitats represent climax communities on-site and persisted here without negative impacts of human activity for long periods. However, even in Slovakia (similarly to the rest of the European countries), the species is gradually vanishing, which is becoming evident when comparing historical occurrence data with recent ones. Historically, about 30 populations were reported from the Slovakian Carpathians, but only about 10 lasted until today.

Evolutionary history of *Adenophora liliifolia*

We attempted to answer questions related to the Quaternary history of *A. liliifolia* involving different modern analytical methods, but we succeeded only partially. The species diversity center for the genus *Adenophora* is East Asia (Lammers 2007b); however, this region is probably not the place of the genus origin. The most recent common ancestor of the genus *Adenophora* split from its relatives from the rest of Campanuloideae ca. 14.8 Ma somewhere on the border of Europe/W Asia (SW Siberia). A swarm of newly formed taxa from this region migrated eastward to Asia, where the genus further diversified (Yoo *et al.* 2018). Yoo *et al.* (2018) further state that one representative (*A. liliifolia*) later migrated back, westward, to Europe. Our data and observations support the assumption that the most likely area of *A. liliifolia* origin is the area of Southern Siberia/Ural Mountains, from where the taxon later expanded to Europe. The following arguments support this assumption: 1) presence of haplotype lineage *hap_14*, which was suggested as probably the most ancestral lineage; 2) presence of highest haplotype diversity in the investigated Russian area; 3) a verified record of *A. liliifolia* occurrence from the area of Altai mountains is missing (Vaculná *et al.* 2021). The presence of ancestral haplotype lineage close to the assumed place of the genus origin, together with high haplotypic diversity, suggests the location of the species origin should be localized there. We are rather skeptical of Yoo *et al.* (2018) statement regarding *A. liliifolia* backward migration from East Asia through Europe/SW Siberia border to Europe. Their assumption is based on two samples of Asiatic "*A. liliifolia*," which seems to be highly different from the European ones (see below). Although the species is often mentioned as part of native flora in many Asiatic countries (e.g., China – Hong & Ge 2010, Hong *et al.* 2011), and is also a part of published research datasets (e.g., Kim & Yoo 2011; Cheon & Yoo 2013; Cheon 2014; Yoo *et al.* 2018) we are convinced of the incorrect taxonomic determination of the material used in these studies. Overall low nucleotide diversity and sequence divergence of the investigated loci (Vaculná *et al.* 2021) suggest the species is evolutionarily relatively young, or the westward migration took place recently with only a limited number of genotypes which necessarily resulted in a bottleneck effect evident within the Central European populations. Such movement out of the place of origin could be enhanced by global climate changes and the spread of steppe and forest-steppe habitats at the end of Tertiary and during the transition of Tertiary – Quaternary. Molecular dating would help address this question; however, the above-mentioned low sequence divergence and taxonomic uncertainties hinder this task. At this time, we can rely only on data from other studies. Biogeographical analysis of Asian Campanuloids estimated the main diversification of the genus *Adenophora* into Miocene – Pliocene (Yoo *et al.* 2018). This study also includes *A. liliifolia,* and based on the chronogram from the study of Yoo *et al.* (2018), *A. liliifolia* belongs among the youngest species within the genus originated at the turn of Pliocene – Pleistocene (~ 4–2 Myr). However, we are rather cautious in strict conclusions due to doubts about the correct taxonomic determination. Estimates based on more variable chloroplast and nuclear loci are needed for more precise and reliable dating.

Our study revealed the shallow genetic differentiation of haplotype lineages and their spatial distribution within the Central-East European region. We also witnessed a limited differentiation across the investigated 3 600 km transect. The identified areas of higher haplotype diversity [1) Central-East Europe: Western Carpathians; 2) South-East Carpathians; 3) Alps-Dinarides; 4) Central Russia] might represent refugial regions. However, these regions should not be considered as sites where the species retreated from the worsening conditions during the onset of the Ice age and from where the recolonisation took place during the release of harsh conditions. Instead, they should be understood as places where the species found the best available habitats, which allowed further diversification even within the otherwise monotonous landscape. Russian and Central-East Europe regions have experienced slightly different conditions during the glaciation. An ice sheet did not cover the area of Central Russia south of Moscow during the LGM (Svendsen *et al.* 2004), and general cooling in this period did not dramatically change available habitats (Tikhomirov & Kulikova 1987). Therefore, several habitats suitable for *A. liliifolia* remained present, including boreal forest, forest-steppe, or steppe (Markova *et al.* 2002; Hurka *et al.* 2019). Regarding the habitats in Europe, it is generally accepted nowadays that not only steppe but also other types of habitats were available, particularly in the climatically favorable locations (e.g., Willis *et al.* 2000; Magri *et al.* 2006; Magri 2008; Juřičková *et al.* 2014). Janská *et al.* (2017) reconstructed the position of European vegetation types during the LGM using modern analogs from southern Siberia. Their results show the presence of several habitat types within Europe, and Russia (e.g., temperate light-coniferous forest, or pine and larch boreal forests), where *A. liliifolia* could persist (Fig. 1).

Habitats of most of the investigated W Carpathians *A. liliifolia* populations (where the species currently experience the best conditions) represent limestone beech forests with relict *Pinus* islets on rocky outcrops (Prausová *et al.* 2016). These habitats could be present here also during the late Pleistocene (Magri *et al.* 2006; Jankovská & Pokorný 2008; Kuneš *et al.* 2008). The relict character of forests of W Carpathians also supports the presence of a Tertiary relict and stenoendemic *Daphne arbuscula* Čelak. on the Muránska planina (SK_D in Vaculná *et al.* 2021) locality (Erdelská & Turis 1995), or the presence of *Cyclamen purpurascens* subsp. *immaculatum* (Hrabětová) Halda et Soják, a narrow endemic of the W Carpathians (Kučera *et al.* 2013). In SE Carpathians, *A. liliifolia* grows in meso-xerophile subcontinental meadows-steppes (Ukraine), thermophilous oak forests, and intermittently wet *Molinia* meadows (Romania). The investigated Slovenian populations are situated in beech forest (SL_A in Vaculná *et al.* 2021) and on river gravel banks in willow scrubs (SL_B in Vaculná *et al.* 2021). Italian habitats are similar to the Slovenian and are represented mainly by beech and black pine forests and calcareous rocky outcrops (EUNIS 2021). Contrary, the remaining investigated populations represent mostly intermittently wet *Molinia* meadows (AT, CZ, HU), riparian mixed gallery forests (HU, RU), thermophilous oak forests (CZ, PL), oak-hornbeam forest (CZ), or mountain meadows (AT, HU) and thick beech forest (AT; see Prausová *et al.* 2016, and Vaculná *et al.* 2021). Our data show elevated haplotype diversity and DW values in W Carpathians, SE Carpathians, and Alps-Dinarides – populations occurring in mainly beech forests or relict *Molinia* meadows. Contrastingly, oak-related habitats harbored limited haplotype diversity and generally lower DW (Vaculná *et al.* 2021). From these findings, we can conclude that populations associated with beech forests might represent the most relict and refugial populations within forest habitats where the species found the best conditions. In contrast, the oak-related habitats and *Molinia* meadows represent secondary habitats that progressively replaced otherwise widespread Pleistocene steppes. Pleistocene steppe habitats offered only a limited number of ecological niches/opportunities compared to retained forests of deep valleys in high mountains. This could lead to a marked reduction of genetic diversity and selection of a genotype best fitted to steppe conditions. Consequently, this process of gradual adaptations to new habitat led to the selection of general-purpose genotype represented by haplotype lineage *hap_01* (Vaculná *et al.* 2021), which gradually expanded into widespread steppes, forest-steppes. This lineage is absent in the investigated Russian territory as well as it is absent from the Southeastern Carpathians. We, therefore, infer its origin in the area of Central Europe.

Based on all of this information mentioned above, and notably the elevated haplotype diversity in given areas, we infer that the W Carpathians, SE Carpathians, and the Alps-Dinarides might have served as glacial refugia for *A. liliifolia*. The assumption was repeatedly proved for several other taxa in these areas (e.g., *Rosa pendulina* L., *Hypochaeris uniflora* Vill., *Cicerbita alpina* (L.) Wallr in the Carpathians). However, the species distribution modeling (SDM) supported our refugial predictions only for the area of Alps-Dinarides. This fact is probably due to the limited resolution of data layers used and the complicated projection of micro-habitat conditions of, e.g., valleys of W Carpathians, into the computing algorithm. Based on the presumed climatic data for Holocene, the SDM also showed significant habitat expansion, which probably occurred during this period (associated with the rapid spread of the species, notably the lineage *hap_01*).

However, it has to be noted here that our data revealed a low level of genetic variation and sequence divergence; therefore, we are careful in the evaluation of the Quaternary history of the species.

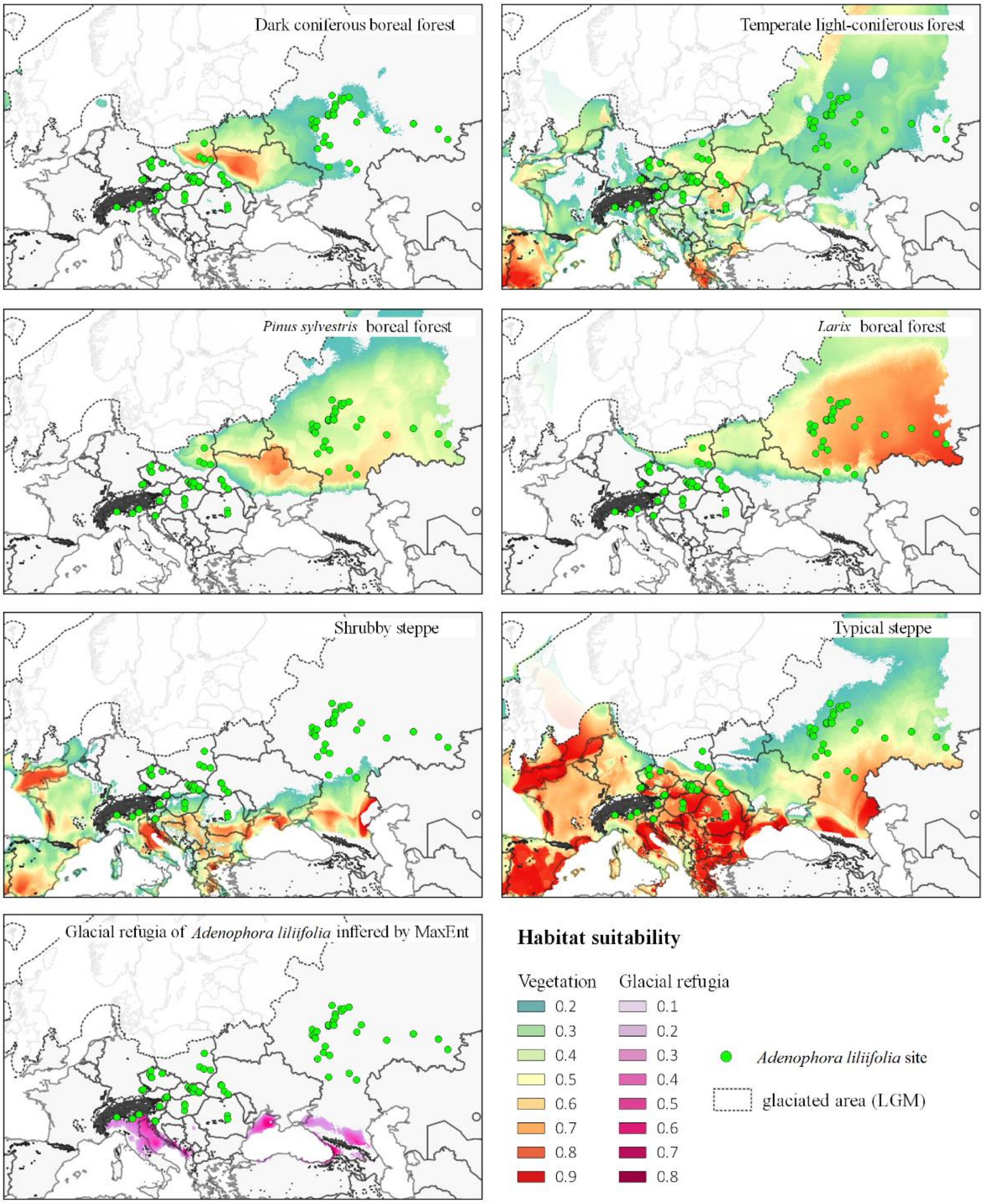


Fig. 1. Palaeodistribution modeling of European vegetation types at the Last Glacial Maximum (LGM) and the position of *A. liliifolia* populations (light green dots) investigated in Vaculná *et al.* (2021). Figures show the spatial distribution of particular vegetation types relevant for *A. liliifolia* (i.e., habitats where the species could persist). Primary vegetation data layers are from Janská *et al.* (2017) and were provided by the author. Maps arranged in QGIS by L. Vaculná.

The need for the whole genus taxonomic revision

In the European context, *Adenophora liliifolia* represents a well-defined taxon, significantly different from other Campanulaceae representatives. Nevertheless, the taxonomy of the genus within its center of diversification in East Asia is highly complicated and unresolved. There are around 62–65 accepted species (Lammers 2007b; Hong & Ge 2010). Flora of China reports 62 species, of which 23 are endemic for China (Hong *et al.* 2011). It also lists *A. liliifolia* as a native species, but this is doubtful information as we were not able to confirm the occurrence of the species at Altai mountains (Vaculná *et al.* 2021). To support our suspicion about the incorrect taxonomic determination of „Asiatic" *A. liliifolia*, we performed a phylogenetic analysis based on ITS1-5.5SrDNA-ITS2 locus using all available GenBank records (Fig. 2, Supplementary material 7). Although this locus is not well suited to infer the phylogeny of the genus due to low sequence divergence, it has enough power to infer clusters of taxa with highly similar sequences. The results show that only one GenBank record (DQ304581.1) can be considered *A. liliifolia* (sensu European taxonomic concept). This is mostly because this sample originated in Europe. The remaining two GenBank records (HQ704551.1 and KU983920.1; both Kim and Yoo, unpublished) were revealed to represent different genus taxa. Except the phylogenetic analysis supports our suspicions about *A. liliifolia*, it also clearly shows that many species are not monophyletic; thus, particular species names are misused. This highlights the need for the whole-genus taxonomic revision.

The problematic taxonomy of the genus results from an uncritical description of a large number of distinct taxa and the high morphological plasticity of particular taxa. A large number of specific and infraspecific taxa were described based on a single morphological character. Many species and varietal names have already been reduced to synonymy. The complexity of the taxonomy and the high morphological variation of this genus make species identification difficult, especially for widely distributed species, such as *A. stricta* Miquel, *A. polydentata* Nakai, and *A. capillaris* Hemsley (Tian *et al.* 2016 and literature cited therein). Variability in morphological characters such as leaf shape is a prominent feature of *Adenophora* genus, and this wide range of variation in this genus has been discussed several times (Hong & Ge 2010 and literature cited therein). Broad morphological variation was also recorded during our field monitoring, when different shapes of the leaves within *A. liliifolia* were recognized, even in closely located individuals within a particular locality (Fig. 3). A possible solution to taxonomic uncertainties in the genus *Adenophora* would be the use of molecular barcoding. Our investigation showed that intraspecific sequence variation in commonly used nuclear and cpDNA markers is not high even across large geographic regions. Based on this experience, particular taxa should be easily defined using a few genomic loci, e.g., ITS from ncDNA, and cpDNA loci with elevated nucleotide diversity, e.g., *ndh*F-*trn*L(UAG), *ndh*B-*trn*I(CAU), *ycf*3-*rpo*B, *psb*B-*rpl*20 (Cheon *et al.* 2017). This could be a proper strategy, how to set up sustainable taxonomy of the genus *Adenophora* in the future.

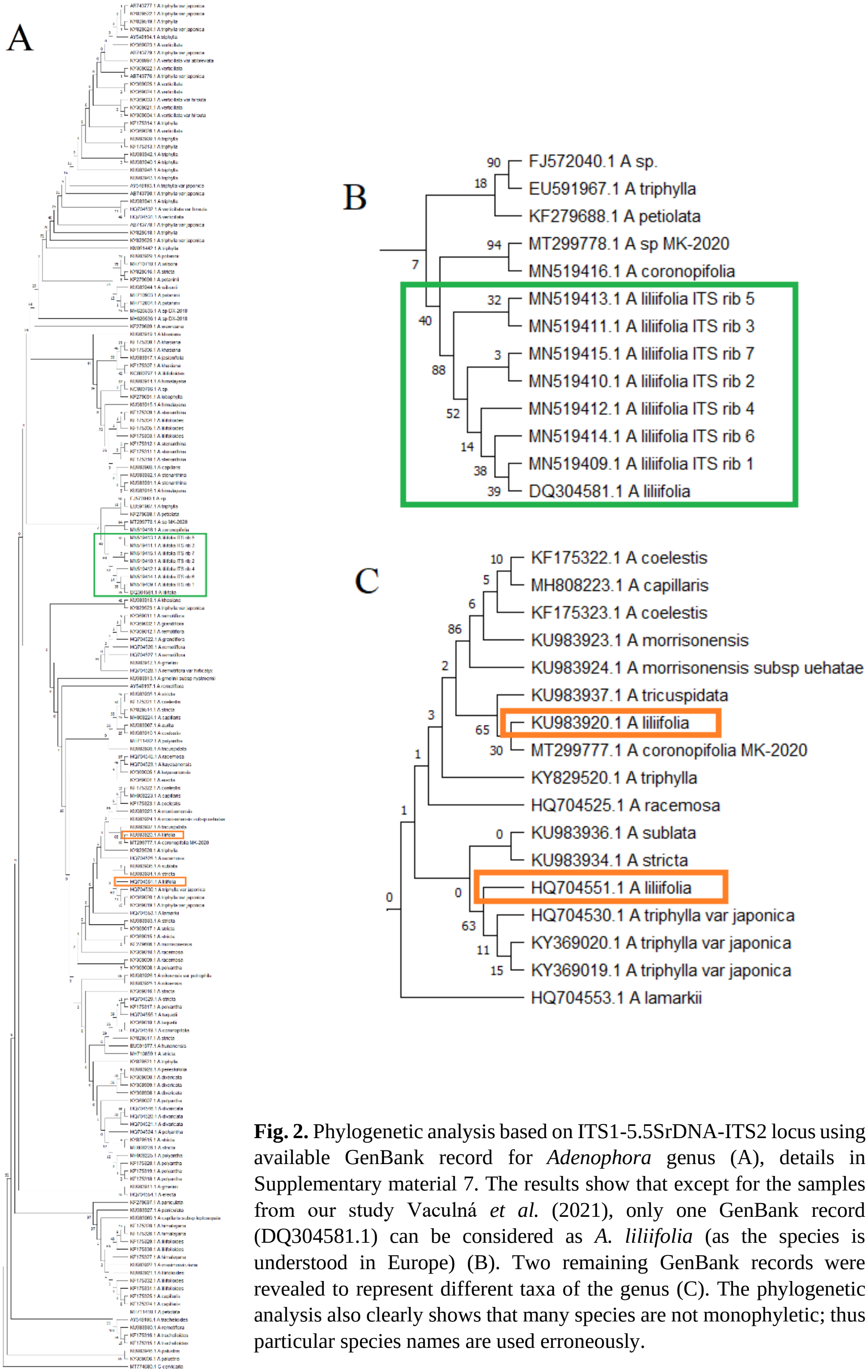


Fig. 2. Phylogenetic analysis based on ITS1-5.5SrDNA-ITS2 locus using available GenBank record for *Adenophora* genus (A), details in Supplementary material 7. The results show that except for the samples from our study Vaculná *et al.* (2021), only one GenBank record (DQ304581.1) can be considered as *A. liliifolia* (as the species is understood in Europe) (B). Two remaining GenBank records were revealed to represent different taxa of the genus (C). The phylogenetic analysis also clearly shows that many species are not monophyletic; thus particular species names are used erroneously.

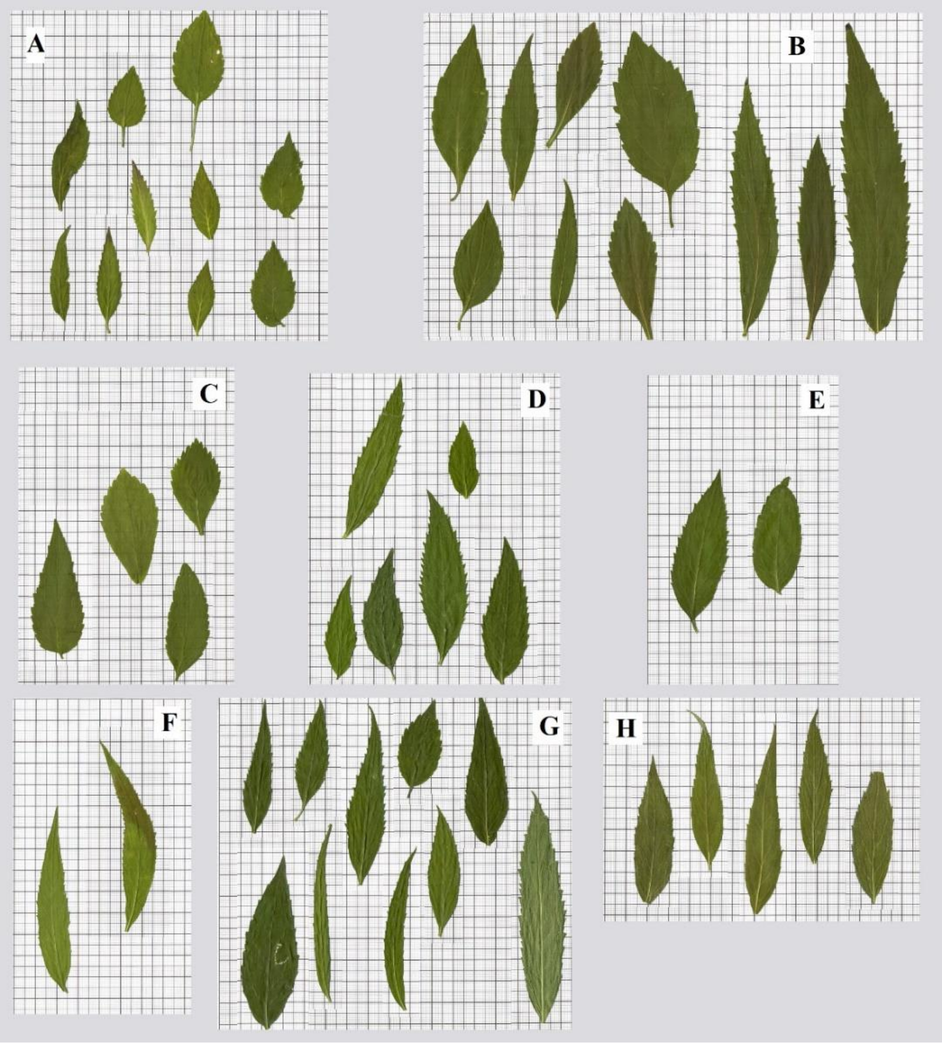
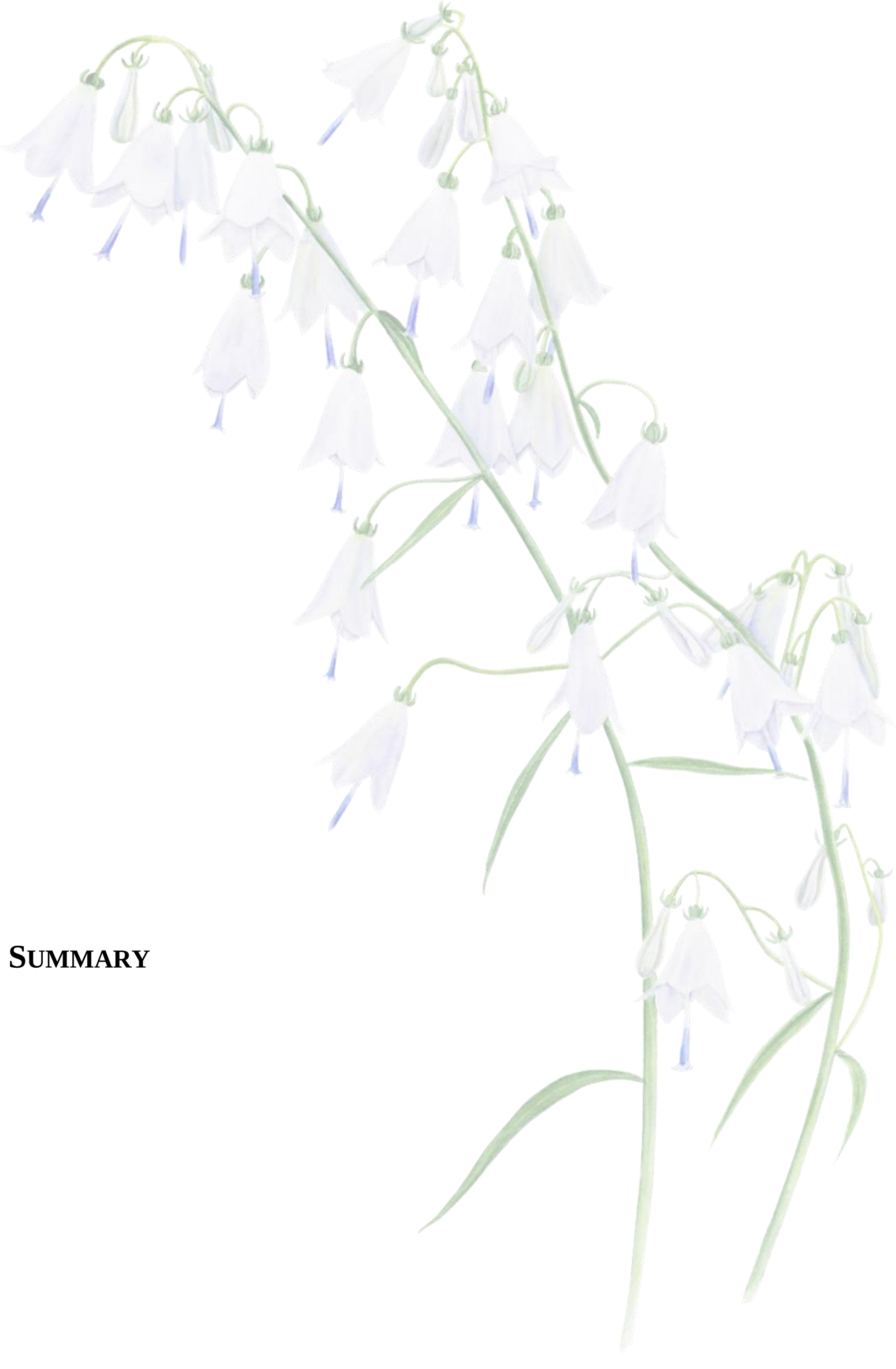


Fig. 3. Example of high morphological variability in leaf shape of *Adenophora liliifolia*. Leaves collected from the middle part of the stem. This wide variation is a prominent feature of this genus and has been discussed several times in literature. Babinské louky, CZ (A); Džbán, CZ (B); Karlické údolí, CZ (C); Dabrowa, PL (D); Kisielany, PL (E); Niedzieliski Las, PL (F); Krzemionki Opatowskie, PL (G); Zagryazhskoye, RU (H). Author of photographs: Lucie Vaculná.



SUMMARY

The submitted thesis brings an overview of information related to the critically endangered species *Adenophora liliifolia*, its habitat characteristics, population condition, and conservation management relevant to the species, particularly in the Czech Republic (Chapter **2**) and Poland (Chapter **3**). A summary of species ecological and phytosociological demands (Chapter **4**), a range of novel findings of genetic diversity and genetic structure of the species within Europe (Chapters **4**, **5**), and hints on species history and future (Chapter **5**) is presented. The comprehensive view and synthesis of partial fragments of *A. liliifolia* biology are essential for the appropriate conservation strategy and requested for National Action plans already implemented or prepared in the European region.

The current conditions of *A. liliifolia* populations in Europe regarding its habitat are, according to NATURA 2000 methodology, assessed as unfavourable, except for Slovenia and Slovakia. Our data proved in detail the situation in Slovakia, where the species grows mainly in places distant from human settlement, on rocky outcrops, and well-lit edges of beech or relict pine forests. Such associations represent climax communities on-site. Altogether, six vegetations units within Central Europe in which the species grow were identified (Chapter **4**). However, species preservation in these habitats is conditioned mostly by appropriate conservation measures, mitigating undesirable effects of the abandonment of traditional site management, ongoing on-site succession, eutrophication, expansion of oppressing nitrophilous species, change of the habitat light condition, or the overpopulated wildlife (Chapter **2**, **3**, **4**).

Our data detected rather low genetic diversity values in *A. liliifolia* populations. Such a situation may be related to the species' young evolutionary origin, alternatively, by the repeated populations' contractions and expansions accompanied by bottlenecks during Quaternary climatic fluctuations. All of this could reduce the variability of the genetic pool that (re)colonised Europe and further depleted during the Pleistocene. The values of genetic diversity determined by the two AFLP based papers show 75 %, respectively 78 % of the total variation represent variation inside the studied populations. The absence of a more robust genetic differentiation and overall similarity of the investigated populations, together with the longevity of plants, may indicate on gradual fragmentation of a larger genetic pool of the populations densely connected still in the relatively recent past within Central Europe. Elevated pairwise F_{ST} values were reported for specific pairs of geographically more distant populations, suggesting their ongoing isolation. Probably only due to the species biology, life cycle, and particularly the longevity, the conservation situation of the species regarding the genetic diversity is not critical yet (Chapter **4**, **5**).

We identified three spatially differentiated areas of elevated haplotype diversity in Central-East Europe: Western Carpathians (Slovakia), South-East Carpathians (Romania, Ukraine), Alps-Dinarides (Italy, Switzerland, Slovenia); and fourth on the Russian territory. Regarding the species history in Central Europe, the three above-mentioned areas might serve as glacial refugia. The rest of Europe is almost all (87 % of samples from Czechia, Germany, Austria, Hungary, Poland) inhabited by the widespread haplotype *hap_01*. This *hap_01* might represent the lineage of "general-purpose," evolutionarily selected as the most advantageous during the fast spread in the early Holocene. Alternatively, the *hap_01* might represent the lineage persisting on-site in the climatically favourable places during the glaciation. The lineage is absent east from the Southeastern Carpathians and probably originate from Central Europe (Chapter **5**).

Our data further support the assumption that *A. liliifolia* most likely originates from the area of Southern Siberia/Ural Mountains, from where it expanded westward to Europe. We based this conclusion on detecting probable ancestral lineage (marked as *hap_14*) currently present in some populations from Russia and Central Europe (e.g., Poland, Slovakia, Ukraine) and elevated haplotype diversity found in Russia. We also found only a low variability along the 3 600 km long transect when sequencing the nuclear ITS marker and several chloroplast regions (Chapter **5**).

The species distribution modeling helped us identify the glacial refugia located south of the Alps, where elevated haplotype diversity was observed. Based on the presumed climatic data for Holocene, it also shows significant habitat expansion, which probably occurred during this period (associated with the rapid spread of the species, notably its haplotype lineage *hap_01*, as described above). Predictions of the Maxent algorithm applied when evaluating present habitat conditions show much broader suitable habitat areas than the one actually realised. This might be caused by many biotic and abiotic factors affecting the species, which are hard to be converted into the data layers (e.g., traditional management abandonment or ongoing secondary succession changing the light conditions). However, the data layers used helped to elucidate some preferences (e.g., optimal temperature and precipitation range, percentage of soil base saturation) *Adenophora liliifolia* has. The SDM results further suggest that ongoing climate change will worsen the situation and influence the future distribution of *A. liliifolia* in Europe. Scenarios for years 2050 and 2070 show gradual habitat constrictions. Under the most stringent course (rcp85), prediction shows a noticeable shift of suitable habitats towards the northeast in Russia. The vastness of Russian territory seems to currently preserve much broader options for *A. liliifolia* survival than Central Europe. However, the species currently face habitat loss and is listed as endangered also in numerous Russian regions (Chapter **5**).

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SUPPLEMENTARY MATERIAL

Supplementary Material 1

SM 1a. AFLP primer combinations used for selective and pre-selective amplification.

Primer combinations – selective amplification:	
Set A	D2-EcoRI primer E-ACC / MseI primer M-ACT
Set B	D3-EcoRI primer E-ACT / MseI primer M-AAC
Set C	D4-EcoRI primer E-AGC / MseI primer M-AAT
Set D	D2-EcoRI primer E-ACC / MseI primer M-AAC
Set E	D3-EcoRI primer E-ACT / MseI primer M-AAT
Set F	D4-EcoRI primer E-AGC / MseI primer M-ACT
Pre- selective amplification primers:	
EcoRI primer: 5´-GACTGCGTACCAATTCA-3´	
MseI primer: 5´-GATGAGTCCTGAGTAAC-3´	

SM 1b. List of tested nuclear and chloroplast regions; F (forward primer), I (internal primer), R (reverse primer).

Region		**Primer sequence**	**Reference**
*trn*S^{GCU}-*trn*G^{UUC} intergenic spacer	F	AGATAGGGATTCGAACCCTCGGT	Shaw *et al.* 2005
	R	GTAGCGGGAATCGAACCCGCATC	
	I	TTTTACCACTAAACTATACCCGC	
trnS^{UGA}-*trnG*^{GCC} intergenic spacer	F	GAGAGAGAGGGATTCGAACC	Shaw *et al.* 2005
	R	CATAACCTTGAGGTCACGGG	
*psb*D-*trn*T^{GGU} intergenic spacer	F	CTCCGTARCCAGTCATCCATA	Shaw *et al.* 2007
	R	CCCTTTTAACTCAGTGGTAG	
*trn*C-*ycf*6	F	CCAGTTCRAATCYGGGTG	Shaw *et al.* 2005
	R	GCCCAAGCRAGACTTACTATATCCAT	
*ycf*6 – *psb*M	F	ATGGATATAGTAAGTCTYGCTTGGGC	Shaw *et al.* 2005
	R	ATGGAAGTAAATATTCTYGCATTTATTGCT	
*psb*A–*trn*H^{GUG} intergenic spacer	F	GTTATGCATGAACGTAATGCTC	Ragupathy *et al.* 2009
	R	CGCGCATGGTGGATTCACAAATC	
*pet*L – *psb*E intergenic spacer	F	AGTAGAAAACCGAAATAACTAGTTA	Shaw *et al.* 2007
	R	TATCGAATACTGGTAATAATATCAGC	
*ndh*J–*trn*F-*trn*L	F	ATGCCYGAAAGTTGGATAGG	Shaw *et al.* 2007
	R	GGTTCAAGTCCCTCTATCCC	Taberlet *et al.* 1991
rps16-*trn*K^{UUU}	F	AAAGTGGGTTTTTATGATCC	Shaw *et al.* 2007
	R	TTAAAAGCCGAGTACTCTACC	
rpl32-*trn*L^{UAG}	F	CAGTTCCAAAAAAACGTACTTC	Shaw *et al.* 2007
rpl32-*trn*L^{UAG}	R	CTGCTTCCTAAGAGCAGCGT	
*mat*K	F	TAATTTACGATCAATTCATTCA	Dunning & Savolainen (2010)
	R	CAAATAATATCCAAATACCAA	
*rbc*L	F	ATGTCACCACAAACAGAGACTAAAGC	Levin *et al.* 2003
	R	GTAAAATCAAGTCCACCRCG	Kress & Erickson 2009
ITS	F	TCCGTAGGTGAACCTGCGG	White *et al.* 1990
	R	TCCTCCGCTTATTGATATGC	

SM 1c. PCR reaction conditions for the sequenced regions.

***trn*S-*trn*G spacer**			***psb*D-*trn*T**		
30 x	95 °C	3 min	35x	95 °C	2 min
	95 °C	30 s		95 °C	1 min
	66.5 °C	2 min		60 °C	1 min
	72 °C	1 min		72 °C	2 min
	72 °C	10 min		72 °C	5 min
***trn*S-*trnf*M**			***ITS***		
33x	94 °C	3 min	36x	95 °C	5 min
	94 °C	45 s		95 °C	30 s
	64 °C	1 min		56 °C	45 s
	72 °C	2 min 30 s		72 °C	45 s
	72 °C	5 min		72 °C	10 min

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Supplementary Material 2

SM 2a. List of identified haplotypes/ribotypes in analysed *A. liliifolia* samples. Electronic appendix of the thesis.

SM 2b. Details on herbarium vouchers used in the study.

ID	Herbarium voucher	Species	Sampling year	Country	Geographical region	GPS lat (N)	GPS lon (E)
RU_MW-01	MW0531301	*A. liliifolia*	1979	Russia	Central region	56.46	41.53
RU_MW-02	MW0531304	*A. liliifolia*	2008	Russia	Central region	55.74	42.00
RU_MW-03	MW0531305	*A. liliifolia*	2008	Russia	Central region	55.73	42.01
RU_MW-05	MW0531307-1	*A. liliifolia*	1913	Russia	Central region	55.20	41.81
RU_MW-06	MW0531308	*A. liliifolia*	1913	Russia	Central region	55.20	41.81
RU_MW-07	MW0531310	*A. liliifolia*	1996	Russia	Central region	53.75	38.88
RU_MW-08	MW0531311	*A. liliifolia*	1996	Russia	Central region	53.75	38.88
RU_MW-09	MW0531316	*A. liliifolia*	1990	Russia	Central region	54.73	41.01
RU_MW-10	MW0531318	*A. liliifolia*	1996	Russia	Central region	53.75	38.88
RU_MW-11	MW0531319	*A. liliifolia*	1992	Russia	Central region	53.75	38.88
RU_MW-12	MW0531321	*A. liliifolia*	1968	Russia	Central region	54.80	40.97
RU_MW-13	MW0531327	*A. liliifolia*	1928	Russia	Central region	54.08	38.98
RU_MW-15	MW0531333	*A. liliifolia*	1990	Russia	Central region	53.89	38.52
RU_MW-17	MW0531336	*A. liliifolia*	1929	Russia	Central region	54.23	39.24
RU_MW-18	MW0531339	*A. liliifolia*	1970	Russia	Central region	54.82	41.65
RU_MW-19	MW0531340	*A. liliifolia*	1973	Russia	Central region	54.80	41.01
RU_MW-20	MW0531341	*A. liliifolia*	1967	Russia	Central region	54.80	40.97
RU_MW-21	MW0531342	*A. liliifolia*	1956	Russia	Central region	53.61	39.02
RU_MW-22	MW0531343	*A. liliifolia*	2010	Russia	Central region	56.18	42.86
RU_MW-24	MW0531345	*A. liliifolia*	1970	Russia	Central region	54.82	41.65
RU_MW-25	MW0531346	*A. liliifolia*	1977	Russia	Central region	54.71	40.98
RU_MW-26	MW0531347	*A. liliifolia*	1977	Russia	Central region	54.78	40.99
RU_MW-27	MW0531348	*A. liliifolia*	1973	Russia	Central region	54.77	41.82
RU_MW-28	MW0531350	*A. liliifolia*	1961	Russia	Moscow region	54.40	38.53
RU_MW-29	MW0531365	*A. liliifolia*	1929	Russia	Central forest region	57.31	43.17
RU_MW-31	MW0531368	*A. liliifolia*	1874	Russia	Central forest-and-steppe region	51.73	39.35
RU_MW-32	MW0531376	*A. liliifolia*	1970	Russia	Central forest-and-steppe region	52.06	41.17
RU_MW-37	MW0531421	*A. liliifolia*	2003	Russia	Central forest-and-steppe region	52.89	40.50

ID	Herbarium voucher	Species	Sampling year	Country	Geographical region	GPS lat (N)	GPS lon (E)
RU_MW-38	MW0531424	*A. liliifolia*	1986	Russia	Central forest-and-steppe region	49.99	41.02
RU_MW-39	MW0531429	*A. liliifolia*	1934	Russia	Central forest-and-steppe region	52.32	40.23
RU_MW-40	MW0531434	*A. liliifolia*	1927	Russia	Volga-Kama region	56.32	44.10
RU_MW-41	MW0531436	*A. liliifolia*	1971	Russia	Volga-Kama region	56.00	43.02
RU_MW-42	MW0531439	*A. liliifolia*	1926	Russia	Volga-Kama region	56.20	43.36
RU_MW-43	MW0531451	*A. liliifolia*	1963	Russia	Middle Volga region	53.45	49.80
RU_MW-44	MW0531458	*A. liliifolia*	1990	Russia	Middle Volga region	54.17	45.28
RU_MW-45	MW0531460	*A. liliifolia*	1982	Russia	Middle Volga region	54.81	45.96
RU_MW-46	MW0531479	*A. liliifolia*	1949	Russia	Lower Volga region	49.72	45.23
RU_MW-47	MW0531482	*A. liliifolia*	1935	Russia	Lower Volga region	50.36	44.12
RU_MW-49	MW0531508	*A. liliifolia*	1958	Russia	Eastern region	53.54	57.63
RU_MW-50	MW0531509	*A. liliifolia*	2007	Russia	Eastern region	52.60	59.05
RU_MW-51	MW0531527	*A. liliifolia*	1958	Russia	Eastern region	53.54	57.63
RU_MW-52	MW0202805	*A. liliifolia*	2012	Russia	Eastern region	54.02	53.84
RU_BRNU-17	BRNU 653148	*A. liliifolia*	2013	Russia	Omsk region	54.55	71.80
RU_BRNU-01	BRNU 576154	*A. coronopifolia*	2004	Russia	Khakasiya	53.04	91.14
KZ_MW-76	MW0880873	*Adenophora* sp.	1979	Kazakhstan	Dzungarian Alatau & Tarbagatai	47.30	81.49

Supplementary Material 3

SM3. Haplotype alignments of three cpDNA loci and ITS region in ‘fasta’ format. Electronic appendix of the thesis.

Supplementary Material 4

SM 4a. Geographic coordinates of 78 *A. liliifolia* records used in Maxent modeling.

Species	Longitude	Latitude
Adenophora liliifolia	16.46	48.02
Adenophora liliifolia	16.52	48.03
Adenophora liliifolia	16.24	47.91
Adenophora liliifolia	15.43	47.07
Adenophora liliifolia	14.13	50.60
Adenophora liliifolia	13.90	50.26
Adenophora liliifolia	14.26	49.95
Adenophora liliifolia	14.17	49.96
Adenophora liliifolia	15.82	50.34
Adenophora liliifolia	12.74	48.70
Adenophora liliifolia	12.97	48.78
Adenophora liliifolia	20.56	48.52
Adenophora liliifolia	21.46	48.54
Adenophora liliifolia	21.40	48.43
Adenophora liliifolia	19.20	46.63
Adenophora liliifolia	19.27	47.17
Adenophora liliifolia	19.21	47.29
Adenophora liliifolia	8.95	45.93
Adenophora liliifolia	12.33	46.22
Adenophora liliifolia	11.35	45.77
Adenophora liliifolia	22.21	52.25
Adenophora liliifolia	22.15	50.77
Adenophora liliifolia	23.13	50.68
Adenophora liliifolia	21.50	50.98
Adenophora liliifolia	25.71	46.12
Adenophora liliifolia	25.74	45.73
Adenophora liliifolia	42.04	55.48
Adenophora liliifolia	71.80	54.55
Adenophora liliifolia	53.84	54.02
Adenophora liliifolia	41.53	56.46
Adenophora liliifolia	42.00	55.74
Adenophora liliifolia	42.01	55.73
Adenophora liliifolia	41.75	55.15
Adenophora liliifolia	41.80	55.20
Adenophora liliifolia	38.88	53.75
Adenophora liliifolia	40.99	54.71
Adenophora liliifolia	41.01	54.73
Adenophora liliifolia	40.97	54.80
Adenophora liliifolia	38.98	54.08
Adenophora liliifolia	38.52	53.89
Adenophora liliifolia	39.24	54.23
Adenophora liliifolia	41.65	54.82
Adenophora liliifolia	41.01	54.80
Adenophora liliifolia	39.02	53.61
Adenophora liliifolia	42.86	56.18
Adenophora liliifolia	40.98	54.71
Adenophora liliifolia	40.99	54.78
Adenophora liliifolia	41.82	54.77
Adenophora liliifolia	38.53	54.40
Adenophora liliifolia	39.35	51.73
Adenophora liliifolia	41.17	52.06
Adenophora liliifolia	40.50	52.89
Adenophora liliifolia	41.02	49.99
Adenophora liliifolia	40.23	52.32
Adenophora liliifolia	44.10	56.32
Adenophora liliifolia	43.02	56.00
Adenophora liliifolia	43.36	56.20
Adenophora liliifolia	49.80	53.45
Adenophora liliifolia	45.28	54.17
Adenophora liliifolia	45.96	54.81
Adenophora liliifolia	45.23	49.72
Adenophora liliifolia	44.12	50.36
Adenophora liliifolia	57.63	53.54
Adenophora liliifolia	59.05	52.60
Adenophora liliifolia	20.55	48.57
Adenophora liliifolia	20.23	48.89
Adenophora liliifolia	19.75	49.01
Adenophora liliifolia	20.06	48.76
Adenophora liliifolia	20.13	48.81
Adenophora liliifolia	20.38	48.96
Adenophora liliifolia	20.25	48.88
Adenophora liliifolia	20.70	48.91
Adenophora liliifolia	14.72	45.57
Adenophora liliifolia	14.81	45.50
Adenophora liliifolia	25.22	48.60
Adenophora liliifolia	25.80	48.29
Adenophora liliifolia	24.84	49.17
Adenophora liliifolia	24.70	49.23

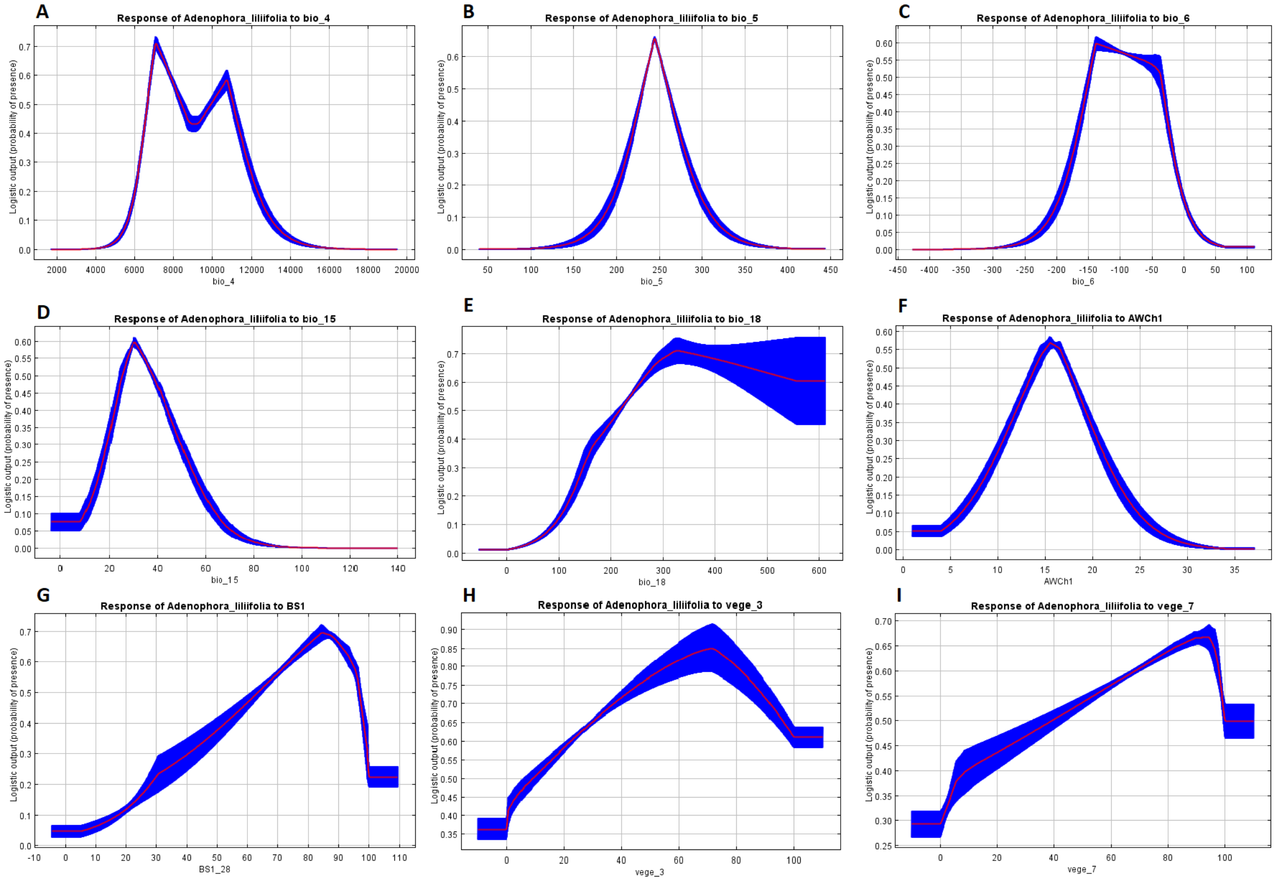
SM 4b. Summary of selected WorldClim Global Circulation Models (GCM) and data resolution used for Maxent modeling.

GCM	code	LGM (2.5 min)	Mid-Holocene (30s)	2050 (30s)	2070 (30s)
CCSM4	CC	*	*	-	-
CNRM-CM5	CN	-	*	*	*
GFDL-CM3	GF	-	-	*	*
HadGEM2-CC	HG	-	*	-	-
HadGEM2-ES	HE	-	*	*	*
MIROC5	MC	-	-	*	*
MIROC-ESM	MR	*	-	-	-
MPI-ESM-LR	MP	-	-	*	*
MPI-ESM-P	ME	*	*	-	-

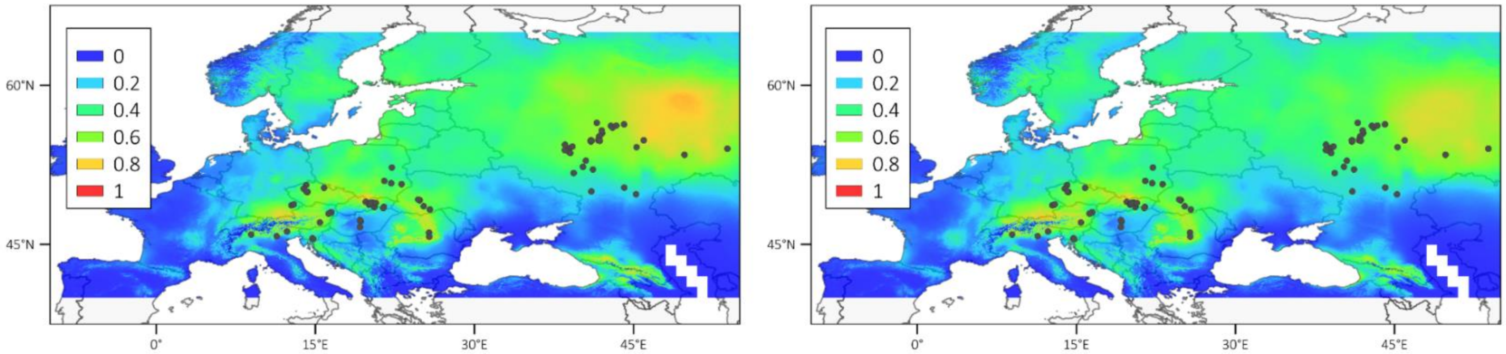
SM 4c. Bioclimatic and environmental variables used for Maxent modeling.

Code	Database	Environmental variable	Unit	% contribution (Present)	Present (30s)	LGM	Mid-Holocene	2050	2070
Bio4	WorldClim	Temperature Seasonality (st. dev. × 100)	Coeff. of Var	14.7	*	*	*	*	*
Bio5	WorldClim	Max Temperature of Warmest Month	°C	6.5	*	*	*	*	*
Bio6	WorldClim	Min Temperature of Coldest Month	°C	35.2	*	*	*	*	*
Bio15	WorldClim	Precipitation Seasonality	Coeff. of Var	4.4	*	*	*	*	*
Bio18	WorldClim	Precipitation of Warmest Quarter	mm	21.7	*	*	*	*	*
AWCh1	ISRIC	Available Soil Water Capacity	%	0.5	*	-	-	-	-
BS1_28	GlobalChange	Base Saturation	%	10.6	*	-	-	-	-
vege_3	EarthEnv	Deciduous Trees cover	%	3.3	*	-	-	-	-
vege_7	EarthEnv	Cultived & Managed vegetation cover	%	3.2	*	-	-	-	-

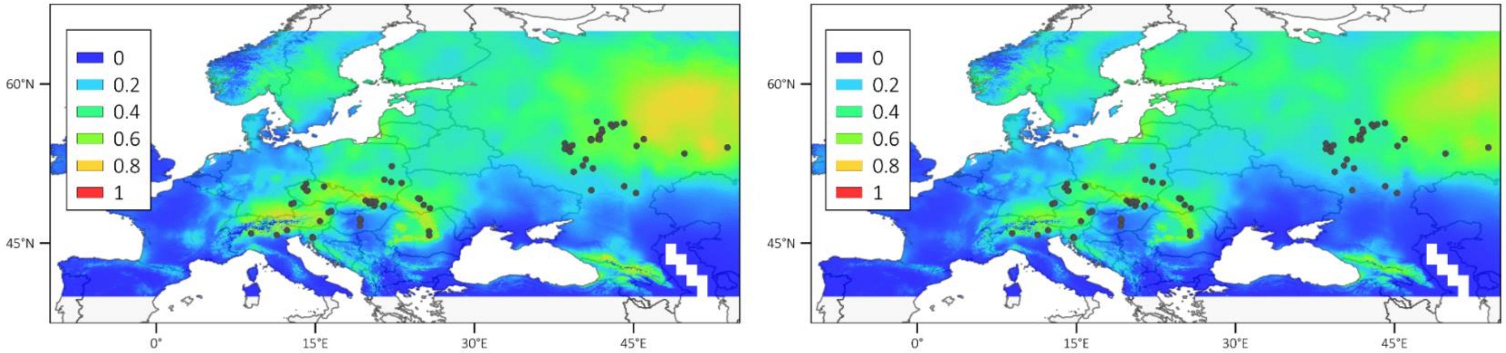
SM 4d. Response of *A. liliifolia* to variables used in Maxent modeling. A) Temperature Seasonality (Coeff. of Var; bio_4), B) Max Temperature of Warmest Month (°C x 10; bio_5), C) Min Temperature of Coldest Month (°C x 10; bio_6), D) Precipitation Seasonality (Coeff. of Var, bio_15), E) Precipitation of Warmest Quarter (mm; bio_18), F) Available Soil Water Capacity (%; Awch1), G) Base Saturation (%; BS1), H) Deciduous Trees cover (%; vege_3), I) Cultived & Managed vegetation cover (%; vege_7).



SM 4e. Maxent prediction of future habitat suitability for *A. liliifolia* populations based on ensemble models: A) rcp45 - 2050; B) rcp45 - 2070; C) rcp85 - 2050; D) rcp85 - 2070. Suitability is expressed between 0 and 1; higher values (represented by warmer colours) indicate an increased habitat suitability. Dots on the map represent verified populations sampled within the present study.


A) ensemble model for rcp45 2050

B) ensemble model for rcp45 2070


C) ensemble model for rcp85 2050

D) ensemble model for rcp85 2070

Supplementary Material 5

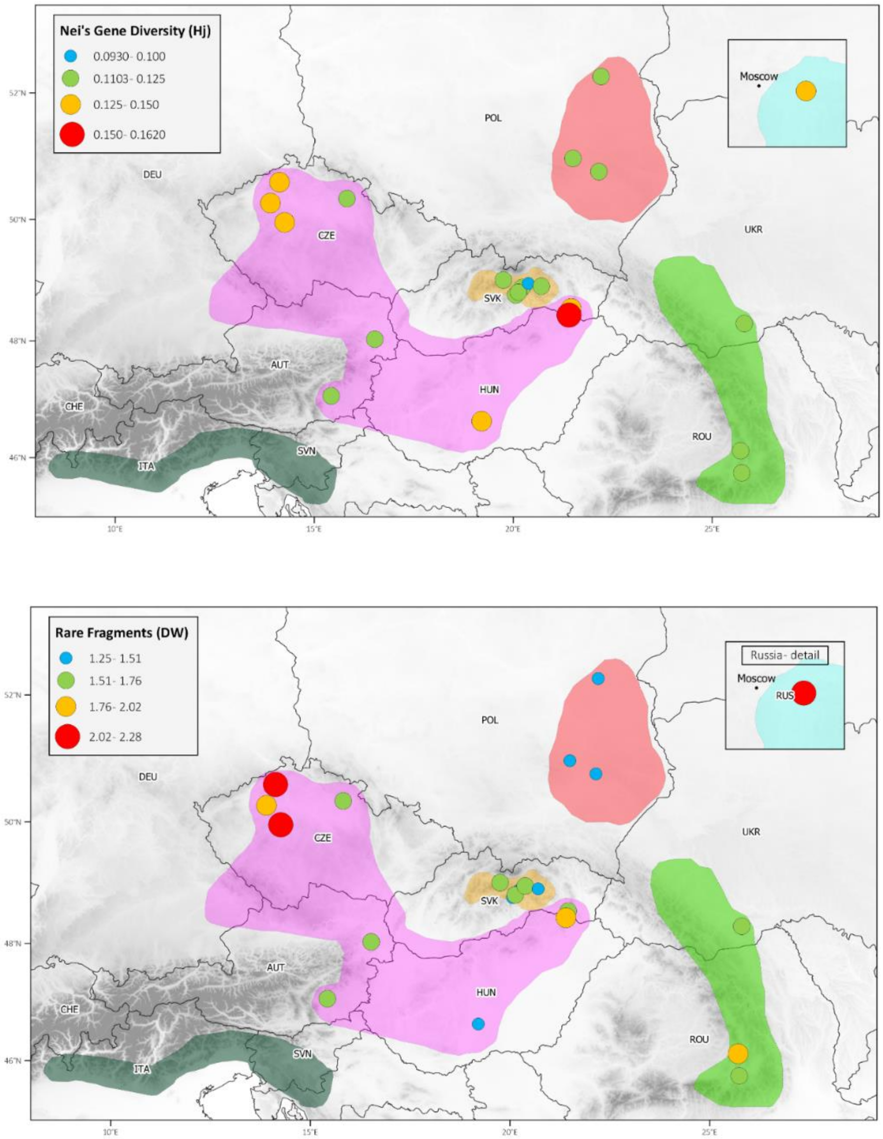
SM 5a. Statistic based on AFLP and concatenated three cpDNA loci. nAFLP (number of samples used for AFLP analysis), PLP (percentage of polymorphic loci), nFB (fixed band number), nPB (private band number), nFPB (fixed private band number), Hj (Nei's Gene Diversity), I (Shannon's index), N_E (number of effective alleles), DW (frequency-down-weighted marker value [rare fragments]), nSeq (number of samples sequenced for three cpDNA loci), $N°_H$ (number of different haplotypes), H_D (haplotype diversity), Pi (nucleotide diversity), Tajima's $D^{[P]}$ (Tajima's D test, significance of the test is shown as the superscript, ns = not significant), cpDNA (haplotype observed for the concatenated three intergenic spacers; + frequency of observed haplotype), SE (standard error), † (indicates populations with the presence of suggested ancestral haplotype.

Region	nAFLP	PLP	nFB	nPB/ nFPB	Hj (SE)	I (SE)	Ne (SE)	DW	Seq	N_H	H_D	Pi	Tajima's D[P]	cpDNA (frequency)
Central Europe	**88**	**48.6**	**3**	**8/ 0**	**0.159 (0.008)**	**0.263 (0.012)**	**1.241 (0.015)**	**1.71**	**119**	**4**	**0.23**	**0.0002**	**-1.081^{ns}**	**hap_01 (0.87), hap_02 (0.02), hap_04 (0.08), hap_05 (0.03)**
AT_A	9	38.3	38	0/ 0	0.120 (0.009)	0.167 (0.012)	1.173 (0.016)	1.51	17	n.a.	n.a.	n.a.	n.a.	hap_01 (0.88) + hap_02 (0.12)
AT_B	2	n.a.	n.a.	n.a./ n.a.	n.a.	n.a.	n.a.	n.a.	2	n.a.	n.a.	n.a.	n.a.	hap_01
AT_C	8	39.1	44	0/ 0	0.122 (0.009)	0.168 (0.012)	1.166 (0.015)	1.65	11	n.a.	n.a.	n.a.	n.a.	hap_01
CZ_A	7	46.3	24	0/ 0	0.146 (0.010)	0.204 (0.012)	1.204 (0.016)	2.07	11	n.a.	n.a.	n.a.	n.a.	hap_01
CZ_B	12	36.0	27	0/ 0	0.141 (0.009)	0.206 (0.012)	1.208 (0.016)	1.88	12	n.a.	n.a.	n.a.	n.a.	hap_01 (0.25), hap_04 (0.75)
CZ_C	5	38.6	35	0/ 0	0.145 (0.010)	0.186 (0.012)	1.192 (0.016)	2.26	7	n.a.	n.a.	n.a.	n.a.	hap_01
CZ_D	1	n.a.	n.a.	n.a./ n.a.	n.a.	n.a.	n.a.	n.a.	4	n.a.	n.a.	n.a.	n.a.	hap_01
CZ_E	12	31.1	40	0/ 0	0.122 (0.009)	0.176 (0.012)	1.176 (0.015)	1.54	12	n.a.	n.a.	n.a.	n.a.	hap_01
CZ_F	0	n.a.	n.a.	n.a./ n.a.	n.a.	n.a.	n.a.	n.a.	2	n.a.	n.a.	n.a.	n.a.	hap_01
DE_A	3	n.a.	n.a.	n.a./ n.a.	n.a.	n.a.	n.a.	n.a.	3	n.a.	n.a.	n.a.	n.a.	hap_01
DE_B	2	n.a.	n.a.	n.a./ n.a.	n.a.	n.a.	n.a.	n.a.	2	n.a.	n.a.	n.a.	n.a.	hap_01
HU_A	1	n.a.	n.a.	n.a./ n.a.	n.a.	n.a.	n.a.	n.a.	1	n.a.	n.a.	n.a.	n.a.	hap_01
HU_B	8	38.3	31	0/ 0	0.131 (0.010)	0.179 (0.012)	1.191 (0.017)	1.60	10	n.a.	n.a.	n.a.	n.a.	hap_01
HU_C	8	48.0	21	0/ 0	0.162 (0.011)	0.224 (0.012)	1.244 (0.018)	1.79	10	n.a.	n.a.	n.a.	n.a.	hap_01

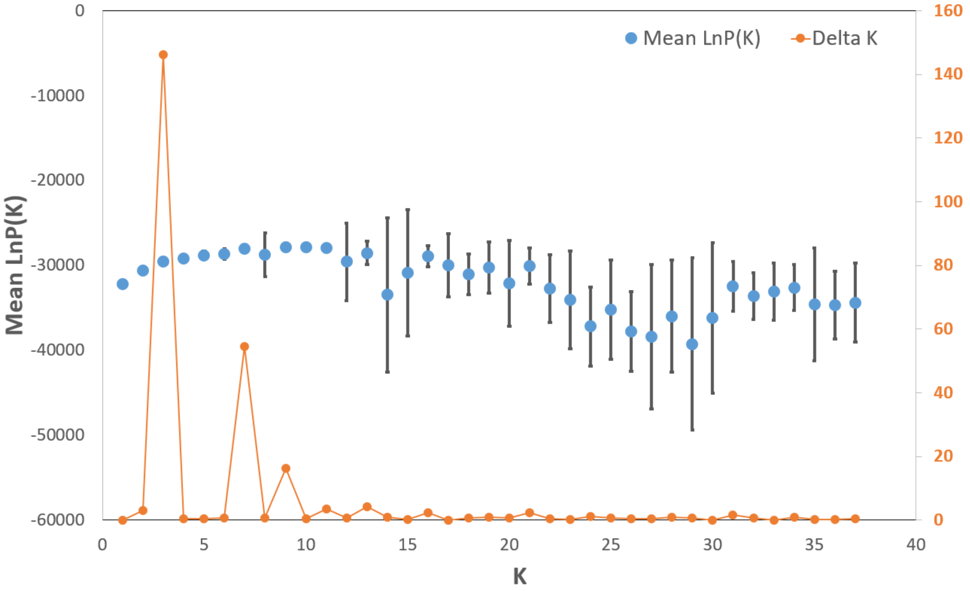
Region	nAFLP	PLP	nFB	nPB/ nFPB	Hj (SE)	I (SE)	Ne (SE)	DW	Seq	N_H	H_D	Pi	Tajima's D[P]	cpDNA (frequency)
HU_D	8	35.4	25	0/ 0	0.131 (0.010)	0.176 (0.012)	1.198 (0.018)	1.25	10	n.a.	n.a.	n.a.	n.a.	hap_01
HU_E	0	n.a.	n.a.	n.a./ n.a.	n.a.	n.a.	n.a.	n.a.	2	n.a.	n.a.	n.a.	n.a.	hap_01 (0.50), hap_05 (0.50)
HU_F	3	n.a.	n.a.	n.a./ n.a.	n.a.	n.a.	n.a.	n.a.	3	n.a.	n.a.	n.a.	n.a.	hap_01
Alps-Dinarides	**12**	**35.7**	**23**	**0 / 0**	**0.134 (0.009)**	**0.200 (0.013)**	**1.194 (0.015)**	**2.02**	**15**	**5**	**0.75**	**0.0006**	**0.304^{ns}**	**hap_01 (0.13), hap_03 (0.07), hap_06 (0.2), hap_07 (0.13), hap_18 (0.47)**
IT_A	4	n.a.	n.a.	n.a./ n.a.	n.a.	n.a.	n.a.	n.a.	4	n.a.	n.a.	n.a.	n.a.	hap_06 (0.50), hap_07 (0.50)
IT_B	3	n.a.	n.a.	n.a./ n.a.	n.a.	n.a.	n.a.	n.a.	3	n.a.	n.a.	n.a.	n.a.	hap_01 (0.67), hap_06 (0.33)
SL_B	3	n.a.	n.a.	n.a./ n.a.	n.a.	n.a.	n.a.	n.a.	6	n.a.	n.a.	n.a.	n.a.	hap_18
CH_A	1	n.a.	n.a.	n.a./ n.a.	n.a.	n.a.	n.a.	n.a.	1	n.a.	n.a.	n.a.	n.a.	hap_03
Poland	**41**	**40.6**	**10**	**0 / 0**	**0.122 (0.008)**	**0.199 (0.012)**	**1.181 (0.014)**	**1.38**	**57**	**3**	**0.20**	**0.0001**	**-0.925^{ns}**	**hap_01 (0.89), hap_14 (0.07), hap_19 (0.04)**
PL_A	22	32.9	12	0/ 0	0.123 (0.009)	0.195 (0.012)	1.182 (0.015)	1.40	26	n.a.	n.a.	n.a.	n.a.	hap_01
PL_B †	9	40.9	22	0/ 0	0.122 (0.009)	0.174 (0.012)	1.176 (0.015)	1.47	17	n.a.	n.a.	n.a.	n.a.	hap_01 (0.70), hap_14 (0.18), hap_19 (0.12)
PL_C	3	n.a.	n.a.	n.a./ n.a.	n.a.	n.a.	n.a.	n.a.	4	n.a.	n.a.	n.a.	n.a.	hap_01
PL_D †	7	36.6	23	0/ 0	0.117 (0.009)	0.162 (0.012)	1.164 (0.015)	1.33	10	n.a.	n.a.	n.a.	n.a.	hap_01 (0.90), hap_14 (0.10)
W Carpathians	**45**	**37.4**	**22**	**0 / 0**	**0.120 (0.008)**	**0.196 (0.012)**	**1.177 (0.014)**	**1.54**	**61**	**9**	**0.75**	**0.0005**	**-0.347^{ns}**	**hap_01 (0.34), hap_06 (0.03), hap_14 (0.36), hap_18 (0.08), hap_19 (0.02), hap_23 (0.07), hap_24 (0.07), hap_25 (0.02), hap_26 (0.02)**
SK_A	3	n.a.	n.a.	n.a./ n.a.	n.a.	n.a.	n.a.	n.a.	8	n.a.	n.a.	n.a.	n.a.	hap_01
SK_B †	5	21.7	47	0/ 0	0.105 (0.010)	0.119 (0.012)	1.140 (0.016)	1.46	7	n.a.	n.a.	n.a.	n.a.	hap_01 (0.29), hap_14 (0.14), hap_23 (0.57)
SK_C †	7	30.0	51	0/ 0	0.102 (0.009)	0.132 (0.012)	1.135 (0.014)	1.51	7	n.a.	n.a.	n.a.	n.a.	hap_01 (0.43), hap_14 (0.57)
SK_D †	6	30.3	41	0/ 0	0.111 (0.009)	0.142 (0.012)	1.150 (0.015)	1.46	10	n.a.	n.a.	n.a.	n.a.	hap_14 (0.90), hap_19 (0.10)
SK_E	8	35.1	35	0/ 0	0.112 (0.009)	0.152 (0.012)	1.157 (0.015)	1.51	10	n.a.	n.a.	n.a.	n.a.	hap_18 (0.50), hap_24 (0.40), hap_25 (0.10)

Region	nAFLP	PLP	nFB	nPB/ nFPB	Hj (SE)	I (SE)	Ne (SE)	DW	Seq	N_H	H_D	Pi	Tajima's D[P]	cpDNA (frequency)
SK_F †	6	28.0	46	0/ 0	0.093 (0.008)	0.120 (0.012)	1.113 (0.012)	1.69	5	n.a.	n.a.	n.a.	n.a.	hap_01 (0.20), hap_06 (0.40), hap_14 (0.20), hap_26 (0.20)
SK_G †	4	n.a.	n.a.	n.a./ n.a.	n.a.	n.a.	n.a.	n.a.	7	n.a.	n.a.	n.a.	n.a.	hap_01 (0.14), hap_14 (0.86)
SK_H †	6	28.3	43	0/ 0	0.107 (0.009)	0.135 (0.012)	1.143 (0.015)	1.36	7	n.a.	n.a.	n.a.	n.a.	hap_01 (0.86), hap_14 (0.14)
SE Carpathians	**21**	**34.3**	**21**	**0 / 0**	**0.129 (0.008)**	**0.205 (0.012)**	**1.189 (0.015)**	**1.73**	**36**	**6**	**0.58**	**0.0004**	**-0.721** ^{ns}	**hap_14 (0.03), hap_16 (0.06), hap_17 (0.65), hap_20 (0.06), hap_21 (0.1), hap_22 (0.1)**
RO_A	5	27.7	45	0/ 0	0.111 (0.009)	0.134 (0.012)	1.142 (0.015)	1.98	6	n.a.	n.a.	n.a.	n.a.	hap_17 (0.67), hap_20 (0.33)
RO_B	8	37.1	35	0/ 0	0.112 (0.009)	0.157 (0.012)	1.154 (0.014)	1.64	8	n.a.	n.a.	n.a.	n.a.	hap_17 (0.75), hap_21 (0.12), hap_22 (0.12)
UA_A	1	n.a.	n.a.	n.a./ n.a.	n.a.	n.a.	n.a.	n.a.	1	n.a.	n.a.	n.a.	n.a.	hap_17
UA_B	7	37.4	36	0/ 0	0.118 (0.009)	0.161 (0.012)	1.162 (0.015)	1.73	19	n.a.	n.a.	n.a.	n.a.	hap_16 (0.10), hap_17 (0.58), hap_21 (0.16), hap_22 (0.16)
UA_C †	0	n.a.	n.a.	n.a./ n.a.	n.a.	n.a.	n.a.	n.a.	1	n.a.	n.a.	n.a.	n.a.	hap_14
UA_D	0	n.a.	n.a.	n.a./ n.a.	n.a.	n.a.	n.a.	n.a.	1	n.a.	n.a.	n.a.	n.a.	hap_17
Russia	**5**	**32.3**	**37**	**1 / 0**	**0.126 (0.010)**	**0.158 (0.012)**	**1.169 (0.016)**	**2.28**	**48**	**11**	**0.65**	**0.0004**	**-0.862** ^{ns}	**hap_08 (0.58), hap_09 (0.06), hap_10 (0.02), hap_11 (0.02), hap_12 (0.02), hap_13 (0.02), hap_14 (0.10), hap_15 (0.04), hap_16 (0.04), hap_17 (0.04), hap_18 (0.04)**
RU_A	5	32.3	37	1 / 0	0.126 (0.010)	0.158 (0.012)	1.169 (0.016)	2.28	5	n.a.	n.a.	n.a.	n.a.	hap_08 (0.60), hap_09 (0.40)
herbarium BRNU + MW	0	n.a.	n.a.	n.a./ n.a.	n.a.	n.a.	n.a.	n.a.	43	n.a.	n.a.	n.a.	n.a.	hap_08 (0.58), hap_09 (0.02), hap_10 (0.02), hap_11 (0.02), hap_12 (0.02), hap_13 (0.02), hap_14 (0.12), hap_15 (0.05), hap_16 (0.05), hap_17 (0.05), hap_18 (0.05)

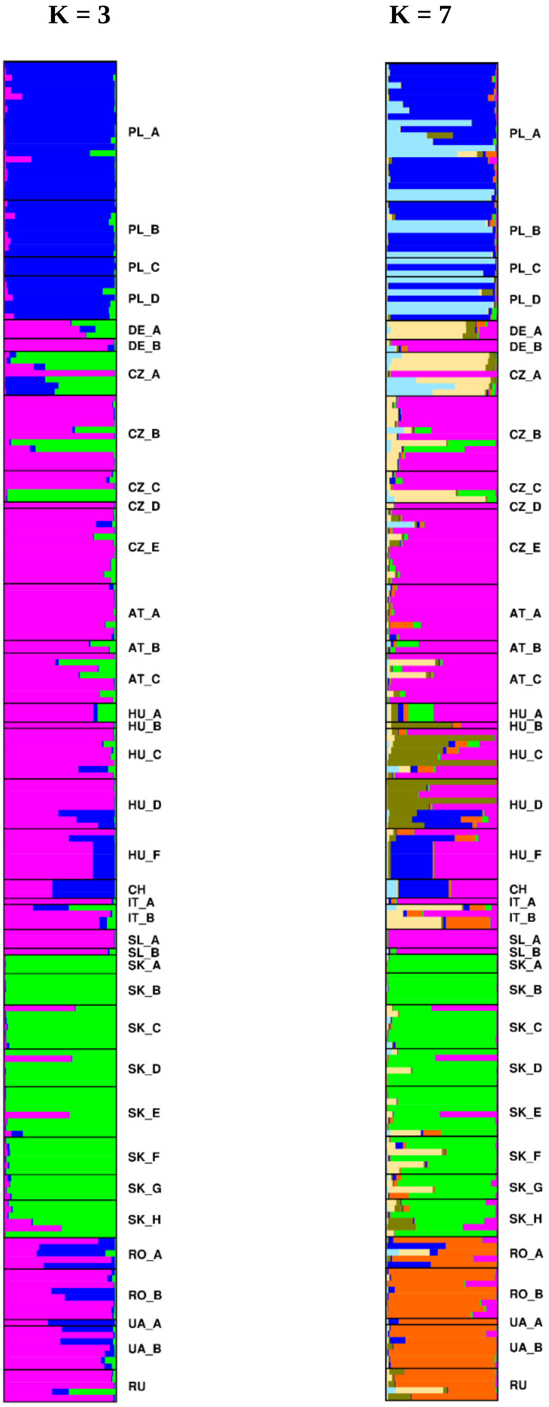
SM 5b. Visualization of A) the spatial distribution of Nei’s Gene Diversity (Hj), B) the frequency-down-weighted (DW) marker across the sampled populations (n >5). Coloured polygons show defined regions: Central Europe (magenta), Poland (red), Western Carpathians (orange), Alps - Dinarides (dark turquoise), Southeastern Carpathians (green), Russia (light blue).



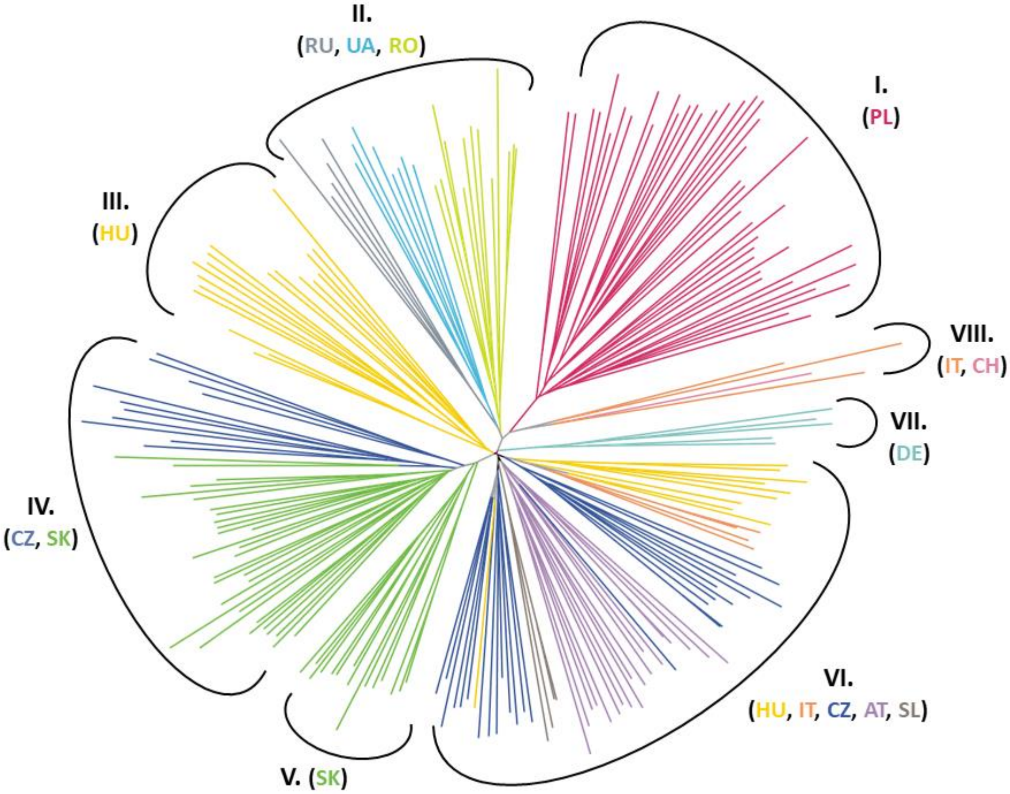
SM 5c. Result of Bayesian clustering performed in STRUCTURE and processed by STRUCTURE HARVESTER. The plot of mean likelihood L(K) and variance per each *K* value and plot of deltaK for detecting the number of *K* best fitting analysed data.



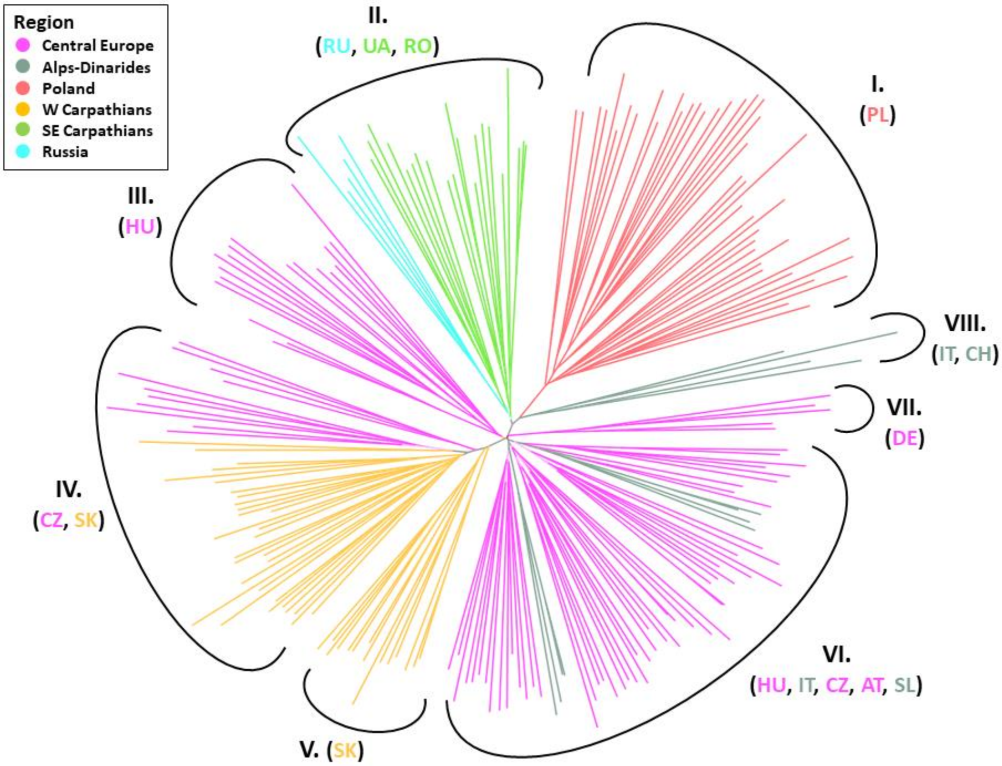
SM 5d. Graphical output of STRUCTURE clustering analysis for K = 3, and K = 7. Individuals are represented by a single vertical line broken into colored segments representing different genetic groups, where the length of each shade (x-axis) is proportional to the assignment to particular cluster.



SM 5e. Unrooted Neighbor-joining dendrogram based on 350 AFLP markers, showing the division of 213 samples into eight clusters. Branches colored according to the country of origin. Bootstrap values on all nodes were below 50% and are not shown.



SM 5f. Unrooted Neighbor-joining dendrogram based on 350 AFLP markers, showing the division of 213 samples into eight clusters. Branches coloured according to the samples' affiliation between the six defined regions. Bootstrap values on all nodes were below 50% and are not shown.



Supplementary Material 6

SM 6a. Table of pairwise F_{ST} values based on AFLP data. Darker colour means lower pairwise FST. Sampling sites highlighted in pink indicate sites with less than 5 individuals. Significance levels: * (p < 0.05), *** (p < 0.001).

		CH	IT_A	IT_B	SL_A	SL_B	DE_A	DE_B	CZ_A	CZ_B	CZ_C	CZ_D	CZ_E	AT_A	AT_B	AT_C	SK_A	HU_A	HU_B	HU_C	HU_D	HU_F	RO_A	RO_B	UA_A	UA_B	PL_A	PL_B	PL_C	PL_D	RU	SK_B	SK_C	SK_D	SK_E	SK_F	SK_G	SK_H
1	CH	0	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
2	IT_A	0.339	0	*	-	*	*	-	*	***	*	-	***	***	-	*	*	-	*	*	***	*	*	*	-	*	***	***	*	*	*	*	***	*	*	*	*	*
3	IT_B	-0.193	0.210	0	-	-	-	-	*	*	*	-	*	*	-	***	-	-	*	*	*	-	*	*	-	*	***	*	-	*	*	*	*	*	*	*	*	*
4	SL_A	1.000	0.321	0.000	0	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	*	-	-	-	-	-	-	-	-	-	-	-
5	SL_B	0.216	0.285	0.199	-0.290	0	-	-	*	*	-	-	*	*	-	*	-	-	*	*	*	-	*	*	-	*	***	*	-	*	*	*	*	*	*	*	*	*
6	DE_A	0.222	0.391	0.225	0.226	0.329	0	-	*	*	*	-	*	*	-	*	-	-	*	*	*	-	*	*	-	*	***	*	-	*	*	*	*	*	*	*	*	*
7	DE_B	0.375	0.387	0.238	0.355	0.359	0.215	0	*	*	-	-	*	*	-	*	-	-	*	*	*	-	*	*	-	*	*	*	-	*	*	*	*	*	*	*	-	*
8	CZ_A	-0.014	0.255	0.160	0.137	0.216	0.182	0.237	0	***	-	-	***	***	-	*	*	-	***	*	***	*	***	***	-	***	***	***	*	***	*	***	***	***	***	*	*	***
9	CZ_B	0.088	0.178	0.206	0.118	0.183	0.265	0.183	0.182	0	*	-	***	***	*	***	***	-	***	***	***	***	*	***	-	***	***	***	***	***	***	***	***	***	***	***	***	***
10	CZ_C	-0.012	0.189	0.138	-0.015	0.112	0.166	0.172	0.053	0.079	0	-	***	***	-	*	*	-	***	*	***	*	*	*	-	***	***	***	*	*	*	*	***	*	***	*	*	*
11	CZ_D	1.000	0.283	0.111	1.000	0.188	0.282	0.412	0.169	-0.017	-0.006	0	-	-	-	-	-	-	-	-	-	-	-	-	-	-	*	-	-	-	-	-	-	-	-	-	-	-
12	CZ_E	0.156	0.230	0.230	0.129	0.161	0.267	0.204	0.186	0.137	0.117	0.178	0	***	*	***	*	-	***	***	***	***	***	***	-	***	***	***	*	***	***	***	***	***	***	***	***	***
13	AT_A	0.104	0.161	0.207	0.063	0.152	0.317	0.216	0.205	0.176	0.131	0.203	0.135	0	-	***	*	-	***	***	***	*	***	***	-	***	***	***	*	***	***	***	***	***	***	***	***	***
14	AT_B	0.062	0.199	0.138	0.094	0.168	0.243	0.191	0.103	0.128	0.020	0.124	0.123	0.000	0	-	-	-	-	-	*	-	*	*	-	*	*	*	-	*	*	-	*	*	*	*	-	-
15	AT_C	0.135	0.206	0.205	0.084	0.136	0.277	0.233	0.175	0.133	0.070	0.121	0.138	0.106	0.062	0	***	-	***	***	***	*	*	***	-	***	***	***	*	***	*	***	***	***	***	***	*	***
16	SK_A	0.427	0.412	0.320	0.430	0.316	0.427	0.458	0.170	0.226	0.141	0.483	0.263	0.263	0.292	0.236	0	-	*	*	*	-	*	*	-	*	***	*	-	*	*	*	*	*	*	*	*	*
17	HU_A	1.000	0.433	0.044	1.000	0.238	0.226	0.412	0.094	0.121	0.037	1.000	0.167	0.187	0.138	0.103	0.442	0	-	-	-	-	-	-	-	-	*	-	-	-	-	-	-	-	-	-	-	-
18	HU_B	0.195	0.250	0.237	0.141	0.204	0.254	0.259	0.152	0.168	0.140	0.183	0.155	0.181	0.136	0.143	0.271	0.134	0	*	***	*	***	***	-	***	***	***	*	***	*	***	***	***	***	***	*	***
19	HU_C	0.010	0.178	0.163	0.014	0.147	0.177	0.176	0.110	0.137	0.100	0.006	0.095	0.141	0.071	0.128	0.217	-0.081	0.049	0	***	*	***	***	-	***	***	***	*	***	***	*	***	***	***	***	*	***
20	HU_D	0.076	0.161	0.207	0.214	0.250	0.280	0.255	0.194	0.142	0.147	0.223	0.131	0.170	0.131	0.167	0.290	0.193	0.125	0.104	0	*	***	***	-	***	***	***	*	***	***	***	***	***	***	***	*	***
21	HU_F	0.241	0.343	0.217	0.288	0.300	0.357	0.335	0.214	0.163	0.173	0.330	0.162	0.199	0.246	0.192	0.395	0.333	0.219	0.142	0.129	0	*	*	-	*	***	*	-	*	*	*	*	*	*	*	*	*
22	RO_A	0.210	0.336	0.213	0.294	0.325	0.366	0.324	0.251	0.266	0.230	0.372	0.270	0.223	0.224	0.251	0.414	0.353	0.259	0.230	0.219	0.297	0	***	-	*	***	***	*	***	*	*	***	*	***	*	*	***
23	RO_B	0.145	0.257	0.218	0.180	0.226	0.324	0.307	0.223	0.227	0.153	0.268	0.201	0.168	0.132	0.211	0.330	0.268	0.181	0.168	0.212	0.243	0.160	0	-	***	***	***	*	***	***	***	***	***	***	***	*	***
24	UA_A	1.000	0.361	-0.009	1.000	0.270	0.266	0.355	0.010	0.123	0.021	1.000	0.160	0.133	0.078	0.121	0.459	1.000	0.170	0.010	0.119	0.288	0.162	0.095	0	-	-	-	-	-	-	-	-	-	-	-	-	-
25	UA_B	0.191	0.264	0.254	0.227	0.253	0.325	0.273	0.195	0.214	0.183	0.260	0.222	0.191	0.161	0.205	0.325	0.186	0.208	0.177	0.200	0.227	0.203	0.151	-0.161	0	***	***	*	***	-	***	***	***	***	*	*	***
26	PL_A	0.036	0.318	0.251	0.261	0.289	0.339	0.324	0.209	0.250	0.219	0.317	0.265	0.249	0.196	0.256	0.324	0.247	0.278	0.247	0.230	0.227	0.200	0.218	0.019	0.208	0	-	-	-	***	***	***	***	***	***	***	***
27	PL_B	-0.003	0.322	0.221	0.243	0.277	0.324	0.320	0.199	0.243	0.207	0.303	0.269	0.247	0.204	0.247	0.320	0.228	0.267	0.223	0.238	0.261	0.212	0.230	-0.001	0.217	0.019	0	-	-	***	***	***	***	***	***	***	***
28	PL_C	0.118	0.418	0.208	0.388	0.356	0.366	0.422	0.170	0.265	0.201	0.432	0.330	0.320	0.263	0.299	0.411	0.399	0.313	0.244	0.271	0.350	0.279	0.279	0.147	0.256	-0.009	0.026	0	-	*	*	*	*	*	*	*	*
29	PL_D	0.043	0.349	0.218	0.276	0.303	0.325	0.338	0.142	0.247	0.188	0.338	0.267	0.267	0.216	0.251	0.314	0.253	0.254	0.227	0.237	0.250	0.245	0.246	0.045	0.214	0.016	0.001	-0.004	0	***	*	***	***	***	***	*	***
30	RU	0.179	0.291	0.218	0.241	0.291	0.261	0.320	0.170	0.256	0.200	0.323	0.251	0.250	0.209	0.253	0.370	0.253	0.182	0.166	0.197	0.281	0.278	0.222	0.069	0.178	0.266	0.264	0.277	0.249	0	*	***	*	***	*	*	***
31	SK_B	0.380	0.410	0.334	0.427	0.338	0.393	0.444	0.187	0.248	0.178	0.465	0.264	0.303	0.253	0.261	0.202	0.406	0.263	0.229	0.302	0.421	0.428	0.344	0.406	0.354	0.322	0.332	0.379	0.306	0.356	0	***	*	***	*	*	***
32	SK_C	0.304	0.320	0.303	0.318	0.277	0.364	0.327	0.194	0.189	0.151	0.344	0.205	0.251	0.213	0.210	0.143	0.359	0.245	0.206	0.233	0.303	0.362	0.312	0.324	0.288	0.302	0.322	0.346	0.276	0.325	0.155	0	-	***	***	*	***
33	SK_D	0.220	0.301	0.249	0.225	0.233	0.331	0.353	0.146	0.185	0.088	0.299	0.214	0.229	0.203	0.193	0.126	0.258	0.203	0.176	0.221	0.305	0.355	0.268	0.270	0.263	0.278	0.279	0.297	0.240	0.286	0.148	0.074	0	***	*	*	*
34	SK_E	0.332	0.328	0.273	0.324	0.297	0.347	0.348	0.192	0.207	0.142	0.351	0.234	0.271	0.205	0.204	0.173	0.303	0.223	0.197	0.252	0.323	0.358	0.290	0.289	0.283	0.294	0.314	0.300	0.274	0.309	0.160	0.161	0.106	0	***	*	***
35	SK_F	0.211	0.336	0.241	0.301	0.291	0.335	0.376	0.150	0.213	0.135	0.385	0.223	0.249	0.230	0.222	0.199	0.313	0.223	0.185	0.247	0.310	0.355	0.259	0.257	0.253	0.256	0.250	0.307	0.234	0.279	0.184	0.165	0.091	0.153	0	*	*
36	SK_G	0.148	0.271	0.177	0.154	0.213	0.256	0.280	0.130	0.199	0.100	0.178	0.227	0.226	0.135	0.192	0.167	0.115	0.196	0.121	0.232	0.287	0.317	0.238	0.178	0.256	0.278	0.267	0.261	0.226	0.247	0.128	0.138	0.098	0.136	0.142	0	*
37	SK_H	0.246	0.294	0.230	0.271	0.253	0.313	0.312	0.140	0.176	0.102	0.313	0.173	0.209	0.160	0.162	0.182	0.302	0.169	0.145	0.204	0.281	0.326	0.258	0.279	0.275	0.272	0.268	0.305	0.245	0.305	0.151	0.131	0.088	0.140	0.101	0.106	0

Legend:		**Fst < 0.5**	**Fst < 0.4**	**Fst < 0.3**	**Fst < 0.2**	**Fst < 0.1**			**n samples < 5**		*** (p < 0.05)**	***** (p < 0.001)**

Supplementary material 7. Maximum likelihood (ML) analysis was done using MEGA X. For the analysis, 168 sequences of ITS1-5.8SrDNA-ITS2 locus deposited in GenBank (https://www.ncbi.nlm.nih.gov/genbank/) plus seven ribotypes identified within the study Vaculná *et al.* (2021) were used. *Campanula cervicaria* (GenBank accession MT774680) was used as outgroup to root the final tree. The selection of the appropriate model of DNA evolution for the ML analysis was performed in MEGA X (ITS: GTR+G). The statistical support for the inferred topology was tested using 1000 bootstrap resampling.

Sequence ID	Species
KU983907	*A. aurita*
MH808223	*A. capillaris*
KU983908	*A. capillaris*
MH808224	*A. capillaris*
KF175324	*A. capillaris*
KF175325	*A. capillaris*
KU983909	*A. capillaris* subsp. *leptosepala*
KF175322	*A. coelestis*
KF175323	*A. coelestis*
KF175321	*A. coelestis*
KU983910	*A. coelestis*
HQ704519	*A. coronopifolia*
MN519416	*A. coronopifolia*
MT299777	*A. coronopifolia* MK-2020
HQ704521	*A. divaricata*
HQ704520	*A. divaricata*
HQ704546	*A. divaricata*
KY369000	*A. divaricata*
KY368998	*A. divaricata*
KY368999	*A. divaricata*
KY369001	*A. erecta*
HQ704554	*A. erecta*
KU983911	*A. gmelinii*
KU983912	*A. gmelinii*
KU983913	*A. gmelinii subsp. nystroemii*
HQ704522	*A. grandiflora*
KY369002	*A. grandiflora*
KU983915	*A. himalayana*
KU983916	*A. himalayana*
KU983914	*A. himalayana*
KF175326	*A. himalayana*
KF175328	*A. himalayana*
KF175327	*A. himalayana*
EU591977	*A. hunanensis*
KU983917	*A. jasionifolia*
HQ704523	*A. kayasanensis*
KY369005	*A. kayasanensis*
KU983919	*A. khasiana*
KU983918	*A. khasiana*
KF175307	*A. khasiana*
KF175306	*A. khasiana*
KF175308	*A. khasiana*

Sequence ID	Species
HQ704553	*A. lamarkii*
HQ704551	*A. liliifolia*
KU983920	*A. liliifolia*
DQ304581	*A. liliifolia*
MN519409	*A. liliifolia* ITS rib_1
MN519410	*A. liliifolia* ITS rib_2
MN519411	*A. liliifolia* ITS rib_3
MN519412	*A. liliifolia* ITS rib_4
MN519413	*A. liliifolia* ITS rib_5
MN519414	*A. liliifolia* ITS rib_6
MN519415	*A. liliifolia* ITS rib_7
KF175303	*A. liliifolioides*
KF175304	*A. liliifolioides*
KF175305	*A. liliifolioides*
KC880797	*A. liliifolioides*
KF175331	*A. liliifolioides*
KF175332	*A. liliifolioides*
KU983921	*A. liliifolioides*
KF175329	*A. liliifolioides*
KF175330	*A. liliifolioides*
KF279691	*A. lobophylla*
KU983922	*A. maximowicziana*
KU983923	*A. morrisonensis*
KF279686	*A. morrisonensis*
KU983924	*A. morrisonensis* subsp. *uehatae*
KU983925	*A. nikoensis*
KU983926	*A. nikoensis* var. *petrophila*
KU983946	*A. palustris*
KY369006	*A. palustris*
KU983946	*A. palustris*
KU983927	*A. paniculata*
KF279687	*A. paniculata*
KU983928	*A. pereskiifolia*
MH711418	*A. petiolata*
KF279688	*A. petiolata*
KY369008	*A. polyantha*
HQ704524	*A. polyantha*
KF175317	*A. polyantha*
KF175318	*A. polyantha*
KF175319	*A. polyantha*
KF175320	*A. polyantha*
KY369007	*A. polyantha*

Sequence ID	**Species**
MH808225	*A. polyantha*
MH711482	*A. polyantha*
MH712604	*A. potaninii*
KU983929	*A. potaninii*
MH710903	*A. potaninii*
KF279690	*A. potaninii*
HQ704545	*A. racemosa*
KY369009	*A. racemosa*
KY369010	*A. racemosa*
HQ704525	*A. racemosa*
HQ704526	*A. remotiflora*
HQ704527	*A. remotiflora*
KY369011	*A. remotiflora*
KY369012	*A. remotiflora*
KU983930	*A. remotiflora*
AY548197	*A. remotiflora*
HQ704528	*A. remotiflora* var. *hirticalyx*
KC880796	*A.* sp.
FJ572040	*A.* sp.
MH626536	*A.* sp. DX-2018
MH626535	*A.* sp. DX-2018
MT299778	*A.* sp. MK-2020
KF175309	*A. stenanthina*
KF175310	*A. stenanthina*
KF175311	*A. stenanthina*
KF175312	*A. stenanthina*
KU983931	*A. stenanthina*
KU983932	*A. stenanthina*
KY829515	*A. stricta*
KU983933	*A. stricta*
KU983934	*A. stricta*
KY369015	*A. stricta*
KY369017	*A. stricta*
KY829517	*A. stricta*
MH710859	*A. stricta*
HQ704529	*A. stricta*
MH808226	*A. stricta*
KU983935	*A. stricta*
KY829514	*A. stricta*
KY369016	*A. stricta*
KY829516	*A. stricta*
KU983936	*A. sublata*
HQ704555	*A. taquetii*
KY369018	*A. taquetii*
AY548196	*A. trachelioides*
KF175315	*A. trachelioides*
KF175316	*A. trachelioides*
KU983938	*A. tricuspidata*
KU983937	*A. tricuspidata*
EU591967	*A. triphylla*
KY829520	*A. triphylla*
KY829521	*A. triphylla*
KU983941	*A. triphylla*
KF175313	*A. triphylla*
KF175314	*A. triphylla*
KM051442	*A. triphylla*
KU983939	*A. triphylla*
KU983940	*A. triphylla*
KU983942	*A. triphylla*
KU983943	*A. triphylla*
KU983945	*A. triphylla*
KY829518	*A. triphylla*
KY829519	*A. triphylla*
AY548194	*A. triphylla*
HQ704530	*A. triphylla* var. *japonica*
KY369019	*A. triphylla* var. *japonica*
KY369020	*A. triphylla* var. *japonica*
KY829523	*A. triphylla* var. *japonica*
AB743776	*A. triphylla* var. *japonica*
AB743777	*A. triphylla* var. *japonica*
AB743778	*A. triphylla* var. *japonica*
AB743779	*A. triphylla* var. *japonica*
AB743780	*A. triphylla* var. *japonica*
KY829522	*A. triphylla* var. *japonica*
KY829524	*A. triphylla* var. *japonica*
AY548193	*A. triphylla* var. *japonica*
KY829525	*A. triphylla* var. *japonica*
HQ704531	*A. verticillata*
KY369021	*A. verticillata*
KY369022	*A. verticillata*
KY369023	*A. verticillata*
KY369024	*A. verticillata*
KY369025	*A. verticillata*
KY369026	*A. verticillata*
KY368997	*A. verticillata* var. *abbreviata*
HQ704532	*A. verticillata* var. *hirsuta*
KY369003	*A. verticillata* var. *hirsuta*
KY369004	*A. verticillata* var. *hirsuta*
KF279689	*A. wawreana*
MH710710	*A. wilsonii*
KU983944	*A. wilsonii*
MT774680	*C. cervicaria*

Palacký University Olomouc
Faculty of Science
Department of Botany

Adenophora liliifolia - present, past and future of the critically endangered species in Europe

P1527 Biology – Botany

Summary of the Ph.D. thesis

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The summary of Ph.D. thesis was sent for distribution on ……………

The oral defence will take place on 24. 3. 2022 at 10:00 in front of the Commission for Ph.D. thesis of the Study Program Botany at building 47, auditorium 137, Šlechtitelů 27, Olomouc.

The Ph.D. thesis will be deposited in the Library of the Biological Departments of Faculty of Science at Palacký University, Šlechtitelů 27, Olomouc.

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1 Introduction

Adenophora liliifolia (L.) A. DC is the only representative of species-rich Asian genus *Adenophora* in Europe, where it represents the European-South Siberian floristic element and a probable relic species of the late glacial - early Holocene. The distribution of the species extends from southwestern Siberia and the Ural Mountains in the East to Western Europe, where the populations are highly fragmented. The species is still relatively abundant in the Carpathians (Slovakia), but population numbers in Europe are gradually declining. At present, *A. liliifolia* has the status of an endangered or critically endangered species in most countries of occurrence. In EU territory, *A. liliifolia* is listed among the species of the European Community interest (Directive 1992), whose conservation requires the designation of special areas of conservation (SAC; NATURA 2000 sites); out of the EU territory is listed in nearly 40 actual Red Books of several East-European regions (e.g., Belarus, several regions in Ukraine, and numerous regions in Russia; Plantarium 2021).

The light-demanding, long-lived, herbaceous perennial species is in the Central-European context threatened mainly by the loss of suitable habitats due to changes in habitat management, ongoing secondary succession, over-populated wildlife, and the intensive competitive pressure of nitrophilous species. Its persisting in recent European habitats is conditioned mainly by the active conservation measures, including opening up the canopies, early thinning of young stands, control of expansive nitrophilous species, undergrowth mowing, and protection of plants from wildlife grazing.

Several countries (e.g., CZ, PL) have already launched National Action Plans, which aim to develop appropriate techniques for *in situ* and *ex situ* preservation. One of the key conditions for such a challenge and successful conservation strategy is to reveal species genetic diversity and inter-population relationships.

2 Aims of the thesis

During my research, I focused on the following questions:

1) What are the current conditions of *A. liliifolia* populations in Central Europe? Which factors threaten the species, and what type of appropriate management could be suggested for species' prosper?

2) What is the level of genetic diversity and what is its spatial distribution within Europe? What are the relationships among extant *A. liliifolia* populations?

3) Is it possible to draw conclusions about the presence of potential glacial refugia and putative routes of post-glacial (re)colonisation of Europe based on the current distribution of genetic diversity and population structure?

4) Can species distribution modeling answer questions related to the history and future of *A. liliifolia* remnant populations?

3 Material and Methods

This section contains a detailed description of the methods that have been performed by myself (if not stated otherwise in the text). Other methods I have participated in (vegetation survey, yearly habitat monitoring, conservation measures, etc.) are delineated in individual publications and manuscript of the thesis.

Plant material and DNA extraction

For the purpose of the doctoral study, we managed to collect the leaf material (collected in the field and silica-gel dried) from 293 individuals, representing 41 actual and verified sampling sites within Europe (Austria, Czech Republic, Germany, Hungary, Italy, Slovenia, Slovakia, Switzerland, Poland, Romania, Russia, Ukraine). These samples were used for analyses based on amplified fragment length polymorphism (AFLP) and DNA sequencing. Additional 80 voucher specimens from the Moscow University Herbarium (Russia; MW) and eight from the Department of Botany and Zoology at Masaryk University in Brno (the Czech Republic; BRNU) were used for DNA sequencing, covering the easternmost part of the species distribution area.

For the first study based on AFLP markers only (Prausová *et al.* 2016), the subset of 84 individuals covering 23 populations was used. The whole sample set (293 individuals) was used in Vaculná *et al.* (2021). Sampling was done either directly by me or by co-authors of publications or colleagues involved in the species protection in particular countries. The permissions from corresponding government authorities allowed the collection of 1-2 leaves per individual plant to prevent unnecessary harm to the populations composed frequently of 1-5 plants.

Genomic DNA was extracted using a modified CTAB protocol (Doyle and Doyle 1987). The integrity and quality of extracted DNA were assessed using 1.5% agarose gel electrophoresis, and concentration was determined using a NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies, Delaware, USA). For the control of repeatability and error rate calculation, 10% of samples were double extracted and represented replicates in all subsequent analyses.

AFLP analyses

AFLP analyses were performed according to the procedure of Vos *et al.* (1995) with modifications described in Ali *et al.* (2017), using eight or six primer combinations respectively (Prausová *et al.* 2016, Vaculná *et al.* 2021), selected by preliminary testing. The first study (Prausová *et al.* 2016) was performed on 6 %, 0.4 mm-thick denaturing polyacrylamide gels (0.5×TBE buffer) using 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. In this case, silver staining was used to detect the AFLP fragments after electrophoretic separation. In the second study, AFLP fragments were fluorescently labelled and separated by capillary electrophoresis (GenomeLab CEQ/GeXP DNA, Beckman Coulter, USA). Raw data were analysed with CEQ 8000 software using an incorporated dominant scoring algorithm (bin 1-2 bp, threshold 100), where peaks ranging from 70 to 550 bp were scored as either present (1) or absent (0) (Vaculná *et al.* 2021).

Nuclear and chloroplast DNA sequencing

Altogether, 13 genomic loci [12 chloroplast (cpDNA) and one nuclear (internal transcribed spacer, ITS)] were screened for polymorphism in a 46-sample subset (i.e., at least one randomly selected sample per each sampled site) (Vaculná *et al.* 2021). Based on observed variation, only three variable intergenic spacers (*trn*S-*trn*G, *trnS-trnfM, and psb*D-*trn*T) were used in further analyses. The PCR reaction consisted of template DNA (75 ng), 0.4 μM of each primer, 1× KAPA Taq buffer (containing 1.5 mM $MgCl_2$), 0.2

mM dNTPs, 0.4 U of KAPA *Taq* polymerase (Kapabiosystems, Boston, USA), and PCR-quality water in a 20 µl total volume. The reaction

conditions were optimized for each of the three selected cpDNA loci separately. The PCR products were sequenced at the Biodiversity and Climate Research Centre (BiK-F) (Frankfurt am Main, Germany) or by Macrogene Europe (Amsterdam, The Netherlands). The contig assembly from partial reads, base call editing, sequence alignment, and sequence concatenation were performed in Geneious 7.1.8 (Biomatters Ltd., Auckland, New Zealand; Vaculná *et al.* 2021).

Molecular data analyses

AFLP

From the final AFLP binary matrix, basic population genetic indices were calculated (e.g., Nei's gene diversity, rare fragments, percentage of polymorphic loci), and various clustering methods have been applied, using the following software: AFLP-SURV v.1.0 (Vekemans *et al.* 2002), Arlequin 3.5.2.2 (Excoffier and Lischer 2010), GenAlEx 6.5 (Peakall and Smouse 2006, 2012), the R-script AFLPdat (Ehrich 2006; R Core Team 2020), FAMD 1.31 (Schlüter and Harris 2006), FigTree v1.4.0 (Rambaut 2016), STRUCTURE 2.3.4 (Falush, Stephens and Pritchard 2007), STRUCTURE HARVESTER (Earl and von Holdt 2012), CLUMPP (Jakobsson and Rosenberg 2007), and DISTRUCT (Rosenberg 2004). The CLUMPP output was also used for the admixture visualization in QGIS (Quantum GIS Development Team 2019).

DNA Sequencing

DnaSP v.6 (Rozas et al. 2017) software was used for estimation of sequence diversity; polymorphic, singleton, and parsimony-informative sites; a number of haplotypes (N_H); haplotype diversity (H_D; Nei 1987); nucleotide diversity (Pi; Tajima 1983); and to test if subpopulations are genetically differentiated. The presence of bottleneck event and/or population contraction/expansion within defined geographic regions was tested by Tajima's D test. The haplotype network was constructed by statistical parsimony method using R package Haplotypes (Aktas 2015). Most of the statistics were calculated by Miloslav Kitner and Ľuboš Majeský.

Species distribution modeling

Maxent (version 3.3.3; Phillips, Anderson and Schapire 2006) was used for habitat suitability modeling, analysing environmental data from a total of 78 cross-checked occurrence points (GBIF 2018; Plantarium 2021). The present-day climate data (1970 – 2000) as well as different Global Circulation Models (GCMs) for the Last Glacial Maximum (LGM; 22,000 BP), Mid-Holocene (6,000 BP), and models for the years 2050 and 2070 were extracted from the WorldClim database (Hijmans *et al.* 2005). Data resolution was 30s except for LGM (2.5m). Soil data were accessed from the ISRIC (Hengl *et al.* 2017) and GlobalChange (Shangguan *et al.* 2014) portals and vegetation cover data from the EarthEnv database (Tuanmu and Jetz 2014). Two possible emissions scenarios were tested. Emissions in RCP 4.5 peak around 2040, then decline. Whereas RCP 8.5 emissions continue to rise throughout the 21st century (Meinshausen *et al.* 2011). The multicollinearity of the contributing environmental variables was tested by the ENMTools (Warren *et al.* 2010). To prevent misleading signals, highly correlated variables with Pearson's $r > 0.8$ were excluded. For the final modeling of the present distribution, nine ecologically meaningful variables were used. Models were assembled in the Dismo R package (Hijmans *et al.* 2017), and their predictions were visualized using QGIS.

4 Publications included in the thesis

4.1 *Adenophora liliifolia*: Condition of its populations in Central Europe

Romana Prausová*, Lucie Marečková*, Adam Kapler, Ľuboš Majeský, Tünde Farkas, Adrian Indreica, Lenka Šafářová, Miloslav Kitner

(* These two authors contributed equally to this work)

Acta Biologica Cracoviensia Series Botanica 58/2: 83–105, 2016
DOI: 10.1515/abcsb-2016-0018

Abstract

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 genetic structure was observed (22% at the interpopulation level, AMOVA analysis), with moderate values for gene diversity (Hj = 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, and 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.

Key Words: AFLP, Campanulaceae, the European periphery distribution, declining population, European-South-Siberian geoelement, genetic variability, vegetation

4.2 The use of *Adenophora liliifolia* seeds collected at Dabrowa near Zaklików (Rzeszów voievodship) to reinforce the declining population in Niedzieliski Forest (Lublin voievodship)

Adam Kapler, Maciej Niemczyk, Jerzy Puchalski, Adam Rapa, Bogusław Radliński, Tomasz Bajdak, Marcin Kołodziej,
Lucie Marečková

Chrońmy Przyrodę Ojczystą 75 (2): 125–134, 2019

Abstract

One of the conservation measures included in the "Infrastructure & Environment Operational Programme – Implementation of protection tasks for Natura 2000 sites and nature reserves in the Lublin province" was to reinforce the declining population of *Adenophora liliifolia* at the Niedzieliski Forest Natura 2000 site, using local seeds. In 2013, seeds from populations located in the Lublin and Podkarpacie provinces were collected. The experiments realized in the Powsin Seed Bank Laboratory showed a lack of germination ability of seeds from the Lublin region. The only exception was one seedling obtained from the seeds collected at the Lasy Strzeleckie natural site. However, this single seedling soon died. On the other hand, seeds collected in the Dąbrowa near Zaklików Natura 2000 area (Podkarpacie province) showed a good germination rate of 42–65%. Therefore, authorities supervising the conservation project decided to use *A. liliifolia* plants obtained from the seeds collected in the Dąbrowa near Zaklików Natura 2000 area to reinforce the declining population Niedzieliski Forest.

Keywords: active protection, introduction, vitality of seeds, genetic rescue strategy.

4.3 Genetic structure of endangered species *Adenophora liliifolia* and footprints of postglacial recolonisation in Central Europe

Lucie Vaculná, Ľuboš Majeský, Tahir Ali, Alexey P. Seregin, Romana Prausová, Adam Kapler, Dmytro Iakushenko, Marco Thines, Miloslav Kitner

Conservation Genetics 22(6): 1069–1084
DOI: 10.1007/s10592-021-01396-5

Abstract

Quaternary climatic fluctuations changed the floristic composition of large areas and forced biota to repeated movements following gradients of their ecological tolerance. Different and contrasting patterns of responses were reported for various species. In this study we focused on *Adenophora liliifolia*, a perennial herbaceous species representing a south Siberian floristic element in European flora, well adapted to a continental climate. We investigated the genetic diversity and phylogeography of the species within Europe. The results show the absence of stronger sequence differentiation across the investigated 3600 km long transect, which reflects the young evolutionary origin of the species and/or repeated population contractions/expansions accompanied by bottleneck during the Quaternary climatic fluctuations. Along with this, the results suggest several regions located in Carpathians, Alps-Dinarides, and in non-glaciated parts of Central Russia with high haplotype diversity, which probably served as refugia. Low genetic diversity, the prevalence of a single cpDNA haplotype, and the genetic composition of the investigated European sites suggest re-colonization of Central Europe from a refugium located in southern Europe. The distribution of genetic variation and moderate genetic differentiation of the investigated sites may indicate the process of fragmentation of the common genetic pool in Central Europe. Projections of ongoing climate change during the 21st century are not favorable for species persistence in Central Europe. In the case of *ex-situ* conservation actions, we recommend protection and proper management of rapidly vanishing populations and maintenance of genetic diversity, especially in regions with high haplotype diversity.

Keywords: AFLP – cpDNA sequencing – glacial refugia – NATURA 2000 – niche modeling – Quaternary history.

5 Survey of results

Two genetic studies were performed during my doctoral studies. The first one investigated the situation in East-Central Europe, dealing with 84 samples from 23 populations by the AFLP analysis. The second study extended the sample set to cover as much of the *A. liliifolia* distribution area as possible, using AFLP and DNA sequencing. The results of both studies point to the following facts: overall low values of genetic diversity; majority of genetic variation present within the investigated populations; higher genetic similarity of the investigated populations and the absence of a robust genetic differentiation.

We did not detect any fixed private bands (nFPB) and only a low number of private bands (nPB) at the regional level. Also, the frequency-down-weighted marker (DW) values were low, ranging from 1.7–4.2 for the particular localities in the first study, respectively 1.38 (Poland) to 2.28 (Russia) in the second study. This index may be linked with the duration of the isolation of populations. In our case, the values are rather low and suggest that *A. liliifolia* populations diverged relatively recently. The Bayesian clustering approach implemented in STRUCTURE suggested the presence of three ($K = 3$) or seven ($K = 7$) clusters. The clustering for $K = 3$ differentiated mainly sampled sites from Western Carpathians (1st cluster), Poland (2nd cluster), while all the remaining sites represented the 3rd cluster. Clustering for $K = 7$ further refined genetic groups' distribution defined by $K = 3$ and primarily showed further sub-separation of populations from Hungary and Russia. The AMOVA results ($P < 0.001$) showed the greatest proportion of genetic variation 75.38% (respectively 78% in the first study) present within sampling sites, 11.91% (22%) present among sampling sites within the geographical regions, and 12.71% present among the six regions defined in Vaculná *et al.* (2021). The overall differentiation among the sampled sites in the first, Central-European study was $F_{ST} = 0.125$. In the second study, among 41 populations, it increased to $F_{ST} = 0.246$ ($P < 0.001$). The pairwise F_{ST} values ranged between 0.00075 (geographical distance; GGD = 50 km) and 0.428 (GGD = 540 km).

Analyses of herbarium vouchers led to successful sequencing of 50 out of 80 MW (62.5%) and two of 8 BRNU vouchers. Their nucleotide sequences were compared with those obtained from recently occurring samples. As a result, we confirmed taxonomic determination of 43 *A. liliifolia* samples from MW, while eight samples represented different, not determined *Adenophora* taxon, and one sample represented taxon *A. coronopifolia*.

We found only a low variability along the 3600 km long transect, sequencing the nuclear ITS marker and several chloroplast regions. From the 13 loci tested, only three (*trn*S-*trn*G, *trnS-trnfM,* and *psb*D-*trn*T) showed some variability, which was low (e.g., gene diversity ranging from 0.053 for *psb*D-*trn*T to 0.596 for *trn*S-*trn*G; based on the whole dataset). Among 336 samples sequenced, 28 different cpDNA haplotypes (26 *A. liliifolia*: *hap_1* to *hap_26*; one *A. coronopifolia – hap_27*, and one *A*. sp. – *hap*_28) were distinguished.

Haplotype network displays an interconnected topology reflecting low sequence divergence of identified lineages. Lineage *hap_14* seems to represent ancestral lineage to all the European lineages based on its geographical location (Poland, Russia, Western Carpathians, and Ukraine) and a direct connection with two outgroup lineages *hap_27* and *hap_28*.

Three spatially differentiated areas of elevated haplotype diversity were identified in Central-East Europe: Western Carpathians (Slovakia; $H_D = 0.759$), Southeastern Carpathians (Romania, Ukraine; $H_D = 0.576$), Alps-Dinarides (Italy, Switzerland, Slovenia; $H_D = 0.752$); and fourth on the Russian territory ($H_D = 0.650$). The lowest haplotype diversity was recorded for Poland ($H_D = 0.197$).

The most common haplotype within the whole dataset was *hap_01* found nearly in all sampled sites except Southeastern Carpathians and Russia. Within the region Central Europe its frequency reached 87%, respectively 89% in region Poland. This haplotype is only one mutational step apart from the suggested ancestral haplotype lineage *hap_14*. The lineage *hap_01* is absent in the Russian area (and in general east from the Southeastern Carpathians).

The species distribution modeling (SDM) helped us identify the glacial refugia located south of the Alps, where elevated haplotype diversity was observed. Based on the presumed climatic data for early Holocene, it also shows significant habitat expansion, which probably occurred during this period (associated with the rapid spread of the species, notably its haplotype lineage *hap_01*, as described above). Predictions of the current distribution of *A. liliifolia* by the Maxent algorithm show much broader suitable habitat areas than are actually realised. This might be caused by many biotic and abiotic factors affecting the species on-site, which are hard to be converted into the data layers (e.g., traditional management abandonment or ongoing secondary succession changing the light conditions). However, the data layers used helped to elucidate some ecological preferences of *Adenophora liliifolia* (e.g., optimal temperature and precipitation range, percentage of soil base saturation, etc.). The SDM results further suggest that ongoing climate change will worsen the situation and influence the future distribution of *A. liliifolia* in Europe. Scenarios for years 2050 and 2070 show gradual habitat constrictions. Under the most stringent course (rcp85), prediction shows a noticeable shift of suitable habitats towards the northeast in Russia. The vastness of Russian territory seems to currently preserve much broader options for *A. liliifolia* survival than Central Europe. However, the species currently face habitat loss and is listed as endangered in numerous Russian regions.

6 Conclusions

The submitted thesis provides information about the critically endangered species *Adenophora liliifolia*, its habitat characteristics, population conditions, and conservation management relevant to the species, particularly in the Czech Republic and Poland. A summary of species ecological and phytosociological demands, a range of novel findings of genetic diversity and population genetic structure of the species within Europe, and hints on species history and future are presented. The comprehensive view and synthesis of partial fragments of *A. liliifolia* biology are essential for the appropriate conservation strategy and requested for National Action plans already implemented or prepared in the European region.

The current conditions of *A. liliifolia* populations in Europe regarding its habitat are, according to NATURA 2000 methodology, assessed as unfavourable, except for Slovenia and Slovakia. Our data proved the situation in Slovakia in detail, where the species grows mainly in places distant from human settlement, on rocky outcrops, and well-lit edges of beech or relict pine forests. Such associations represent climax communities on-site. Altogether, six vegetations units within Central Europe in which the species grow were identified. However, species preservation in these habitats is conditioned mostly by appropriate conservation measures, mitigating undesirable effects of the abandonment of traditional site management, ongoing on-site succession, eutrophication, expansion of oppressing nitrophilous species, change of the habitat light condition, or the overpopulated wildlife.

Our data support the assumption that *A. liliifolia* most likely originates from the area of Southern Siberia/Ural Mountains, from where it expanded westward to Europe. We based this conclusion on the spatial distribution of identified haplotype lineages. Further, we detected low genetic diversity in *A. liliifolia* populations and low sequence divergence (in all sequenced loci) along the 3600 km long transect. This situation may be related to the species’ young evolutionary origin, alternatively, by the repeated populations’ contractions and expansions accompanied by bottlenecks during Quaternary climatic fluctuations. All of this could reduce the variability of the genetic pool which (re)colonized Europe and was further depleted during the Pleistocene. The absence of a more robust genetic differentiation and overall similarity of the investigated populations may indicate on gradual fragmentation of a larger genetic pool of populations (densely) connected in the relatively recent past within Central Europe. Probably only due to the species biology, life cycle, and particularly the longevity of plants, the conservation situation of the species regarding the genetic diversity is not critical yet.

We identified three spatially differentiated areas of elevated haplotype diversity in East-Central Europe: Western Carpathians (Slovakia), Southeastern Carpathians (Romania, Ukraine), Alps-Dinarides (Italy, Switzerland, Slovenia); and fourth on the Russian territory. Regarding the species history in Central Europe, the first three above-mentioned areas might serve as glacial refugia. The rest of Europe is inhabited by the widespread haplotype *hap_01*. This *hap_01* might represent the lineage of "general-purpose," evolutionarily selected as the most advantageous for late-Glacial/early Holocene habitats. The lineage is absent east from the Southeastern Carpathians and probably originated in Central Europe.

The species distribution modeling helped us identify the glacial refugia located south of the Alps, where elevated haplotype diversity was observed. Based on the presumed climatic data for Holocene, it also showed significant habitat expansion, which probably occurred during this period (associated with the rapid spread of the species, notably its haplotype lineage *hap_01*, as described above). The results further suggest that ongoing climate change will worsen the situation and influence the future distribution of *A. liliifolia* in Europe.

7 Souhrn (Summary in Czech)

Název práce: *Adenophora liliifolia* – současnost, minulost a budoucnost kriticky ohroženého druhu v Evropě

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Adenophora liliifolia (L.) A. DC (zvonovec liliolistý) je jediný zástupce početného asijského rodu *Adenophora* v Evropě, kde představuje evropsko-jihosibiřský floristický element a pravděpodobný relikt pozdního glaciálu – raného Holocénu. Celkový areál druhu sahá od jihozápadní Sibiře a pohoří Ural na východě po západní Evropu, kde jsou populace značně fragmentované. Druh je stále poměrně hojný v Západních Karpatech (Slovensko), ale celkové počty populací v Evropě klesají. V současnosti má zvonovec liliolistý ve většině zemí svého výskytu status ohroženého nebo kriticky ohroženého druhu.

Ve své disertační práci jsem se zabývala zejména studiem genetické variability a vzájemných vztahů evropských populací. Mým cílem bylo také pokusit se odpovědět na otázky jak a kdy druh migroval ze svého asijského centra do Evropy, a zda mohl přežít chladné období Pleistocénu v refugiích lokalizovaných ve Střední Evropě. K zodpovězení těchto otázek jsem použila dva typy molekulárních markerů – metodu AFLP a sekvenování vybraných regionů chloroplastové a jaderné DNA. Jako pomocnou metodu pro interpretaci genetických dat jsem použila predikci vhodných stanovišť v různých časových obdobích pomocí algoritmu MaxEnt. Součástí předložené práce jsou také informace o velikostech populací, podmínkách stanovišť a vegetačních jednotkách, ve kterých zvonovec liliolistý roste ve střední Evropě, s detailními informacemi o lokalitách v České republice.

Výsledky mé práce podporují předchozí očekávání, že zvonovec liliolistý nejpravděpodobněji pochází z oblasti jihozápadní Sibiře a Uralu, odkud se později rozšířil do Evropy. Bližší určení období, ve kterém k tomuto procesu došlo, však nebylo kvůli celkově nízké diverzitě sekvenovaných lokusů možné. Zjištěná centra zvýšené haplotypové diversity v západních Karpatech, jihovýchodních Karpatech, a v oblasti Alp a Dinárských hor mohla představovat glaciální refugia druhu. Ostatní evropské populace se vyznačují výraznou převahou jediné haplotypové linie, která pravděpodobně představuje linii nejlépe adaptovanou na podmínky pozdně-glaciální/raně-holocénní středoevropské krajiny. Genetická diverzita evropských populací je spíše na nižší až střední úrovni, výraznější populačně genetickou strukturu lze pozorovat mezi geograficky vzdálenějšími populacemi. To může, společně s dlouhověkostí rostlin, poukazovat na započatou fragmentaci populací, které byly propojeny ještě v relativně nedávné minulosti. Za procesem fragmentace může stát také činnost člověka, vedoucí k úbytku stanovišť vhodných pro přežití druhu. Lze tedy konstatovat, že zvonovec liliolistý není primárně ohrožen ztrátou genetické diverzity, ale spíše, jak vyplývá z terénních pozorování, ztrátou vhodných stanovišť. Výsledky této práce také identifikují několik oblastí v Evropě, které by z hlediska zachování genetické diverzity měly mít prioritu při zavádění ochranářských opatření.

Výsledky této disertační práce přinesly řadu nových poznatků o genetické diverzitě evropských populací zvonovce liliolistého a jejich vzájemných vztazích. Poodhalily také historii druhu v Evropě, a nastínily pravděpodobný ústup druhu ze Střední Evropy v souvislosti s hrozbou klimatické změny. Množství otázek spjatých s budoucností (ale i minulostí) zvonovce liliolistého ale zůstává nezodpovězeno. V České republice a Polsku je druh ohrožen především ztrátou vhodných biotopů. Velké riziko představuje minimální přirozená obnova jedinců v existujících populacích, bez které nelze dosáhnout zlepšení stavu a přirozené udržitelnosti populací. Další výzkum by tedy měl být zaměřen na studium faktorů, které obnově populací mohou pomoci (např. testy viability semen, experimenty s klíčivostí semen získaných z dalších lokalit, pokusy s výsevem semen a výsadbou mladých rostlin).

8 List of author’s publications

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