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THE GENUS *SYMPHYTUM* L. IN THE CENTRAL EUROPE: CHOROLOGY, CYTOGEOGRAPHY AND TAXONOMY

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“If you truly love nature, you will find beauty everywhere.” – Vincent Van Gogh

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ABSTRACT

In recent decades, the plant research has provided a satisfying picture of plant evolution, with polyploidization being recognised as a significant driver affecting the speciation and diversification of flowering plants. This thesis deals with the genus *Symphytum* (Boraginaceae), wherein polyploidy considerably shaped its evolution. The first part of the thesis aims at the revision of the genus *Symphytum* in the Central Europe, with the special focus to the Czech Republic. The distribution of particular taxa was studied based on the revised herbarium specimens, as well as own field research. As a result, nine taxa have been confirmed, with two alien taxa (i.e. *S. grandiflorum* and *S.* ×*hidcotense*) being reported for the first time. A detailed morphological description, habitat requirements, distribution and the history of cultivation (in the case of alien taxa) are provided for all *Symphytum* taxa from the studied area. In addition, two local (Czech Republic, Slovakia) identification keys have been compiled. The second part of the thesis investigates the morphological and ecological consequences of polyploidy within two taxonomically and evolutionary complicated polyploid complexes, namely *S. officinale* complex and *S. tuberosum* complex. With respect to the taxonomy of both groups, frequent polyploidy together with a high morphological variation often makes the delimitation of individual taxa quite problematic. In more detail, the ploidy level variation, cytotypes distribution patterns, morphological variation and ecological preferences of these two species' groups were studied using flow cytometry, multivariate morphometric analyses and ecological niche modelling, primarily on a Central European scale. In light of the results obtained, the taxonomic concept has been suggested for each group. Within the *S. officinale* complex, three cytotypes should be treated as separate species, i.e. diploid *S. bohemicum* F.W. Schmidt, tetraploid *S. officinale* s. str. and hypotetraploid *S. tanaicense* Steven. In contrast, the taxonomic treatment of cytotypes as subspecies has been proposed for the *S. tuberosum* complex, i.e. tetraploids as *S. tuberosum* subsp. *angustifolium* and dodecaploids as *S. tuberosum* subsp. *tuberosum*.

To sum up, the present thesis documents a complete overview of the representatives of the genus *Symphytum* in Central Europe. The results highlighted the cytotype diversity among Central-European members of the genus. Moreover, they emphasise the significance of

polyploidy and determined its direct morphological and ecological consequences, with regard to the taxonomy of both studied groups. Further studies should aim to support the proposed taxonomic value of both groups and to reveal the origin (auto- vs. allopolyploid) of given taxa using the methods of molecular biology.

Keywords: Boraginaceae, ecology, geographical distribution, herbarium revision, flow cytometry, morphology, polyploidy
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ABSTRAKT

V posledních desetiletích poskytl výzkum relevantní obraz o evoluci rostlin, přičemž polyploidie byla prokázána jako významná hnací síla ovlivňující speciaci a diverzifikaci kvetoucích rostlin. První část práce se věnuje revizi rodu kostival (*Symphytum*, čel. Boraginaceae) ve střední Evropě, především na území České republiky. Rozšíření jednotlivých druhů bylo zpracováno na základě revize herbářových položek, ale i vlastního terénního pozorování. Celkem byl v rámci této oblasti potvrzen výskyt devíti taxonů, z nichž dva (tzn. nepůvodní *S. grandiflorum* a *S.* ×*hidcotense*) představují první záznam pro českou flóru. Pro všechny taxony byl vypracován podrobný morfologický popis, specifikovány stanovištní nároky, popsáno celkové rozšíření a shrnuta historie pěstování nepůvodních, zavlečených taxonů. Navíc byly nově vytvořeny determinační klíče pro Klíč ke květeně České republiky a budoucí Malou flóru Slovenska. Druhá část práce se následně zabývá detailním studiem morfologických a ekologických důsledků polyploidie v rámci dvou taxonomicky komplikovaných polyploidních skupin, konkrétně okruhem k. lékařského (*S. officinale* agg.) a k. hlíznatého (*S. tuberosum* agg.). S ohledem na taxonomii obou skupin činí častá polyploidie, spolu s vysokou morfologickou variabilitou, vymezení jednotlivých taxonů značně problematické. Podrobněji byla v rámci obou skupin studována variabilita ploidní úrovně, struktura distribuce dílčích cytotypů, jejich morfologická variabilita a ekologické nároky, a to zejména za pomoci průtokové cytometrie, vícerozměrných morfometrických analýz a modelování ekologických nik. Na základě získaných výsledků byl pro každou skupinu navržen taxonomický koncept. V rámci okruhu k. lékařského jsou všechny cytotypy považovány za samostatné druhy, tj. diploidní *S. bohemicum* F.W. Schmidt, tetraploidní *S. officinale* s. str. a hypotetraploidní *S. tanaicense* Steven. Naproti tomu pro okruh k. hlíznatého bylo navrženo taxonomické hodnocení cytotypů pouze na úrovni poddruhů, tj. tetraploidní *S. tuberosum* subsp. *angustifolium* (A. Kern.) Nyman a dodekaploidní *S. tuberosum* subsp. *tuberosum*.

Předložená disertační práce představuje kompletní přehled zástupců rodu *Symphytum* ve středoevropské krajině. Získané výsledky zdůraznily význam polyploidie v evoluci rodu (vysoká cytotypová diverzita) a poukázaly na její přímé morfologické a ekologické důsledky s ohledem na taxonomii obou skupin. Výsledky vybízí k podrobnému zkoumání za pomoci

molekulárních metod, a to zejména za účelem podpory navržených taxonomických konceptů obou skupin a k odhalení původu (auto- vs. alopolyploidie) daných taxonů.

Klíčová slova: Boraginaceae, ekologie, geografické rozšíření, herbářová revize, morfologie, polyploidie, průtoková cytometrie
Počet stran: 184
Jazyk: anglický

DECLARATION

Hereby, I declare that this thesis is my own work and that I wrote this thesis independently using the mentioned references.

Olomouc ……..… ……..……

Mgr. Lucie Kobrlová

AUTHOR CONTRIBUTION STATEMENT

I declare that I have contributed to all papers included in this thesis and my contributions to particular chapters are as follows:

CHAPTER 1 General introduction and aims of the thesis.

Author wrote this part.

CHAPTER 2 **Kobrlová, L.**, Hroneš, M., Koutecký, P., Štech, M., Trávníček, B. 2016. *Symphytum tuberosum* complex in central Europe: cytogeography, morphology, ecology and taxonomy. *Preslia* 88: 77–112.

Study design, field sampling, lab work, data analyses, manuscript writing; author's contribution 55%.

CHAPTER 3 **Kobrlová, L.**, Mandáková, T., Hroneš, M. 2018. Taxonomic status and typification of a neglected name *Symphytum leonhardtianum* from the *Symphytum tuberosum* complex (Boraginaceae). *Phytotaxa* 349: 225–236.

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CHAPTER 4 **Kobrlová, L.**, Duchoslav, M., Hroneš, M. subm. Morphological, ecological and geographic differences between diploids and tetraploids of *Symphytum officinale* (Boraginaceae) justify both cytotypes as separate species. *AoB Plants.*

Field sampling, lab work, data analyses, manuscript writing; author's contribution 60%.

CHAPTER 5 **Kobrlová, L.**, Hroneš, M. 2019. *Symphytum* L. In: Kaplan, Z., Danihelka, J., Chrtek, J. Jr., Kirschner, J., Kubát, K., Štech, M., Štěpánek, J., eds. *Key to the flora of the Czech Republic [Klíč ke květeně České Republiky]. Ed. 2*. Academia: Praha, 817–818.

Author's contribution 70%.

CHAPTER 6 **Kobrlová, L.** subm. *Symphytum* L. In: Letz, D. R., ed. *Key to the flora of Slovakia [Malá flóra Slovenska – Kľúč na určovanie cievnatých rastlín].* Veda: Bratislava.

Author's contribution 100%.

CHAPTER 7 Distribution of the genus *Symphytum* L. in the Czech Republic – A three-part series of articles published in *Zprávy České Botanické Společnosti*, here shortened and translated into English.

SUBCHAPTER 7.1 **Kobrlová, L.**, Hroneš, M., Trávníček, B. 2016. The genus *Symphytum* (Comfrey) in the Czech Republic. I. *S. tuberosum* agg. [Rod *Symphytum* (kostival) v České republice. I. *S. tuberosum* agg.] *Zprávy České botanické společnosti* 51: 221–256.

Field sampling, herbarium revisions, lab work, data processing, manuscript writing; author's contribution 65%.

SUBCHAPTER 7.2 **Kobrlová, L.** 2017. The genus *Symphytum* (Comfrey) in the Czech Republic II. *S. officinale* agg. [Rod *Symphytum* (kostival) v České republice II. *S. officinale* agg.] *Zprávy České botanické společnosti* 52: 175–223.

Field sampling, herbarium revisions, lab work, data processing, manuscript writing; author's contribution 100%.

SUBCHAPTER 7.3 **Kobrlová, L.**, Hroneš, M. 2017. The genus *Symphytum* L. (Comfrey) in the Czech Republic III. Introduced and cultivated species [Rod *Symphytum* L. (kostival) v České republice III. Nepůvodní a pěstované druhy] *Zprávy České botanické společnosti* 52: 225–248.

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CHAPTER 8 General discussion.

Author wrote this part.

CHAPTER 9 Conclusion and future outlook.

Author wrote this part.

SUPPLEMENTARY FILE 1 Distribution of the genus *Symphytum* in the Czech Republic was also compiled and published as a part of the series *Distributions of vascular plants in the Czech Republic*:

Kaplan, Z., Danihelka, J., Lepší, M., Lepší, P., Ekrt, L., Chrtek, J., Kocián, J., Prančl, J., **Kobrlová, L.**, Hroneš, M., Šulc, V. 2016. Distributions of vascular plants in the Czech Republic. Part 3. *Preslia* 88: 459–544.

Fieldwork and herbarium revisions, data processing, distribution maps and comments relating to the genus Symphytum.

Other supporting files are attached as electronic supplements on CD-ROM.

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CONTENTS

Chapter 1

General introduction and aims of the thesis

Kobrlová Lucie

1. Polyploidy – the hidden force in plant evolution

Polyploidy, a state of having more than two complete sets of chromosomes in the nuclear genome, has been studied over 100 years (Husband *et al.* 2013, Soltis *et al.* 2014, Barker *et al.* 2016), and thanks to improving and sophisticated approaches (i.e. cytogenetical advances like flow cytometry and *in situ* hybridization, Kron *et al.* 2007, Han *et al.* 2015, Jiang 2019; or genomics methods, e.g. NGS, Illumina, Aversano *et al.* 2012, Dufresne *et al.* 2014, Kyriakidou *et al.* 2018) mysteries of this process have gradually been revealed, but still not satisfactory elucidated. Its great importance to evolution has frequently been proved in association with the diversification and speciation in plants (Soltis & Soltis 1999, Otto & Whitton 2000, Soltis *et al.* 2009, 2010, Jiao *et al.* 2011, Lavania 2020, Levin 2020), representing a driving force of biodiversity. Nowadays, polyploidy is generally considered to be a widespread phenomenon that has been involved in the evolution of flowering plants, and even more, recent studies have suggested that all angiosperms (except of *Amborella* Baill. with only the ancestral ancient polyploidy event shared, but no lineage-specific genome duplications, Amborella Genome Project 2013) have undergone at least one round of polyploidy in their evolutionary history (Soltis *et al.* 2009, Jiao *et al.* 2011, Weiss-Schneeweiss *et al.* 2013, Soltis *et al.* 2016, Wendel *et al.* 2016, van de Peer *et al.* 2017). Furthermore, from a global perspective, the relevance of polyploidy in the domestication process and crop improvement cannot be left out (Renny-Byfield & Wendel 2014). Traditionally, two paths of polyploid formation are recognised, i.e. (1) autopolyploidy (intraspecific polyploidy), arising within individuals of a single species as a result of the doubling of one chromosome set, and (2) allopolyploidy (interspecific polyploidy), involving hybridization, the merging of the chromosome sets of different species, and subsequent doubling (Soltis & Soltis 2009, van de Peer *et al.* 2017), of which the first-mentioned have generally been understudied (cf. Soltis *et al.* 2003, 2007, 2014, Parisod *et al.* 2010). However, the true nature of polyploids often does not enable an unambiguous interpretation and the genome evolution may be far more complex and challenging (cf. Soltis *et al.* 2003).

The consequences of polyploidy are complex, affecting many key features from genome to organismal level (e.g. Soltis & Soltis 2000, Levin 2002, Adams & Wendel 2005), and usually, polyploids differ from their diploid ancestors in phenotype, physiology, ecology or distribution pattern (Levin 2002, Ramsey & Schemske 2002). In this respect, polyploids often show higher ecological flexibility and improved stress response (cf. Hörandl 2022), appear to be more tolerant to abiotic (e.g. Saleh *et al.* 2008, Manzaneda *et al.* 2012, Hao *et al.* 2013, Dong *et al.* 2020, Ulum *et al.* 2021) as well as biotic stressors (van de Peer *et al.* 2021) in comparison with their diploid ancestors. Therefore, polyploids are often more widespread with increased competitive abilities and broader ecological niches (Levin 2002), also inhabit more extreme/diverse habitats (Brochmann *et al.* 2004, Marques *et al.* 2017, Rice *et al.* 2019) and frequently become invasive (Pandit *et al.* 2011, 2014, te Beest *et al.* 2012). Naturally, this is not a general rule regarding to all polyploids (e.g. niche contraction, Theodoridis *et al.* 2013, Kirchheimer *et al.* 2016). However, it seems that species with multiple, ecologically differentiated ploidy levels may be favoured during the colonization of large geographic ranges with heterogeneous environmental conditions (Manzaneda *et al.* 2012, te Beest *et al.* 2012, Hao *et al.* 2013).

In this context, the detailed study of geographic distribution patterns (both, global and local) of cytotypes in polyploid complexes is useful not only to detect the incidence of polyploids (e.g. rare, minority cytotypes, Mandáková & Münzbergová 2006, Trávníček *et al.* 2012, Rejlová *et al.* 2019), but also to outline the spatial arrangement of cytotypes and mechanisms affecting it, e.g. primary and secondary contact zones (*sensu* Petit *et al.* 1999, e.g. Kolář *et al.* 2009, Morgan *et al.* 2020, 2021), intermediate cytotypes (e.g. Kolář *et al.* 2009, Sabara *et al.* 2013, Zozomová-Lihová *et al.* 2015) or ploidy-mixed populations (e.g. Duchoslav *et al.* 2010, Trávníček *et al.* 2011, reviewed in Kolář *et al.* 2017). From a biological (and scientific) point of view, highly polyploid complexes with several ploidy levels (e.g. Balao *et al.* 2009, Sonnleitner *et al.* 2010, Duchoslav *et al.* 2020), and those with highly dynamic contact zones (e.g. Castro *et al.* 2018, Morgan *et al.* 2020, Čertner *et al.* 2022) are of special interest, allowing us to better understand to the regularities of polyploid formation/establishing and coexistence.

Besides that, the process of polyploidy often mediates morphological and physiological shifts in newly formed cytotypes, generally in the positive association (cf. Chansler *et al.* 2016, Pegoraro *et al.* 2019). Typically, polyploidy enlarges cell sizes (e.g. Otto & Whitton 2000, Gregory 2001, Levin 2002, Beaulieu *et al.* 2008), affects the size of organs (e.g. leaves, flowers, seeds) or overall habit (e.g. Balao *et al.* 2011, Tunbridge *et al.* 2011, Wu *et al.* 2012, Baker *et al.* 2017) and/or increases the number of structures (e.g. flowers per inflorescence, Vamosi *et al.* 2007). Consequently, it results in a high morphological variation in many polyploid groups. This is somehow challenging, since the practical botanical handbooks are based on the morphological features. Therefore, the taxonomic classification of polyploids still remains a matter of debate for taxonomists. Given the divergent morphology from their diploid ancestors, allopolyploids are usually considered as distinct taxa (see e.g. Hartmann *et al.* 2021), while autopolyploids are often regarded as conspecific with diploids (see e.g. Mandáková & Münzbergová 2008, Španiel *et al.* 2008) due to their high morphological similarity (reviewed in Soltis *et al.* 2007, see also Ramsey &Ramsey 2014). In the review of Soltis et al. (2007), authors argued for taxonomic recognition of autopolyploids after the careful revision and examination of the studied complex. However, such studies are still relatively sparse (e.g. Judd *et al.* 2007, Sosa & Dematteis 2014, Laport & Ramsey 2015).

Although we have learned a lot about polyploidy over the years (e.g. Levin 2002, Soltis *et al.* 2010, Weiss-Schneeweiss *et al.* 2013, Ramsey & Ramsey 2014, Wendel *et al.* 2016), there are still a lot of unknowns and questions to be answered. This is also linked to the fact, that new polyploids have constantly been discovered across plant kingdom and/or various biomes and world regions. However, the modern genomic era enables us to get a deeper insight into genome evolution and better understand the evolutionary role of polyploidy and its direct consequences and effects on distributional patterns, ecological requirements or morphological variations of polyploid plants. Therefore, taxonomic concepts may be properly revisited and/or newly proposed.

2. The family Boraginaceae

General description

Given the ongoing uncertainty over the exact placement and classification of the family Boraginaceae Juss. (e.g. Långström & Chase 2002, Nazaire & Hufford 2012, Weigend *et al.* 2013, Cohen 2014, summarised in Chacón *et al.* 2016), as well as the order Boraginales Juss. ex Bercht. et J. Presl (summarised in Luebert *et al.* 2016, recognised by APG IV 2016), its systematics has been discussed for a long time. Recently, the evaluation of traditional classifications of borage family using molecular approaches provided a very good resolution and supported the main clades of the family, including the placement of several problematic genera (Chacón *et al.* 2016). The family Boraginaceae includes about 95 genera and around 1600–1700 species of subcosmopolitan distribution and it is particularly diverse in temperate regions of the northern hemisphere (Chacón *et al.* 2016, 2017, Luebert *et al.* 2016, Weigend *et al.* 2016). Morphologically, it is readily distinguished by its mostly herbaceous habit, generally scorpioid inflorescences (= cincinni), characteristic pubescent, sericeous or hispid indumentum and especially by gynobasic style and the ovary subdivided into (1–)4(–8) eremocarpids in flower, developing into (1–2)–4 1-seeded, rarely of two 2-seeded, nutlets (Fig. 1.1; Buys & Hilger 2003, Luebert *et al.* 2016, Weigend *et al.* 2016). Furthermore, it is also known and widely studied due to the presence of various secondary metabolites (pyrrolizidine alkaloids as typical), because of their former and/or present use for medicinal preparations and dyes (e.g. Smith & Culvenor 1981, Ober & Hartmann 1999, Frölich *et al.* 2007, Damianakos *et al.* 2016, Weigend *et al.* 2016 and references therein, Dresler *et al.* 2017). A recent study of Chacón *et al.* (2016) resolved three major clades in Boraginaceae: (1) subfamily Echiochiloideae Weigend, the basal clade with an American–northern to northern African/western Asian trans-Atlantic disjunction between the Old World and the New World (Långström & Chase 2002), (2) a predominantly Mediterranean subfamily Boraginoideae Arn., and (3) subfamily Cynoglossoideae Weigend, the largest, widespread and most variable group by far. The last two subfamilies are further subdivided into several tribes and subtribes (see e.g. in Figs 1.1, 1.2; Chacón *et al.* 2016). Subsequently, the dated phylogeny estimated the Paleocene diversification of borage family initiated in Western Europe, followed by range expansions to the Irano-Turanian region and Eastern Asia and multiple events of long-distance dispersals to the Americas, Australia, and New Zealand (Chacón *et al.* 2017).

The evolutionary pitfalls – the lesson on the karyological variation within the borage family

Many botanists have been fascinated by the species diversity of the borage family, especially thanks to the heterogeneous morphology (Fig. 1.2). Traditionally, the morphological (incl. anatomy, micromorphology and palynology) and karyological studies have been widely used to elucidate the systematics and taxonomy of Boraginaceae members (e.g. Johnston 1924, 1927, 1954, Retief & Van Wyk 1997, Selvi & Bigazzi 2001, Peruzzi & Passalacqua 2008). In recent decades, various sophisticated methods (DNA markers, cytogenetic analyses) have also been successfully applied within the borage family, to provide a better insight into the plant

evolution and diversification. Thanks to these modern approaches, traditional taxonomic concepts of many borage groups have been revised, leading to substantial changes and clearer picture of relationships within given groups, as well as to the re-evaluation of genera delimitations based on their morphology (e.g. Selvi *et al.* 2002, Thomas *et al.* 2008, Cecchi & Selvi 2009, Weigend *et al.* 2010, Cohen 2011, Hasenstab-Lehman & Simpson 2012, Huang *et al.* 2013, Meudt *et al.* 2013, Cecchi *et al.* 2014, Otero *et al.* 2014, Coppi *et al.* 2015, Holstein *et al.* 2016a,b, Serrano *et al.* 2016, Selvi *et al.* 2017, Simpson *et al.* 2017, Mabry & Simpson 2018, Chacón *et al.* 2019, Cohen 2021).

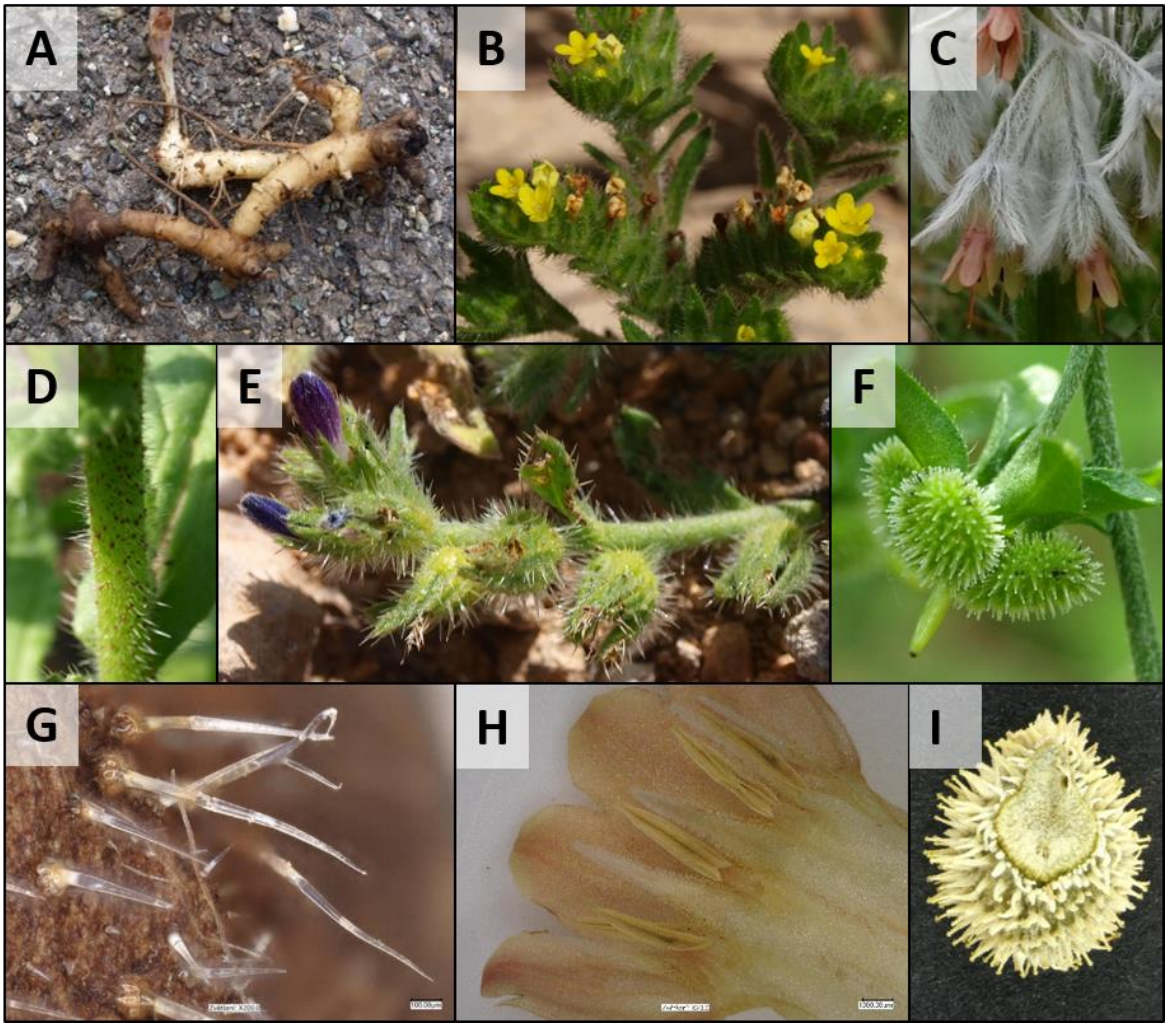

FIGURE 1.1. Basic morphological features of the borage family. (**A**) Tuberous rhizomes with spaced bulb-like thickenings of *Symphytum tuberosum* agg. (Boragineae Rchb.). (**B**) Double cymoids of *Neatostema apulum* (L.) I.M. Johnst. (Lithospermeae Dumort.). (**C**) Densely sericeous indumentum of *Rindera umbellata* (Waldst. & Kit.) Gürke (Cynoglosseae W.D.J. Koch). (**D**) Irritant hairs (Ensikat *et al.* 2021) of *Borago officinalis* L. (Boragineae). (**E**) Hispid indumentum of *Echium sabulicola* Pomel (Lithospermeae). (**F**) Schizocarp of four mericarpids (= nutlets) of *Cynoglossum hungaricum* Simonk. (Cynoglosseae) with glochidiate surface. (**G**) A detail of hairs on calyx of *Pulmonaria montana* subsp. *montana* (Boragineae). (**H**) Five scale-shaped intrusions (= faucal scales, fornices) near corolla throat alternating with anthers of *Symphytum tuberosum* agg. (**I**) Glochidiate nutlet of *Cynoglossum cheirifolium* L. (Cynoglosseae).

From the karyological point of view, the borage family appears to be a suitable group to study since it possesses a considerable variation in terms of chromosome number, ploidy level and karyotype morphology together with a range of chromosomal aberrations (e.g. Britton 1951, Luque 1992, Štěpánková 1996, Bigazzi *et al.* 1999, Selvi & Bigazzi 2002, Bigazzi & Selvi 2003, Coppi *et al.* 2006, Selvi *et al.* 2006, 2009, Mártonfi *et al.* 2008, summarised in Weigend *et al.* 2016). However, the number of species investigated to date is still scanty (ca 35%, Weigend *et al.* 2016), and the karyological diversity in the majority of the taxa is unknown. This is well illustrated by the absence of any chromosome report in the subfamily Echiochiloideae (cf. Weigend *et al.* 2016). At the tribe level, the greatest chromosomal variation has been reported within the tribe Boragineae (subfam. Boraginoideae, according to Chacón *et al.* 2016; e.g. Coppi *et al.* 2006, reviewed in Weigend *et al.* 2016), which is at the same time the most comprehensively studied clade (Weigend *et al.* 2016). Apart from this, the study of chromosomes often provides taxonomically valuable data and also a relevant insight into the possible mechanisms involved in speciation of given groups (e.g. Luque & Valdés 1984, 1986, Štěpánková 1993a,b, Selvi & Bigazzi 2001, 2002, Bigazzi & Selvi 2003, Mártonfi *et al.* 2008).

Based on several studies, the presence of polyploidy seems to be associated with the chromosome variation in a number of groups (e.g. Strey 1931, Britton 1951, Murín & Májovský 1982, Selvi & Bigazzi 2002, 2003, Cecchi & Selvi 2009). So far, the high frequency of polyploids among Boraginaceae relatives has been documented within the genera *Anchusa* s. l. (2*x*–6*x*), *Buglossoides* Moench (2*x*–6*x*), *Lappula* Moench (2*x*–6*x*), *Myosotis* L. (2*x*–9*x*), *Nonea* Medik. (2*x*–10*x*), *Paramoltkia* Greuterand (~14*x*) and *Symphytum* L. (2*x*–21*x*), with the last-mentioned genus being karyologically most variable (Kobrlová unpubl.). The published data indicating/confirming the presence of polyploidy are based only on a few chromosome counts reported for given taxa; almost no systematic studies have been published yet (but see e.g. Selvi *et al.* 2006). In general, the auto- or allopolyploid origin of most of the taxa is unknown, or possibly only hypothesised. Likewise, the knowledge of the diversity and geographical distribution of cytotypes in nature is almost missing. From the morphological, ecological and karyological viewpoints, an allopolyploid origin of some taxa has been suggested, for example within the genera *Phyllocara* Gușul. (Bigazzi *et al.* 1999) and *Nonea* (Selvi & Bigazzi 2002, 2003). In the genus *Onosma* L., the evolutionary significance of hybridization and an allopolyploid speciation have also been supported by molecular data (Kolarčik *et al.* 2010, 2014). Besides that, autopolyploidization event appears to be also involved in the evolution of the genus *Onosma* (*O. fastigiata* Braun-Blanq., Kolarčik *et al.* 2015). Furthermore, autopolyploidy is the assumed path of the cytotype formation also within *Lithodora fruticosa* (L.) Griseb. (Luque & Valdés 1984) and *Borago pygmaea* (DC.) Chater et Greuter (Selvi *et al.* 2006). Although the studies examining the cytogeographic pattern of polyploid groups within the Boraginaceae family are scarce, there is evidence of the sympatric (*Nonea persica* Boiss., Bigazzi & Selvi 2003) and allopatric distribution (*Myosotis lamottiana* Braun-Blanq., Štěpánková 2001; *Buglossoides arvensis* s. l., cf. Coppi *et al.* 2006; *Borago pygmaea*, Selvi *et al.* 2006) of diploid–polyploid populations. Last but not least, in some taxa (e.g. *Pulmonaria* spp.), the chromosome number variation has possibly arisen as a result of dysploid or aneuploid changes (e.g. Grau 1968, 1971, Sauer 1975, Bolliger 1982, Luque & Valdés 1984, Selvi *et al.* 2006, 2009).

In recent years, the method of flow cytometry (FCM) is the most common way of DNA-ploidy level, genome size (GS) and DNA base composition (GC content) estimations (Doležel *et al.* 2007, Šmarda *et al.* 2008, 2012, Bourge *et al.* 2018, and references therein), which foreshadows indisputable benefits in the study of polyploids and/or cryptic species (e.g. Prančl *et al.* 2014, 2020, Čertner *et al.* 2017, 2022, Padilla-García *et al.* 2018, Duchoslav *et al.* 2020). Although it has been successfully applied in many plant groups (Leitch *et al.* 2019), the borage family is still among the least studied plant groups (Kobrlová & Hroneš 2019). In this respect, the family Boraginaceae belongs to somehow challenging group, due to the characteristic hairy indumentum and the presence of various chemical compounds (Kolarčik *et al.* 2018, Kobrlová & Hroneš 2019). Hence the long-term sample storage is sometimes impossible, the isolation of nuclei is more difficult and the quality of analysis is often substandard. Even so, several studies using FCM have been published (see Kobrlová & Hroneš 2019 and references therein). Moreover, the cytological data known so far suggest the greatest GS variability of Boraginaceae among lamiids (Kobrlová unpubl.). In general, the GS variation can arise due to changes in chromosome numbers (genome duplications, aneuploidy, dysploidy), hybridization events or activity of transposable elements (e.g. Macas *et al.* 2015, Wendel *et al.* 2016, Siljak-Yakovlev *et al.* 2017, Senderowicz *et al.* 2021). The pilot study exploring the DNA content of Czech Boraginaceae suggested the proliferation of transposable elements and other types of repeats as the major driving force of GS variability (Kobrlová & Hroneš 2019). Taxonomically, FCM data may serve as an additional and reliable marker (quickly acquired) for species determination (Chumová *et al.* 2005, Prančl *et al.* 2014, 2020).

In view of the karyotype morphology, except of A chromosomes, the occurrence of supernumerary B chromosomes (see e.g. Camacho *et al.* 2000, Jones & Houben 2003, Palestis *et al.* 2004, Houben *et al.* 2014) has also been observed in several genera: *Cynoglottis* (Guşul.) Vural et Kit Tan (Markova & Goranova 1995), *Nonea* (Bigazzi & Selvi 2003), *Onosma* (Teppner 1971, 1991, Peruzzi & Passalacqua 2008), *Paramoltkia* (Cecchi & Selvi 2009), *Pulmonaria* L. (Sauer 1975), *Solenanthus* Ledeb. (Constantinidis & Kamari 1995), *Symphytum* (Grau 1971, Gadella & Kliphuis 1967, 1978). The role of B chromosomes in the karyotype and their evolutionary relatedness within borage family is not clarified. Moreover, they can be overlooked since their occurrence within a species is not regular and they can vary in number between (and within) individuals. Therefore, their presence in individuals of one species as well as a distribution pattern among species depends on the intensity of study, as shown by the example of the variation of B chromosomes among Dutch populations of *Symphytum bohemicum* F.W. Schmidt: $2n = 24 + 0–4B$, Gadella & Kliphuis 1967). Among flowering plants, B chromosomes are more likely to occur in outcrossing species than in inbred ones and, conversely, they seem not to be more frequent in polyploids than in diploids and not even in species with multiple ploidies or with small genomes (Palestis *et al.* 2004, Trivers *et al.* 2004, Levin *et al.* 2005).

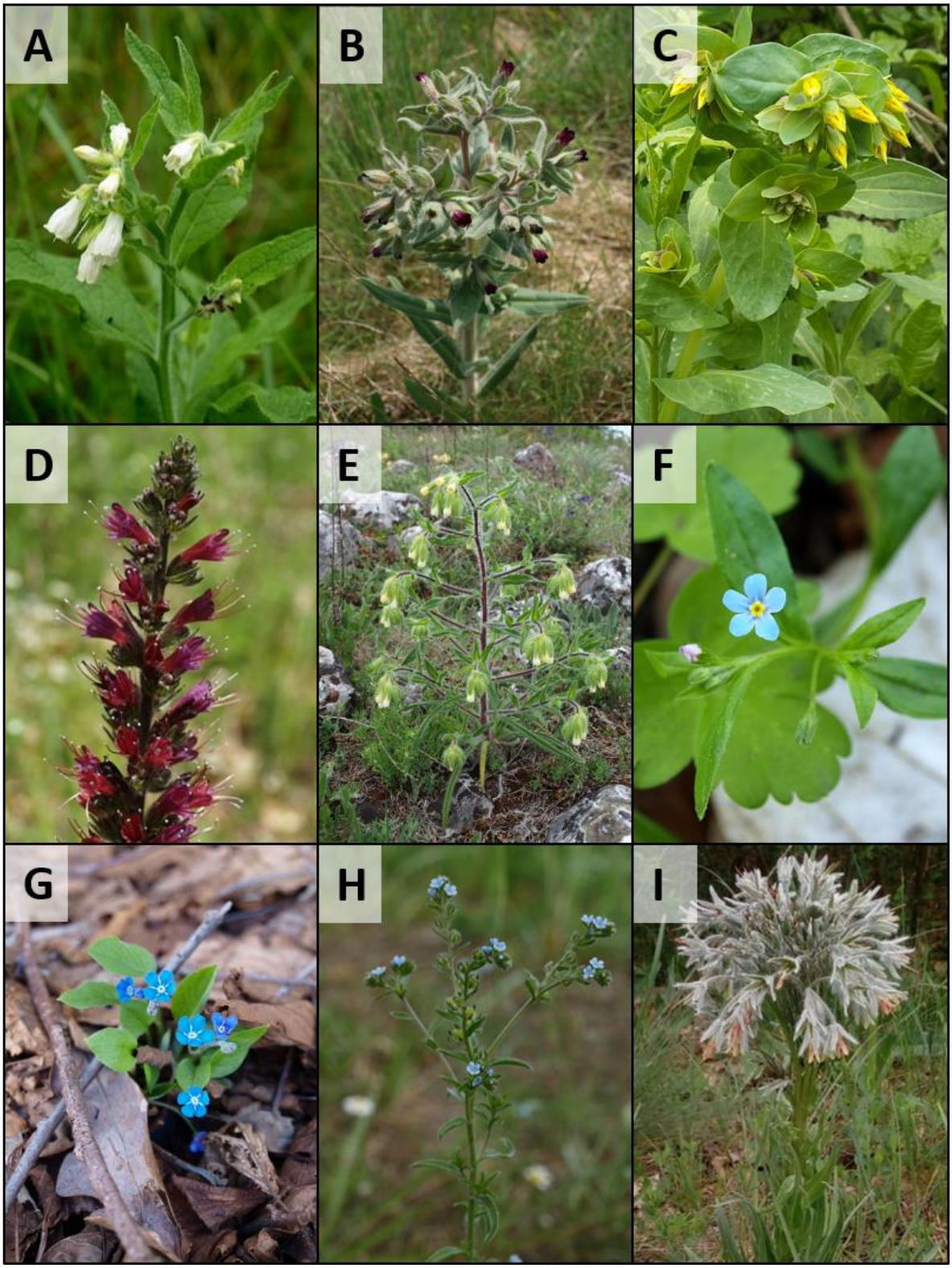

FIGURE 1.2. The morphological diversity in the Boraginaceae family. (**A**) *Symphytum bohemicum*. (**B**) *Nonea pulla* (L.) DC (both Boragineae). (**C**) *Cerinthe minor* L. (**D**) *Pontechium maculatum* (L.) Böhle & Hilger. (**E**) *Onosma visianii* Clem. (all Lithospermeae). (**F**) *Memoremea scorpioides* (Haenke) Otero, Jim.-Mejías, Valcárcel & P.Vargas (Asperugeae Zakirov ex Ovczinnikova). (**G**) *Omphalodes verna* Moench. (Omphalodeae Weigend). (**H**) *Lappula squarrosa* (Retz.) Dumort (Rochelieae DC.). (**I**) *Rindera umbellata* (Cynoglosseae).

3. The genus *Symphytum*

General description

The genus *Symphytum* (subfam. Boraginoideae, Boragineae, *sensu* Chacón *et al.* 2016) is an Eurasian genus whose range includes almost the whole Europe, Asia Minor and part of Southwest Asia and Siberia (Bucknall 1913, Wickens 1978, Hultén & Fries 1986, Malyschev 1997), with the centre of distribution in the Pontic area and in the western parts of the Irano-Turanian region (cf. Gadella & Kliphuis 1978, Wickens 1978). Thus, the majority of taxa occur in Turkey and the adjacent areas (cf. Wickens 1969, 1978). In addition, some species have been reported as introduced to North (Gadella 1984) and South America (Ariza-Espinar 2006), China (Zhu *et al.* 1995) or New Zealand (Hultén & Fries 1986), mainly due to their economic importance (medicinal and nectar-bearing plants, livestock fodder or ornamentals).

Morphologically, the genus is well-defined by creeping, often fleshy rhizomes, alternate leaves, double scorpioid cymes (= double boragoids) with almost completely united tubular corollas and five, triangular to lanceolate fornices inside the flowers (Fig. 1.3; Pawłowski 1972). In general, *Symphytum* species mostly represent mesophytic forest herbs (Weigend *et al.* 2016), but some species also inhabit wet meadows and wetlands (e.g. Gadella & Kliphuis 1973, Peruzzi *et al.* 2001), rock crevices (e.g. Runemark 1967, Stearn 1985) and can occasionally be found also in ruderal and disturbed habitats, such as road verges, railways and grasslands in settlements (cf. Bomble & Schmitz 2013, Kniely 2015).

With approximately 40 species (cf. Bucknall 1913, Wickens 1978), it represents the fourth largest genus of the subfamily, after the genera *Onosma* (150), *Lithospermum* L. (80) and *Echium* L. (60, Chacón *et al.* 2016). Historically, the genus has been divided into several sections (and series), according to the given author (Bucknall 1913, Pawłowski 1961, 1971a,b, Wickens 1969, Sandbrink *et al.* 1990). In this context, the recent molecular analyses of the genus suggested the division into nine sections (Table 1.1, Hacıoğlu & Erik 2011). The most widespread and cultivated species are the members of the sections *Officinalia* Buckn. (i.e. *S. officinale* L.), *Coerulea* Buckn. (i.e. *S. asperum* Lepech. and *S.* ×*uplandicum* Nyman) and *Lingulata* Pawł. (i.e. *S. grandiflorum* DC. and *S. orientale* L.; Pawłowski 1961). On the other hand, the distribution range of several species is restricted only to a narrow area, e.g. *S. caucasicum* Bieb. (i.e. Caucasus Mts., Wickens 1969) and *S. tanaicense* Steven (i.e. scattered across Europe, esp. eastern parts, Peruzzi *et al.* 2001), with some taxa being even endemic, e.g. *S. pseudobulbosum* Azn. (i.e. Asiatic side of the Bosporus, Wickens 1969, Kurtto 1985), *S. sylvaticum* Boiss. (i.e. Turkey, Wickens 1978, Özgişi & Tarıkahya-Hacıoğlu 2021) and *S. davisii* s. l. (i.e. Cyclades and East Aegean Islands, Pawłowski 1971a, Stearn 1985; the last-mentioned being a member of the former genus *Procopiania* Guşul., see below). In addition, the distribution pattern of some taxa is uncertain due to taxonomic confusion and frequent misidentifications (e.g. *S. bohemicum*).

Like some other genera of the borage family, the genus *Symphytum* (especially *S. officinale* and *S.* ×*uplandicum*) has been traditionally used in practical herbal medicine (comfrey cream as typical), mainly in relation to the musculoskeletal system (e.g. joint disorders, pulled muscles and bone fractures). Apart from the external application, the use of tea for liver problems, ulcers and haemorrhoids has also been documented (Mei *et al.* 2005, Frost *et al.* 2013). However, plants contain some level of pyrrolizidine alkaloids (PAs),

known to be hepatotoxic (Culvenor *et al.* 1980, Smith & Culvenor 1981, Yeong *et al.* 1990, Rode 2002, Mei *et al.* 2005, 2010, Malik *et al.* 2021). Nevertheless, the poor transcutaneous absorption of PAs contributes to the safety of herb comfrey cream (Kuchta & Schmidt 2020). Similarly, seed oil has become more popular (together with *Echium* and *Borago* oils, respectively) since it is very rich in highly unsaturated fatty acids and tocopherols, while is very low in PAs (e.g. Guil-Guerrero *et al.* 2003, Weigend *et al.* 2016). As within other PA-producing species, the PAs are constitutively produced as a part of the plant's chemical defence strategy (e.g. Ober & Hartmann 1999, Macel 2011, reviewed in Schramm *et al.* 2019). Recently, the specific dynamics of PA biosynthesis and accumulation during the inflorescence development have been described within the species *S. officinale* (Stegemann *et al.* 2019). Last but not least, several studies on closely related *Symphytum* taxa, using the PAs content as an additional chemo-taxonomical marker, have been published (Huizing *et al.* 1982, 1983, Gadella *et al.* 1983, Jaarsma *et al.* 1989, 1990).

Section	**N species**	**2*n***	***x***	**Reference**
Officinalia Buckn.	3–4	24, 40, 48	12	e.g. Gadella & Kliphuis (1967), Basler (1972), Wcisło (1972), Gadella *et al.* (1983), Wille (1998), Peruzzi *et al.* (2001)
Coerulea Buckn. (incl. sec. *Caucasica* Gviniasch.)	5–7	24, 32, 36, 40	12	e.g. Basler (1972), Gviniashvili (1972), Gadella *et al.* (1983), Gagnidze *et al.* (2015)
Albida Buckn.	3	24	12	e.g. Gviniashvili (1972), Gadella & Kliphuis (1978), Gagnidze *et al.* (2015)
Orientalia Buckn.	3–4	32	8	e.g. Markova (1983), Markova & Goranova (1995), Bottega *et al.* (2001)
Suborientalia Buckn.	3	?	?	–
Tuberosa Buckn.	2–4	32, 64, 96, 128, 144	8	e.g. Grau (1968), Wcisło (1972), Murín & Májovský (1982), Jaarsma *et al.* (1990), Bottega & Garbari (2003)
Cordata Buckn.	2–3	60, 120	10	e.g. Gviniashvili (1972), Wcisło (1972), Murín & Májovský (1982), Gagnidze *et al.* (2015)
Bulbosa Kuzn.	3	48, 84, 96, 104, 120 20 (*S. ottomanum*)	8 10	e.g. Grau (1971), Gadella & Kliphuis (1978), Markova & Goranova (1995), Johnson & Brandham (1997), Bottega *et al.* (2001), Peruzzi (2003), Coppi *et al.* (2006)
Procopiania (Guşul.) Wick.	4–6	28, 30	7	Pawłovski (1982), Runemark (1967)
Graeca Pawl.	1–2	30	10	Runemark (1967)

TABLE 1.1. Infrageneric classification of the genus *Symphytum*, compiled and adapted according to Bucknall (1913), Pawłowski (1961, 1971a,b), Wickens (1969), Sandbrink *et al.* (1990) and Hacıoğlu & Erik (2011). N species – number of species, adapted in relation to the Euro+Med Plantbase (Valdés 2011); 2*n* – the main, prevailing chromosome counts for given section; *x* –assumed basic chromosome number for given section.

Taxonomical challenges of the genus

Despite the abundant literature on taxonomy of the genus *Symphytum* (e.g. Bucknall 1913, Pawłowski 1961, Wickens 1969, Pawłowski 1971a,b, Stearn 1985, Sandbrink *et al.* 1990), studies dealing with the evolutionary insight of the relationships among species are almost missing (but see Sandbrink *et al.* 1990, Hacıoğlu & Erik 2011, Özgişi & Tarıkahya-Hacıoğlu 2021). Therefore, the taxonomic value of some described taxa is still uncertain because of taxonomic difficulties and unclear delimitations, and thus, detailed revisions are required. Unfortunately, this kind of studies, using multi-approach revisions, is still rare in terms of the whole family (but see e.g. Selvi & Bigazzi 2001, Thomas *et al.* 2008, Kolarčik *et al.* 2010, Meudt *et al.* 2013). Within the genus *Symphytum*, only a few studies revisiting controversial taxonomic treatments of the given group have been published so far (Kurtto 1982, 1985, Akcin & Baki 2007, Tarıkahya & Erik 2010, Özgişi & Tarıkahya-Hacıoğlu 2021). Mostly, these studies are based on the comparison of morphological characters, in order to evaluate their ranges of variation, with the disentanglement of phylogenetic relationships being rare (but see Tarıkahya & Erik 2010, Özgişi & Tarıkahya-Hacıoğlu 2021).

At the beginning of the 20th century, the separate genus *Procopiania*, closely resembling the genus *Symphytum*, was described (divided from the genus *Trachystemon* D. Don, Gușuleac 1928). The genus *Procopiania* is characterised by deeply divided corollas, corolla lobes being longer than corolla tube (sometimes spirally contorted above) and long-exserted stamens (Pawłowski 1971b, 1972). In more recent studies, the genus *Procopiania* was accepted by some authors (Riedl 1963, Pawłowski 1971b, 1972, Stearn 1985), but not by others (Runemark 1967, Wickens 1969, 1978, Sandbrink *et al.* 1990). These authors considered *Procopiania* to be congeneric with *Symphytum* and the section *Procopiania* (Gușul.) Wick. (Wickens 1969) was proposed. Later on, Hilger *et al.* (2004) showed that the members of section *Procopiania* are nearly identical to the relatives of *Symphytum* s. str. in both nuclear (ITS) and chloroplast (*trn*L_{UAA} intron) sequences, that was subsequently confirmed by the study of Hacıoğlu & Erik (2011).

As mentioned above, the great variation in terms of karyology has been documented in the borage family. Within the genus *Symphytum*, intra- and inter-specific variation of chromosome numbers have also been reported (e.g. Gadella & Kliphuis 1971, Basler 1972, Gadella & Kliphuis 1973, 1978). Moreover, available literature data indicate that it is probably the karyologically most complex genus of the whole family (cf. Weigend *et al.* 2016). In particular, polyploidization and interspecific hybridization have considerably shaped the evolutionary patterns within the genus (e.g. Grau 1968, Gadella & Kliphuis 1967, 1969, 1974, 1978, Grau 1971, Basler 1972). The high frequency of polyploidy in several European species groups of *Symphytum* has been documented in the literature (e.g. Grau 1971, Wcisło 1972, Gadella & Kliphuis 1978, Murín & Májovský 1982), but the data available are far from comprehensive. Moreover, little is known about the auto- or allopolyploid origin of any given species (e.g. Gadella & Kliphuis 1972, 1973).

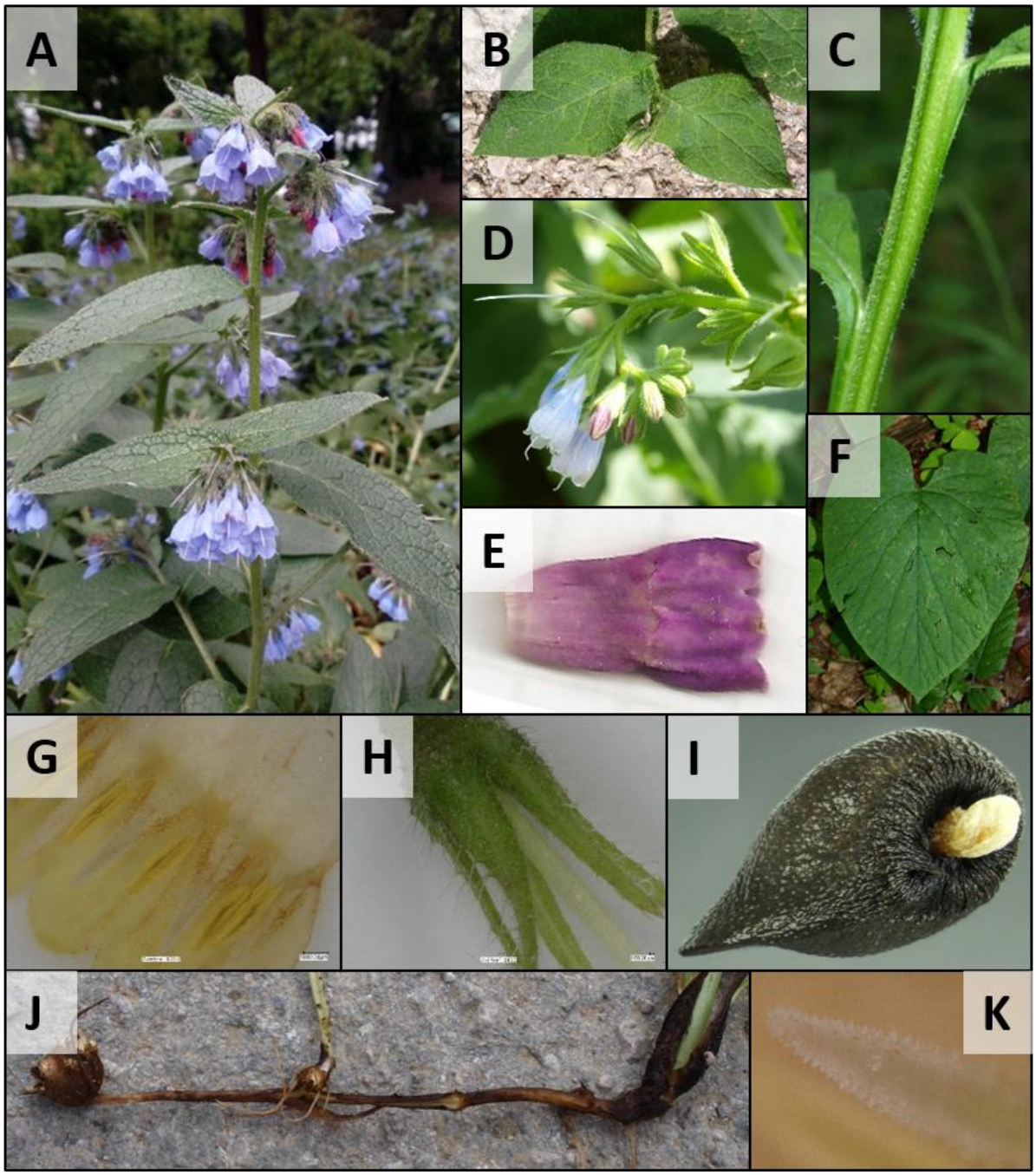

FIGURE 1.3. Several relevant morphological features of the genus *Symphytum*. (**A**) Perennial, softly hirsute, greyish plant of *S. caucasicum* with blue corollas. (**B**) Sessile upper cauline leaves forming bracts of the inflorescence of *S. tuberosum* agg. (**C**) Shortly winged stem from decurrent leaf bases of *S. bohemicum*. (**D**) Scorpioid cyme (= boragoid) with cylindrical to campanulate corollas of *S. asperum*. (**E**) Tubular-infundibuliform corolla of *S. officinale*. (**F**) Large, cordate leaf of *S. cordatum* Waldst. et Kit. ex Willd. (**G**) Five, triangular fornices alternating with anthers of *S. tuberosum* agg. (**H**) Detail of the campanulate calyx divided into five calyx lobes of *S. tuberosum* agg. (**I**) Smooth, shiny nutlet of *S. officinale*. (**J**) Tuberous rhizomes with spaced bulb-like thickenings of *S. bulbosum* K.F. Schimp. (**K**) Papillate apex of the faucal scale of *S. tuberosum* agg.

Altogether, up to 34 various chromosome counts covering approximately half of the genus have been published so far (Table 1.1, Kobrlová unpubl.). Judging from these counts, there is a controversy over the basic chromosome number and therefore, more than one base number is suggested by various authors, i.e. x = 8, 9, 10, 12, 14, 15, 18 (e.g. Strey 1931, Britton 1951, Markova & Ivanova 1970, Grau 1971, Gadella & Kliphuis 1978, Murín & Májovský 1982, Shirato *et al.* 1985, Luque 1989). Moreover, small chromosomes, their conspicuous stickiness and a high degree of polyploidy make it difficult to count chromosomes accurately (Strey 1931, Wcisło 1972, Mekki *et al.* 1987). In addition, the occurrence of supernumerary B chromosomes has also been documented in the karyotype of *S. bohemicum* (e.g. Gadella & Kliphuis 1966, 1967, 1972, 1978). To date, this is the only *Symphytum* species with reported B chromosomes, but it is most probably due to intensive karyological investigations of *S. officinale* complex (e.g. Gadella & Kliphuis 1967, 1969, 1972). Considering all published chromosome records, x = 8 and x = 12 best fit to the majority of the previous karyological surveys (Table 1.1, Kobrlová unpubl.). Remarkably, the most of the chromosome counts suggest some level of polyploidy with diploids ($2n$ = 20, 24) being very rare ($2n$ = 24: *S. bohemicum*, *S. caucasicum* and *S. ibericum* Steven; $2n$ = 20: *S. ottomanum* Friv., e.g. Gadella & Kliphuis 1967, 1978, Markova & Ivanova 1970, Gviniashvili 1972, Markova & Goranova 1995, Wille 1998, Coppi *et al.* 2006, Gagnidze *et al.* 2015). Besides that, Tarnavschi (1948) published another diploid count ($2n$ = 18, the lowest chromosome number of the genus at all) for four taxa, namely *S. cordatum*, *S. ottomanum*, *S. tauricum* Willd. and *S. tuberosum*. However, this chromosome count is most likely erroneous (e.g. *S. cordatum* possesses, in fact, $2n$ = 120, e.g. Wcisło 1972, Murín & Májovský 1982) and has never been established again for any of the species mentioned. In view of this fact, in several polyploid species groups (e.g. *S. orientale*, *S. bulbosum* and *S. tuberosum* complexes), the diploid congeners have not yet been documented at all (Kobrlová unpubl.). This may be related to the absence of a detailed systematic study of these species' groups and thus, the existence of diploid congener has not been revealed yet. Alternatively, diploid progenitors may already be eliminated in populations and/or completely excluded by polyploids (cf. Kolář *et al.* 2017). Generally, this would be aided by the speciation genetics, leading to determinate the origin of polyploid groups and to provide an insight into the mechanisms of formation of polyploids and, therefore, to reveal their evolutionary history.

Aims of the thesis

The main goal of the thesis is the revision of the genus *Symphytum* in the Central Europe, with a special focus on the Czech Republic. Based on field and herbarium investigations, a systematic synthesis of the genus *Symphytum* in the Czech Republic is provided, including all native, naturalised and occasionally cultivated taxa. A detailed overview of morphological characters, ecological differentiation and habitat preferences are given for each species, together with an updated identification key. In addition, distribution maps are outlined and a list of vouchers and field data are included. Furthermore, an analytical key of the genus for Slovak flora has been revised, based on new discoveries and novel information on the taxonomy of *Symphytum* relatives in the Central Europe.

The general aim of this thesis, i.e. the revision of the genus *Symphytum* in the Central Europe and compilation of determination keys and materials to local Floras (i.e. Czech Republic and Slovakia), follows specific objectives:

- What is the diversity of the genus *Symphytum* in the Central Europe?
- How many species occur in the Czech Republic? How are these species distributed?
- Which non-native species have been introduced to the Central Europe, and especially to the Czech Republic?
- Which morphological characters are the most useful for species determination?
- How frequent is hybridization among the (Central) European species?

In the Central Europe, the genus *Symphytum* is particularly represented by members of the two polyploid and taxonomically complicated groups, namely *S. officinale* and *S. tuberosum* complexes, respectively.

Symphytum officinale complex

A widespread perennial herb *Symphytum officinale* and its closest relatives constitute an intricate diploid–tetraploid complex with unresolved taxonomy. Polyploidy and dysploidy are considered the major evolutionary forces shaping the diversity of this group. Several cytotypes, especially diploids ($2n = 24$), hypotetraploids ($2n = 40$) and tetraploids ($2n = 48$), have been reported (e.g. Gadella & Kliphuis 1969, 1972, Gadella 1972, Murín & Májovský 1982, 1987). According to published records, only the tetraploid populations should be considered as *S. officinale* s. str., whereas diploids correspond to *S. bohemicum* and hypotetraploids to *S. tanaicense* (syn. *S. uliginosum* A. Kern. or *S. officinale* subsp. *uliginosum* (A. Kern.) Nyman, Gadella & Kliphuis 1969, Smejkal 1978, Gadella *et al.* 1983, Májovský & Hegedüšová 1993). However, taxonomic concept of this species group is still not satisfactorily resolved and thus, only one polymorphic species *S. officinale* is generally accepted.

Symphytum tuberosum complex

The *Symphytum tuberosum* complex, encompassing perennial, yellow-flowered, mesophytic forest herbs with stolon tubers (Weigend *et al.* 2016), belongs to the most polyploid and morphologically variable groups of the genus. Given all published chromosome data, eight ploidy levels and several additional aneuploid counts are reported for this complex. Even more surprisingly, the existence of the diploid ancestor is disputable and has not yet been confirmed (Kobrlová unpubl.). Moreover, up to 10 taxa (in chronological order: *S. tuberosum* L. s. str., *S. mediterraneum* W.D.J. Koch, *S. angustifolium* A. Kern., *S. nodosum* Schur, *S. foliosum* Rehmann, *S. gussonei* F.W. Schultz, *S. floribundum* Shuttlew. ex Nyman, *S. leonhardtianum* Pugsley, *S. besseri* Zaver., *S. popovii* Dobrocz.; Linnaeus 1753a, Koch 1837, Kerner 1863, Schur 1866, Rehmann 1868, Schultz 1872, 1875, Nyman 1881, Pugsley 1931, Zaverucha 1962, Dobroczajeva 1968) have been described so far, but only two taxa are currently recognised (Valdés 2011). Thus, its taxonomic treatment is challenging and needs to be revised.

Within the scope of these two groups, a detailed revision at Central-European landscape is provided, to gather consistent cytological, morphological and ecological information aiming to reinforce the existing knowledge and indicate the evolutionary relationships within both groups, and thus to support the taxonomic revision. Specifically, the following objectives are addressed:

- What is the cytotype diversity of *S. officinale* and S. *tuberosum* complexes? What is the pattern of their distribution in the Central Europe?
- How frequent are mixed-ploidy populations? Which cytotypes participate in their composition?
- How strong is the niche differentiation between different cytotypes? Is there a different pattern of niche shift with increasing ploidy level?
- What are the morphological differences between cytotypes of both complexes?
- How significant are all of these findings for the taxonomy of both complexes?

The thesis consists of the following parts that aims to answer/discuss the above-mentioned questions:

CHAPTER 2 *Symphytum tuberosum* complex in central Europe: cytogeography, morphology, ecology and taxonomy

This part uncovers patterns of the hidden diversity of the *S. tuberosum* complex in the Central Europe. Based on flow cytometric screening, two major cytotypes (4*x*, 12*x*) are detected. In more detail, their geographic distributions, morphological differences and habitat requirements are evaluated. In view of the results, a taxonomic classification of these cytotypes as subspecies is proposed, i.e. *S. tuberosum* subsp. *tuberosum* (12*x*) and *S. tuberosum* subsp. *angustifolium* (4*x*).

CHAPTER 3 Taxonomic status and typification of a neglected name *Symphytum leonhardtianum* from the *Symphytum tuberosum* complex (Boraginaceae)

The third chapter aims to investigate the taxonomic identity of the name *S. leonhardtianum* (described from the vicinity of Vienna, Austria). Considering all available evidence, plants from the locus classicus of *S. leonhardtianum* do not differ substantially from the nominate subspecies of *S. tuberosum*. Therefore, this name is proposed as a heterotypic synonym of *S. tuberosum* subsp. *tuberosum*.

CHAPTER 4 Morphological, ecological and geographic differences between diploids and tetraploids of *Symphytum officinale* (Boraginaceae) justify both cytotypes as separate species

In this part, the polyploid *S. officinale* complex is studied, to explore the cytotype diversity in Europe and its effect on the taxonomy. Using flow cytometry, two main cytotypes (2*x*, 4*x*) are identified. Both of them are morphologically well differentiated and ecologically segregated. In addition, cytotypes show a diffuse parapatric pattern of distribution, with rare mixed-ploidy populations, suggesting reproductive isolation between them. Therefore, both cytotypes are considered as separate species, i.e. *S. bohemicum* (2*x*) and *S. officinale* (4*x*).

CHAPTER 5 An identification key of the genus *Symphytum* in the Czech Republic

The chapter five provides an updated version of the identification key of the genus *Symphytum* in the Czech Republic, according to the results of Chapters 2 and 4.

CHAPTER 6 An identification key of the genus *Symphytum* in Slovakia

This chapter presents a draft of the new identification key of the genus *Symphytum* in Slovakia, which is compiled for the new edition of the Key to the flora of Slovakia.

CHAPTER 7 Distribution of the genus *Symphytum* L. in the Czech Republic

This chapter summarises the historical and current distribution of the genus *Symphytum* in the Czech Republic. In addition, an overview of morphological characters, habitat preferences and overall distribution are provided for all given taxa.

SUBCHAPTER 7.1 The genus *Symphytum* (Comfrey) in the Czech Republic. I. *S. tuberosum* agg.

The first part deals with the *S. tuberosum* complex in the light of the results presented in the Chapter 2.

SUBCHAPTER 7.2 The genus *Symphytum* (Comfrey) in the Czech Republic II. *S. officinale* agg.

The second part aims to reveal the distribution patterns of the species of the *S. officinale* complex.

SUBCHAPTER 7.3 The genus *Symphytum* (Comfrey) in the Czech Republic III. Introduced and cultivated species

The last part of this series summarises the history of the cultivation of this genus in our country and provides the revision of the naturalised and cultivated taxa. Furthermore, other species cultivated in Central Europe are also discussed.

Chapter 2

Symphytum tuberosum complex in central Europe: cytogeography, morphology, ecology and taxonomy

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Symphytum tuberosum complex in central Europe: cytogeography, morphology, ecology and taxonomy

***Symphytum tuberosum* ve střední Evropě – cytogeografie, morfologie, ekologie a taxonomie**

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Abstract

The *Symphytum tuberosum* complex is a highly polyploid and taxonomically intriguing group. At least eight ploidy levels were recorded previously within this complex. Based on flow cytometric screening of 271 central-European populations, two dominant ploidy levels were revealed: tetraploid ($2n = 4x = 32$) and widespread dodecaploid ($2n = 12x = 96$). The tetraploid cytotype is mainly distributed along the southern and south-western margins of the West Carpathians where they abut the Pannonian basin, and found only in Slovakia, the Czech Republic (south-eastern Moravia) and Hungary; our findings represent the first records of this ploidy level for the latter two countries. In contrast, the dodecaploid cytotype occurs throughout the whole area studied. In addition to their geographic distributions, differences between the cytotypes in morphology and habitat requirements were detected using a multivariate morphometric analysis and analysis of a phytosociological database, respectively. Based on this information and taking certain overlaps in morphological traits and habitat requirements into account, we propose treating the dominant cytotypes as subspecies: *S. tuberosum* subsp. *tuberosum* (dodecaploids) and *S. tuberosum* subsp. *angustifolium* (tetraploids). In some populations, aneuploids and several minority ploidy levels were also detected, including DNA-hexaploids (only within populations of tetraploids), DNAdecaploids and DNA-tetradecaploids (both only within populations of dodecaploids).

Keywords: cytotype distribution, ecology, flow cytometry, morphology, multivariate morphometrics, polyploidy, *Symphytum tuberosum* subsp. *angustifolium*, taxonomy

Introduction

The family Boraginaceae Juss. is known for its considerable chromosome variation, which is a consequence of various cytological processes, such as chromosome fusion or fragmentation, polyploidy or aneuploidy (Britton 1951, Coppi *et al.* 2006). These processes seem to be common in the family and play a crucial role in the evolution of many genera, such as *Borago* L. (Selvi *et al.* 2006), *Cerinthe* L. (Selvi *et al.* 2009), *Myosotis* L. (Štěpánková 2001, 2006), *Nonea* Medik. (Selvi *et al.* 2002, Bigazzi & Selvi 2003), *Onosma* L. (Mártonfi *et al.* 2008), *Omphalodes* Mill. (Grau 1967) and *Pulmonaria* L. (Sauer 1975). In addition, the occurrence of B chromosomes is quite common (Gadella 1972, Sauer 1975, Bigazzi & Selvi 2003, Bedini *et al.* 2012). All these processes are also important for genome evolution in the genus *Symphytum* L. (Grau 1968, 1971, Gadella & Kliphuis 1978, Murín & Májovský 1982). Gadella & Kliphuis (1978) report a high frequency of polyploids in comparison with other genera of Boraginaceae with the occurrence of polyploidy, as in *Onosma*, *Myosotis* and *Pulmonaria*. This phenomenon is well illustrated by the four ploidy levels reported for the *Symphytum officinale* complex (e.g. Markova & Ivanowa 1970, Gadella & Kliphuis 1978) or, even more surprisingly, the eight ploidy levels reported in the *Symphytum tuberosum* complex (Murín & Májovský 1982), which range from presumably diploid ($2n = 2x = 18$) up to octodecaploid cytotypes ($2n = 18x = 144$).

The Old World genus *Symphytum* L. belongs to the tribe Boragineae Bercht. et J. Presl, a major monophyletic group within the family Boraginaceae (Hilger *et al.* 2004). With approximately 40 species, it is one of the largest genera in this tribe (Bucknall 1913, Sandbrink *et al.* 1990). It includes perennial, roughly hirsute plants, which are morphologically well characterised by creeping, mostly fleshy rhizomes, alternate leaves, double scorpioid cymes (= boragoids) with tubular flowers and five corolla appendages (= fornices) inside the flower. The geographical range of the genus covers almost the whole of Europe and Asia Minor, as well as part of Western Asia and Siberia (Bucknall 1913). The centre of its diversity is situated in the Pontic area and in the western parts of the Irano-Turanian region, primarily in the mountain ranges around the Black Sea (Gadella & Kliphuis 1978, Davis 1988).

In central Europe, the following native species are recognised: *S. cordatum* Waldst. et Kit., the *S. officinale* complex (including *S. bohemicum* F.W. Schmidt, *S. officinale* s. str. and *S. tanaicense* Steven) and the *S. tuberosum* complex (including *S. angustifolium* A. Kern.; Kerner 1863, Murín & Májovský 1982, Marhold & Hindák 1998; and *S. tuberosum* L.; Pawłowski 1963, Gams 1966, Smejkal 1978, Májovský & Hegedüšová 1993, Slavík 2000, Danihelka *et al.* 2012). Five additional non-native species originating mostly from eastern Europe, the eastern Mediterranean and the Caucasus are also reported: *S. asperum* Lepech., *S. bulbosum* K. F. Schimp., *S. caucasicum* M. Bieb., *S. orientale* L. and *S. tauricum* Willd. (Pawłowski 1963, Gams 1966, Smejkal 1978, Danihelka *et al.* 2012, Bomble & Schmitz 2013).

Traditionally, the genus is divided into 2–9 sections, based on various infrageneric classifications (Boissier 1879, Kuznetsov 1910, Bucknall 1913, Pawłowski 1961, Wickens 1969, Sandbrink *et al.* 1990). The *S. tuberosum* complex belongs to the widely accepted section *Tuberosa* Buckn., which is characterised by (i) mostly tuberous rhizomes; (ii)

triangular, densely papillose fornices that do not protrude from corollas; and (iii) yellow flowers (Bucknall 1913, Pawłowski 1961, Wickens 1969, Sandbrink *et al.* 1990). Species in this section occur almost all over the European continent and adjacent Anatolia, except for the cold regions of northern Europe (Bucknall 1913, Murín & Májovský 1982). The section is a taxonomically difficult and still unresolved group with high-level polyploids and considerable morphological variation (Gadella & Kliphuis 1978, Murín & Májovský 1982).

In total, there are up to 10 taxa described within the *S. tuberosum* complex (in chronological order): *S. tuberosum* L. s. str., *S. mediterraneum* W.D.J. Koch, *S. angustifolium* A. Kern., *S. nodosum* Schur, *S. foliosum* Rehmann, *S. gussonei* F.W. Schultz, *S. floribundum* Shuttlew. ex Nyman, *S. leonhardtianum* Pugsley, *S. besseri* Zaver. and *S. popovii* Dobrocz. (Koch 1837, Kerner 1863, Schur 1866, Rehmann 1868, Schultz 1872, 1875, Nyman 1881, Pugsley 1931, Zaverucha 1962, Dobroczajeva 1968). However, the treatments of *Symphytum* in Flora Europaea and the Euro+Med Checklist recognise only two species: the Sicilian endemic *S. gussonei* and the widespread *S. tuberosum*, the latter comprising the western European subsp. *tuberosum* and central- and eastern-European subsp. *angustifolium/nodosum* (Pawłowski 1972, Valdés 2011). There is no clear relationship between the known karyological variation of *S. tuberosum* and its two subspecies on a European scale, and thus, only one broadly defined species, *S. tuberosum*, without any infra-specific units is usually recognised in recent floras (Mirek *et al.* 2002, Fischer *et al.* 2008, Jäger 2009, Király *et al.* 2011, Danihelka *et al.* 2012). The only exception is Slovakia, where the two known cytotypes are treated as separate species: *S. tuberosum* (dodecaploids) and *S. angustifolium* (tetraploids; Májovský & Hegedüšová 1993, Marhold & Hindák 1998).

In addition to the unresolved taxonomy, there is also controversy over the basic chromosome number of the *S. tuberosum* complex and the whole genus. The authors of the first karyological study suggest $x = 18$ as the basic chromosome number of *Symphytum* (Strey 1931). With the increasing number of counts, other basic numbers were proposed ($x = 8, 9, 10, 12, 14, 15$; e.g. Britton 1951, Markova & Ivanova 1970, Gadella & Kliphuis 1978, Luque 1989), and $x = 12$ is widely considered to be the basic chromosome number of this genus (Grau 1971, Murín & Májovský 1982, Slavík 2000). However, Murín &Májovský (1982) show that $x = 8$ best fits their comprehensive karyological survey of the *S. tuberosum* complex in the eastern part of central Europe.

The aim of this study was to examine the karyological variation and cytogeography of the *S. tuberosum* complex in central Europe and their relationship to morphological variation and habitat preferences. Based on the results, a revised taxonomic treatment of this complex in this region is proposed.

Material and methods

Field sampling

Plant material was collected in the Czech Republic (207 populations), Slovakia (24 populations), Austria (24 populations), Hungary (9 populations), Poland (4 populations) and Germany (3 populations; Electronic Appendix 2.1) between 2011 and 2014. A total of 1693 plants from 271 populations (1–26 plant per population, depending on population size) were

collected for DNA ploidy level estimation by flow cytometry. For the morphometric analyses, only well-developed plants with at least five flowers were selected. The collected plants were transplanted into the experimental garden of Palacký University (Olomouc, Czech Republic) or processed as standard herbarium vouchers and deposited in the Herbarium of the Palacký University in Olomouc (OL).

Estimation of DNA ploidy level

The DNA ploidy level was determined using a Partec CyFlow MLflow cytometer (Partec GmbH, Münster, Germany) equipped with a diode-pumped solid state green laser (532 nm, 100 mW, Cobolt Samba; Cobolt AB, Stockholm, Sweden), a BD Accuri C6 flow cytometer (BD Biosciences, San Jose, California) equipped with a blue laser (488 nm, 20 mW, BD Accuri™; BD Biosciences, San Jose) or a Partec PA II flow cytometer (Partec GmbH., Münster, Germany) equipped with a mercury arc lamp. In the first two instruments, propidium iodide (PI) was used as a stain; in the last instrument, DAPI was used for staining. However, note that even the measurements with PI should be considered only as estimates of the ploidy level, as we have not fulfilled (and did not intend to fulfill) all the requirements for an exact estimate of genome size (e.g. repeated measurements on different days; all samples were measured only once; Doležel *et al.* 2007). Data calibration was done using measurements of the same individuals cultivated in a greenhouse using different instruments.

The samples stained with PI were prepared using a simplified protocol with LB01 isolation buffer (Doležel *et al.* 2007). Approximately 0.5 cm^2 of fresh leaf tissue (from cultivated or plants growing in the field stored in plastic bags for a maximum of few days in a refrigerator) was chopped together with an appropriate amount of the internal reference standard with a razor blade in a Petri dish containing 1 ml of the isolation buffer. *Pisum sativum* L. 'Ctirad' (2C = 9.09 pg; Doležel *et al.* 1998) was used as the primary standard. However, due to a peak overlap of tetraploids with *Pisum sativum*, we used *Zea mays* 'CE-777' (2C = 5.92 pg, value calibrated against *Pisum sativum* 'Ctirad') as the secondary standard. The isolation buffer was supplemented with PVP-40 (20 mg/ml) to suppress the phenolic compounds interfering with DNA staining (Doležel & Bartoš 2005). The solution was filtered through a 42-μm nylon mesh and incubated for ~1 min. at room temperature. Then, a flow-through fraction was stained with fluorochrome PI (50 μg/ml). Samples were run on the flow cytometer immediately after staining and the relative fluorescence intensity of at least 3000 particles was recorded. Each individual was analysed separately.

For samples stained with DAPI, a simplified two-step protocol (Doležel *et al.* 2007) was used. Fresh leaves were chopped with *Bellis perennis* L. leaf tissue as the internal standard (the ratio of mean relative fluorescence compared to *Pisum sativum* 'Ctirad' is 0.429) in a Petri dish containing 0.5 ml of ice-cold Otto I buffer (0.1 M citric acid, 0.5% Tween-20). The suspension was filtered through a 42-μm nylon mesh and then incubated for ~3 min. at room temperature. After incubation, 1 ml of the Otto II buffer (0.4 M $Na_2HPO_4 \cdot 12H_2O$) supplemented with 2-mercaptoethanol (2 μl/ml) and DAPI (4 μg/ml) was added. Samples were run after ~1 min. of staining, and the fluorescence intensity of 3000–5000 particles was recorded. Usually, 3–5 individuals from the same population were analysed together; if the occurrence of more ploidy levels or genome size variation in the

bulked sample was suspected, each individual was re-analysed separately. The ploidy level of each sample was determined by the position of its G0/G1 peak relative to the G0/G1 peak of the internal standard. Generally, for measurements using both PI and DAPI, histograms with coefficients of variation (CV) for the G0/G1 peaks of the analysed sample and the standard less than 5% were accepted.

Chromosome counts

To calibrate the results of the flow cytometry measurements, the chromosome numbers of two tetraploid (populations 11 and 84) and three dodecaploid (populations 5, 18B and 33; Electronic Appendix 2.1) plants were determined. Chromosomes in the metaphase cells of the root meristems of cultivated plants were counted. The root tips were pre-treated with α-bromonaphthalene for 4 hours at room temperature, fixed in cold acetic acid/ethanol (1:3) overnight and stored in 70% ethanol at 4°C. Then, samples were macerated for 5 min. in 1 M hydrochloric acid at 60°C (Krahulcová & Krahulec 1999). The apical part of the root tip was cut and squashed in lacto-propionic orcein. Metaphases were observed under 1000× overall magnification.

Morphometric analyses

Only flowering plants with at least five flowers and known ploidy levels were used for morphometric analysis. Plants of minority cytotypes could not be included because they were either poorly developed or were only recorded by cytometric screening after anthesis. In total, 522 individuals (196 tetraploids and 326 dodecaploids) from 40 populations (at least 10 individuals per population) were analysed (Electronic Appendix 2.1). For each individual, 19 vegetative and generative characters were measured, and four ratios were calculated (Table 2.1). All quantitative characters were measured on fresh material using a digital calliper except for characters inside the corolla, which were measured on dried flowers under a binocular microscope. For generative characters, five flowers per plant were measured and median values used in all analyses.

The morphological dataset was analysed using NCSS 2007 (Hintze 2008) and CANOCO for Windows 4.5 (ter Braak & Šmilauer 2002) software. Initially, univariate analyses were used. Spearman correlation coefficients were computed to reveal pairs of highly correlated characters. One-way ANOVA was used to compare the mean values of characters between cytotypes. Because multiple tests were performed, Bonferroni correction of the significance values was applied. Principal component analysis (PCA; Sneath & Sokal 1973) based on the correlation matrix was used to display the overall pattern of variation. Canonical discriminant analysis (CDA; Legendre & Legendre 1998) was used to determine the extent of morphological separation between the cytotypes. Parametric classificatory discriminant analysis was used to estimate the percentage of plants correctly assigned to the predetermined groups (cytotypes) based on the morphological characters measured. The character ‘branching of stem’, due to its qualitative nature, was separately analysed using a contingency table and hence not included in the above-described statistical procedures. All analyses were performed with individual plants as objects.

Ecological differences among cytotypes

To understand the ecological differentiation and phytosociological affinity of the cytotypes in the Czech Republic, phytosociological relevés comprising *Symphytum tuberosum* were analysed. Relevés were obtained from the Czech National Phytosociological Database (ČNFD; Chytrý & Rafajová 2003), and 18 additional relevés were recorded. As only the species *S. tuberosum* is included in the database taxonomic list, all records must be considered as potentially referring to either cytotype. We therefore selected only relevés from localities that we screened using flow cytometry; for dodecaploids, relevés from Bohemia and northern Moravia, where tetraploids are unlikely to occur, were also included. In total, 520 phytosociological relevés were analysed (162 and 358 relevés related to tetraploids and dodecaploids, respectively).

The relevés were classified by an expert system using the Cocktail method (Kočí *et al.* 2003, Chytrý 2007) in JUICE 7.0 software (Tichý 2002); this expert system allows classification of the rank of association following the Vegetation of the Czech Republic series (Chytrý 2007, 2009, 2013). Average Ellenberg's indicator values (light, temperature, continentality, moisture, soil reaction and nutrients; Ellenberg *et al.* 1992) were calculated for individual relevés using JUICE 7.0. Differences in the average Ellenberg's indicator values between the two dominant cytotypes were analysed using one-way ANOVA with the permutation significance test instead of the parametric method; presence/absence data and the R function summary.aov.iv (Zelený & Schaffers 2012) with 999 permutations were used, and the Bonferroni correction of the P-values was applied. The computation was performed in R 3.1.2 (R Core Team 2014).

The main trends in the composition of the vegetation with *S. tuberosum* cytotypes were analysed using two approaches. First, fidelity (i.e. species concentration in vegetation units; Chytrý *et al.* 2002) was calculated in JUICE 7.0 using Fisher's exact test with a significance level $P < 0.01$ (Chytrý *et al.* 2002). Second, multivariate ordination techniques were employed using Canoco forWindows 4.5 (ter Braak & Šmilauer 2002). Data were exported from TURBOVEG using the van der Maarel transformation of species abundances from Braun-Blanquet to the ordinal scale (Kočí *et al.* 2003). The length of the gradient was tested in DCA. Because the length of the gradient was greater than 4 SD units, unimodal techniques were used (ter Braak & Šmilauer 2002). Canonical correspondence analysis (CCA; ter Braak 1986) was done using individual relevés as objects and the ploidy level as the sole explanatory variable. A Monte Carlo permutation test with 499 permutations was used to assess the significance of the explanatory variable (Lepš & Šmilauer 2003).

Results

Chromosome numbers, cytotype diversity and distribution

Two dominant cytotypes were found in the 271 populations sampled, corresponding to previously published tetraploid and dodecaploid chromosome counts and confirmed by our own chromosome counts (Figs 2.1, 2.5, Tables 2.2, 2.3). Tetraploids ($2n = 4x = 32$) were detected in central and southern Moravia, southern and south-western Slovakia and northern

Hungary. For the Czech Republic and Hungary, this ploidy level is reported for the first time. Dodecaploids ($2n = 12x = 96$) occurred in all countries included in this study (Fig. 2.2).

The dodecaploid cytotype was more frequent and occurred at 70.8% (192 populations) of the localities sampled, while the tetraploid cytotype occurred at 27.7% of the localities (75 populations). Mixed populations were rare, occurring in only four cases (1.5% of all localities): three adjacent populations in south-eastern Moravia (the northern White Carpathians Mts) and one population in Slovakia (the Tríbeč hills). However, in some other areas at the northern limit of the tetraploids' distribution, both cytotypes occurred in close proximity (less than 1 km; Fig. 2.3). In addition to the dominant cytotypes, three minority ploidy levels were detected using FCM in certain populations (Tables 2.2, 2.3). As the exact chromosome numbers of these cytotypes were not established, we refer to them henceforth as DNA-hexaploids ($\approx 6x$), DNA-decaploids ($\approx 10x$) and DNA-tetradecaploids ($\approx 14x$; Fig. 2.4; Tables 2.2, 2.3). DNA-hexaploids were found admixed in populations of the tetraploid cytotype, whilst DNA-decaploids and DNA-tetradecaploids occurred within the dodecaploid populations. The minority cytotypes were rare (2.6% of all analysed individuals analysed). We also detected aneuploidy. An aneuploid chromosome number was successfully established for a plant from a dodecaploid population 5 ($2n = 94$). Given the associated variation in the mean relative fluorescence both between and within populations of both dominant cytotypes (~23% for tetraploids and ~10% for dodecaploids), aneuploid plants may be quite frequent. In certain cases, the difference between individual plants was corroborated by bifurcated peaks in joint FCM analyses (Fig. 2.5).

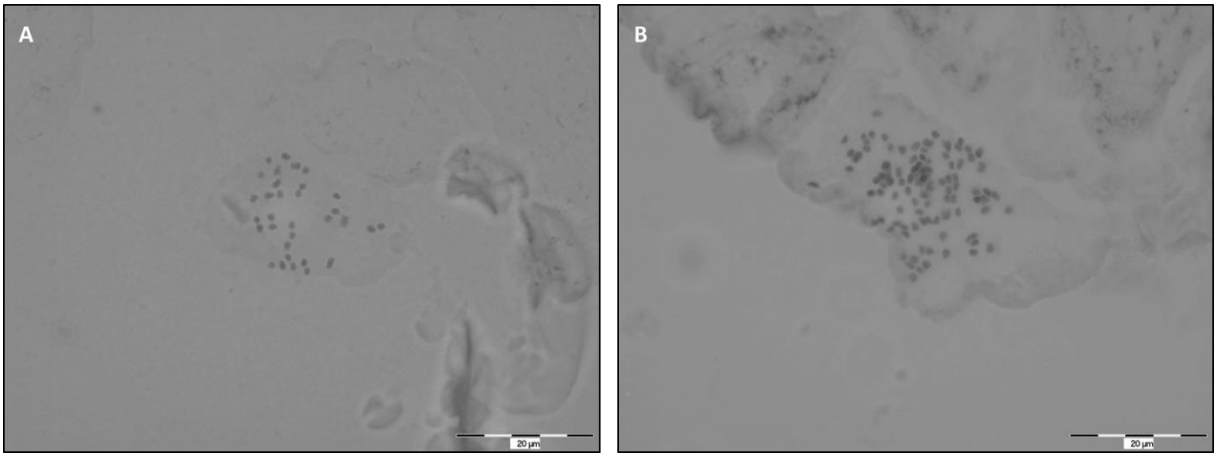

FIGURE 2.1. Mitotic metaphase chromosomes of (**A**) *Symphytum tuberosum* subsp. *angustifolium* ($2n = 4x = 32$; population 84, Velká nad Veličkou) and (**B**) *S. tuberosum* subsp. *tuberosum* ($2n = 12x = 96$; population 18, Tavíkovice).

Morphometric analyses

In total, 522 plants from 40 populations were used for the morphological analyses. No pairs of highly correlated characters ($r > |0.95|$) were found, and thus, the entire dataset was used in the multivariate analyses. The two dominant cytotypes significantly differed in most of the

morphological characters studied, except for the length of the lowermost leaf, length of pedicel and length of calyx (see Table 2.1, Fig. 2.6). However, most of the ranges in variation overlap and no single character can be used for unambiguous determination of the cytotypes.

The only qualitative character used in our morphometric study ('branching of stem') was separately analysed using a contingency table. The pattern of branching was significantly different between the two cytotypes (Pearson's $\chi 2\ 2 = 57.67$; DF = 3; $P < 0.01$), although all character states were found in both cytotypes and only their frequencies were somewhat different. Thus, this character can only be used as a supplement to quantitative characters. The two cytotypes differ in the proportion of unbranched individuals (23% of tetraploids, 53% of dodecaploids). The frequency of plants branched in the upper part is similar (9% tetraploids and 8% of dodecaploids), while the frequency of individuals that branched in the middle or lower part of the stem was higher in tetraploids (18% and 50%, respectively) than dodecaploids (4% and 35%, respectively).

PCA based on individuals (Fig. 2.7) revealed partial separation of the dominant cytotypes. The characters most correlated with the first component were the widths of the uppermost, middle and lowermost leaves and lengths of the uppermost and middle leaves, whereas the ratios of the length of the corolla to the narrow part of the corolla, the length to width of the lowermost leaves and the height of the plant were mainly correlated with the second component. Canonical discriminant analysis (CDA) based on individual plants resulted in a clear morphological separation between the two cytotypes ($F = 76$; num. DF 22; denum. DF 499; $P < 0.01$; Fig. 2.8). The greatest weight included the length of the corolla to the narrow part of the corolla ratio, the length of the corolla and the width of the filament. The parametric method of classificatory discriminant analysis based on probability models resulted in a high number of plants being correctly classified to the cytotype (97.0% of tetraploids and 97.2% of dodecaploids).

TABLE 2.1. Descriptive statistics (arithmetic mean with standard deviation, minimum and maximum) of the morphological characters of the major cytotypes of the *Symphytum tuberosum* complex in central Europe. Differences among cytotypes were tested by one-way ANOVA (DF = 1; 521), P shows significant differences after Bonferroni correction (n.s. = not significant). Abbreviations of characters given in parentheses correspond to those used in Fig. 2.7.

Morphological character	4*x* (N = 196)			12*x* (N = 326)			F	p
(abbreviations used in Fig. 2.7)	mean (SD)	min	max	mean (SD)	min	max		
Height of plant (cm; height)	33.1 (7.2)	15.6	52.0	30.2 (7.9)	8.5	51.7	18.12	< 0.001
Length of uppermost leaf (cm; l_leaf_U)	5.2 (1.6)	1.9	10.5	6.4 (2.1)	2.0	13.6	38.27	< 0.001
Width of uppermost leaf (cm; w_leaf_U)	1.7 (0.7)	0.5	3.8	2.6 (1.1)	0.7	8.5	115.23	< 0.001
Length to width ratio of uppermost leaf (r_leaf_U)	3.4 (0.7)	1.2	5.5	2.6 (0.5)	0.4	4.7	185.98	< 0.001
Length of middle leaf (cm; l_leaf_L)	9.7 (2.3)	4.2	19.1	11.8 (2.9)	5.2	19.3	72.8	< 0.001
Width of middle leaf (cm; w_leaf_M)	2.6 (0.7)	1.3	4.5	4.2 (1.2)	2.0	8.4	281.46	< 0.001
Length to width ratio of middle leaf (r_leaf_M)	3.9 (0.8)	1.7	6.1	2.9 (0.5)	1.4	4.7	335.78	< 0.001
Length of lowermost leaf (cm; l_leaf_L)	10.0 (2.6)	2.8	17.9	10.5 (3.7)	2,0	22.1	3.08	n.s.
Width of lowermost leaf (cm; w_leaf_L)	2.50 (0.8)	0.7	5.3	4.0 (1.2)	1.5	8.7	251.1	< 0.001
Length to width ratio of lowermost leaf (r_leaf_L)	4.2 (1,0)	1.3	8.7	2.6 (0.7)	1.0	5.5	411.29	< 0.001
Length of pedicel (mm; fl_stalk)	8.35 (1.94)	4.16	14.13	8.46 (2.01)	3.99	14.97	0.33	n.s.
Length of calyx (mm; calyx)	7.46 (0.96)	5.37	9.46	7.68 (1.02)	5.24	11.27	5.93	n.s.
Length of corolla (mm; corrolla)	15.24 (1.11)	12.32	17.59	16.03 (1.19)	12.34	18.82	57.97	< 0.001
Length of narrowed part of corolla tube (mm; cor_tube)	7.60 (0.68)	5.52	9.66	8.37 (0.85)	5.71	10.76	117	< 0.001
Length of corolla to narrowed part of corolla ratio (r_cor_ct)	2.01 (0.12)	1.72	2.45	1.92 (0.13)	1.66	2.37	54.58	< 0.001
Length of style (mm; style)	15.98 (1.78)	10.15	19.37	17.79 (1.58)	10.67	21.83	145.3	< 0.001
Length of filament (mm; l_fil)	0.86 (0.09)	0.57	1.10	0.96 (0.12)	0.53	1.30	71.6	< 0.001
Length of free part of filament (mm; free_fil)	0.05 (0.01)	0.03	0.78	0.06 (0.01)	0.03	0.08	18.7	< 0.001
Width of filament (mm; w_fil)	0.21 (0.04)	0.14	0.29	0.23 (0.05)	0.09	0.34	26.51	< 0.001
Length of fornice (mm; cor_sca)	1.23 (0.11)	0.89	1.51	1.29 (0.14)	0.93	1.65	31.46	< 0.001
Length of anther (mm; l_thec)	0.33 (0.04)	0.23	0.46	0.34 (0.04)	0.25	0.44	30.16	< 0.001
Width of anther (mm; w_thec)	0.07 (0.01)	0.05	0.08	0.08 (0.01)	0.05	0.09	225.43	< 0.001

TABLE 2.2. Relative DNA content of the individual cytotypes of the *Symphytum tuberosum* complex in Central Europe assessed using flow cytometry; PI was used as a stain. All values are calculated relative to the internal standard *Pisum sativum* 'Ctirad'. Recalculation to *Zea mays* 'CE-777' based on reciprocal calibration of the two standards using PI as a stain is also provided. Note that tetraploids and hexaploids were analysed with *Zea mays*; the result was then recalculated to *Pisum sativum*. N = number of samples analysed; SE = standard error of mean. Variation is calculated as the difference between the most extreme values expressed in % of the mean value.

DNA ploidy level	N	Standard = *Pisum sativum*			Standard = *Zea mays*
		Mean ratio to the standard ± SE	Range	Variation (%)	Calculated mean ratio to the standard
4*x*	381	0.246 ± 0.010	0.221–0.278	22.9	0.373
6*x*	6	0.384 ± 0.012	0.365–0.404	10.2	0.582
10*x*	13	0.584 ± 0.019	0.569–0.598	12.7	0.889
12*x*	739	0.663 ± 0.017	0.628–0.698	10.6	1.005
14*x*	17	0.762 ± 0.010	0.748–0.783	4.5	1.155

TABLE 2.3. Relative DNA content of the individual cytotypes of the *Symphytum tuberosum* complex in Central Europe assessed using flow cytometry; DAPI was used as a stain. All values are calculated relative to the internal standard *Bellis perennis* (2C = 3.62 pg), which is given the unit value. Recalculation to *Pisum sativum* 'Ctirad' based on reciprocal calibration of the two standards using DAPI as a stain is also provided. Note that hexaploids overlap with *Bellis perennis* and were analysed with *Pisum sativum*; the result was then recalculated to *Bellis perennis*. N = number of samples analysed (note that the number of measured individuals is higher due to use of bulked samples); SE = standard error of mean. Variation is calculated as the difference between the most extreme values expressed in % of the mean value.

DNA ploidy level	N	Standard = *Bellis perennis*			Standard = *Pisum sativum*
		Mean ratio to the standard ± SE	Range	Variation (%)	Calculated mean ratio to the standard
4*x*	36	0.650 ± 0.003	0.623–0.702	12.2	0.279
6*x*	1	0.982			0.421
10*x*	1	1.539			0.660
12*x*	129	1.842 ± 0.003	1.746–1.954	11.3	0.790
14*x*		not analysed			

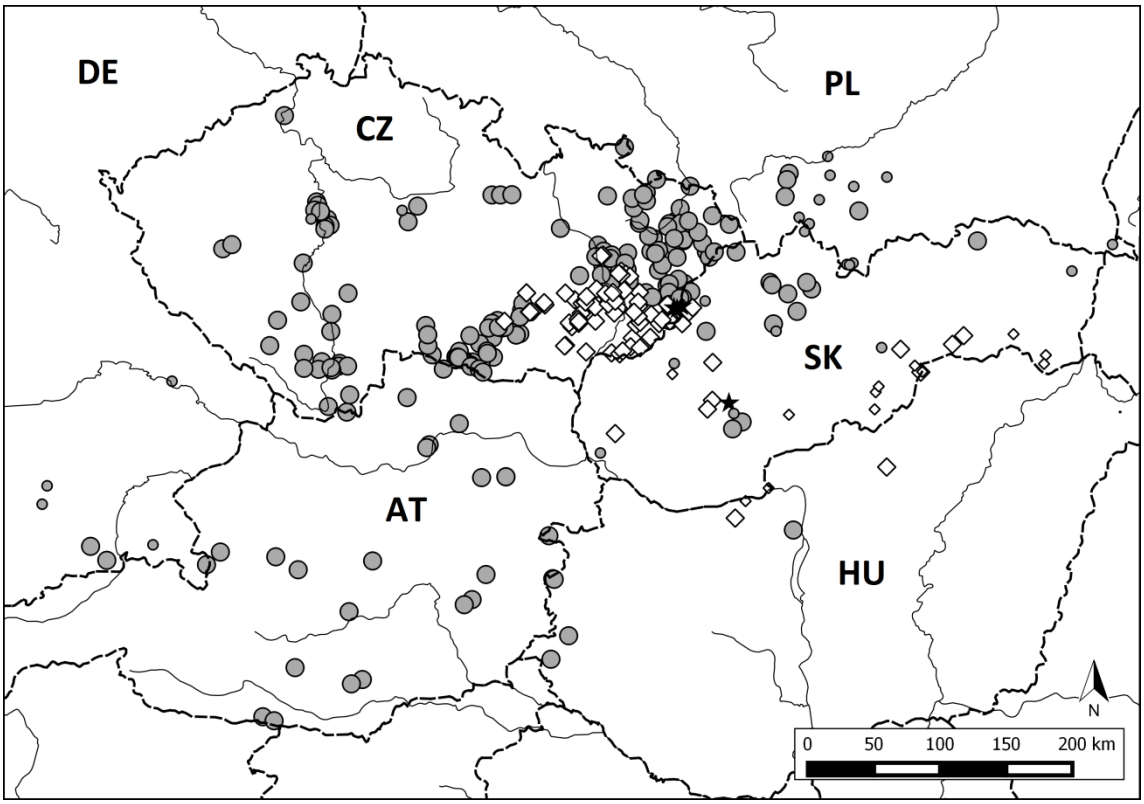

FIGURE 2.2. Map showing the locations of the populations sampled; ◊ *Symphytum tuberosum* subsp. *angustifolium*, ● subsp. *tuberosum*, ★ mixed populations of both subspecies. Additional localities from previous karyological studies by Grau (1968), Wcisło (1972), Májovský (1976), Gadella & Kliphuis (1978), Murín & Májovský (1982), Javůrková-Jarolímová & Měsíček (1992) and Lippert (2006) are indicated by smaller symbols.

Ecological differences of cytotypes

Altogether, 520 phytosociological relevés (162 with tetraploids and 358 with dodecaploids) were analysed. All relevés were successfully classified by the expert system. Tetraploids were present mostly in oak-hornbeam forests, thermophilous and acidophilous oak forests and beech forests of the *Carici pilosae-Fagetum sylvaticae* association (in contrast to dodecaploids; see Table 2.4) and in semi-dry grasslands and herbaceous communities along forest edges. Dodecaploids were present mostly in humid, broadleaved floodplain forests, ravine and cliff forests, mesic and nutrient-rich beech and coniferous forests. They also occurred in the vegetation along river banks, on gravel deposits and alluvial sediments. In addition, they pervaded ruderal vegetation and disturbed forest sites. In Bohemia, dodecaploids were also common in thermophilous oak and oak-hornbeamwoodlands (particularly *Galio sylvatici-Carpinetum betuli*; Table 2.4).

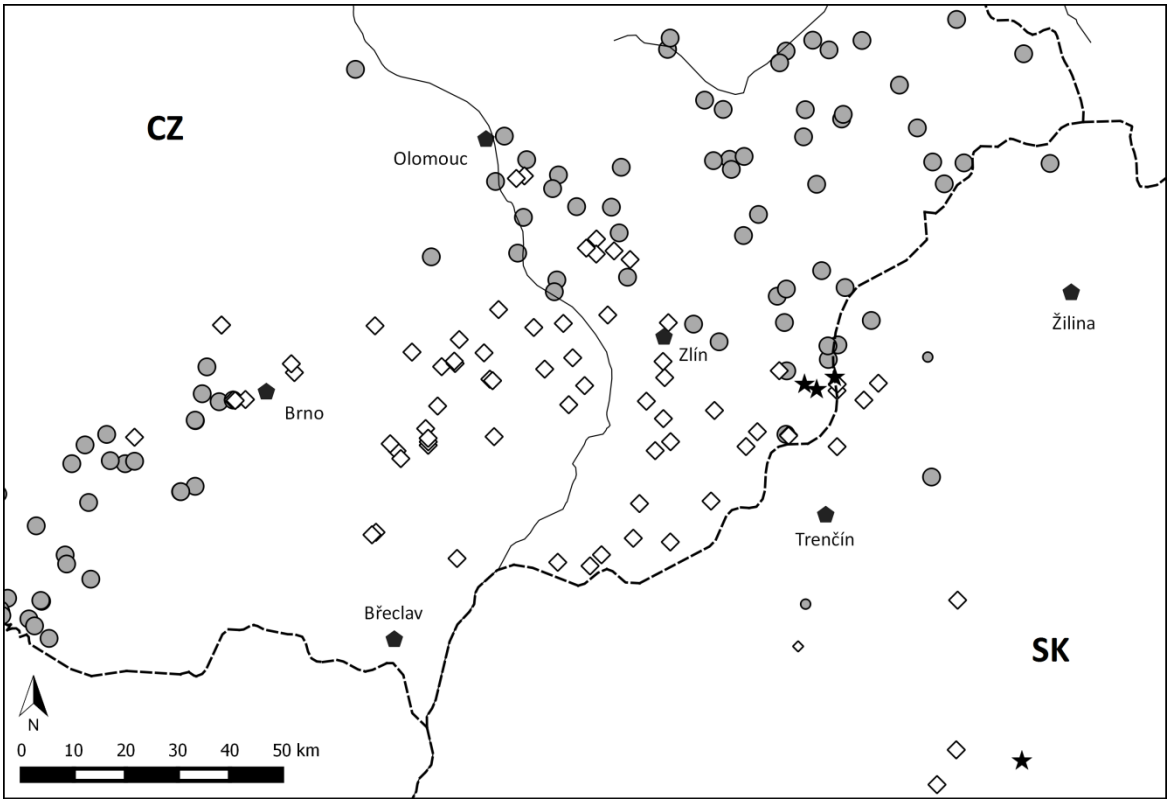

FIGURE 2.3. A detailed map of the contact zone between the two subspecies of *Symphytum tuberosum* in Moravia and western Slovakia; ◊ *Symphytum tuberosum* subsp. *angustifolium*, ◉ subsp. *tuberosum*, ★ mixed populations of both subspecies.

The two sets of relevés with individual cytotypes significantly differ in Ellenberg's indicator values for temperature (P = 0.030 after Bonferroni correction), moisture (P = 0.042) and nutrients (P = 0.006); other indicator values would be insignificant even without the application of the Bonferroni correction. Tetraploids grew at sites with, on average, higher temperature and lower moisture and nutrients, than the dodecaploids (Fig. 2.9).

The plants with the highest fidelity (> 45) to relevés with tetraploids were *Carex pilosa, Quercus petraea* agg. and *Lathyrus niger*, and the highest fidelity of those to relevés with dodecaploids included *Urtica dioica* (see Electronic Appendix 2.2 for more species). The main differences in the composition of the vegetation with *S. tuberosum* cytotypes were also confirmed by the CCA (P = 0.002, F = 8.31; Fig. 2.10). Nitrophilous, hygrophilous and sciophylous species, such as *Urtica dioica, Aegopodium podagraria, Geranium robertianum, Galeobdolon luteum* agg. and Anemone nemorosa, correlated most strongly with the presence of dodecaploids. Among relevés associated with tetraploids, two groups of species were identified, the first are species of thermophilous woodlands (*Lathyrus niger, Melittis melissophyllum* and *Carex montana*), and the second those of slightly thermophilous grasslands (e.g. *Betonica officinalis, Potentilla alba* and *Filipendula vulgaris*).

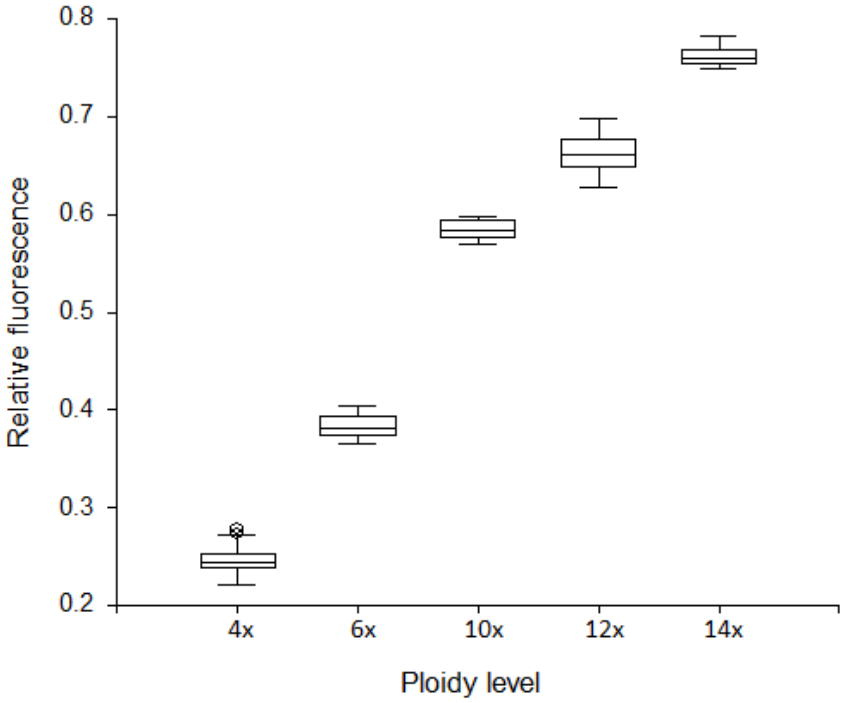

FIGURE 2.4. Relative fluorescence (ratio to the internal standard *Pisum sativum* 'Ctirad') for individual cytotypes of the *Symphytum tuberosum* complex in central Europe assessed using flow cytometry; the stain was PI (4*x* – 381 samples, 6*x* – 6 samples, 10*x* – 13 samples, 12*x* – 739 samples, 14*x* – 17 samples). Median, quartiles, non-outlier range and outliers are depicted.

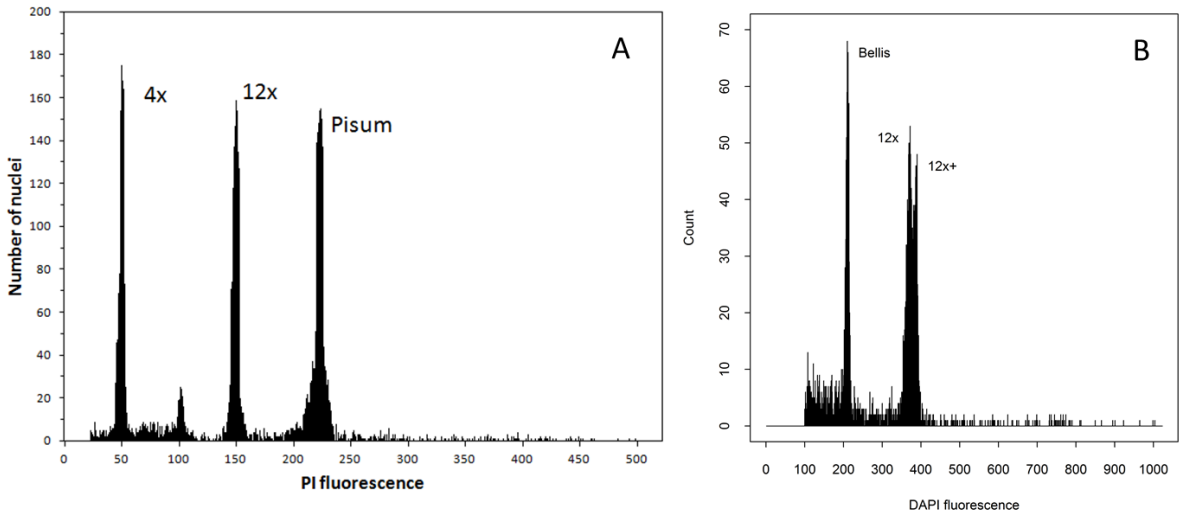

FIGURE 2.5. Flow cytometric histograms of the relative DNA content of: (**A**) simultaneous analysis of PI-stained nuclei isolated from tetraploid and dodecaploid plants of the *Symphytum tuberosum* complex, with the internal standard *Pisum sativum* 'Ctirad', (**B**) DAPI-stained nuclei showing variation in the relative fluorescence of two individuals of the dodecaploid ploidy level with *Bellis perennis* as the internal standard.

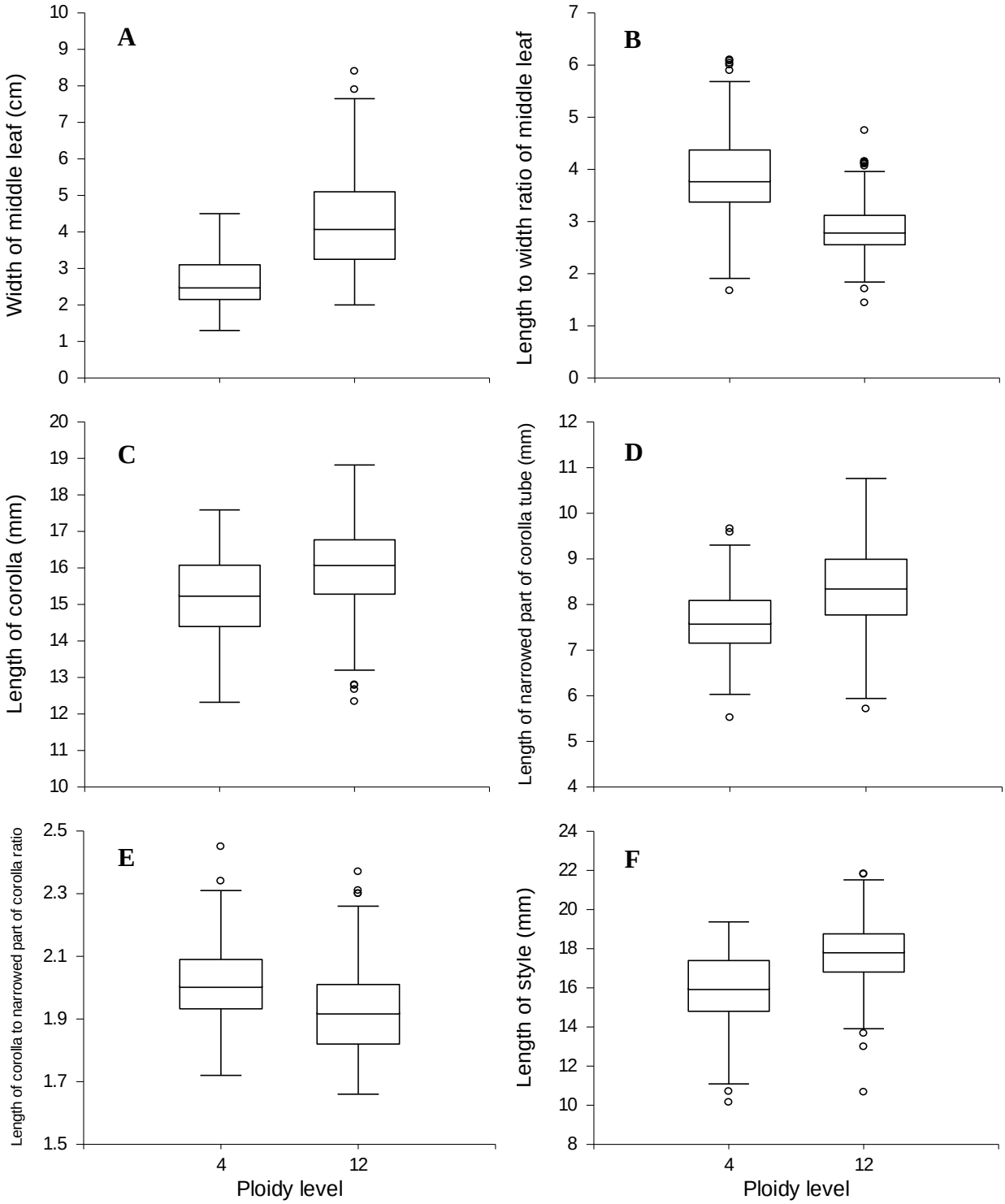

FIGURE 2.6. Variation in the quantitative characters of tetraploid and dodecaploids cytotypes of the *Symphytum tuberosum* complex in central Europe: (**A**) width of the middle stem leaf, (**B**) length to width ratio of the middle stem leaf, (**C**) length of the corolla, (**D**) length of narrow part of the corolla tube, (**E**) ratio of the length of corolla to that of the narrow part of the corolla and (**F**) length of the style.

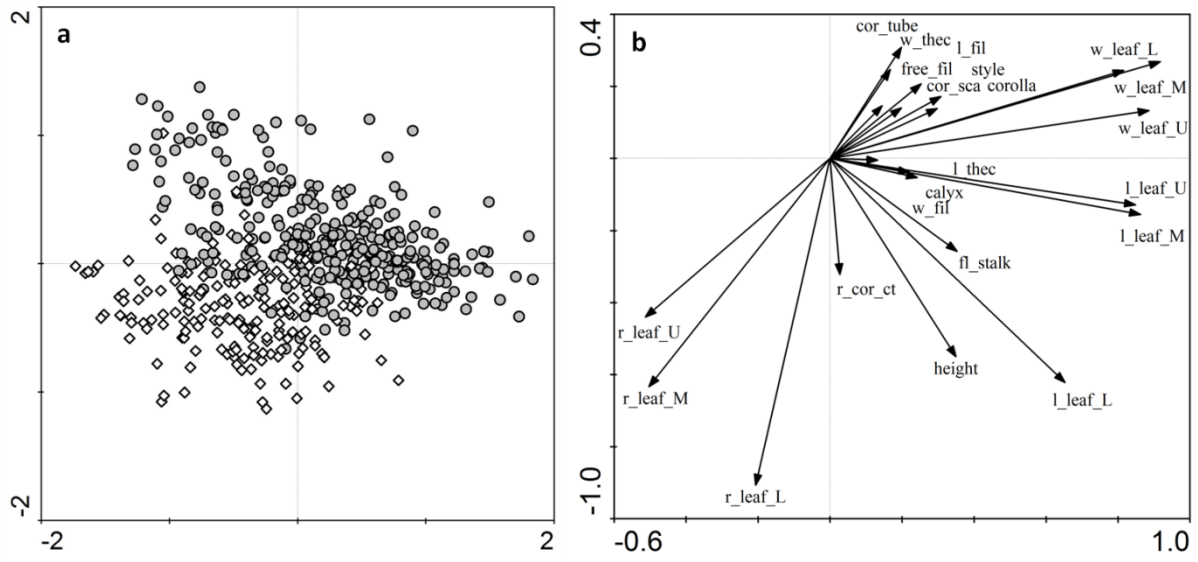

FIGURE 2.7. PCA of morphological characters of the two cytotypes of *Symphytum tuberosum*: (**A**) distribution of individuals in ordination space (◊ 4*x*, ● 12*x*), (**B**) fit of the 18 morphological characters and four ratios studied to the ordination axes (abbreviations of morphological characters explained in Table 2.1). The first and the second ordination axis explain 48% and 20% of the variation, respectively.

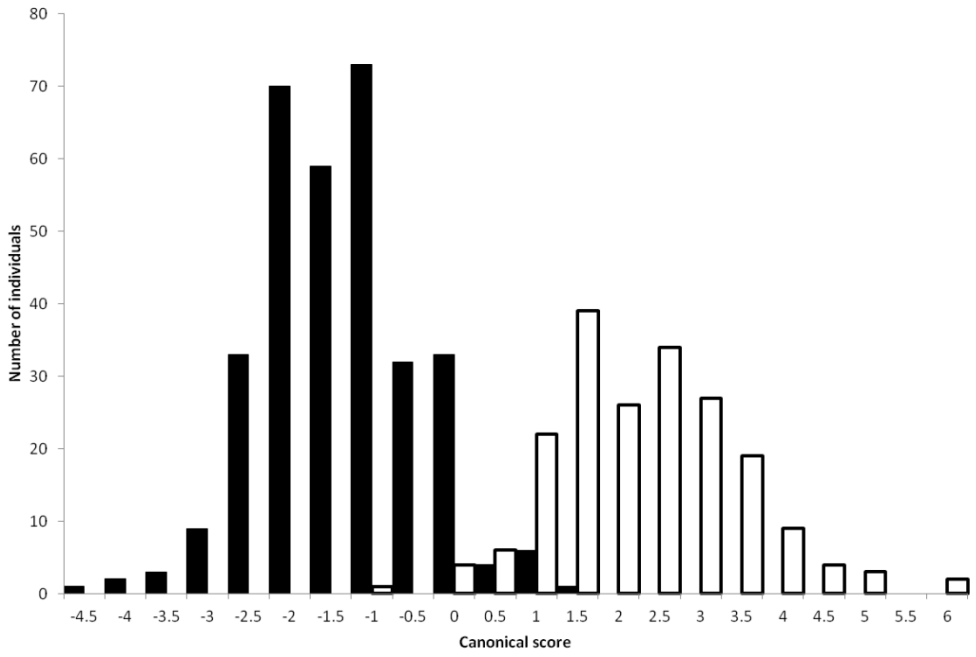

FIGURE 2.8. Histogram of canonical scores of linear discriminant analysis of individuals (N = 522) of the *Symphytum tuberosum* complex in central Europe (□ tetraploids; ■ dodecaploids). All quantitative characters were used in this analysis.

Discussion

Cytotype distribution

A detailed screening of the cytotype diversity of the *Symphytum tuberosum* complex in central Europe revealed two dominant (tetraploid and dodecaploid) and three minority cytotypes (DNA-hexaploids, DNA-decaploids and DNA-tetradecaploids). The dominant cytotypes correspond well with the previously published chromosome counts (Wcisło 1972, Murín & Májovský 1982, Javůrková-Jarolímová & Měsíček 1992). Overall, the cytotype diversity of the *S. tuberosum* complex is similar to other thoroughly studied polyploid complexes in the central-European flora, such as *Senecio carniolicus* (Suda *et al.* 2007b) and *Gymnadenia conopsea* (Trávníček *et al.* 2012), both with five cytotypes, or *Allium oleraceum* (Duchoslav *et al.* 2013), with four cytotypes.

The most frequent cytotype of the *S. tuberosum* complex in central Europe is dodecaploid, which occurs throughout the whole area. Apparently, it is the prevalent cytotype throughout the whole distribution range of the complex (e.g. Grau 1968, Wcisło 1972, Gadella & Kliphuis 1978, Murín & Májovský 1982, Luque 1989). In contrast, tetraploids are only recorded in Slovakia (Murín &Májovský 1982). In this paper, we record them for the first time from two other adjacent countries (Czech Republic, Hungary). Based on published chromosome counts for the whole *S. tuberosum* complex and our own unpublished data from different parts of Europe, it seems that tetraploids might be a very restricted central-European element, with a possible overlap into the Balkans.

Within the area studied, the distribution of the cytotypes is parapatric and mirrors their different habitat preferences. Dodecaploids do not occur in the warmest and driest part of the area studied, which is at the periphery of the Pannonian Lowlands in southern Moravia (south-eastern part of the Czech Republic) and south-western and southern Slovakia. Nevertheless, the rare occurrence of dodecaploids in the Pannonian Lowlands cannot be completely ruled out, as there are reports of S. *tuberosum* s. l. from alluvial forests along large rivers (e.g. Májovský & Hegedüšová 1993) that might represent dodecaploid plants dispersed by floods from higher altitudes; during this study, however, we failed to find any of these small and probably temporary populations. In contrast, tetraploids mainly occur in hilly (not flat) landscapes at the northern border of the Pannonian Lowlands and the lower parts of the Western Carpathians and from a phytogeographic perspective may be considered to be a Matrian-Praecarpathian floristic element (similar to e.g. *Chamaecytisus virescens, Dorycnium pentaphyllu*m s. l., *Glechoma hirsuta, Iris graminea* or *Pseudolysimachion orchideum*). Due to sparse sampling, the exact southern and especially eastern limits of the tetraploids' distribution require further study.

In the narrow contact zone, the two dominant cytotypes are spatially intermixed, but only four mixed populations (three in the Czech Republic and one in Slovakia) were discovered, despite intensive sampling. This result may be due to the different habitat requirements of the cytotypes (see below). In mixed populations, no intermediate (octoploid) individuals were detected, which might indicate limited or no gene flow between the two cytotypes. A similar parapatric distribution due to (at least partly) different habitat requirements with only rare mixed populations and breeding barriers between the cytotypes is reported for other polyploid complexes: for example, in central Europe, the *Aster amellus*

complex (Münzbergová *et al.* 2013), the *Centaurea phrygia* group (Koutecký *et al.* 2012) or *Galium valdepilosum* (Kolář *et al.* 2014).

Differences in the ecology of the cytotypes

Numerous studies also report that cytotypes in a wide range of polyploid complexes differ ecologically (e.g. Rothera & Davy 1986, Hülber *et al.* 2009, Kolář *et al.* 2014). Cytogeographic data (see above) and our detailed analysis of phytosociological relevés from the Czech Republic indicate there is also a clear differentiation in the *S. tuberosum* complex. The latter is consistent with the results of the study by Murín & Májovský (1982) in Slovakia, which indicates that the tetraploid cytotype is a typical element of thermophilous oak and oak-hornbeam woodlands, whereas the dodecaploid cytotype is a sub-montane or montane element of beech forests with only occasional occurrence in oak woodlands. The four mixed populations all occurred in the contact zones between semi-dry meadows (three cases) or oak forests (one case; typical habitat of tetraploids) and more mesophilous vegetation along small streams (marginal habitat of dodecaploids). Finally, there is a habitat differentiation in the altitudinal distributions of the cytotypes. Tetraploids occur in warmer areas and only rarely occur at higher altitudes (in this study the recorded maximum is 710 m a.s.l. in the Mátra Mts), whereas dodecaploids frequently grow in colder areas and at high altitudes; in the Slovakian Carpathians, Májovský & Hegedüšová (1993) record a maximum of ~1650 m a.s.l., in this study, the locality Turracher Höhe in Austria is at 1725 m a.s.l., and some populations in the Alps may occur at even higher altitudes.

Minority cytotypes and aneuploidy

Three minority cytotypes were rarely detected in populations of the two dominant cytotypes. In four out of the 75 tetraploid populations studied (see Electronic Appendix 2.1), DNAhexaploid plants were discovered. The origin of DNA-hexaploids can be explained by the fusion of an unreduced and a reduced gamete produced by tetraploids. An analogous scheme seems to occur quite frequently in plants and is assumed, for example, in mixed 4*x* + 6*x* populations of *Allium oleraceum* (Šafářová & Duchoslav 2010), *Hypericum perforatum* (Qu *et al.* 2010) and *Molinia caerulea* (Dančák *et al.* 2012). Within dodecaploid populations, DNA-decaploid (in seven populations) and DNA-tetradecaploid (in 10 out of 192 populations) plants were detected. There is no simple explanation of their origin as in the case of DNA-hexaploids, and either the presence of other undiscovered cytotypes or aneuploidy/dysploidy must be hypothesised. Dysploidy is expected to be important in the karyotype evolution of several genera of Boraginaceae, such as *Nonea* (Selvi & Bigazzi 2002) and *Pulmonaria* (Sauer 1987). Moreover, we recorded significant variation in relative fluorescence reflecting genome size variation in both dominant cytotypes. This finding could be explained by the occurrence of aneuploidy or the presence of B chromosomes, both of which are recorded in *Symphytum* (Grau 1971, Gadella & Kliphuis 1978). Indeed, aneuploidy in dodecaploids was directly confirmed by the chromosome count of 2*n* = 94, recorded for one divergent individual. In general, his is more likely to occur in high-level polyploids because they possess multiple gene copies, and the gain/loss of some of them may not have a serious effect on individual viability (Leitch & Leitch 2008).

Vegetation unit	***S. tuberosum* subsp. *angustifolium***	***S. tuberosum* subsp. *tuberosum***
Alnion incanae	1.2%	19.0%
Carpinion betuli	55.9%	17.9%
Fagion sylvaticae		
as. Carici pilosae-Fagetum sylvaticae	13.7%	1.1%
other associations	–	18.2%
Tilio platyphylli-Acerion	0.6%	16.5%
Quercion petraeae	9.9%	–
Quercion roboris	1.7%	4.3%
Petasition hybridi	–	4.5%
Aegopodion podagrariae	–	8.9%
Fragarion vescae	–	2.5%
Bromion erecti	5.6%	0.3%
Trifolion medii	2.5%	0.3%

TABLE 2.4. The most frequent vegetation units comprising the subspecies of *Symphytum tuberosum* in Czech Republic based on classification of 520 phytosociological relevés (162 related to tetraploids and 358 to dodecaploids). The proportion of both subspecies in each unit is displayed.

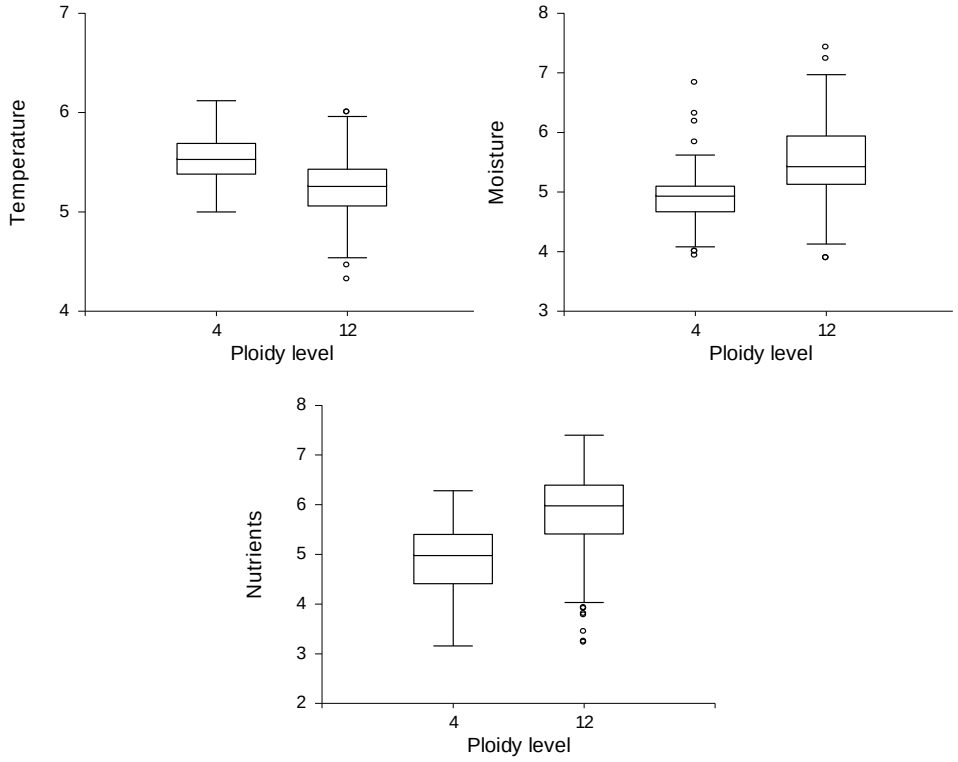

FIGURE 2.9. Selected average Ellenberg's indicator values of vegetation plots from the Czech Republic containing *Symphytum tuberosum* subspecies/cytotypes.

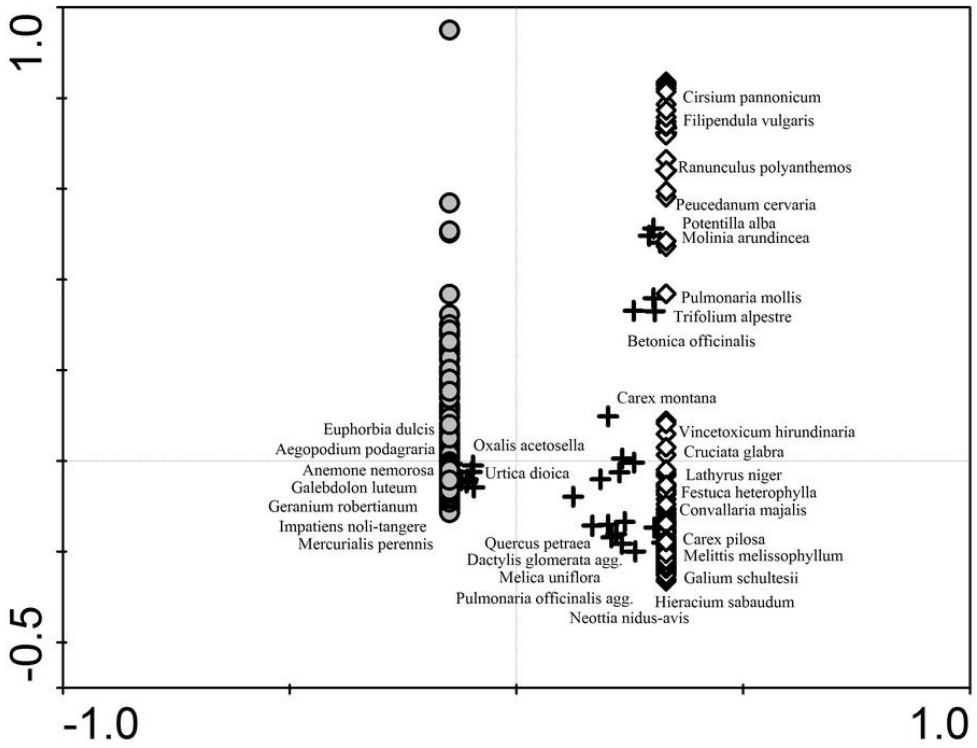

FIGURE 2.10. Canonical correspondence analysis (CCA) of phytosociological relevés from the Czech Republic (● relevés with 12*x* cytotype, ◊ relevés with 4*x* cytotype) with 33 most important species displayed (i.e. species with best fit to the first axis, depicted by black crosses).

Morphological differences between cytotypes

Polyploidy is a common source of taxonomical problems, mainly due to the formation of difficult polyploid complexes/series and/or the origin of taxa by reticulate evolution (Rieseberg 1991, Marhold & Lihová 2006, Soltis *et al.* 2009). In many cases, cytotypes of polyploid complexes do not clearly differ morphologically, such as in *Allium oleraceum* (Fialová *et al.* 2014), *Juncus bufonius* (Rooks *et al.* 2011) and *Pseudolysimachion maritimum* (Trávníček *et al.* 2004). In such cases, individual cytotypes are usually not recognised as autonomous taxa. Even if there is some morphological differentiation among cytotypes, there may be no correlation between quantitative and other characters, and the ploidy level (e.g. the *Centaurea phrygia* group, Koutecký *et al.* 2012). In contrast, there is a clear trend of enlarging organs with increasing ploidy level in some polyploid complexes (e.g. the *Cerastium pumilum* group, Letz *et al.* 2012; the *Molinia caerulea* complex, Dančák *et al.* 2012). Our results indicate that the *S. tuberosum* complex also fits the latter case: tetraploids have narrower leaves, smaller corollas, shorter styles, and so on (Table 2.1), although a certain overlap occurs, mostly because of the greater morphological variability of the dodecaploid cytotype. In general, our results are similar to those of Májovský & Hegedüšová (1993) for generative characters, but the vegetative characters are more variable in both

cytotypes than previously reported; this can be explained by the more extensive sampling used in this study.

Taxonomic treatment of Symphytum tuberosum in central Europe

Symphytum tuberosum s. l. is a morphologically and karyologically variable complex (Gadella & Kliphuis 1978, Murín & Májovský 1982). In addition to the ploidy levels revealed in this study, other cytotypes exist in south-eastern and southern Europe (e.g. Grau 1968, Markova & Ivanova 1970, Jaarsma *et al.* 1990, Bottega *et al.* 2001). There seems to be no clear relation between karyology and taxonomic classification on a European scale; instead, western-European plants are classified as *S. tuberosum* subsp. *tuberosum* and central- and eastern-European plants as *S. tuberosum* subsp. *angustifolium/nodosum* (Pawłowski 1972, Bottega & Garbari 2003, Valdés 2011). In contrast, both the study of Murín & Májovský (1982) and this study clearly show that there are two elements in central Europe that merit taxonomic classification, which correspond to the tetraploid and dodecaploid cytotypes. Thus, the current taxonomic treatment of the whole complex is challenged and needs to be revised.

Two dominant cytotypes of the *Symphytum tuberosum* complex in central Europe can be recognised based on morphology and differ in their habitat requirements and geographic distribution. Their distribution is parapatric, with a narrow contact zone. Mixed populations are rare, and no intermediate (hybrid) cytotypes were discovered. However, because the ranges in the variation of most of the morphological characters of the different cytotypes overlap and their habitat requirements are not completely distinct, we propose treating the cytotypes as subspecies. Murín & Májovský (1982) suggest that the widespread dodecaploid cytotype is *S. tuberosum* L., 1753, s. str., i.e. the type subspecies in our treatment. This view is consistent with the original location in the protologue ("*Germania australi*") for which dodecaploid chromosome counts are known (surroundings of München, Grau 1968; however, more chromosome counts from this area are needed) and also with the morphology of the lectotype (LINN 185.3; but note that plants could have originated from cultivation; Pugsley 1931). For tetraploids, Murín & Májovský (1982) propose the name *S. angustifolium* A. Kern., 1863 [*S. tuberosum* subsp. *angustifolium* (A. Kern.) Nyman, 1881], of which the type material (WU 69896–69899, high-resolution images available at http://herbarium.univie.ac.at) collected by Anton Kerner in the Pilis Mts (northern Hungary) corresponds well with the tetraploids. The name *S. angustifolium* is sometimes synonymized with *S. nodosum* Schur, 1866 [*S. tuberosum* subsp. *nodosum* (Schur) Soó, 1941], based on plants from southern Romania. The latter name, however, probably belongs to a different ploidy level and cannot be applied to central-European tetraploids. Moreover, even if it belonged to the same taxon, the names *S. angustifolium* and *S. tuberosum* subsp. *angustifolium* have priority in their respective ranks.

Identification key and taxonomic treatment of the *Symphytum tuberosum* group in central Europe

The central-European subspecies of *S. tuberosum* can be identified using the following key. If possible, several plants from a population should be studied and the average values used.

1a Rhizome stout; stem fleshy, thick; leaves elliptic, broadly ovate to ovate lanceolate, obtuse to acute; middle stem leaves 8–15.5 cm long and 2.5–5 cm wide, 2.3–3.5× long as wide; corolla yellow to dark yellow, somewhat robust, with lower narrowed part of the tube 7.3–9.5 mm long; style 15.8–19.8 mm long ..... *S. tuberosum* subsp. *tuberosum*
1b Rhizome rather slender; stem rather thin; leaves ovate lanceolate to narrowly lanceolate, acuminate; middle stem leaves 7–13 cm long and 1.6–3.6 cm wide, 3–4.8× long as wide; corolla pale yellow, smaller, with lower narrowed part of the tube 6.7–8.4 mm long; style 13.5–18.2 mm long ..... *S. tuberosum* subsp. *angustifolium*

Symphytum tuberosum Linnaeus, Sp. Pl. 136, 1753.

Typus: LINN 185.3 (lectotypus Stearn 1985: 177).

Symphytum tuberosum L. subsp. *tuberosum* (Fig. 2.11A–C)

DESCRIPTION: Perennial plants. Rhizomes stout, creeping, horizontal to oblique, tuberous. Stem 20–41(–52) cm tall, erect, fleshy, roughly hairy, simple or branched. Lower leaves petiolate, upper leaves sessile. Leaf blade of middle stem leaves elliptic, broadly ovate to ovate lanceolate, (5.1–)8.0–15.5(–19.3) cm long, (2.0–)2.5–5(–8.4) cm wide, 2.3–3.5× long as wide, obtuse to acute, densely hairy. Corollas dark yellow, (12.3–)14.5–17.5(–18.8) mm long, with lower narrowed part of the tube (5.7–)7.3–9.5(–10.8) mm long. Style (10.7–)15.8–19.8(–21.8) mm long. Filaments (0.5–)0.8–1.1(–1.3) mm long, anthers (0.25–)0.30–0.38(–0.44) mm long. Fornices triangular, (0.9–)1.1–1.5(–1.7) mm long. Mericarpids dark brown, densely verrucose. Flowers from late April to early June. $2n = 12x = 96$; DNA-decaploid and DNA-tetradecaploid individuals are rarely detected within populations using FCM.

DISTRIBUTION: Europe except for Scandinavia, rarely in western Mediterranean regions (southern border is unclear due to unresolved taxonomy of the other ploidy levels). In central Europe, it is widespread in Austria, the Czech Republic, southern Poland, northern Slovakia and western Hungary, but rare or absent in the warmest and driest areas, such as the Pannonian Lowlands.

ECOLOGY: Mesic deciduous woodlands, usually in shady places with humid soils, also along streams and on river alluvia or in ruderal vegetation.

Symphytum tuberosum subsp. *angustifolium* (A. Kern.) Nyman, Consp. Fl. Eur. 510, 1881. (Fig. 2.11D–F)

Typus: Hungary, Pilis, Slanitzka bei Csaba, Kerner, s.d., WU0069897 (lectotypus Bottega & Garbari 2003: 247).
≡ *Symphytum angustifolium* A. Kern., Österr. Bot. Z. 13: 227, 1863.
= *Symphytum tuberosum* auct. medioeur. pro parte

DESCRIPTION: Perennial plants. Rhizomes slender, creeping, oblique, interruptedly tuberous. Stem 24–43(–52) cm tall, erect, with short appressed rough hairs throughout, simple or branched from the middle or from the base. Lower leaves long petiolate, upper almost sessile. Petioles narrowly winged and shortly descending to the stem. Leaf blade of middle stemleaves ovate lanceolate to narrowly lanceolate, (4.2–)7–13(–19) cm long, (1.3–)1.6–3.6(–4.5) cm wide, 3.0–4.8× long as wide, acuminate, sparsely to densely roughly hairy. Peduncles hairy, hairs often with bulbous base. Corollas pale yellow, (12.3–)13.9–16.8 (–17.6) mm long, with lower narrowed part of the tube (5.5–)6.7–8.4(–9.7) mm long. Style (10.2–)13.5–18.2(–19.4) mm long. Filaments (0.6–)0.7–1.0(–1.1) mm long, anthers (0.23–)0.29–0.37(–0.46) mm long. Fornices narrowly triangular, (0.9–)1.1–1.4(–1.5) mm long. Mericarpids light brown, shiny, finely wrinkled. Flowers from early May to early June. $2n = 4x = 32$; DNA-hexaploid individuals are rarely detected within populations using FCM.

DISTRIBUTION: Northern margin of the Pannonian Basin and adjacent part of the Western Carpathians; known from the Czech Republic (central and south-eastern Moravia), southern Slovakia and northern Hungary. For detailed information on the distribution in Slovakia, see Murín & Májovský (1982).

ECOLOGY: It is more thermophilous than the type subspecies, grows in deciduous forests (especially oak-hornbeam or Carpathian beech forests with *Carex pilosa*), at their fringes and in semi-dry grasslands, on intermittently wet soils.

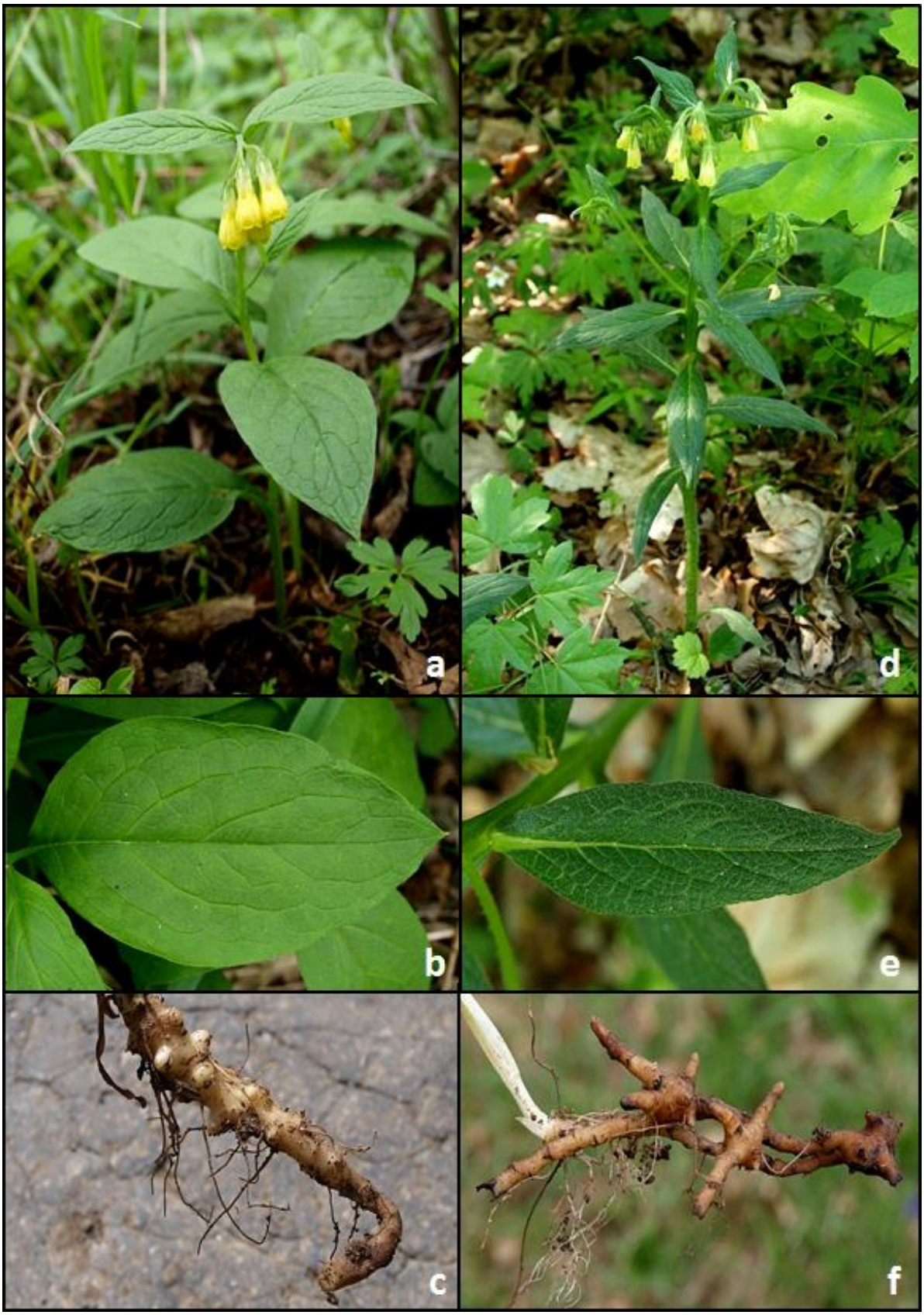

FIGURE 2.11. Typical plants of *Symphytum tuberosum* subsp. *tuberosum* (**A** – general habit, **B** – middle stem leaf, **C** – rhizome) and subsp. *angustifolium* (**D** – general habit, **E** – middle stem leaf, **F** – rhizome).

Chapter 3

Taxonomic status and typification of a neglected name in the *Symphytum tuberosum* complex (Boraginaceae)

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Taxonomic status and typification of a neglected name *Symphytum leonhardtianum* from the *Symphytum tuberosum* complex (Boraginaceae)

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Abstract

Symphytum leonhardtianum, a member of the *S. tuberosum* complex, is investigated. This taxon was described by Pugsley in 1931, from the vicinity of Vienna, Austria. Nevertheless, it is generally not accepted in European floras. In this study, we conducted an evaluation of this taxon using flow cytometry, karyology and morphological analysis. Flow cytometric and karyological investigations of plants from the type locality of *S. leonhardtianum* revealed only dodecaploids ($2n = 12x = 96$), a ploidy level corresponding to the *S. tuberosum* subsp. *tuberosum*. The chromosome number of the *S. tuberosum* from Austria is here recorded for the first time. Morphological comparison of Central European populations of *S. tuberosum* complex showed that *S. leonhardtianum* did not differ significantly from *S. tuberosum* subsp. *tuberosum*. Based on our findings, we propose treating the name *S. leonhardtianum* as a heterotypic synonym of *S. tuberosum* subsp. *tuberosum*. The lectotype of *S. leonhardtianum* is designated.

Keywords: Central Europe, flow cytometry, karyology, lectotypification, morphology, polyploidy

Introduction

The *Symphytum tuberosum* complex belongs to one of the most complicated groups within the genus *Symphytum* Linnaeus (1753a: 136) in Europe, mainly due to an occurrence of polyploidy and associated extensive morphological variability (Chapter 2; Gadella & Kliphuis 1978, Murín & Májovský 1982). Despite current progress, the taxonomy of *S. tuberosum* is still not satisfactorily resolved. The members of this complex are distributed across Europe and Asia Minor (Chapter 2; Bucknall 1913, Murín & Májovský 1982, and a total of ten taxa have been described within this complex, three of them from Central Europe: *Symphytum*

tuberosum Linnaeus (1753a: 136), *Symphytum angustifolium* A. Kerner (1863: 227) and *Symphytum leonhardtianum* Pugsley (1931: 95).

Symphytum tuberosum is one of the three species of Symphytum distinguished by Linnaeus. The original description is based on plant material apparently originating from southern Germany (Linnaeus 1753a). It is traditionally accepted as a wide-ranging European species. Plants from southern Germany were shown to have a dodecaploid cytotype ($2n = 96$; Chapter 2).

Symphytum angustifolium was described from the plant material collected in the Pilis Mountains in northern Hungary as a narrow-leaved morph of *S. tuberosum* (Kerner 1863). Later, it was also discovered in Slovakia and in the south-eastern part of the Czech Republic. It has been shown to have a tetraploid chromosome number ($2n = 32$; Murín & Májovský 1982, Chapter 2). Nevertheless, there has been much confusion surrounding this name, and it has been often synonymised with *S. nodosum* Schur (1866: 468) or applied to all populations of the *S. tuberosum* complex from East and Central Europe (cf. Pawłowski 1972, Smejkal 1978, Valdés 2011).

Symphytum leonhardtianum was described from specimens collected in Haltertal near Vienna, Lower Austria and was originally differentiated from *S. tuberosum* s. str. by its slender rhizomes, shorter and less branched stems, fewer and broader leaves, shorter and more strongly ciliate calyx lobes, brightly coloured corollas and smaller and paler mericarpids (Pugsley 1931). According to Pugsley (1931) the species is mainly confined to Central Europe, with its range extending from the French Alps and Pyrenees to Russia and Balkan Peninsula. However, *S. leonhardtianum* has been neglected in most European floras and only the Soviet and Ukrainian floras (Popov 1953, Dobroczajeva 1957) and some Ukrainian studies (Zaverucha 1962, Dobroczajeva 1968) recognise it.

Kobrlová *et al.* (Chapter 2) recently showed that two members of the *S. tuberosum* complex should be recognised in Central Europe: the widespread dodecaploid ($2n = 12x = 96$) and broad-leaved taxon corresponding to *S. tuberosum* subsp. *tuberosum* (thereafter *S. *tuberosum*) and the tetraploid ($2n = 4x = 32$) narrow-leaved taxon corresponding to *S. tuberosum* subsp. *angustifolium* (A. Kern.) Nyman (1881: 510; thereafter *S. *angustifolium*), which shows an affinity to the northern regions of the Pannonian Basin (Chapter 2, 4). Unfortunately, the name *S. leonhardtianum* was omitted from their study and its analysis is therefore provided here.

The aims of the present study are (i) to determine DNA-ploidy level, the number of chromosomes and morphological variation of the populations from the locus classicus of *S. leonhardtianum* and its close vicinity and (ii) to infer the relationship of these populations within the *S. tuberosum* complex in the Central Europe.

Material and Methods

Plant material and morphometric analyses

Plant material for *S. leonhardtianum* was collected in the locus classicus (i.e., Haltertal) and its vicinity in western surroundings of Vienna (Pugsley 1931). In total, five populations (37 individuals) were collected (see Electronic Appendix 3.1). Additional four populations (32

individuals) of *S. *angustifolium* (two from the locus classicus in Pilis Mts., northern Hungary and two from Moravia) were also collected. Voucher specimens are deposited in the Herbarium of the Palacký University in Olomouc (OL). A morphological investigation was conducted on 64 individuals from eight populations and added to the dataset used in Chapter 2. Altogether, 50 populations of the *S. tuberosum* complex from Central Europe were morphologically evaluated. For each individual, 19 vegetative and generative characters were studied (Table 3.1), i.e. the same set of morphological traits that was already used for differentiation of Central European populations of *S. tuberosum* (Chapter 2). Other characters, such as rhizome slenderness and colour of flowers and mericarpids were compared later in the herbaria and are not included in the analyses.

Morphological character (unit)	**Code**
Height of plant (cm)	height
Length to width ratio of uppermost leaf	shape_U
Length of uppermost leaf (cm)	
Width of uppermost leaf (cm)	
Length to width ratio of middle leaf	shape_M
Length of middle leaf (cm)	
Width of middle leaf (cm)	
Length to width ratio of lowermost leaf	shape_L
Length of lowermost leaf (cm)	
Width of lowermost leaf (cm)	
Length of pedicel (mm)	l_ped
Length of calyx (mm)	calyx
Length of corolla (mm)	corolla
Length of narrow part of corolla tube (mm)	cor_tube
Length of style (mm)	style
Length of filament (mm)	l_fill
Width of filament (mm)	w_fill
Length of free part of filament (mm)	l_ffill
Length of fornice (mm)	l_forn
Length of anther (mm)	l_anth
Width of anther (mm)	w_anth

TABLE 3.1. List of the morphological characters analysed, and their codes used in the descriptive statistics (Fig. 3.3).

Flow Cytometry (FCM)

DNA-ploidy amounts were estimated using a Partec PAS flow cytometer equipped with a green solid-state laser. Samples were prepared following the simplified protocol with LB01 isolation buffer and propidium iodide (Sigma-Aldrich, St Louis, MO, USA) staining (Doležel *et al.* 2007). Details for sample preparation are given in Chapter 2. *Pisum sativum* 'Ctirad' (2C = 9.09 pg; Doležel *et al.* 1998) and *Zea mays* 'CE-777' (2C = 5.92 pg, value calibrated against *Pisum sativum* 'Ctirad') were used as the internal standards. Each plant was analysed

separately and the fluorescence intensity of at least 3,000 particles was recorded. The resulting values were determined by the position of its G0/G1 peak relative to the G0/G1 peak of the internal standard. Histograms with a coefficient of variation less than 5 % were accepted.

Chromosome counts

Actively growing, young roots were harvested from the cultivated plants, pre-treated with ice-cold water for 24 h, fixed in ethanol/acetic acid (3:1) fixative for 24 h at 4°C and stored at -20°C until further use. Selected root tips were rinsed in distilled water (twice for 5 min) and citrate buffer (10 mM sodium citrate, pH 4.8; twice for 5 min), and digested in 0.3% cellulase, cytohelicase and pectolyase (all Sigma-Aldrich, St Louis, MO, USA) in citrate buffer at 37°C for 90 min. After digestion, individual root tips were dissected on a microscope slide in approximately 10 µl acetic acid and covered with a cover slip. The cell material was then spread evenly using tapping, thumb pressing and gentle flame-heating. Finally, the slide was quick frozen in liquid nitrogen and the cover slip flicked off with a razor blade. Slides were fixed in ethanol/acetic acid (3:1) and air-dried. Chromosomes were counterstained with 2 µg/ml DAPI in Vectashield. Preparations were photographed using Zeiss Z2 epifluorescence microscope and CoolCube CCD camera.

Statistical analyses

All studied morphological characters were used except for the length and width of the leaves from which ratios were calculated. The morphological dataset therefore contained 12 measured morphological characters and three ratios (Table 3.1). The dataset was analysed using a set of R functions contained in MorphoTools version 1.01 (Koutecký 2015). Basic descriptive statistics (average, minimum, maximum) were calculated for each morphological character and studied taxon. Tukey-Kramer multiple comparison tests at $p \leq 0.01$ for all three putative taxa (*S. *angustifolium*, *S. leonhardtianum*, *S. *tuberosum*) were calculated to determine which characters show significant differences among groups. Population averages were calculated and used as operational taxonomic units (OTUs) for multivariate analyses. Logarithmic transformations of several characters were applied, i.e. natural logarithmic transformations (log) of the pedicel length and the fornice length and common logarithmic transformations (log10) for the style length and the anthers width. Correlations of morphological characters were tested using Pearson's correlation coefficient. A Principal component analysis (PCA; Sneath & Sokal 1973) was used to test the morphological homogeneity within three putative taxa. The character 'branching of stem', due to its qualitative nature, was separately analysed using subdivided contingency tables (Zar 1996) in NCSS 9 (Hintze 2013).

Typification process

Name was typified following the instructions of the International Code of Nomenclature for algae, fungi and plants (Melbourne Code; McNeill *et al.* 2012).

Results

Flow Cytometry

FCM data were newly obtained for 69 plants from nine populations. All five populations from the vicinity of the locus classicus of *S. leonhardtianum* had DNA-dodecaploid ploidy level. Additional four populations of *S. *angustifolium* were all DNA-tetraploids (Table 3.2).

Chromosome counting

Two individuals of *S. leonhardtianum* (from populations 455 and 456; Electronic Appendix 3.1) were counted to calibrate the results from FCM. Both counts resulted in $2n = 96$ (Fig. 3.1).

Morphometric analyses

The extent of the morphological variability of *S. leonhardtianum* was generally similar to the variability of the morphological traits of *S. *tuberosum*. The average value of several morphological characters of *S. leonhardtianum* measured, e.g. corolla length, corolla tube length, style length, significantly exceeded the average value detected for the same characters of *S. *tuberosum* (Table 3.3, Fig. 3.2). No pairs of highly correlated characters ($r > 0.95$) were found. Therefore, the entire dataset was used in the multivariate analyses. Two groups corresponding to *S. *angustifolium* and *S. *tuberosum* were separated along the first component axis in the principal component analysis (the first, second and third axis explaining 42.6 %, 15.7 % and 13.6 % of variation, respectively). All five studied populations putatively belonging to *S. leonhardtianum* were grouped together with *S. *tuberosum* in the PCA diagram (Fig. 3.3). The pattern of branching was significantly different between the three taxa ($\chi 2 = 63.24$; DF = 6; $P < 0.01$). Subdivided contingency tables showed that *S. leonhardtianum* and *S. *tuberosum* have very similar branching pattern ($\chi 2 = 5.09$; DF = 3; $P = 0.17$) and they both differ significantly from *S. *angustifolium* ($\chi 2 = 58.78$; DF = 3; $P < 0.01$).

	DNA ploidy level	N	Mean ratio to the standard ± SE	Range	Variation (%)	Mean 2C-value (pg) ± SE
A	4*x*	413	0.247 ± 0.011	0.222–0.278	22.9	2.03 ± 0.104
B	12*x*	739	0.663 ± 0.017	0.628–0.698	10.6	6.03 ± 0.171
C	12*x*	37	0.662 ± 0.020	0.623–0.698	11.3	6.02 ± 0.178

TABLE 3.2. Relative DNA content of the *Symphytum tuberosum* complex in Central Europe assessed using flow cytometry. A) *S. *angustifolium* (Chapter 2, including four populations from this study), B) *S. *tuberosum* (Chapter 2) and C) populations from the locus classicus of *S. leonhardtianum*. All values are calculated relative to the internal standard *Pisum sativum* 'Ctirad'. Tetraploids were analysed with *Zea mays* 'CE-777'; the result was then recalculated to *Pisum sativum*. N = number of samples analysed; SE = standard error of mean. Variation is calculated as the difference between the most extreme values expressed in % of the mean value.

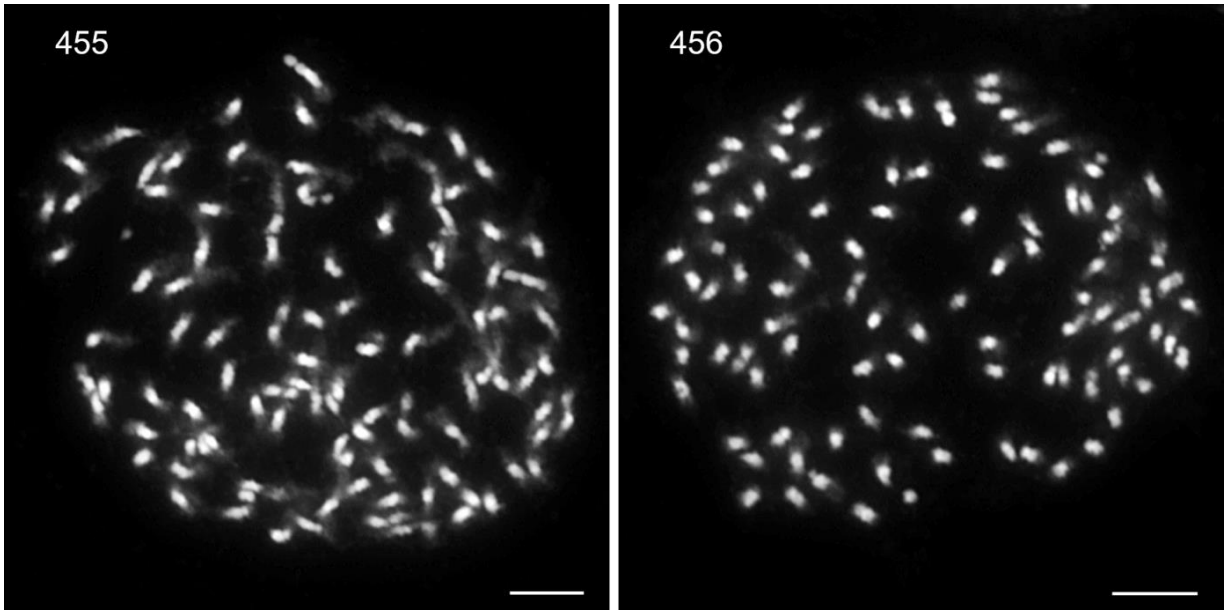

FIGURE 3.1. Micrographs of somatic metaphase chromosomes of two individuals from the locus classicus of *Symphytum leonhardtianum* (455) and its vicinity (456) near Vienna, Austria. Scale bar = 10 μm.

	S. *angustifolium		***S. leonhardtianum***		***S. *tuberosum***	
	(min)mean(max)	**±SD**	**(min)mean(max)**	**±SD**	**(min)mean(max)**	**±SD**
height	(156)339(593)*	76	(140)275(390)	60	(85)303(517)	79
shape_U	(1.2)3.3(5.5)*	0.7	(1.5)2.5(4.1)	0.6	(1.6)2.6(4.7)	0.5
shape_M	(1.7)3.8(6.1)*	0.8	(1.9)2.7(3.9)	0.5	(1.4)2.8(4.8)	0.5
shape_L	(1.3)4.1(8.7)*	1.0	(1.3)2.8(4.4)	0.6	(1.5)2.8(5.5)	0.5
l_ped	(4.2)8.2(14.8)	1.9	(3.6)7.3(11.9)**	2.0	(4.0)8.5(15.0)**	2.0
calyx	(4.6)7.6(11.9)	1.1	(6.1)7.8(10.7)	0.9	(5.2)7.7(11.3)	1.1
corolla	(12.3)15.3(17.6)*	1.1	(13.6)16.7(20.7)*	1.6	(12.3)16.1(18.8)*	1.2
cor_tube	(5.5)7.7(9.7)*	0.7	(6.4)9.3(12.2)*	1.2	(5.7)8.4(10.8)*	0.8
style	(10.2)16.1(20.2)*	1.7	(15.3)18.8(22.2)*	1.3	(10.7)17.8(21.8)*	1.6
l_fill	(0.57)0.88(1.48)*	0.09	(0.77)1.07(1.38)*	0.15	(0.53)0.96(1.30)*	1.22
w_fill	(0.03)0.05(0.08)**	0.01	(0.03)0.05(0.07)	0.01	(0.03)0.06(0.08)**	0.01
l_ffill	(0.13)0.21(0.29)*	0.04	(0.16)0.27(0.34)*	0.05	(0.09)0.23(0.34)*	0.05
l_forn	(0.89)1.23(1.52)*	0.11	(1.07)1.41(1.75)*	0.16	(0.93)1.30(1.65)*	0.14
l_anth	(0.23)0.33(0.46)*	0.03	(0.27)0.35(0.45)	0.04	(0.21)0.34(0.44)	0.04
w_anth	(0.05)0.07(0.10)*	0.01	(0.06)0.07(0.08)	0.01	(0.06)0.07(0.09)	0.01

TABLE 3.3. Basic descriptive statistics for each taxon. (min)mean(max) = minimal, average and maximal value of morphological character, in millimetres; SD = standard deviation; asterisk = means significantly different from each of all groups and two asterisks denote two groups significantly different at $p \leq 0.01$ in Tukey-Kramer multiple comparison test.

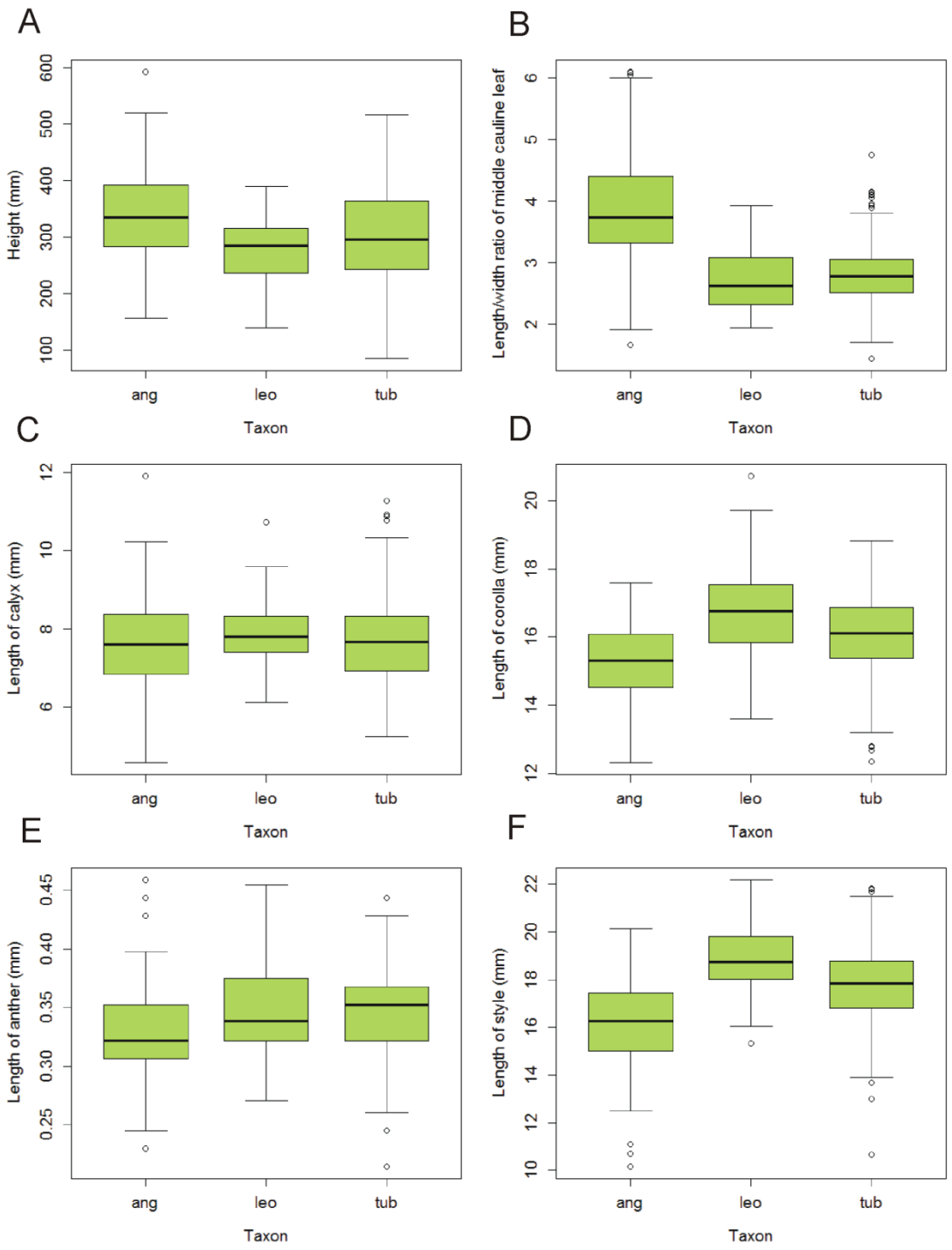

FIGURE 3.2. Variation of selected morphological characters and ratios. Rectangles define the 25th and 75th percentiles, horizontal lines show the median, whiskers are from the 10 to 90 percentiles, circles show extreme values. (**A**) height of plants. (**B**) length to width ratio of middle cauline leaves. (**C**) length of calyx. (**D**) length of corolla. (**E**) length of anther. (**F**) length of style.

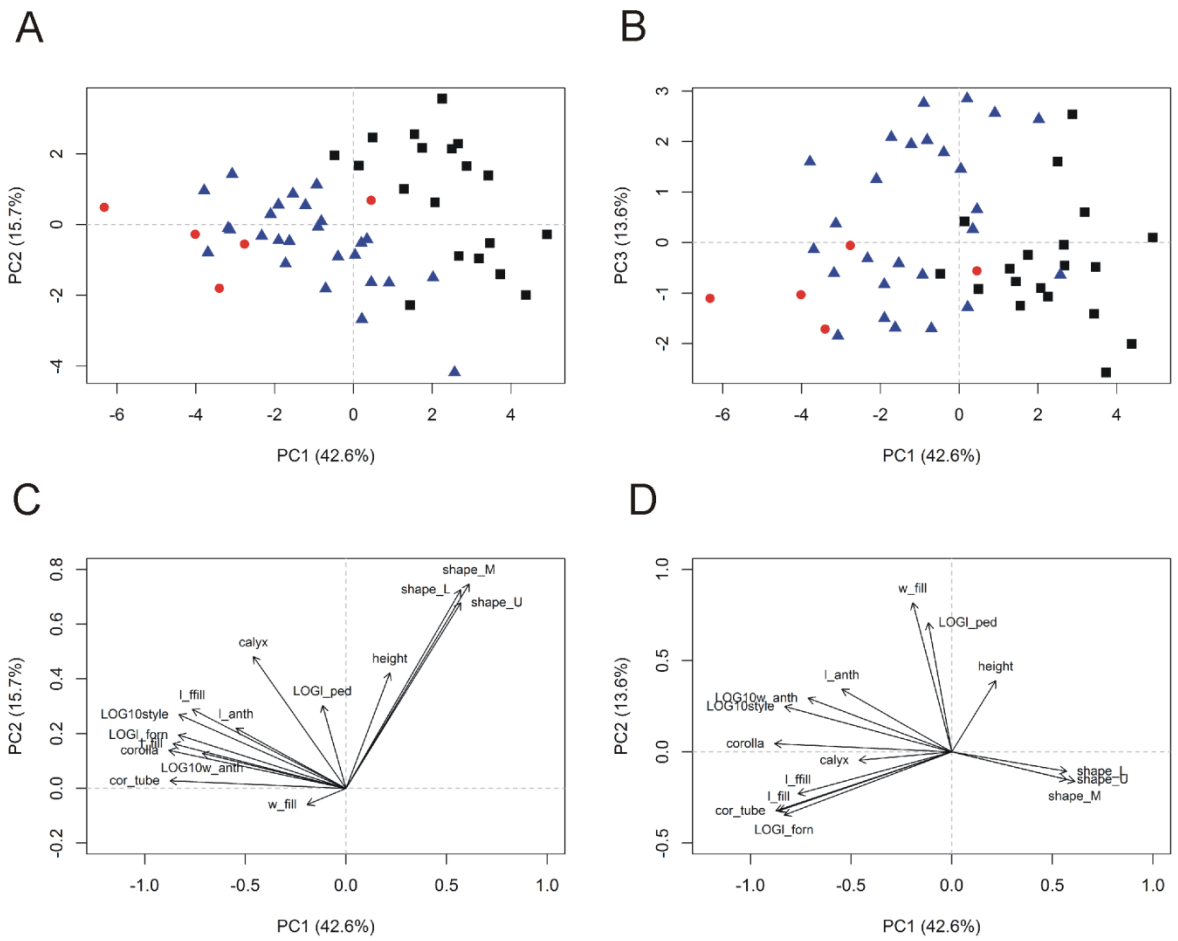

FIGURE 3.3. Principal component analysis (PCA) of 50 populations based on 12 morphological characters and three ratios. Squares correspond to *S. *angustifolium*, triangles to *S. *tuberosum* and circles to *S. leonhardtianum*. (**A**) PCA of populations, first and second axes displayed. (**B**) PCA of populations, first and third axes displayed. (**C**) fit of the morphological characters and ratios to the ordination axes (abbreviations of morphological characters are explained in Table 3.1), first and second axes displayed. (**D**) fit of the morphological characters and ratios to the ordination axes, first and third axes displayed.

Discussion

The morphological variability within the *S. tuberosum* complex is high (cf. Chapter 2). We assume that a substantial part of this variation is probably caused by morphological plasticity, rather than genetic variability. Moreover, this variation is often increased by ecological conditions, especially by the availability of water and nutrients, sometimes resulting in atypical local entities, which deviate from the typical form (i.e. dwarfed plants, plants with unusual proportion of leaves and with sparse inflorescences). However, more detailed investigations are necessary in order to confirm this hypothesis. Nevertheless, the variation found in several morphological traits is correlated with the ploidy level and as such it has its taxonomical value (Chapter 2).

The taxon *S. leonhardtianum* was distinguished from *S. tuberosum* by the British amateur botanist H.W. Pugsley (Pugsley 1931, Lousley 1948) based on his knowledge of *S. tuberosum* from England, which he considered to be the true origin of the Linnean type (instead of southern Germany, Pugsley 1931, Stearn 1985). He observed dwarfed and more ornamental plants of *S. tuberosum* near Salzburg (Austria) and later in herbaria elsewhere from Central Europe and decided to describe them as a new species based on A. Kerner's Flora Exsiccata Austro-Hungarica no. 3710. Based on his conviction that the "true" *S. tuberosum* grows in England, he distinguished *S. leonhardtianum* from *S. tuberosum* mainly on the basis of shorter stems, broader leaves and more conspicuous flowers (Pugsley 1931). However, our analysis showed that *S. leonhardtianum* from its locus classicus is indistinguishable from *S. tuberosum* s. str. in most of these morphological traits (Table 3.3, Fig. 3.3). Similarly, McClintock (1968) and his colleagues when revising material of the *S. tuberosum* complex that was determined by Pugsley in the British Museum, considered *S. leonhardtianum* as inseparable from *S. tuberosum*.

Analysed individuals of *S. leonhardtianum* did not differed from individuals of *S. *tuberosum* in several morphological characters used by Pugsley (1931) for distinction of these two taxa (i.e., height of stems, width of leaves and length of calyx; Table 3.3). Likewise, the pattern of stem branching was similar to the branching in *S. *tuberosum*, i.e. prevailing of plants unbranched and branched in the lower part of the stem. According to Pugsley (1931), *S. leonhardtianum* is also distinctive by its slender rhizomes. Although, we have not evaluated the character of rhizomes, based on our observations, rhizomes of *S. leonhardtianum* are the same as in *S. *tuberosum* which is characterised by stout, creeping, horizontal to oblique and tuberous rhizomes (Chapter 2). Other morphological characters used by Pugsley such as hairiness of calyx and colour nuance of flowers and mericarpids are very hard to quantify and therefore not very useful for species distinction. However, the comparison of herbarium specimens collected at loci classici of both taxa yielded no substantial differences in these traits. Quite surprisingly, the plants from four out of five of the populations studied in the close vicinity of Vienna (i.e., locus classicus of *S. leonhardtianum*) were found to have corollas and associated characters (i.e., length of fornices, styles and filaments) slightly larger in average (i.e., 2 mm) than all other plants evaluated from Central Europe. The size of flowers may be to some extent affected by ecological conditions or these populations may represent a local morph with somewhat larger flowers. However, such small differences in size of flowers were not considered as important trait for taxonomy in any morphological analysis of the *Symphytum* (Gadella *et al.* 1983, Sandbrink *et al.* 1990).

In absence of a clear morphological distinction, *S. leonhardtianum* was not recognised in most of the European floras. In most cases, it was synonymised with other member of the *S. tuberosum* group, usually with *S. *angustifolium* (e.g., Pawłowski 1961, Pawłowski 1963, Soó 1968, Stearn 1985, Sandbrink *et al.* 1990, Bottega & Garbari 2003, Fischer *et al.* 2008, Valdés 2011). The only exceptions are the Soviet (Popov 1953) and Ukrainian floras (Dobroczajeva 1957) and the studies of the Ukrainian botanists Zaverucha (1962) and Dobroczajeva (1968), who recognised *S. leonhardtianum* as a separate species. However, the new editions of the Russian Floras do not follow this concept and either refer the *S. leonhardtianum* only as a synonym of *S. popovii* Dobrocz. (1968: 59; Fedorov 2001) or do not mention this name at all (Czerepanov 2007).

The FCM analyses of Central European populations revealed two ploidy levels in the studied material: significantly less common tetraploids ($2n = 4x = 32$) growing only in the Czech Republic, Slovakia and Hungary and widespread dodecaploids ($2n = 12x = 96$), occurring throughout the whole Central Europe (Chapter 2). These findings are in agreement with previously reported chromosome numbers by e.g. Májovský (1976), Gadella & Kliphuis (1978), Murín & Májovský (1982) and Javůrková-Jarolímová & Měsíček (1992) for dodecaploid and by Murín & Májovský (1982) for tetraploid plants. Unfortunately, no chromosome records of *S. leonhardtianum* were published. Additionally, there is no evidence about the chromosome counts of any *S. tuberosum* from Austria (cf. Dobeš & Vitek 2000). Our study therefore presents first chromosome counts for this country. Only two karyological studies mentioned the name *S. leonhardtianum* as a synonym of another member of the *S. tuberosum* complex (Grau 1968, Wcisło 1972). Both these studies reported dodecaploid chromosome counts from countries (i.e., Germany and Poland), where only *S. *tuberosum* is present according to Chapter 2. All studied populations of *S. leonhardtianum* from the vicinity of Vienna belong to a dodecaploid cytotype (i.e., the same as in *S. *tuberosum*). Moreover, this is the only cytotype detected in Austria up to now and there is no evidence about the presence of another cytotype (Chapter 2).

Finally, there are also no specific differences in habitat preferences of *S. leonhardtianum* as we found all plants growing generally in the same conditions as *S. *tuberosum*, i.e. mesic deciduous and shady woodlands and in ruderal vegetation along road (Chapter 2).

Therefore, when considering all available evidence, we assume that the plants from the locus classicus of *S. leonhardtianum* do not differ substantially from *S. tuberosum* subsp. *tuberosum sensu* Kobrlová *et al.* (Chapter 2) and therefore should not be considered as a separate species, even though they may represent a specific local form with somewhat larger flowers.

Conclusions

Altogether three taxa of the *Symphytum tuberosum* complex (*S. tuberosum*, *S. angustifolium* and *S. leonhardtianum*) have been reported from Central Europe, however, our study confirms the presence of only two taxonomic entities: the narrow-leaved, tetraploid *S. tuberosum* subsp. *angustifolium* and the widespread, dodecaploid and broad-leaved *S. tuberosum* subsp. *tuberosum* (see also Chapter 2) with *S. leonhardtianum* included as a synonym of the latter taxon.

Taxonomic treatment

Symphytum tuberosum Linnaeus (1753: 136). Lectotype (designated by Stearn 1985: 177):—GERMANY. "*Germania australi*", C. Linnaeus s.n. (LINN 185.3!).

Symphytum tuberosum subsp. *tuberosum*
= *Symphytum leonhardtianum* Pugsley (1931: 95). Lectotype (designated here):—AUSTRIA. Vienna: "*Austria inferior, Haltertal prope Vindobonam (Wien) [Vienna], in dumetis*", A. Kerner s.n. (BM no. 000752614!, Fig. 3.4; known isolectotypes BRNU!, PRC!).

Notes on typification.—When describing *S. leonhardtianum*, Pugsley did not mention the location of the type. Although several attempts were made, the name *S. leonhardtianum* Pugsley was never properly typified. The first attempt was made by Arto Kurtto when revising specimens of *Symphytum* in BM in 1983. He labelled the specimen no. 000752614 as lectotype with a note stating that the lectotypification would be made in the journal Annales Botanici Fennici, however, to our knowledge this was never done (A. Kurtto pers. communication). The second attempt, made by Bottega and Garbari in 2003, was also not successful because the authors did not include the term "designated here" or its equivalent (Art 7.10; McNeill *et al.* 2012).

Symphytum tuberosum subsp. *angustifolium* (A. Kern.) Nyman (1881: 510). Lectotype (designated by Bottega & Garbari 2003: 247):—HUNGARY. "*Pilis, Slanitzka bei Csaba*", A. Kerner s.n. (WU0069897!).

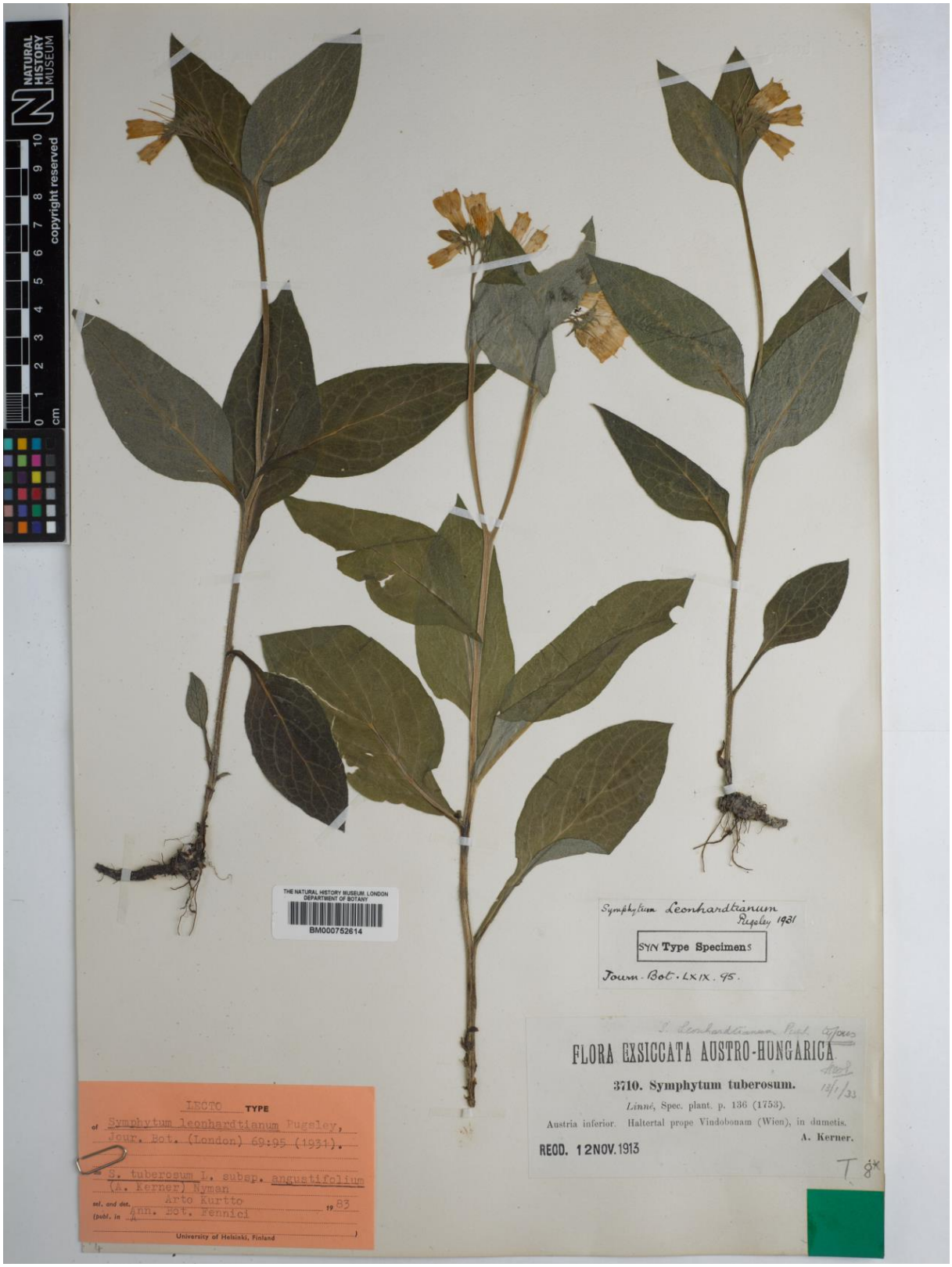

FIGURE 3.4. Lectotype specimen of *Symphytum leonhardtianum* (BM 000752614, from the collections of the Natural History Museum, London).

Chapter 4

Morphological, ecological and geographic differences between diploids and tetraploids of *Symphytum officinale* (Boraginaceae) justify both cytotypes as separate species

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Abstract

Polyploidization is generally considered to be an important evolutionary driver affecting the genetic diversity, that can alter the morphology, phenology, physiology or ecology of plants, which in turn may make the taxonomy of polyploids more difficult. One such example is the *Symphytum officinale* complex, a polyploid species group represented by three major cytotypes: tetraploids (2*n* = 48), and less common, geographically restricted diploids (2*n* = 24) and hypotetraploids (2*n* = 40), with several aneuploids reported as well. In most of European floras only one polymorphic species *S. officinale* is widely recognised while the particular cytotypes are usually considered conspecific. Our study provided a thorough evaluation of the ploidy level diversity, morphological and ecological variation, with a special attempt to clarify status of "white-flowered" diploids. Using flow cytometry, we identified three cytotypes: widespread tetraploids (76.1%); less frequent diploids (23.6%) with scattered distribution across the range of tetraploids and confined only to several areas of Europe; and extremely rare triploids (0.3%). Cytotypes (2*x*, 4*x*) showed diffuse parapatric pattern of distribution, with only four mixed-cytotype populations (2.7%) found, but almost entirely without triploids, suggesting reproductive isolation between di- and tetraploids. Diploids were clearly distinguishable morphologically from tetraploids. Niche of diploids falls nearly completely within the niche of tetraploids that showed niche expansion and an almost complete filling of the diploid niche. Tetraploids also showed a shift in niche optimum toward a less continental and colder climate, coupled with expansion to more disturbance-prone sites with higher nutrient availability. The morphological differentiation of studied cytotypes is obvious and appears to be taxonomically significant, especially in combination with ecological segregation and the apparent presence of hybridisation barriers. Both cytotypes should be treated as separate species (i.e. *S. bohemicum* F.W. Schmidt and *S. officinale* s. str.).

Keywords: autopolyploidy, Boraginaceae, cytogeography, flow cytometry, niche modelling, taxonomy

Introduction

Polyploidy is generally considered as a major evolutionary force in higher plants (Otto & Whitton 2000). Chromosome doubling acts as an immediate strong reproductive barrier and affects many important processes and traits at different levels of organisation from genome to individual plant (Levin 2002). After formation, polyploids often diverge from their diploid progenitors in morphology, physiology, and ecology, which may affect their distribution pattern, resulting in shifts in range between diploid and polyploid relatives (Ramsey & Schemske 2002, te Beest *et al.* 2012, van de Peer *et al.* 2017). However, frequently reported wider or more extreme ranges of polyploids are not a general trend in plants, and in many mixed-ploidy complexes, even opposite relationship is known to occur (Husband *et al.* 2013, Visger *et al.* 2016, Spoelhof *et al.* 2017). One likely reason for the various distribution patterns of the cytotypes might be the participation of different routes leading to polyploidization. Two main paths are usually considered, autopolyploidization

(polyploidization on intraspecific level) and allopolyploidization (polyploidization coupled with interspecific hybridization). Autopolyploidy, in contrast to allopolyploidy, does not inevitably produce transgressive traits to boost adaptive ecological divergence (Parisod *et al.* 2010) and autopolyploids might escape from minority cytotype disadvantage and achieve establishment alternatively also by spatial separation unaccompanied by niche divergence, e.g. by a chance colonization of recently opened (disturbed) habitat (te Beest *et al.* 2012, Godsoe *et al.* 2013). Moreover, there is still different perception of allo- and autopolyploids in taxonomy, as allopolyploids are usually considered as different taxa given their divergent morphology from their diploid ancestors while autopolyploids are regarded as conspecific with diploids due to their high morphological similarity (Soltis *et al.* 2007). In their review, Soltis *et al.* (2007) argued for taxonomic recognition of autopolyploids after the careful examination of the studied complex. However, such studies are still relatively sparse.

In this study, we focus on the *Symphytum officinale* complex, which is the widespread *Symphytum* L. (Boraginaceae, Boragineae; Chacón *et al.* 2016) group in Europe. An extensive cytological variation has been observed in this complex which corresponds to three main cytotypes: diploid ($2n = 24$), tetraploid ($2n = 48$) and dysploid (hypotetraploid, $2n = 40$). Tetraploids are of presumable autopolyploid origin (Gadella & Kliphuis 1972) and represent the most frequently documented ploidy level covering the whole range of the complex, whereas the data on diploids are rather solitary and scattered across Europe (Basler 1972, Gadella & Kliphuis 1972, Wille 1998, Peruzzi *et al.* 2001). Zones with occasional sympatric growth of diploids and tetraploids have been observed in some parts of Europe (Gadella & Kliphuis 1967, Wille 1998), but there are almost no records of triploids ($2n = 36$; Basler 1972). The hypotetraploids are the rarest of the three main ploidy levels and they also have very scattered distribution (Gadella & Kliphuis 1967, Májovský & Uhríková 1985, Peruzzi *et al.* 2001).

The complex is known for its high morphological variability that led to confusion and nonuniformity of taxonomic concepts across European floras (Table 4.1). Flower colour varying from pure white to dark purple, corolla shape and size, and decurrency of leaves to stem are considered as the most important characters for taxonomy of this group (Smejkal 1978, Májovský & Hegedüšová 1993, Peruzzi *et al.* 2001). However, it is not always clear how the morphology is connected with a particular ploidy level. In the Czech Republic and Slovakia, the diploids are linked to the name *S. bohemicum* F.W. Schmidt (Fig. 4.1A) and tetraploids to *S. officinale* L. s. str. (Fig. 4.1B). Elsewhere in Europe, diploids and tetraploids are mainly considered as mere cytotypes of S. officinale, while hypotetraploids are almost exclusively called *S. tanaicense* Steven (Table 4.1, Fig. 4.1C). Dysploidy contrary to autopolyploidy is considered a strong reproductive barrier (Mandáková & Lysák 2018), therefore, *S. tanaicense* is generally regarded as a separate species (Table 4.1).

Here, our objective was to explore the cytotype diversity of the *S. officinalis* complex in Europe and its effect on the taxonomy. We summarised the geographic distribution of the cytotypes and asked whether the observed spatial patterns might be explained by abiotic factors. In addition, we investigated whether morphology correlates with established ploidy levels and therefore can be unequivocally connected with the taxa described. For that, we (1) revised published chromosomal counts (2) investigated the diversity and distribution of cytotypes throughout Europe using flow cytometry, (3) examined the morphological

differences between cytotypes using multivariate morphometrics, and (4) studied the ecological differences between cytotypes on the continental scale using niche modelling and on local spatial scales in the area of sympatry using records of vegetation surrounding occurrence points. More specifically, we placed particular emphasis on the white-flowered plants and their relation to the name *S. bohemicum*, in order to deal with the taxonomic chaos that is connected with this taxon.

Material and methods

Study species

The members of the *Symphytum officinale* complex are traditionally placed in the sect. *Symphytum* characterised by fusiform, ± vertical rhizomes, decurrent leaves, broadly triangular-lanceolate, acute, densely papillate faucal scales, stamens with connectives projecting beyond thecae and smooth, shiny nutlets (Pawłowski 1961). The complex consists of widespread *S. officinale* (Fig. 4.1B) and several local taxa sometimes recognised in regional floras (Table 4.1), i.e. *S. tanaicense* (Fig. 4.1C) from the Don river delta in the south-western Russia, *S. uliginosum* A. Kern. from Hungary and *S. bohemicum* (Fig. 4.1A) from the Elbe basin in the Czech Republic. The conspecificity of *S. tanaicense* and *S. uliginosum* has already been discussed by Degen (1930) who identified the name *S. tanaicense* as the oldest validly described name. *Symphytum officinale* s. str. represents the widest-ranging member of the whole genus, growing in most of Europe to Western Siberia and Central Asia (Meusel *et al.* 1978; Hultén & Fries 1986). It is also cultivated worldwide as a nectar source, fodder plant, or green manure, sometimes escapes from cultivation and becomes naturalised, e.g. in North and South America, China and New Zealand (Gadella 1984, Hultén & Fries 1986, Zhu *et al.* 1995, Jørgensen *et al.* 2014). The possible interploidy hybridization between diploids and tetraploids, based on the intermediate morphology of plants, is rarely documented (Smejkal 1978, Májovský & Hegedüšová 1993, Buch *et al.* 2007), and such plants have been described as *S.* ×*rakosiense* (Soó) Pénzes. However, chromosomes of any of these plants have never been counted.

Plant material

Samples were collected between 2014 and 2021 in Europe, with special attention to Central Europe. In total, 156 populations and 776 individuals were sampled (Electronic Appendix 4.1), and for all of them the DNA-ploidy level was determined by flow cytometry. The number of individuals sampled per population varied from 1 to 15 (mean ± SD: 5 ± 2). Voucher specimens are deposited in the Herbarium of Palacký University in Olomouc (OL).

Flow cytometry and chromosome number revision

DNA-ploidy level (Suda *et al.* 2006) and absolute genome size (AGS; Greilhuber *et al.* 2005) were estimated using flow cytometry. Generally, fresh leaf tissue has been used, but in some cases silica-dried material has also been analysed. Samples were prepared according to the protocol described in Chapter 2 and were carried out on the following flow cytometers using

two different fluorochromes staining: (i) BD Accuri C6 (BD Biosciences, San Jose, CA, USA) – propidium iodide (PI); (ii) Partec PAS (Partec GmbH, Münster, Germany) – PI; (iii) Partec Cy Flow ML (Partec GmbH) – 4,6-diamidino-2-phenylindole (DAPI). *Pisum sativum* L. 'Ctirad' (2C = 9.09 pg; Doležel *et al.* 1998) and *Zea mays* 'CE-777' (2C = 5.92 pg, the value recalculated to the primary standard *Pisum sativum*) were used as internal references. The ploidy level of each sample was determined by the position of its G0/G1 peak relative to the G0/G1 peak of an internal standard. For each sample, the fluorescence intensity of 3000 and 5000 particles was recorded for DNA-ploidy level (relative genome size, RGS) and for AGS (expressed as 2C value) estimations, respectively. For AGS estimation, each sample was prepared and analysed three times. The rule was followed that the between-day variation of the sample does not exceed 2%. The ploidy level was calibrated using population ID 38 from which previous chromosome record exist (Murín & Májovský 1982).

In addition, a complete bibliographic review of published chromosome counts was performed (Electronic Appendix 4.2) to find out the karyological variability of the complex. Together with flow cytometric data, the compiled chromosome counts were used to build a distribution map of the *S. officinale* complex. Only data with given localities were used and georeferenced.

Estimation of environmental and geographic niches at large spatial scale

Climatic and soil data related to different eco-physiological constraints of plant species were downloaded from various open-source databases. The WorldClim 2.1 database (Fick & Hijmans 2017) was used for the extraction of annual trends and extreme limiting conditions related to precipitation, temperature, and solar radiation (bio 1-19 variables; mean annual solar radiation [$kW.m^{-2}$]). Quantitative physical and chemical soil variables were downloaded from the SoilGrid database (Hengl *et al.* 2017). All downloaded variables had a resolution of 30 arcseconds (~1 km).

All data adjustments and calculations were performed on the R platform. To trim the predictor set to reduce collinearity, all downloaded environmental variables were examined for pairwise correlations in ENMTools (Warren *et al.* 2021), using data from the entire study area (5°W–35°E, 40°N–60°N). After evaluation, 13 variables not highly correlated with ecologically interpretable effects ($|r| \leq 0.75$) were retained and used in further analyses.

Georeferenced location data showed highly unequal sampling. After preliminary analyses, different thinning settings were selected for each cytotype. To remove aggregation, occurrences closer than a distance of 5/15 km (diploids/tetraploids) from each other were removed, separately for each cytotype, in humboldt (Brown & Carnaval 2019). This resulted in 224 localities (2*x*, n = 77, 4*x*, n = 147), which were used for subsequent analyses.

The environmental niche space occupied by diploids and tetraploids was accessed using environmental PCA (PCAenv; Broennimann *et al.* 2012). Niche overlap was estimated by Schoener's D calculated directly from environmental niche space (Warren *et al.* 2008). The background area was taken from 200 km buffer zones around thinned occurrences. The number of background points equalled 10.000 per cytotype. Niche equivalency and similarity between diploids and tetraploids were tested by niche equivalency and similarity tests (Broennimann *et al.* 2012).

To compare niches in terms of optima and breadths, 100 random pixels, weighted by density along PC1 and PC2, were sampled in the niche of each cytotype and their scores were extracted (Broennimann *et al.* 2012). The niche optimum and the niche breadth were calculated as the median and the variance of the sampled scores along the PCA axes. This procedure was repeated 100 times. The distributions of values of niche optimum and breadth for each PCA axis were compared between cytotypes. Niche change of tetraploids relative to diploids was estimated using the indices of niche change (Petitpierre *et al.* 2012; Guisan *et al.* 2014): niche expansion (E), i.e. proportion of the niche space of the tetraploids not overlapping the niche of the diploids; niche unfilling (U), i.e. proportion of the niche of the diploids not overlapping the niche of the tetraploids; and niche stability (S_n, S_e), i.e. proportion of the niche of either diploids (S_n) or tetraploids (S_e), shared with the other cytotype. All environmental niche analyses were performed using ecospat (Di Cola *et al.* 2017).

Niche modelling analyses in the geographic space were performed with maximum entropy modelling (MaxEnt) using MAxEnt 3.4.4 (Phillips *et al.* 2006, 2008). Spatial predictive models were calibrated based on the same subset of environmental variables and occurrence data as PCAenv, plus 10 000 pseudo-absences sampled randomly within the predefined study area based on known distribution of *S. officinale* complex, separately for each cytotype. To reduce uncertainty and to produce robust models, we used 10 replicate runs with cross-validation. The presence localities of each cytotype were divided randomly into training (80%) and test (20%) subsets. We used the default settings of the program. Models were evaluated based on the independent accuracy measure AUC of ROC, and combined final model is presented for each cytotype. Relative contribution of each environmental variable to the MaxEnt model was determined for each run and averaged over replicated runs (Table 4.2). Response curves of selected environmental variables with high average percent contribution to the models for both or one of cytotypes were reported. To visualise the relative suitability within studied range, final models with the log-log (clog-log) format were used as model output for each cytotype.

Ecological differences between cytotypes on the local spatial scale

To test ecological differentiation of cytotypes on local spatial scales, the Elbe basin area (Central Bohemia, Czech Republic) has been selected. We acquired 3809 phytosociological relevés from the Czech National Phytosociological Database (Chytrý & Rafajová 2003) with the presence of either *S. bohemicum* or *S. officinale*, which correspond to diploids and tetraploids, as indicated by our results. Subsequently, only the relevés recorded in the localities with the confirmed occurrence of *S. bohemicum* (see Subchapter 7.2) were selected, resulting in 54 relevés. When more than two relevés from the same locality were available, only two relevés were randomly selected. Relevés with the occurrence of *S. officinale* were then selected from the data set based on their position within an approximately 20 km radius from the nearest *S. bohemicum* relevé (considered as sympatric occurrences to *S. bohemicum* relevés), resulting in additional 78 relevés.

The ecological differences of both cytotypes were established using Ellenberg-type indicator values (EIVs) derived for the Czech flora (Chytrý *et al.* 2018). EIVs for nutrients,

light, temperature, moisture, soil reaction and salinity were calculated for each relevé in Juice 7.1 (Tichý 2002), excluding EIVs for both *Symphytum* taxa from the calculation. Differences in cover-unweighted average EIVs between relevés with the presence of either *S. bohemicum* or *S. officinale* were analysed using one-way ANOVA with the modified permutation test with 499 permutations using MoPeT 1.2 (Zelený & Schaffers 2012).

Morphometric analyses

In total, 151 plants (40 diploids, 111 tetraploids) from 18 populations (5 diploid, 13 tetraploid) were morphologically investigated (Electronic Appendix 4.1). Only well-developed plants with at least five flowers were collected. For each plant, 37 quantitative and four qualitative (Table 4.3) morphological characters were measured *in situ* using a digital calliper or retractable meter. Nine additional ratios were calculated and several measured characters were thus excluded from the analyses (Table 4.3).

Descriptive statistics were calculated for each quantitative character and each cytotype. Intercytotype differences in quantitative traits were tested using *t*-test and proportional differences in qualitative traits were tested using χ^2-tests. Bonferroni correction was applied to adjust the *P* values of these tests.

The correlations of quantitative characters of the initial data matrix were tested using the Pearson's correlation coefficient. One character of pair of highly correlated characters ($|r| \geq 0.85$) was excluded from further analyses (Table 4.3). Principal component analysis (PCA) was run to observe the structuring of individuals in the ordination space. We performed PCA both with and without qualitative data and the results were almost identical (not shown), therefore only PCA with qualitative data included is shown here. Canonical discriminant analysis (CDA; Legendre and Legendre 1998) was performed to determine the extent of morphological separation between cytotypes. A step-wise forward selection of characters with 1000 permutations was used to find a set of most important characters used for discrimination. A multi-state quantitative character flower colour was excluded from the dataset prior to CDA performance. PCA and CDA were performed in Morphotools 1.1 (Koutecký 2015) in R (R Core Team 2021). All analyses used individuals as OTUs.

Results

Ploidy variation, genome size and cytogeography

Bibliographic review of 298 chromosome counts (Electronic Appendix 4.2) from the *S. officinale* complex confirmed the occurrence of three major cytotypes in Europe: (1) diploids ($2n = 2x = 24 + 0–4$ B); (2) tetraploids ($2n = 4x = 48$) and (3) hypotetraploids ($2n = 4x- = 40$; Fig. 4.1D) and additional 12 rare cytotypes. The most common and widespread cytotype is tetraploid (154 records, 51.7 %) that occurred across Europe. On the contrary, diploids (76 records, 25.5 %) have a very scattered distribution through Europe and have been reported from Great Britain, France, Netherlands, Italy, Germany, Czech Republic, Poland, Slovakia, and Hungary. Hypotetraploid cytotype (25 records, 8.4 %) has been detected in the Netherlands, Germany, Slovakia and Italy. Furthermore, several aneuploid chromosome counts have also been documented (29 records, 9.7 %: 1 record from diploid and 28 records

from tetraploid populations, respectively), and additional 14 chromosome reports (4.7 %) were assessed as unclear and mostly belonging to other taxa than *S. officinale* complex (Electronic Appendix 4.2).

Three DNA ploidy levels were detected by flow-cytometry: diploids (183 plants/35 populations), triploids (2 plants/1 population) and tetraploids (591 plants/118 populations, Electronic Appendix 4.3). Tetraploids have been confirmed in France, Switzerland, Italy, Slovenia, Germany, Austria, Czech Republic, Slovakia, Hungary, Romania and Ukraine, while diploids have been found in Germany, Czech Republic, Hungary and Italy (Fig.4.1D, Electronic Appendix 4.1). The presence of hypotetraploids has not been confirmed in this study only due to the lack of its samples, not by its absence. The RGS of all ploidy levels formed non-overlapping groups (Fig. 4.2A) which allowed all individuals to be clearly distinguished. Furthermore, in one tetraploid population (ID 68) a large variation (25%) in the relative nuclear DNA amount was recorded (see 4*x* + in Fig. 4.2A, Electronic Appendix 4.1). Considering populations with at least two individuals analysed (149 populations, 95.5%), most of them comprised a single cytotype and only four mixed-ploidy populations (ID 9, 76, 130 – 2*x* + 4*x*; ID 133 – 2*x* + 3*x*) were discovered (Electronic Appendix 4.1).

The mean AGS was 2.46±0.10 pg in diploids and 4.41±0.13 pg in tetraploids, with the mean monoploid genome size (1C*x* value) 1.23 pg and 1.10 pg, respectively (Fig. 4.2B, (Electronic Appendix 4.4).

Environmental and geographic niches at large spatial scale

The first two PCAenv axes explained 35.6% and 25.3% of the total variation in the environmental space available within the studied ranges of cytotypes (Fig. 4.3A). The PC1 axis mirrored a general seasonality (continentality) gradient, in terms of increasing mean (bio1, bio8) and maximal temperatures (bio5) and increasing temperature (bio2, bio7) and precipitation (bio15) seasonalities (Fig. 4.3A). Additionally, soil pH increased along PC1. The PC2 axis mirrored the gradient of soil physical variables, from more clayey soils (clyppt) with higher cation exchange capacity (cecsol) and higher available soil water capacity (WWP) to more sandy soils with lower WWP and cecsol. Both cytotypes avoided the coldest climatic conditions with less seasonality in temperature and precipitation of the available environmental space (Fig. 4.3C, D).

The niche overlap (Schoener's D) between the cytotypes was 0.668, suggesting a moderate to high niche overlap. Niche equivalency test suggested that the niches of di- and tetraploids were nearly different ($P = 0.054$). Niche similarity tests suggested that the niches of cytotypes were significantly more similar than expected by chance, regardless of the direction of the test (all $P < 0.006$). The niche of diploids falls nearly completely within the niche of tetraploids (Fig. 4.3B), i.e. tetraploids have greater niche breadth than diploids (Fig. 4.3F). Consequently, tetraploids showed niche expansion (E = 0.182) and an almost complete filling of the diploid niche ($S_e = 0.818$, U = 0.026). Tetraploids also showed a shift in niche optimum (Fig. 4.3E) toward a less continental and colder climate, occasionally coupled with expansion to more sandy soils with lower cation exchange capacity.

The average Maxent models for diploids and tetraploids had mean (±SD) AUC values of 0.926(±0.038) and 0.863(±0.045), respectively, showing very good predictive ability. The

predicted distributions showed the nestedness of the distribution of diploids within that of tetraploids (Fig. 4.4), except for the Veneto and Po regions in northern Italy, where only diploids were predicted. Across the geographic range studied, the model showed high habitat suitability for diploids in several lowland or hilly country regions of Central and Western Europe, but strict avoidance of Northern and Eastern Europe, and most part of South Europe (Fig. 4.4A). Except for several localities in South-eastern France, the model showed high habitat suitability for currently known locations of diploids. Concerning tetraploids, the model showed high habitat suitability for tetraploids over most part of Western, Central and the western part of Eastern Europe, but also for the Southern Scandinavia (Fig. 4.4B).

Two variables that contributed the most to the average model for both diploids and tetraploids were SRAD and bio1 (Table 4.2). While SRAD had a negative effect on the predicted probability of presence changes in both cytotypes (Electronic Appendix 4.5), bio1 behaved conversely. However, tetraploids were predicted to occur, though with a lower probability, even in areas with low mean annual temperature, while diploids were not. Although tetraploids were predicted to occur in a wide range of bio5 (maximal temperature of the warmest month), the model showed a lower suitability of low bio5 values for diploids (Electronic Appendix 4.5). Regarding bio7 (temperature annual range), diploids were predicted to occur more likely at intermediate values, while tetraploids were predicted to occur in a wide range of low and intermediate values (Electronic Appendix 4.5). Soil variables had a generally low percent contribution to the average models for both cytotypes (Table 4.2). Only the volumetric percentage of coarse fragments (crfvol) in the soil had a positive effect on the suitability for both cytotypes (Electronic Appendix 4.5).

Ecological differences between cytotypes on the local spatial scale

The mean site EIVs for nutrients and salinity but not for light, temperature, moisture, and soil reaction differed significantly between cytotypes (Electronic Appendix 4.6). Diploids grow on heavier and more mineral rich (salinity EIV, $F = 9.388$, $P < 0.05$) and nutrient poorer (nutrients EIV, $F = 22.278$, $P < 0.01$) soils than tetraploids.

Morphological differences between cytotypes

PCA revealed two groups distributed along the first axis corresponding to diploid and tetraploid cytotypes (Fig. 4.5A, B). The first two PCA axes explained 23.1% and 11.2% of the total variation. The colour of flowers and plants, calyx, corolla, peduncle, and style lengths and corolla width were the most responsible for the observed pattern (Fig. 4.5D-I). The cytotypes differed significantly from each other by 22 of 32 quantitative and all four qualitative morphological characters, and 15/4 characters remained significant even after Bonferroni correction, respectively (Table 4.2, see also Electronic Appendix 4.7). However, the ranges of variation of all quantitative and qualitative (with one exception) characters overlap between cytotypes. Therefore, no single character can be used for the unambiguous determination of cytotypes except for the colour of flowers, where yellowish/greenish white corollas are confined solely to diploids, while pure white and all shades of red corollas are confined to tetraploids (Fig. 4.5E). CDA resulted in a clear morphological separation between the two cytotypes ($F = 11.692$, $P = 0.001$, Fig. 4.5C). The contribution of individual

characters to the observed pattern is given in Electronic Appendix 4.8. The most important combination of characters for inclusion in one of the respective groups resulting from forward selection were length of calyx + plant colour + width of wing below lower leaf + length of peduncle + width of lowered part of corolla + width of wing below upper leaf + length/width ratio of middle leaf lamina.

Discussion

Cytotype diversity

Our flow-cytometric ploidy screening and a review of published chromosome counts of *S. officinale* complex revealed the occurrence of fifteen different chromosome counts with three main cytotypes, corresponding to diploids, hypotetraploids, and tetraploids.

As previously reported, the occurrence of triploids ($2n = 36$) is extremely rare, which is consistent with our discovery of only two triploids in a single diploid population. These two individuals probably resulted from the cross of reduced and unreduced diploid gametes. Some of the few published reports of triploids (Strey 1931, Tischler 1935, Májovský 1974, Wille 1998) may not even be based on plants from *S. officinale* complex. As already noted by Gadella and Kliphuis (1972), at least some of these reports represent the hybrid taxon *S.* ×*uplandicum* (*S. officinale* × *S. asperum*) or its backcrosses with one of the parental taxa. Thus, only reliable record of triploid occurrence besides our data is from mixed diploid-tetraploid population from the Netherlands (Basler 1972). The origin of these triploids may be from crossing between diploids and tetraploids as well as from cross of reduced and unreduced gametes of diploids.

Several other chromosome counts ($2n = 54, 56$) have also been published for *S. officinale* (Markova & Ivanova 1970, Gadella & Kliphuis 1978, Gadella *et al.* 1983, van Loon 1987), although the origin of these plants is unclear (Gadella *et al.* 1983). Furthermore, aneuploid chromosome numbers ranging from $2n = 40$ to $2n = 47$ have been discovered in pure tetraploid populations (Gadella & Kliphuis 1967, 1978, Gadella *et al.* 1974, Shirato *et al.* 1985, Wille 1998). Similarly, the published chromosome record of $2n = 26$ (Gadella & Kliphuis 1963) suggests the occurrence of aneuploidy in diploids, however, the same authors abandoned this view in their consequential studies and only reported the presence of B chromosomes (Gadella & Kliphuis 1967, 1970). Supernumerary B chromosomes have been repeatedly observed in the karyotype of diploids, occurring in various numbers (1–4; Gadella & Kliphuis 1967, 1970, Kamari *et al.* 2001, Peruzzi *et al.* 2001), and have never been identified in other cytotypes. However, since chromosomes of *S. officinale* complex are quite small (1.1–2.4 μm, Mekki *et al.* 1987), confusion with A chromosomes cannot be ruled out in other ploidy levels, particularly in tetraploids, where the aneuploid counts can, in fact, represent B chromosomes. Furthermore, the great variation in the nuclear DNA content within tetraploids was detected in our flow cytometric data (Fig. 4.2A), which may be caused by aneuploidy (reviewed in Šmarda & Bureš 2010) or the presence of B chromosomes. In some studies, the positive correlation between genome size and the presence of B chromosomes has even been found (Trivers *et al.* 2004, Levin *et al.* 2005). However, this variation could also be caused by other chromosomal polymorphisms (Greilhuber 1998) or differences in the content

of repetitive DNA (Macas et al. 2015). Last but not least, methodological errors or the effect of secondary metabolites (Loureiro et al. 2006, Kolarčik *et al.* 2018, Kobrlová & Hroneš 2019) cannot be ruled out. Employment of in situ hybridization techniques (FISH, GISH) could shed light on the origin of plants with uncommon chromosome counts.

Genome size can serve as an additional tool for species identification and discrimination between closely related taxa (Zonneveld 2001, Suda *et al.* 2007a, Prančl *et al.* 2014). Since AGS values estimated for diploids and tetraploids were non-overlapping (Fig. 4.2B), the nuclear DNA amount may be useful as a supportive marker for identification of morphologically problematic plants, e.g. white-flowered tetraploids, or plant determination in mixed population. So far, only three studies have been published considering the AGS of *S. officinale* complex (Veselý *et al.* 2013, Kobrlová & Hroneš 2019, Šmarda *et al.* 2019), and their results agree well with our estimates.

Geographic distribution of cytotypes and population cytotype composition

Our study corroborates the common occurrence of tetraploids of the *S. officinale* complex in Europe, which is consistent with previously published chromosome counts (Electronic Appendix 4.2). Compared to the broad geographic distribution of tetraploids, diploids have a scattered distribution throughout Europe and inhabit mainly calcareous fens and moist places in karst areas (Fig. 4.1D). The overall rarity of diploids maybe caused by anthropogenic pressure and consequent loss of their habitats (Janssen *et al.* 2016) or by over competition by tetraploids. We acknowledge that the distributional data presented here are partly geographically biased due to the lack of records from Eastern Europe, but clearly show the general large-scale distribution patterns of cytotypes of *S. officinale* complex. At the same time, it is possible that in some areas, diploids are overlooked and mistakenly associated with white-flowered tetraploids. In addition to these two major ploidy levels, hypotetraploids are even more scattered than diploids (except for the Netherlands) and occupy mineral-rich fens.

Geographic areas involving two and more different ploidy levels are of special interest (Mráz *et al.* 2008, Kolář *et al.* 2009, Duchoslav *et al.* 2020, Melichárková *et al.* 2021), providing the opportunity to study the evolutionary processes within polyploid complexes (Petit *et al.*, 1999, Kolář *et al.* 2017) and dynamics of ploidy coexistence (Čertner *et al.* 2017, 2022, Castro *et al.* 2018). Our results indicate that diploids and hypotetraploids occur primarily in mosaic regional parapatry (*sensu* Kolář *et al.* 2017) with tetraploids. Consequently, mixed-ploidy populations appear to be rare in *S. officinale* complex because out of 156, only three mixed diploid–tetraploid populations analysed by flow cytometry have been detected. At the same time, we have not detected triploids in these ploidy-mixed populations, so the possibility of gene flow appears to be excluded or extremely rare at present. This is consistent with previously published comprehensive cytotaxonomic studies of *S. officinale* complex in the Netherlands with no triploids detected in mixed diploid–tetraploid populations, indicating the existence of a strong reproductive barrier between these cytotypes (Gadella & Kliphuis 1967, 1972). The only exception is the study of Basler (1972), who detected two triploids (both white-flowered) in mixed diploid-tetraploid population in the Schleswig-Holstein region, Northern Germany. However, their origin has never been confirmed by molecular or experimental methods. Similarly, the crossing experiments

resulted in extremely low reciprocal cross-ability of diploids and white-flowered tetraploids of Dutch origin, with only two triploids produced (i.e. 0.1%), which were not able of flowering and thus producing viable seeds (Gadella & Kliphuis 1969, Gadella 1972).

Environmental and geographic niches at various spatial scales

Adaptation of newly established autopolyploids to new ecological niches is considered as a way to avoid competition of their diploid ancestors and consequently an important speciation mechanism (Fowler & Levin 1984). Therefore, it is hypothesised that polyploids will have wider niches and be better adapted to the abiotic extremes (Levin 2002) and this hypothesis was supported by several studies (Spoelhof *et al.* 2017, Baniaga *et al.* 2020). However, the niches of diploids and autopolyploids may differ (Arnold *et al.* 2015, Visger *et al.* 2016), but also overlap or even be equivalent (Godsoe *et al.* 2013, Casazza *et al.* 2016, Duchoslav *et al.* 2020). We nearly rejected the null hypothesis of highly conserved diploid and tetraploid niches of *S. officinale* complex. Visger *et al.* (2016) argued that even slight differences from niche equivalency in autopolyploids may be important for escape from the minority cytotype exclusion process. However, we also found that tetraploids have a much wider niche than diploids, with the niche of diploids almost embedded within the tetraploid niche. Moreover, observed shift towards more extreme abiotic conditions in tetraploids is pronounced by their tendency to occupy also colder areas with lower precipitations and their ability to inhabit also mineral-poor, sandy soils. Better tolerance to lower mean temperatures in polyploids is a commonly reported trait that strengthen their frequent occurrence at higher latitudes and/or altitudes (Husband *et al.* 2013, Rice *et al.* 2019).

Incorporating data from the local scale also suggest that tetraploids prefer nutrient richer soils that are frequently associated with both natural and anthropically disturbed sites, such as gravel bars, riverbanks, road edges, and various types of perennial ruderal vegetation on moist soils, as shown by Kobrlová (Subchapter 7.2), who analysed data on habitat conditions of both cytotypes extracted from herbarium sheets collected in the Czech Republic. Tetraploids can be thus viewed as more generalist with tendency to occupy also places with higher nutrient content, while diploids are a little bit more specialised to mineral richer soils. This perfectly fits with estimated indices of ecological specialization for Czech flora (Zelený & Chytrý 2019) with diploids (i.e. *S. bohemicum*) being more specialised than tetraploids (*S. officinale*). The stronger synanthropic affinity of polyploids, in contrast to their diploid congeners, has recently been reported in several polyploid complexes (Zozomová-Lihová *et al.* 2014, Chung *et al.* 2015, Rejlová *et al.* 2019, Němečková *et al.* 2019, Urfus *et al.* 2021).

Morphological variation of S. officinale in the Czech Republic

There has been a long-lasting debate about the taxonomical identity of diploids that are exclusively "white-flowered", but in most European floras no or only negligible taxonomic significance is attributed to them (Table 4.1). Most of the authors consider diploids to be morphologically indistinguishable from tetraploids, belonging to one polymorphic species *S. officinale* s. str. This is evidenced by Basler (1972), who provided a morphological evaluation of both cytotypes in the Schleswig-Holstein region (Northern Germany) and did not treat diploids and tetraploids as separate taxa; the only detected significant differences he

found were some microscopic features (pollen, stomata, cell size). However, Wille (1998), evaluating a morphological variation of *S. officinale* complex in Southern and Central Hesse (Central Germany), distinguishes between tetraploids (*S. officinale* s. str.) and diploids (*S. bohemicum*) quite well, although not every individual can be unequivocally identified by its morphological characteristics. This difficulty can be easily avoided by evaluating the whole population (Subchapter 7.2). Rather surprisingly, within the framework of the long-time study of *S. officinale* complex provided in the Netherlands, authors never considered diploids as a separate taxon, but only as a morphotype of *S. officinale* (Gadella & Kliphuis 1967, 1972, Gadella 1972, Gadella *et al.* 1970, 1983).

Our results clearly show that diploids and tetraploids are morphologically distinct. The best morphological characters to discriminate between these two cytotypes are the colour of flowers and plants, the width of the wing below lower and upper leaf, the length/width ratio of the middle leaf lamina, the calyx, corolla, peduncle and style lengths and corolla width (Fig. 4.5D-I, Electronic Appendix 4.7). The corollas of diploids are always yellowish to greenish white, never pure white as reported in most works. In contrast, pure-white corollas are rarely and randomly found only in tetraploids (Fig. 4.5E) and have never been observed in hypotetraploids of *S. tanaicense*. The ignorance of the corolla colour differences (yellowish-white vs. pure-white) might stand behind the long-lasting neglection of diploids as a separate taxon. The combination of quantitative and qualitative morphological traits presented here has previously been successfully applied by the first author during the revision of herbarium vouchers of *S. officinale* complex in the Czech Republic (Subchapter 7.2).

In contrast, less abundant hypotetraploids, that have flower colour similar to tetraploids (dark purple or purplish-violet) are distinguished as a separate taxon by most of the authors. Based on previous studies, they differ by generally unbranched stems, not or only very shortly decurrent leaves, both sparsely hairy to almost glabrous, and calyx lobes with long hairs along margins and at midribs (Gadella & Kliphuis 1973, Smejkal 1978, Májovský & Hegedüšová 1993, Peruzzi *et al.* 2001). The taxonomic treatment of polyploids, especially autopolyploids, has often been controversial, and different taxonomists may have various criteria (Soltis *et al.* 2007). On the basis of our findings, the morphological differentiation of all three major cytotypes is obvious and appears to be taxonomically significant, especially in combination with slight ecological segregation and the apparent presence of hybridisation barriers (Gadella & Kliphuis 1969).

Taxonomic implications

We showed that both major cytotypes of the *S. officinale* complex are morphologically well differentiated. Although we did not morphologically evaluate diploids from other parts of their range, our field observations confirm that the diploids are readily distinguishable from tetraploids. As the different ploidy level act as a strong mating barrier, as indicated by rare occurrence of triploids, both cytotypes should be treated as separate species. As far as we know, the oldest validly published name for diploids is *S. bohemicum* F.W. Schmidt (Kirschner *et al.* 2007). Subsequent studies should focus on the evolutionary pathways of the origin of the tetraploid cytotype (single or multiple, which may explain its broader niche) and also on detailed revision (morphology, ecology etc.) of hypotetraploids, in connection with

the name *S. tanaicense*, to support the taxonomic value of this species. The relationships between all cytotypes should be examined also by molecular approaches to shed light on the evolution of this complex and to clarify its taxonomic concept.

TABLE 4.1. Historical overview of taxonomic treatments in *Symphytum officinale* complex.

S. officinale	*S. officinale*	*S. officinale* var. *purpureum*	*S. officinale* subsp. *officinale* var. *officinale*	*S. officinale* subsp. *officinale*	*S. officinale*	*S. officinale* subsp. *officinale*	*S. officinale*	*S. officinale* subsp. *officinale*
-	-	*S. officinale* var. *ochroleucum*	*S. officinale* subsp. *officinale* var. *bohemicum*	-	*S. bohemicum*	*S. officinale* subsp. *bohemicum*	*S. bohemicum*	*S. officinale* subsp. *bohemicum*
-	*S. tanaicense* / *S. uliginosum*	*S. officinale* var. *lanceolatum*	*S. officinale* subsp. *uliginosum*	*S. officinale* subsp. *uliginosum*	*S. tanaicense*	*S. officinale* subsp. *uliginosum*	-	-
Kuznetsov (1910) Pawłowski (1961) Gadella & Kliphuis (e.g. 1967, 1972) Perring (1975) Gadella *et al.* (1983) Fischer *et al.* (2008) Jäger (2009) Király *et al.* (2011) Gracia & Castroviejo (2012)	Bucknall (1913) Gadella (1972) Sandbrink *et al.* (1990) Peruzzi *et al.* (2001) Cecchi & Selvi (2015, 2017)	Popov (1953)	Pawłowski (1963)	Pawłowski (1972)	Smejkal (1978) Májovský & Hegedüšová (1993) Wille (1998) Fedorov (2001) Czerepanov (2007)	Schmeil & Fitschen (1988) Martinčič (2007)	Slavík (2000) Danihelka *et al.* (2012)	Stace (2010)

TABLE 4.2. Mean percent contribution of each environmental variable for mean MaxEnt model describing the probability of di- (2*x*) and tetraploid (4*x*) occurrences in studied range. The percentages are based on a heuristic method that estimates the proportional contribution of each variable to the model training gain for every iteration during model fitting. Values of three variables with highest average percent contribution to the model training for each cytotype are in bold.

Variable	**Percent contribution**	
	2*x*	**4*x***
Mean annual solar radiation (SRAD)	**25.9**	**32.6**
Annual Mean Temperature (bio1)	**17.6**	**15.3**
Max Temperature of Warmest Month (bio5)	**11.3**	0.4
Mean Temperature of Wettest Quarter (bio8)	8.6	2.9
Weight percentage of the clay particles in soil (<0.0002 mm) (clyppt)	7.9	8.0
Temperature Annual Range (bio7)	6.4	12.2
Precipitation Seasonality (bio15)	5.2	**12.3**
Min Temperature of Coldest Month (bio6)	5.1	2.6
Mean Diurnal Range (bio2)	4.0	7.6
Cation Exchange Capacity of soil (cecsol)	2.5	1.9
Available soil water capacity (volumetric fraction) until wilting point (WWP)	2.3	0.7
Precipitation of Driest Quarter (bio17)	2.3	1.0
Volumetric percentage of coarse fragments (>2 mm) (crfvol)	0.7	1.4
pH index measured in water solution (pH)	0.3	1.2

TABLE 4.3. Descriptive statistics of morphological characters and results of *t*-tests and χ^2-tests. *P*-values in bold indicate significant difference after Bonferroni correction.

Quantitative morphological character	Abbreviation	2x			4x			*t*	*P*
		mean [mm]	±SD	min–max [mm]	mean [mm]	±SD	min–max [mm]		
height of stem	ST_h	734	272	389–1512	692	176	401–1152	1.092	0.276
width of stem	ST_w	8	3	4–16	9	2	6–13	-4.272	**<0.001**
width of stem cavity	CAV_w	2	2	0–7	3	1	0–6	-0.500	0.618
width of stem wall	STW_w	5	1	2–9	6	1	3–10	-5.364	**<0.001**
stem/cavity width ratio	r_ST_CAV	4	3	0–15	3	2	0–11	0.812	0.418
number of branches	no_BR	4	2	0–11	5	2	1–10	-4.254	**<0.001**
length of rosette leaf	R_LF_l	363	96	168–644	401	128	160–784	-1.715	0.088
length/width ratio of rosette lamina[B]	R_LAM_r	3	1	2–5	2	0	1–4	2.782	0.006
width of lower petiole wing	PET_WING_w	3	1	1–5	4	2	2–19	-2.873	0.005
length of lower leaf	L_LF_l	325	124	119–687	302	93	146–686	1.243	0.216
length/width ratio of lower leaf lamina[B]	L_LAM_r	3	1	2–5	3	0	2–4	-0.043	0.966
internode length/wing width below lower leaf ratio[B]	r_LINT_WING	2	1	0–7	2	2	0–15	0.265	0.791
width of wing below lower leaf	WING_L_w	1	1	0–2	2	1	0–5	-4.241	**<0.001**
length of middle leaf	M_LF_l	227	65	96–402	225	56	118–386	0.133	0.894
length/width ratio of middle leaf lamina[B]	M_LAM_r	3	1	2–5	3	1	2–5	-2.314	0.022
internode length/wing width below middle leaf ratio[B]	r_MINT_WING	1	0	0–2	1	1	0–3	1.512	0.133
width of wing below middle leaf	WING_M_w	1	1	0–3	3	2	0–8	-6.868	**<0.001**
length of upper leaf	U_LF_l	147	44	75–255	138	32	62–217	1.238	0.218
length/width ratio of upper leaf lamina[B]	U_LAM_r	3	1	2–7	4	1	2–5	-2.972	0.003
internode length/wing width below upper leaf ratio[B]	r_UINT_WING	1	0	0–2	1	0	0–4	3.222	0.002
width of wing below upper leaf	WING_U_w	2	1	0–3	3	1	0–8	-5.531	**<0.001**
number of flowers	no_FLW	51	24	22–108	58	16	32–96	-2.145	0.034
length of peduncle	PED_l	5	1	3–7	8	1	4–13	-11.253	**<0.001**
length of calyx	CAL_l	7	1	6–9	10	1	7–13	-12.454	**<0.001**
length of calyx lobe[A]	CALL_l	5	1	4–7	7	1	5–9	-10.583	**<0.001**
width of calyx lobe	CALL_w	2	0	2–3	3	0	2–4	-6.565	**<0.001**
length of corolla	COR_l	13	1	10–16	15	1	13–18	-9.417	**<0.001**
length of lowered corolla part[A]	CORT_l	7	1	5–9	9	1	7–10	-11.073	**<0.001**
width of corolla	COR_w	6	1	5–8	7	1	5–9	-8.919	**<0.001**
width of lowered corolla part	CORT_w	4	0	3–5	5	1	3–6	-10.205	**<0.001**
length of style	STY_l	15	1	12–18	16	1	13–9	-7.164	**<0.001**
corolla/style length ratio	r_COR_STY	1	0	1–1	1	0	1–1	-2.657	0.009

Qualitative morphological character			Proportion [%]	Proportion [%]	χ^2	*P*
colour of plant	light green	PL_col	87.5	28.0	42.411	**<0.001**
	dark green		12.5	72.0		
branching[A]	unbranched	BR	2.5	3.6	36.662	**<0.001**
	upper stem		50.0	10.8		
	middle stem		32.5	21.6		
	lower stem		15.0	64.0		
flower colour	yellowish white	FLW_col	100	0.0	151.000	**<0.001**
	pure white		0.0	23.0		
	other		0.0	77.0		
shape of style	straight	STY_s	37.5	60.4	17.874	**<0.001**
	intermediate		12.5	23.4		
	hooked		50.0	16.2		

[A] excluded from multivariate analyses.

[B] calculated from length and width of rosette, lower, middle and upper leaf, and length of internode and respective petiole wing below lower, middle and upper leaf. These characters were excluded from all analyses and left only as ratios.

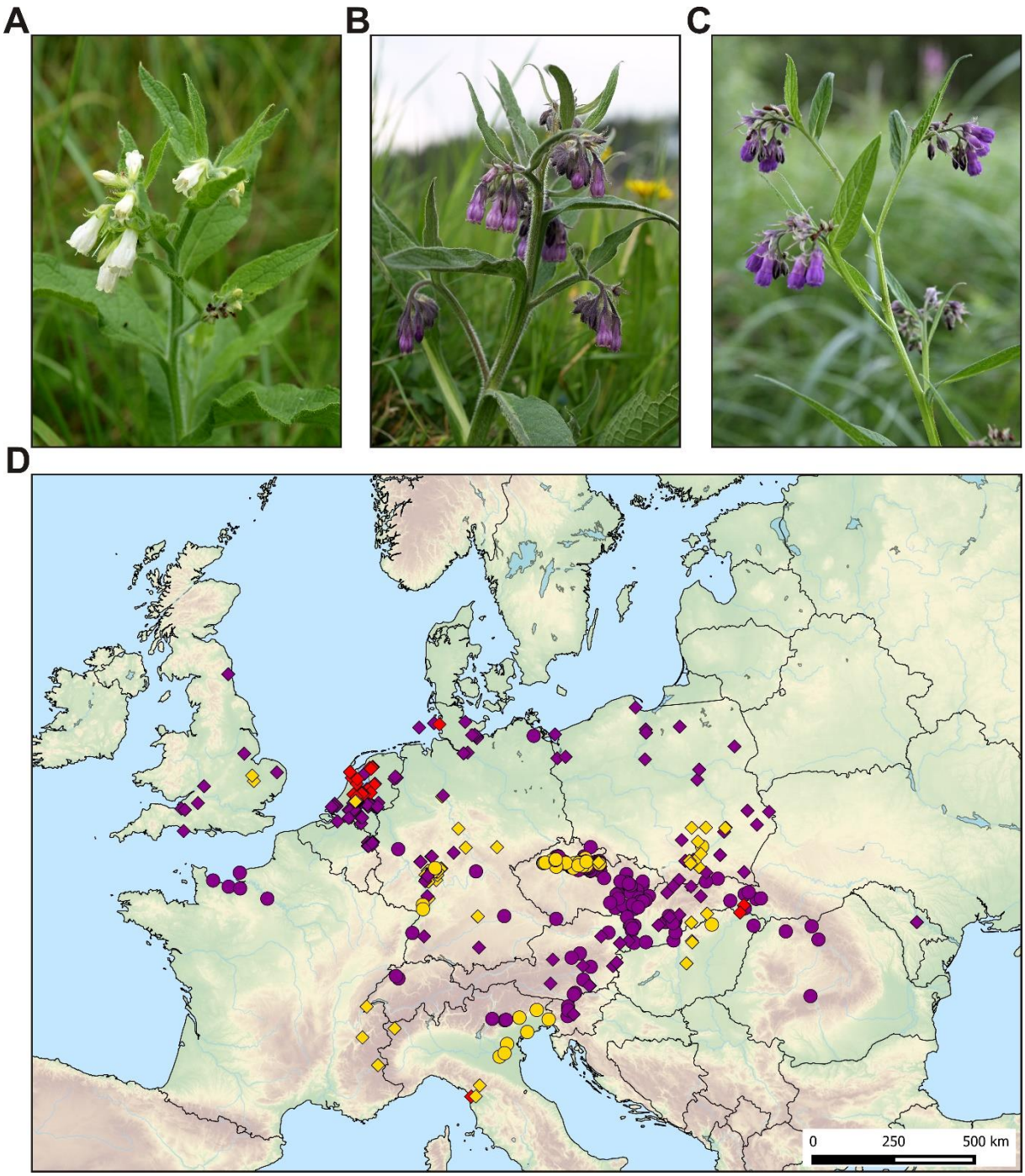


FIGURE 4.1. The members of the *Symphytum officinale* complex. (**A**) *S. bohemicum*. (**B**) *S. officinale*. (**C**) *S. tanaicense*. (**D**) Distribution of cytotypes of *Symphytum officinale* complex in Europe. Diamonds represent chromosome number reports, whereas flow cytometric data are marked with dots. Yellow–diploids; violet–tetraploids; purple–hypotetraploids. Authors of photographs: (**A**) L. Kobrlová, (**B**) M. Duchoslav, (**C**) D. Dítě.

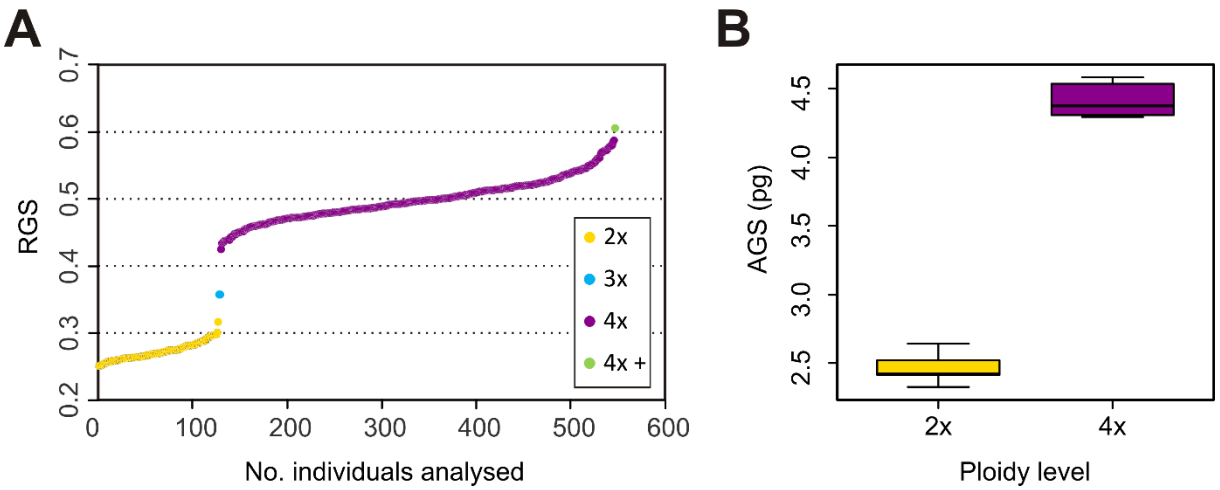
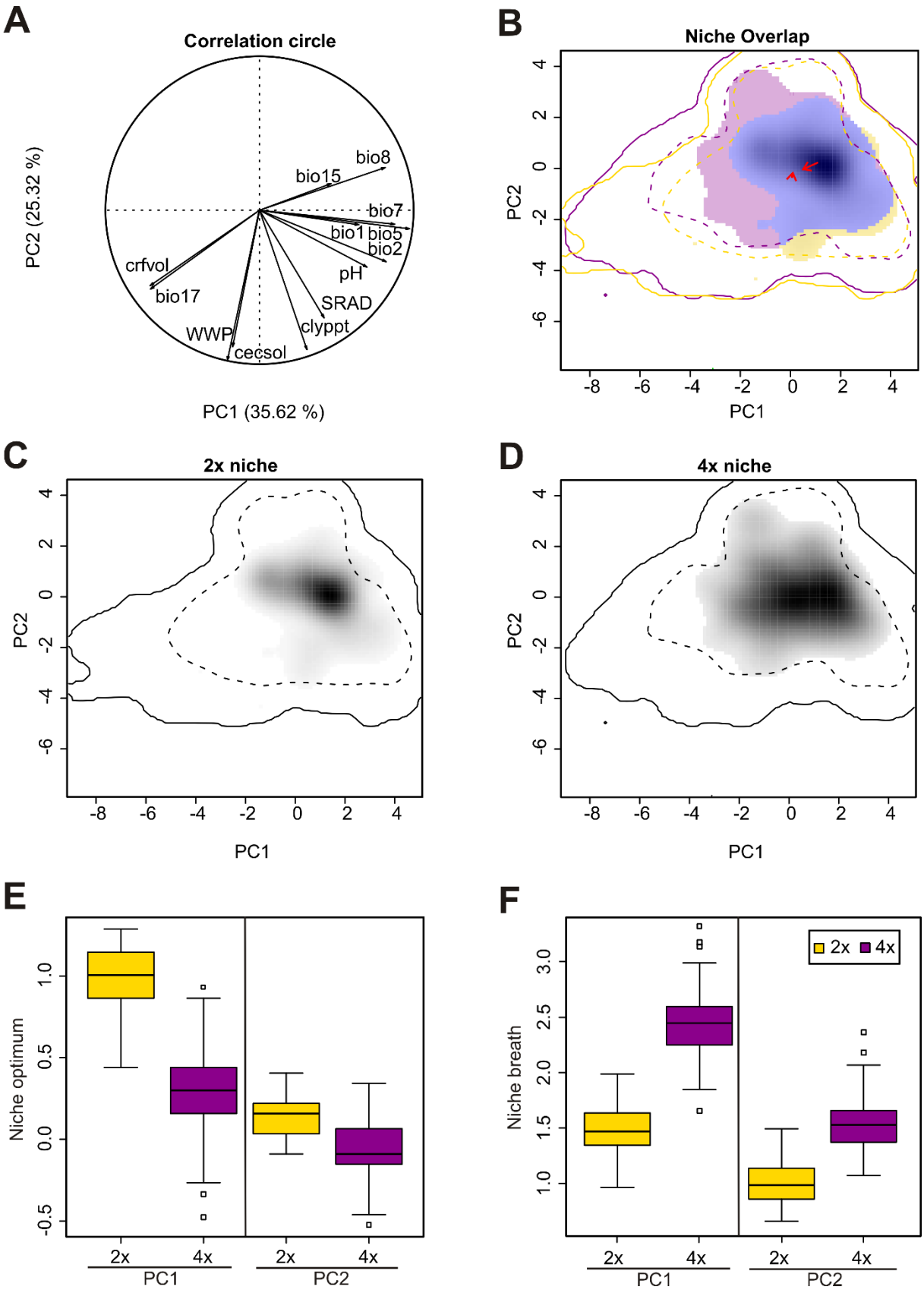


FIGURE 4.2. Variation of the nuclear DNA amount of diploids and tetraploids of the *Symphytum officinale* complex. (**A**) Variation in RGS (the relative nuclear DNA amount), sorted according to increasing relative 2C-values (see Electronic Appendix 4.2), and (**B**) difference in AGS (2C-values) of the respective cytotypes (2*x*, 4*x*).

FIGURE 4.3. Niches of diploids (2*x*) and tetraploids (4*x*) of *Symphytum officinale* complex in the environmental space along the first two axes of PCA (PCAenv). (**A**) the correlation circle shows the loadings of the individual environmental variables to the first two PCA axes. bio1 (mean annual temperature), bio2 (mean diurnal temperature range), bio5 (maximal temperature of warmest month), bio7 (temperature annual range), bio8 (mean temperature of wettest quarter), bio15 (precipitation seasonality), bio17 (precipitation of driest quarter), cecsol (cation exchange capacity of soil, mmol(c)/kg), clyppt (weight percentage of clay particles <0.0002 mm), crfvol (volumetric percentage of coarse fragments >2 mm), pH (soil acidity measured in KCl solution), SRAD (mean annual solar radiation, $kW.m^{-2}$), WWP (available soil water capacity until wilting point, %). (**B**) The niche overlap between diploids and tetraploids and (**C, D**) niches of the respective cytotypes (2*x*, 4*x*). Niche overlap is shown in blue, and parts of niche of the one cytotype unfilled by that of the second are in red (2*x*) and green (4*x*). Shading shows the density of the occurrences of the cytotype. Full and dashed contour lines illustrate 100 and 50%, respectively, of available (background) environments delimited by a 200-km buffer zone around the occurrence points of each cytotype. (**E**) Boxplots of niche optima and (**F**) niche breadths of cytotypes along the first two PCA axes (PC1, PC2).


A
Correlation circle
PC2 (25.32 %)
PC1 (35.62 %)
bio8
bio15
bio7
bio1
bio5
bio2
pH
SRAD
clyppt
cecsol
WWP
bio17
crfvol
B
Niche Overlap
PC2
PC1
C
2x niche
PC2
PC1
D
4x niche
PC2
PC1
E
Niche optimum
2x
4x
2x
4x
PC1
PC2
F
Niche breath
2x
4x
2x
4x
PC1
PC2

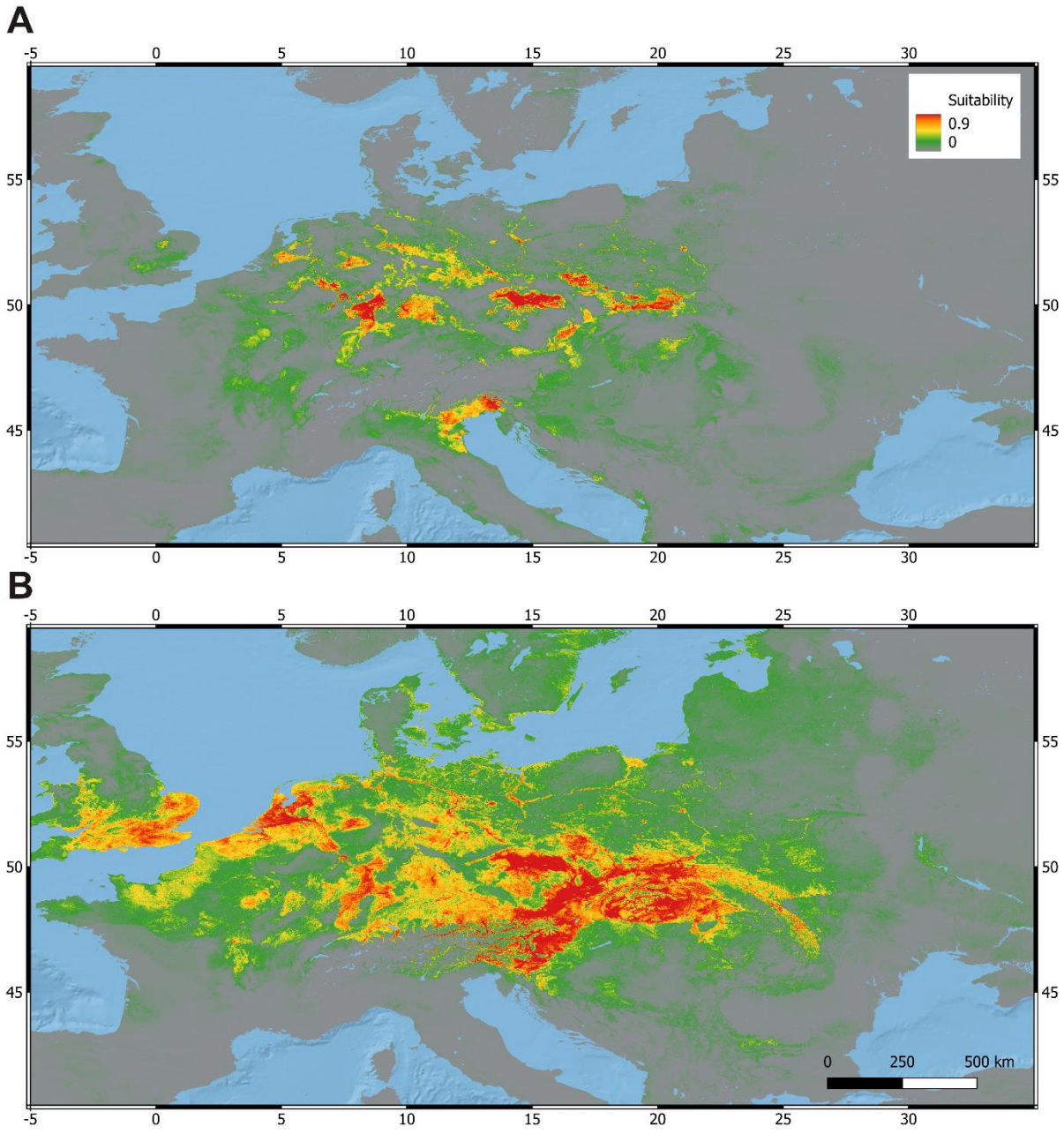


FIGURE 4.4. Predictions of suitability for the occurrence of diploids (**A**) and tetraploids (**B**) of *Symphytum officinale* complex (geographic niches) over studied region (average of 10 replicate MaxEnt runs). Grey to red colour gradient represent increasing probability of occurrence of the cytotype.

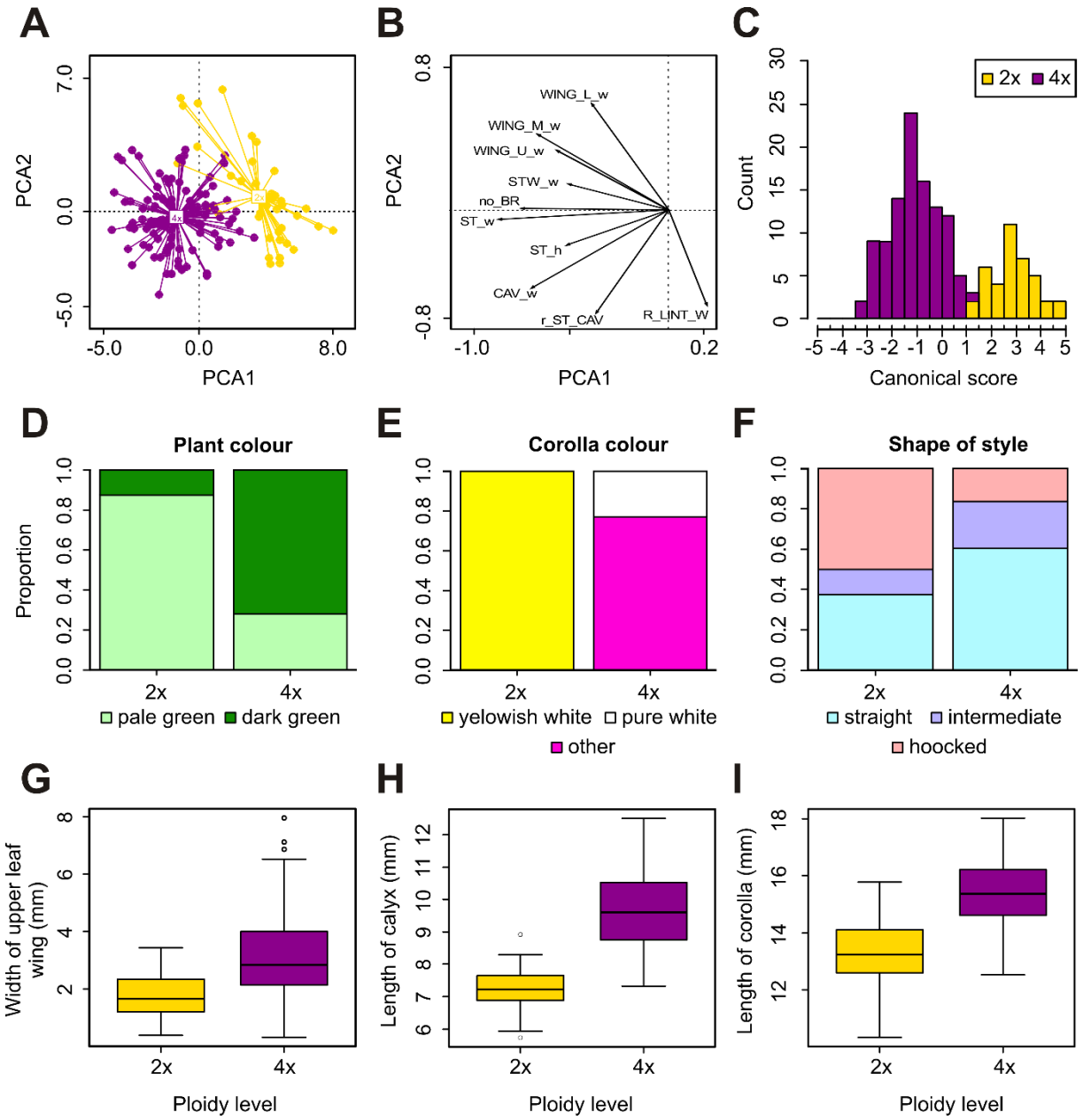


FIGURE 4.5. Morphological variation of diploids and tetraploids of the *Symphytum officinale* complex. (**A**) Spider plot PCA with individuals as OTUs (first and second axis explaining 23.1% and 11.2%, respectively, are displayed). (**B**) PCA of characters (for character abbreviations see Table 4.3). (**C**) Histogram of canonical scores of CDA. (**D–F**) Percentage stacked bar-charts of selected qualitative traits. (**G–I**) Representative box plots of selected quantitative morphological characters.

Chapter 5

An identification key of the genus *Symphytum* in the Czech Republic

Prepared by Kobrlová Lucie, Hroneš Michal (2019) as a part of Kaplan, Z., Danihelka, J., Chrtek, J. Jr., Kirschner, J., Kubát, K., Štech, M., Štěpánek J., eds. *Key to the flora of the Czech Republic [Klíč ke květeně České Republiky]. Ed. 2.* Academia, Praha, 1168 p.

(here translated to English)

14. ***Symphytum*** L. – **kostival ***

1a Leaves strongly decurrent, stamens with connective projecting beyond thecae (fig. 5.1); nutlets smooth, shiny . . . . . [*S. officinale* agg.] 2

b Leaves not or shortly decurrent, stamens with connective not projecting beyond thecae (fig. 5.2); nutlets minutely verrucose, rugose or tuberculate 3

2a C red-violet, violet, blue-violet, rarely pink or white, 13–18 mm long; base of the stem 6–14 mm wide; stems broadly winged from decurrent leaf bases, wings of the middle cauline leaves in the middle of internodes more than 3 mm wide; stems and leaves usually dark green; leaf blades with scattered long and numerous short hairs (0.4–1.2; Hkf, Gf; V–VIII; 2*n* = 48). Ditches, along rivers, wet meadows, humid ruderal habitats (N–Po); abundant . . . . . ***S. officinale*** L., **k. lékařský**

b C greenish white or yellowish white, rarely white, 10–15 mm long; base of the stem 4–10 mm wide; stems narrowly winged from decurrent leaf bases, wings of the middle cauline leaves in the middle of internodes less than 3 mm wide; stems and leaves usually light green; leaf blades with scattered long hairs, short hairs rare or missing (0.3–0.9; Hkf; V–VII; 2*n* = 24). Calcareous fens and adjacent riparian forests, along rivers; (N–Pa); only eastern, central and northern Bohemia, especially along rivers Labe and Ohře (C2) . . . . . ***S. bohemicum*** F. W. Schmidt, **k. český**

3a C white or pale to dark yellow 4

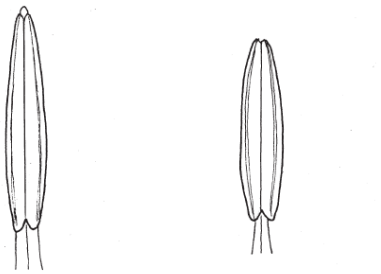
b C red-violet, crimson, purple or blue, rarely pink 5

4a C yellow, faucal scales triangular; rhizomes creeping, ± horizontal, tuberous, thin, with spaced bulb-like thickenings; leaf blades ovate to lanceolate with an acute base. – Only the lowest cauline leaves petiolate, absent at anthesis . . . . . ***S. tuberosum*** L., **k. hlíznatý**

01a Leaf blades elliptic, broadly ovate to ovate lanceolate, obtuse to acute, 2.3–3.5× long as wide, middle cauline leaves 8–15.5 cm long and 2.5–5 cm wide; rhizomes stout; stems thick, fleshy; C yellow to dark yellow, robust, with lower narrowed part of the tube 7.3–9.5 mm long, style 15.8–19.8 mm long (0.2–0.5; Gf; IV–VI; 2*n* = 96). Alder carrs, alluvial, ravine and mesophilous forests, banks of rivers or streams (N–Po); mainly southern and central Bohemia, northern Moravia and Silesia . . . . . ***S. t.*** **subsp.** ***tuberosum***, **k. h. pravý**

01b Leaf blades ovate lanceolate to narrowly lanceolate, acuminate, 3–4.8× long as wide, middle cauline leaves 7–13 cm long and 1.6–3.6 cm wide; rhizomes rather slender; stems rather thin, not fleshy; C pale yellow, smaller, with lower narrowed part of the tube 6.7–8.4 mm long; styles 13.5–18.2 mm long (0.2–0.5; Gf; V–VI; 2*n* = 32). Thermophilous broad-leaved forests, semi-dry grasslands (N–Pa); central and south-eastern Moravia . . . . . ***S. t.*** **subsp.** ***angustifolium*** (A. Kern.) Nyman, **k. h. úzkolistý**

* By L. Kobrlová and M. Hroneš



1 *Symphytum officinale* 2 *S. tuberosum*

b C white of pale yellow, faucal scales linear, rounded; rhizomes fusiform, ± vertical, not tuberous; leaf blades ovate to triangular, with truncate, rounded or slightly cordate base. – The middle and lower cauline leaves long petiolate, persistent (0.2–0.6; Hkf; IV–V; 2*n* = ?). Native to south-eastern Europe, sometimes cult., sporadic garden escape in south-western Bohemia (→neo cas) . . . . . ***S. tauricum*** Willd., **k. krymský**

5a Plants rough, with long, hooked hairs on stems and along middle leaf veins on abaxial side of leaf blades; cauline leaves petiolate or the uppermost ones sessile, not decurrent; C 3–5× longer than K, initially crimson red, later blue; K lobes obtuse or ± rounded at flowering time (0.5–1.5; Hkf; VI–VIII; 2*n* = 32). Native to Caucasus, cult. (neo cas) . . . . . ***S. asperum*** Lepech., **k. drsný**

b Plants less rough, rarely with hooked hairs; uppermost cauline leaves sessile, shortly decurrent, almost amplexicaul; C 2–3× longer than K, permanently red-violet to dark purple; K lobes acute at flowering time (0.7–2; Hkf; VI–VIII; 2*n* = 40). Hybrid taxon with unclear origin; cult.; scattered throughout Bohemia, rare in Moravia (neo nat) [*S. asperum* × *S. officinale*] . . . . . ***S.*** ×***uplandicum*** Nyman, **k. uplandský**

Symphytum grandiflorum DC., **k. velkokvětý**; rhizomes stout, not tuberous, with creeping, rooting shoots; leaf blades cordate; C pale yellow; native to Caucasus, sometimes cult., in 2016 found in Praha-Krč (→neo cas).

Symphytum ×***hidcotense*** P. D. Sell [*S. grandiflorum* × *S.* ×*uplandicum*], **k. trojbarevný**; rhizomes with long creeping, rooting shoots; leaf blades cordate; C initially crimson red, later white-blue; in 2016 found near Ivančice (→neo cas).

Hybrids

S. asperum × *S. officinale* [*S.* ×*uplandicum* Nyman, see 5b]

These hybrids were recorded in literature but have not been reliably proven: *S. bohemicum* × *S. officinale*, *S. officinale* × *S. tuberosum* [*S.* ×*wettsteinii* Sennholz], *S. officinale* × *S.* ×*uplandicum*.

The list of used abbreviations:

Morphological part
C – corolla
K – calyx
The data in parentheses as follows:
Height [m]; Life form (Hkf – hemicryptophyte; Gf – Geophyte);
Flowering period; Chromosome number

Categories of elevational vegetation belts used in the Flora of the Czech Republic
N – lowlands
Pa – colline belt
Po – submontane belt

Threats and protection (Red List 2017, national categories)
C2 – endangered taxon

Taxon origin in the Czech Republic
neo – neophyte
cas – casual
nat – naturalised
cult. – cultivated

Chapter 6

An identification key of the genus *Symphytum* in Slovakia

Prepared by Kobrlová Lucie (2018) for the new edition of the Key to the flora of Slovakia: Letz, D. R. (ed.) *Malá flóra Slovenska – Kľúč na určovanie cievnatých rastlín*, Veda, Bratislava (now in press).

(here translated to English)

XX. ***Symphytum*** L. – **kostihoj ***

1a C red-violet, violet or blue-violet, greenish white, rarely pink or white; stamens with connective projecting beyond thecae; nutlets smooth, shiny . . . . . [1.–3. *S. officinale* agg.] 2

b C yellow, stamens with connective not projecting beyond thecae; nutlets minutely verrucose, rugose or tuberculate 4

2a Leaves not or shortly decurrent 3

b Leaves strongly decurrent. – Plants roughly hirsute; stems many-branched (mostly from the base of the stem), broadly winged from decurrent leaf bases, wings of the middle cauline leaves in the middle of internodes more than 3 mm wide; stems and leaves usually dark green; leaf blades more or less densely hairy and setose (long hairs and short, scattered bristles); C red-violet, violet, blue-violet, rarely pink or white (!4; 0.4–1.2; V–VIII; 2*n* = 48). Ditches, road edges, along rivers, wet meadows, humid ruderal habitats (N–H); abundant . . . . . **1. *S. officinale*** L., **k. lekársky**

3a Leaves not or only very shortly decurrent; K lobes with long hairs along margins and at midribs; C dark purple, campanulate to urceolate. – Stems mostly unbranched, not or only shortly winged from decurrent leaf bases; stems and leaves green, sparsely hairy to almost glabrous (!4; 0.3–0.8(–1); V–VII; 2*n* = 40; NT; !CH). Swamps, marshes or banks of water canals or rivers, riparian forests (N–Pa); Vsl. níž., rare [*S. uliginosum* A. Kern.] . . . . . **2. *S. tanaicense*** Steven, **k. močiarny**

b Leaves not or shortly decurrent; K lobes without long hairs along margins and at midribs; C greenish white or yellowish white, rarely white, tubular. – Stems branched (mostly only in the upper part or from the middle of the stem), narrowly winged from decurrent leaf bases, wings of the middle cauline leaves in the middle of internodes less than 3 mm wide; stems and leaves usually light green, with scattered long hairs, short hairs rare or missing (in contrast to *S. officinale*) (!4; 0.3–0.9; V–VII; 2*n* = 24; VU; !CH). Calcareous fens and adjacent riparian forests, along rivers, water canals, damp ditches (N–Pa); esp. Ip.-rim. brázda, Sl. kras, rare elsewhere . . . . . **3. *S. bohemicum*** F. W. Schmidt, **k. český**

4a Rhizomes not tuberous, cylindrical; leaf blades cordate. – Stems unbranched; C pale yellow; cauline leaves 2–4 (!4; 0.15–0.4(–0.5); IV–VI; 2*n* = 120). Beech, beech-fir forests, mountain stream and river valleys (Ph–H); esp. Pieniny, Spiš. vrchy, V. Besk., Buk. vrchy, rare elsewhere (Z. Besk., MF, VF, ZT, BT) . . . . . **4. *S. cordatum*** Waldst. et Kit. ex Willd., **k. srdcovitolistý**

b Rhizomes tuberous, thin with spaced bulb-like thickenings; leaf blades ovate to lanceolate with cuneate base. – Stems branched; C yellow . . . . . **5. *S. tuberosum*** L., **k. hľuznatý**

01a Leaf blade elliptic, broadly ovate to ovate lanceolate, obtuse to acute, 2.3–3.5× long as wide, middle cauline leaves 8–15.5 cm long and 2.5–5 cm wide; rhizomes

* By L. Kobrlová

stout; stems thick, fleshy; C yellow to dark yellow, robust, with lower narrowed part of the tube 7.3–9.5 mm long, style 15.8–19.8 mm long (!4; 0.2–0.5; IV–VI; 2*n* = 96). Alder carrs, alluvial, ravine and mesophilous forests, banks of rivers or streams (N–Ph); abundant to scattered, Matricum, very rare . . . . . **5a. *S. t.* subsp. *tuberosum*, k. h. pravý**

01b Leaf blade ovate lanceolate to narrowly lanceolate, acuminate, 3–4.8× long as wide, middle cauline leaves 7–13 cm long and 1.6–3.6 cm wide; rhizomes rather slender; stems rather thin, not fleshy; C pale yellow, smaller, with lower narrowed part of the tube 6.7–8.4 mm long; styles 13.5–18.2 mm long (!4; 0.2–0.5; V–VI; 2*n* =32; LC). Thermophilous oak and oak-hornbeam forests and forest fringes, semi-dry grasslands, (N–Pa); Pannon. (exc. Záh. níž.) a Predkarp. (exc. Vih. vrchy), abundant to scattered [*S. angustifolium* A. Kern.] . . . . . **5b. *S. t.* subsp. *angustifolium*** (A. Kern.) Nyman, **k. h. úzkolistý**

Hybrids

S. cordatum × *S. tuberosum* (*S.* ×*ullepitschii*) – very rare; *S. officinale* × *S. tuberosum* subsp. *angustifolium* – very rare.

The list of used abbreviations:

Morphological part

C – corolla
K – calyx
The data in parentheses as follows:
Growth form (!4 – perennial herb); Height [m]; Flowering period; Chromosome number; Threats and protection (NT – near threatened, VU – vulnerable, LC – least concern, !CH – taxon protected by laws of Slovak legislation)

Categories of elevational vegetation belts used in the Flora of Slovakia

N – lowlands
Pa – colline belt
Ph – submontane belt
H – montane belt

esp. – especially
exc. – except

Phytogeographical division of Slovakia

BT – Belianske Tatry
Buk. vrchy – Bukovské vrchy
Ip.-rim. brázda – Ipeľsko-rimavská brázda
MF – Malá Fatra (= Lúčanská + Krivánska Malá Fatra)
Pannon. – Pannonicum
Predkarp. – Predkarpaty
Sl. kras – Slovenský kras
Spiš. vrchy – Spišské vrchy
V. Besk. – Východné Beskydy
VF – Veľká Fatra
Vih. vrchy – Vihorlatské vrchy
Vsl. níž. – Východoslovenská nížina
Z. Besk. – Západné Beskydy
Záh. níž. – Záhorská nížina
ZT – Západné Tatry

Chapter 7

Distribution of the genus *Symphytum* L. in the Czech Republic

A three-part series of articles published in *Zprávy České Botanické Společnosti* (here shortened and translated into English)

Subchapter 7.1. Kobrlová Lucie, Hroneš Michal, Trávníček Bohumil. 2016. *Zprávy ČBS* 51: 221–256.

Subchapter 7.2. Kobrlová Lucie. 2017. *Zprávy ČBS* 52: 175–223.

Subchapter 7.3. Kobrlová Lucie, Hroneš Michal. 2017. *Zprávy ČBS* 52: 225–248.

Distribution of the genus *Symphytum* in the Czech Republic was also published as a part of the series *Distributions of vascular plants in the Czech Republic* (see Supplementary File 1 for maps and comments on the *Symphytum* taxa).

Preslia 88: 459–544, 2016

Distributions of vascular plants in the Czech Republic. Part 3

Rozšíření cévnatých rostlin v České republice. Část 3

Zdeněk Kaplan[1], Jiří Danihelka[1, 2], Martin Lepší[3, 4], Petr Lepší[5], Libor Ekrt[4], Jindřich Chrtek Jr.[1], Jiří Kocián[6], Jan Prančl[1, 7], Lucie Kobrlová[8], Michal Hroneš[8] & Václav Šulc[1]

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The full article is attached as electronic supplement on CD-ROM (Supporting Information_Chapter 7).

Subchapter 7.1

Zprávy Čes. Bot. Společ., Praha, 51: 221–256, 2016 221

Rod *Symphytum* (kostival) v České republice. I. *S. tuberosum* agg.

The genus *Symphytum* (comfrey) in the Czech Republic. I. *S. tuberosum* agg.

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Abstract

A review of the genus *Symphytum* in the Czech Republic is presented, including an identification key and remarks on its distribution and ecology. The first part of this review is focused on the *Symphytum tuberosum* agg. This group is taxonomically difficult and includes high polyploids. Two dominant ploidy levels were found in the Czech Republic: tetraploid (*S. tuberosum* subsp. *angustifolium*) and dodecaploid (*S. tuberosum* subsp. *tuberosum*). *S. tuberosum* subsp. *angustifolium* shows clear affinity to the margins of the Pannonian basin and was found only in Moravia. The more common and widespread *S. tuberosum* subsp. *tuberosum* was demonstrated to occur in the entire country, but is almost absent from the Czech part of the Carpathian Mts. A detailed overview of morphological characters useful for the identification of these taxa, their ecological differentiation and distribution maps are presented.

Keywords: Boraginaceae, Central Europe, ecology, geographical distribution, morphology, polyploidy

Nomenclature of syntaxa: Chytrý (2007, 2009, 2013)

Introduction

The genus *Symphytum* L. (comfrey) comprises perennial, roughly hirsute plants, usually with creeping, fleshy rhizomes, alternate leaves and double scorpioid cymes (= boragoids) with tubular corollas and corolla tubes appendaged near throat with five faucal scales (= fornices).

The distribution range of this genus includes almost the whole Europe, except for Lapland, Asia Minor, Caucasus, Iran and part of Siberia (Bucknall 1913). The centre of diversity is situated in the Pontic area and in the western part of the Irano-Turanian region, primarily in the mountain ranges around the Black Sea (Gadella & Kliphuis 1978, Slavík 2000). According to various taxonomic concepts, about 40 species are distinguished within the genus (Pawłowski 1972, Fedorov 2001, Valdés 2011). In the Czech Republic, this genus is represented by four native and seven naturalised and/or cultivated taxa.

The first part of this series is focused on the *Symphytum tuberosum* complex in the Czech Republic.

***Symphytum tuberosum* agg.**

The *Symphytum tuberosum* complex represents a taxonomically difficult group. Its members occur almost all over the European continent and adjacent Anatolia, except for the cold regions of northern Europe (Bucknall 1913, Murín & Májovský 1982). The taxonomy of this complex is complicated by the occurrence of polyploidy (eight ploidy levels + several aneuploids are documented by previous karyological studies; Chapter 2) and also by a considerable morphological variability. In Central Europe, two ploidy levels are reported: dodecaploids, widely distributed in the whole area and corresponding to the name *S. tuberosum* L., and tetraploids, which are documented only from the Czech Republic, Slovakia and Hungary as *S. tuberosum* subsp. *angustifolium* (A. Kern.) Nyman, or *S. angustifolium* A. Kern. (Murín & Májovský 1982, Chapter 2).

The morphological variability of *S. tuberosum* has already been observed by Carl Linnaeus, since two different morphological forms (narrow- and broad-leaved) have been listed in his works (Linnaeus 1753a, 1753b). However, detailed morphological descriptions of these forms are missing (cf. Linnaeus 1753a,b, Pugsley 1931). The description of this species is based on the plant material most likely originating from southern Germany ("*Germania australi*"; Linnaeus 1753a,b). Unfortunately, type herbarium specimens contain only fragments of plants, i.e. the upper parts of plants with inflorescence, bracts and several cauline leaves (LINN 185.2 and 185.3) and it is not clear to which form they can be connected with. Based on that, several new species were described in various European floras (especially extreme morphotypes of narrow- and broad-leaved plants). In total, ten taxa, including *S. tuberosum* s. str., were described within this complex (Chapter 2). In the 1980s, the herbarium specimen, morphologically corresponding with broad-leaved morphotype, with the code LINN 185.3 was designated as a lectotype (Stearn 1985). Moreover, the study of Kobrlová *et al.* (Chapter 2) revealed the occurrence of dodecaploids only in southern Germany. According to this, we assume that dodecaploid cytotype can be linked with the name *S. tuberosum* L.

For a long time, only the name *Symphytum tuberosum* was used for populations of *S. tuberosum* complex in former Czechoslovakia (cf. Čelakovský 1881, Oborny 1885, Polívka 1901, Laus 1908, Merker 1910, Domin & Podpěra 1928, Domin 1935). From the middle of

the 20th century, the name *S. tuberosum* subsp. *nodosum* (Schur) Soó[1] started to be associated with broad-leaved populations in the Central Europe (cf. Dostál 1950, 1958, Pawłowski 1961, 1963, Soó 1968, Schmeil & Fitschen 1988). By some authors, this name was erroneously used also for narrow-leaved plants (e.g. Pawłowski 1972, Smejkal 1978, Dostál 1989, Májovský & Hegedüšová 1993). This misunderstanding caused considerable confusion in the literature.

The detailed study of morphology, distribution and taxonomy of the genus *Symphytum* in former Czechoslovakia was provided by Smejkal (1978). Within the *S. tuberosum* complex, four morphological types were distinguished:

- f. *angustifolium* A. Kern. with oblong-linear to lanceolate leaves and occurring in Pannonian basin (ca. 3 %)
- f. „*nemophilum*“ (formally not described) with oblong-ovate to ovate-lanceolate leaves and no specific geographical pattern (the most common, ca. 92 %)
- f. *latifolium* (Beck) Guşul. with broadly ovate to elliptic, shortly acute leaves abruptly narrowed towards the base and no specific geographical pattern (ca. 3 %)
- f. *subcanescens* Pawł. with narrow, oblong-linear to lanceolate leaves, densely hirsute on abaxial side of leaf blades and occurring in the Carpathian and rarely also in the Pannonian region (ca. 2 %)

Similar classifications of *S. tuberosum* were also published by Pawłowski (1963) and Soó (1968). However, due to the morphological variability and plasticity of plants throughout the whole area, these classifications do not reflect the real situation as they only focus on regional populations.

The first comprehensive karyological study of the *S. tuberosum* complex was provided by Murín & Májovský (1982) in Slovakia. They revealed two ploidy levels: the previously known dodecaploids ($2n = 12x = 96$) and the newly detected tetraploids ($2n = 4x = 32$). Tetraploid cytotype morphologically corresponds to the plant material from the type locality (Pilis Mts., Hungary) of the taxon *S. angustifolium* A. Kern. (Kerner 1863). Therefore, two species of *S. tuberosum* complex (narrow-leaved *S. angustifolium* and broad-leaved *S. tuberosum* s. str.) are recognised in the Flora of Slovakia (Murín & Májovský 1982, Martinovský *et al.* 1987, Dostál 1989, Májovský & Hegedüšová 1993). In the Czech Republic, only dodecaploid taxon has been recorded for a long time (e.g. Slavík 2000, Kubát *et al.* 2002, Danihelka *et al.* 2012). Based on a detailed flow cytometric revision, the occurrence of tetraploids in the Czech Republic was also confirmed (Chapter 2). Because of some overlaps in morphological traits and habitat requirements, treating of cytotypes as two subspecies, i.e. *S. tuberosum* subsp. *tuberosum* (dodecaploids) and *S. tuberosum* subsp. *angustifolium* (tetraploids) was proposed (Chapter 2).

[1] The species *Symphytum nodosum* Schur was described in 1866 from Romania (Schur 1866). Based on our previous results, this name is connected with other ploidy level occurring in the Southern Carpathians and the Balkan Peninsula (Kobrlová *et al.*, unpubl.).

Material and methods

The distribution of the *Symphytum tuberosum* complex is solely based on examined herbarium specimens deposited in the following national herbaria and some local collections (BRNL, BRNM, BRNU, CB, CESK, FMM, GM, HR, CHOM, LIM, LIT, MJ, MMI, MP, MZ, NJM, OL, OLM, OP, OSM, PL, PR, PRC, ROZ, SUM, VM, ZMT, herb. Česká Lípa; acronyms follow Hradílek *et al.* 1992). No previous field records were accepted. In the case of unclear determination, mainly in the contact zone of both cytotypes, the ploidy level was confirmed using the method of flow cytometry. Likewise, in several populations only flow cytometric data were used. These records are marked by „not.“. In total, 1 500 records were collected for the *S. tuberosum* complex in the Czech Republic. All records were localised using a digital map of the Czech Republic (www.geoportal.gov.cz), GPS coordinates in the WGS-84 system were recorded and used to produce of distribution maps in program DMAP (Morton 1993–1999).

In the list of used records (see Electronic Appendix 7.1.1), partial records were sorted according to phytogeographical (sub) districts (*sensu* Skalický 1988), within districts according to the quadrants of the Central European network mapping and within quadrants from the west to the east. For broadly localised records a question mark was attached to the quadrant number. Records from the same localities were arranged in chronological order. Texts on labels were not modified, only some longer parts were shortened or reformulated. Labels in other than Czech language were translated and are given in square brackets. In some cases when any part of the text of the label was unreadable, it is indicated by „…?“. Records without the collection date are marked by „s. d.“. The cases of the missing and unreadable name of the collector are noted by „s. coll.“ and „coll.?”, respectively. In the case of mixed collections, admixed species are listed with the abbreviation „admixt.“ in brackets. The morphological description and ecology of both taxa follow the study of Kobrlová *et al.* (Chapter 2).

Results and discussion

Symphytum tuberosum L. subsp. *tuberosum* – Tuberous comfrey (kostival hlíznatý pravý)

Symphytum tuberosum Linnaeus, Sp. Pl. 1: 136 (1753)
Symphytum tuberosum subsp. *tuberosum*

Description

Perennial, roughly hirsute, rhizomatous herbs. Rhizomes creeping, horizontal to oblique, tuberous, thick with spaced bulb-like brownish to black thickenings. Stems ca. 25–40(–60) cm tall, erect, fleshy, not winged from decurrent leaf bases, mostly unbranched or branched from the base of the stem, covered with long bristles and short hooked hairs, often roughly hirsute at the base of the stem. Branches at flowering time of the main stem usually short, non-flowering. Leaves alternate, oblong-elliptic, broadly ovate to ovate-lanceolate, obtuse to

acute, 2.3–3.5× long as wide, mostly pale green, rarely dark green, and softly, densely hispid. The middle cauline leaves 8–15.5 cm long and 2.5–5 cm wide. The lower cauline leaves petiolate, upper cauline leaves (forming bracts of inflorescence) sessile. Flowers in dense, double boragoids. Peduncles setaceous, rarely with hooked hairs. Calyces campanulate, divided into five calyx lobes. Corollas yellow to dark yellow, tubular, large, 14.5–17.5 mm long with lower narrowed part of the corolla tube 7.3–9.5 mm long. Anthers 0.32–0.37 mm long. Connectives without apical appendages. Styles erect, 15.8–19.8 mm long, not or only slightly exserted. Nutlets dark brown and densely verrucose.

Flowering time: late April or early May to early June.

$2n = 12x = 96$ [CZ: 41. Střední Povltaví (Gadella & Kliphuis 1978); 65. Kutnohorská pahorkatina (Javůrková-Jarolímová & Měsíček 1992); 68. Moravské podhůří Vysočiny (Chapter 2); 76a. Moravská brána vlastní (Chapter 2)]. A rare minor cytotypes ($10x$ and $14x$) were established in some populations using flow cytometry. Moreover, the occurrence of aneuploidy was also detected ($2n = 94$; Chapter 2).

Variability

Symphytum tuberosum subsp. *tuberosum* is a taxon with considerable morphological variability, especially in vegetative characters (height, thickness of stem, width of leaves, shape of leaves, indumentum). In addition, plants inhabiting drier habitats often have narrow leaves and few-flowered inflorescences (most likely caused by extreme soil aridity) and may resemble plants of *S. tuberosum* subsp. *angustifolium*. In this case, it is necessary to determine more plants from the population, not only one individual, or use the method of flow cytometry. The mixed populations of both subspecies are very rare in the Czech Republic and have only been detected in the north of the Bílé Karpaty Mts.

Ecology

Symphytum tuberosum subsp. *tuberosum* grows from the lowlands to submontane, rarely to the montane zone. It prefers shady, moist and also nutrient-rich habitats. Compared to the next subspecies, it is more tolerant to variation in soil humidity and soil reaction. Most frequently, it inhabits the banks of rivers or streams, forests in deep river valleys, fringes of wet meadows, alder carrs, and alluvial, ravine and mesophilous forests.

Typically, this taxon is a part of the broad-leaved floodplain forests (alliance *Alnion incanae*, mainly association *Ficario vernae-Ulmetum campestris*), ravine forests (alliance *Tilio platyphylli-Acerion*, especially association *Aceri-Tilietum*) and submontane or montane floodplains vegetation dominated by *Petasites* species (association *Petasitetum hybridi*). Besides that, it grows also in mesophilous forests (alliance *Fagion sylvaticae*) and termophilous oak-hornbeam forests (alliance *Carpinion betuli*, especially association *Galio sylvatici-Carpinetum betuli*). In contrast to *S. tuberosum* subsp. *angustifolium*, it was also recorded from ruderal or disturbed places, e.g. roadsides and abandoned wet meadows (mainly vegetation of the association *Aegopodion podagrariae*; Chapter 2).

Distribution in the Czech Republic

In the Czech Republic, *Symphytum tuberosum* subsp. *tuberosum* is mainly distributed in the north-eastern and south-western Moravia and southern and central Bohemia. In southern Bohemia, it occurs predominantly in the area between the Prachatice town and České Budějovice city. Via canyon of the Vltava river, it expands to the surroundings of the Praha city and then continues via canyon of the Labe river to the surroundings of the Ústí nad Labem city. In western Moravia, it grows mainly in the valley of the Dyje river and the Jevišovická pahorkatina hills. In north-eastern Moravia, it is common in the Javorníky and Beskydy Mts. and in the surroundings of the cities of Opava, Ostrava, Olomouc, Přerov and Kroměříž. It is quite rare in south and south-eastern Moravia and most of its populations probably originated from the upper flow of the rivers Morava, Svratka and Dyje. Small, isolated populations occur in the eastern Bohemia (in the surroundings of the town of Choceň), in the surroundings of the town of Kutná Hora (central Bohemia) and in the valley of the Berounka river near the Plzeň city (Fig. 7.1.1).

Distribution

This subspecies is distributed across almost the whole European continent, except for cold Nordic regions (cf. Bucknall 1913). In the southeast, it probably occurs also in Asia Minor (cf. Davis 1988). In the Central Europe, it is common mainly in the submontane zone of the Alps and the Carpathian Mts. (Chapter 2).

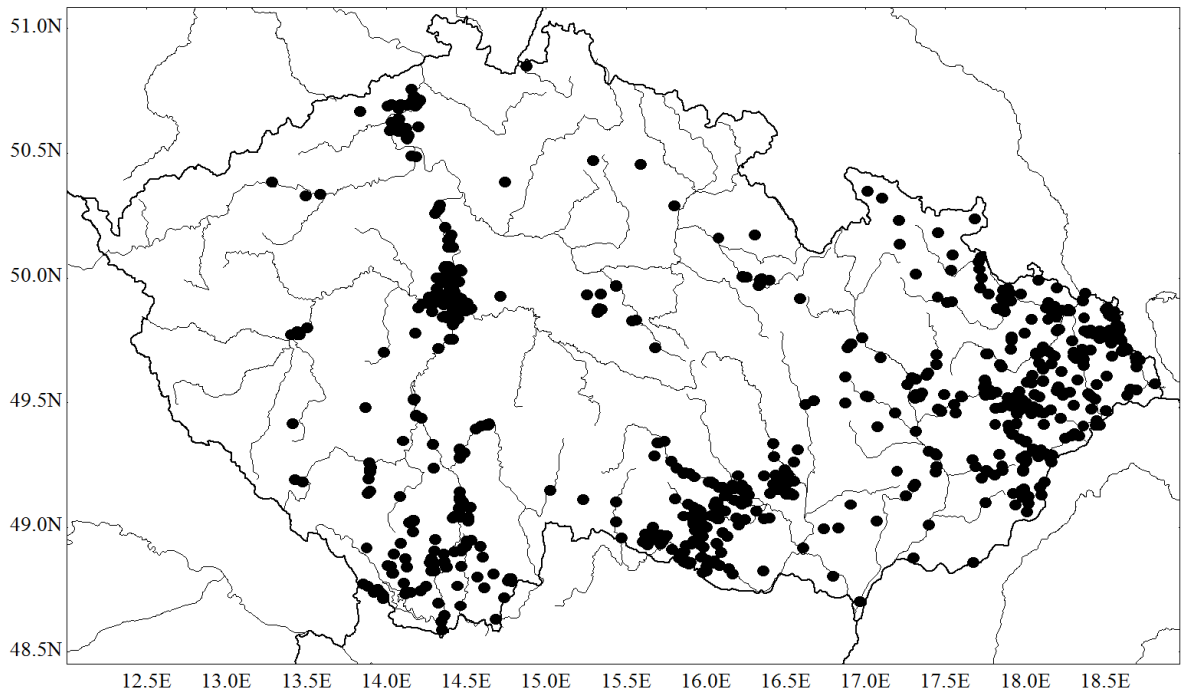

FIGURE 7.1.1. Distribution map of *Symphytum tuberosum* subsp. *tuberosum* in the Czech Republic based on revised herbarium specimens.

***Symphytum tuberosum* L. subsp. *angustifolium* (A. Kern.) Nyman – Narrow-leaved tuberous comfrey (kostival hlíznatý úzkolistý)**

Symphytum tuberosum subsp. *angustifolium (*A. Kerner) Nyman, Consp. Fl. Eur.: 510 (1881)

Syn.: *Symphytum angustifolium* A. Kern., Österr. Bot. Z. 13: 227 (1863). – *S. tuberosum* subsp. *nodosum* sensu auct. medioeur. – *S. nodosum* sensu auct. medioeur.

Description

Perennial, hirsute, rhizomatous herbs. Rhizomes slender (thinner compared to previous subspecies), creeping, tuberous and sparse spaced bulb-like pale brown thickenings. Stems ca. 30–40(–50) cm tall, erect, thin, not winged from decurrent leaf bases, unbranched or branched from the middle or base of the stem, covered with short, appressed bristles. Branches at flowering time of the main stem usually well developed and full flowering. Leaves alternate, ovate-lanceolate to narrowly-lanceolate, acuminate, 3.0–4.8× long as wide, mostly dark green, sparsely to densely roughly pilose. The middle cauline leaves 7–13 cm long and 1.6–3.6 cm wide. The lower cauline leaves petiolate, upper cauline leaves (forming bracts of inflorescence) sessile. Flowers in sparse, double boragoids. Peduncles setaceous, often with hairs with bulbous base. Calyces campanulate, divided into five calyx lobes. Corollas pale yellow, tubular, 13.9–16.8 mm long with lower narrowed part of the corolla tube 6.7–8.4 mm long. Anthers 0.30–0.35 mm long. Connectives without apical appendages. Styles erect, 13.5–18.2 mm long, included. Nutlets bright brown, shiny and verrucose.

Flowering time: early May to early June.

$2n = 4x = 32$ [ČR: 68. Moravské podhůří Vysočiny; 78. Bílé Karpaty lesní (Chapter 2)]. DNA-hexaploid plants were rarely revealed in some populations by the flow cytometry (Chapter 2).

Variability

Less variable than previous subspecies. Plants inhabiting shady and moister habitats are taller, more robust, have wider leaves and can be wrongly identified as nominate subspecies (see above).

Ecology

Compared with the type subspecies, it is more thermophilous and grows from the lowlands to middle elevations, rarely extends to higher altitudes (e.g. through warmer valleys). It inhabits drier and more open habitats, such as thermophilous broad-leaved forests (especially alliances *Carpinion betuli* and *Quercion pubescenti-petraeae* and association *Carici pilosae-Fagetum sylvaticae*), semi-dry grasslands (e.g. association *Brachypodio pinnati-Molinietum arundinaceae*) and thermophilous herbaceous vegetation of ecotonal sites (alliance *Trifolion medii*; Chapter 2).

Distribution in the Czech Republic

Symphytum tuberosum subsp. *angustifolium* represents an important floristic element of the flora of the Czech Republic. It is distributed in the south-eastern Moravia, mainly in the Bílé Karpaty Mts., Litenčické vrchy hills, Chřiby hills and Ždánický les hills. The northern border passes the surroundings of the towns of Valašské Klobouky, Vizovice, Holešov, Bystřice pod Hostýnem and the cities of Přerov and Olomouc. The western border is formed by the foothills of the Tršická pahorkatina hills, eastern part of the Drahanská vrchovina hills and southern part of the Moravian Karst, and by the surroundings of the towns of Bílovice nad Svitavou, Kuřim, Rosice and Ivančice. Quite surprisingly, this taxon is missing from the Pavlovské vrchy Mts. and in the area of the Lower Morava Valley (Fig. 7.1.2).

Distribution

This subspecies shows clear affinity to the margins of the Pannonian basin and was found only in the Czech Republic (south-eastern and central Moravia), Slovakia and in the northern Hungary (Chapter 2). In addition, some isolated populations were also detected in south-western Serbia (Kobrlová *et al.* unpubl.).

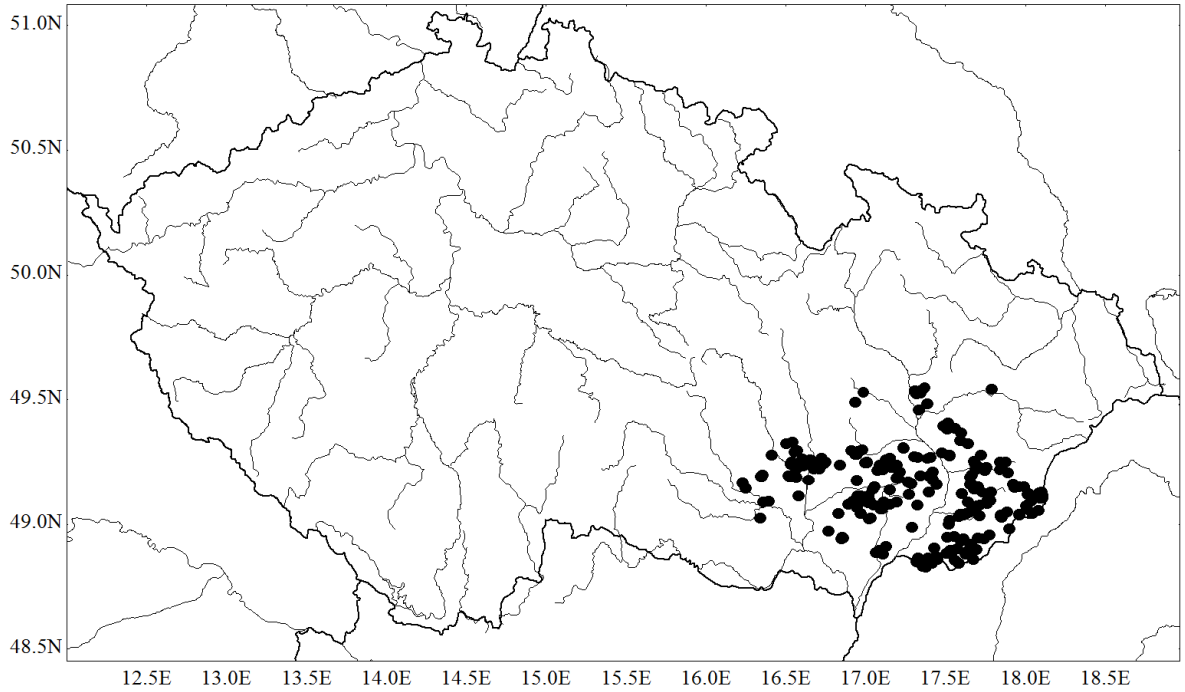

FIGURE 7.1.2. Distribution map of *Symphytum tuberosum* subsp. *angustifolium* in the Czech Republic based on revised herbarium specimens.

Subchapter 7.2

Zprávy Čes. Bot. Společ., Praha, 52: 175–223, 2017 175

Rod *Symphytum* (kostival) v České republice II. *S. officinale* agg.

The genus *Symphytum* (Comfrey) in the Czech Republic II. *S. officinale* agg.

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Abstract

The second part of a revision of the genus *Symphytum* in the Czech Republic deals with *Symphytum officinale* agg. In our flora, this complex is represented by two native species: *S. officinale* s. str. and *S. bohemicum*. *Symphytum officinale* occurs throughout the Czech Republic and grows from the lowlands to the mountains. It is the most common and widespread species of the genus *Symphytum*. In contrast, *S. bohemicum* is classified as endangered and is confined to east, central and north Bohemia (especially to the river basins of the Labe, Ohře, Metuje and Cidlina). An overview of morphological characters, habitat preferences and distribution maps are provided for both taxa.

Keywords: Boraginaceae, Central Europe, ecology, geographical distribution, morphology

Nomenclature: Danihelka et. al. (2012), Chytrý (2007, 2011, 2013)

Introduction

The first part of this series focused on the taxa of the *Symphytum tuberosum* complex (Subchapter 7.1). In this second part, the morphology, ecology and distribution of the polyploid complex of *S. officinale* are described.

Symphytum officinale agg.

The *Symphytum officinale* complex represents the most widespread group within the genus covering almost all Europe and a part of Asia (Meusel *et al.* 1978, Hultén & Fries 1986). Moreover, its members were introduced e.g. to North America (Gadella 1984), China (Zhu *et al.* 1995) and New Zealand (Hultén & Fries 1986), mainly due to their economic importance. This complex belongs to the infrageneric section *Symphytum* (syn. sect. *Officinalia* Buckn.), characterised by fusiform, ± vertical rhizomes, decurrent leaves, broadly triangular-lanceolate, acute, densely papillate faucal scales, stamens with connectives projecting beyond thecae and smooth, shiny nutlets (Pawłowski 1961).

From a taxonomical point of view, the *S. officinale* complex is a quite complicated group with three recognised taxa: diploid *S. bohemicum* F.W. Schmidt ($2n = 24$), tetraploid *S. officinale* L. ($2n = 48$) and hypotetraploid *S. tanaicense* Steven ($2n = 40$). The evolution of this group is mainly driven by polyploidy and hybridization. In comparison with *S. tuberosum* complex (Chapter 2, Subchapter 7.1), hybridization with species from other sections is more frequent. The most common is the hybridization of *S. officinale* with *S. asperum* (sect. *Coerulea* Buckn., Pawłowski 1961), giving arise to a hybrid taxon *S.* ×*uplandicum* Nyman (see the next part of this series). Moreover, backcrossing of *S.* ×*uplandicum* with parental taxa (especially with more common *S. officinale*) occurs (Gadella & Kliphuis 1972), and therefore identification of plants in the field is sometimes very difficult (see below). Within the relatives of *S. officinale* complex, no gene flow was detected (Gadella & Kliphuis 1967, 1969, 1972), which confirms that records of the hybrid *S.* ×*rakosiense* (Soó) Pénzes (*S. officinale* × *S. bohemicum*) in former Czechoslovakia are erroneous (Smejkal 1978). In addition, the occurrence of aneuploidy and B chromosomes also play an important role in karyotype evolution (Gadella & Kliphuis 1967, Májovský 1974, Kamari *et al.* 2001, Peruzzi *et al.* 2001).

The taxonomic concept of this polyploid complex has been frequently discussed, mainly because of the unclear definition of characters given in the literature and the existence of untypical morphological forms (cf. Smejkal 1978). Therefore, only *S. officinale* (in a broader sense) is accepted in many floras. At the end of the 18th century, the white-flowered species *S. bohemicum* was described (Schmidt 1794, Kirschner *et al.* 2007). Despite the fact that the description of *S. bohemicum* is based on the plant material from the Czech Republic (wet meadows in the Elbe Basin near the town of Mělník, Schmidt 1794), some Czech botanists do not accept this species (cf. Domin 1935). By some authors, white-flowered plants are considered as a subspecies or only as a variety of *S. officinale* (cf. Opiz 1839, Čelakovský 1881, Polívka 1901, Merker 1910, Dostál 1950, 1958). At the species taxonomic rank, it is recognised by e.g. Domin & Podpěra (1928), Smejkal (1978), Murín & Májovský (1982), Martinovský *et al.* (1987), Dostál (1989), Májovský & Hegedüšová (1993), Marhold & Hindák (1998), Slavík (2000) and Danihelka *et al.* (2012).

The third member of this complex is *S. tanaicense* (cf. Smejkal 1978, Murín & Májovský 1982, Májovský & Hegedüšová 1993), sometimes recognised as *S. uliginosum* A. Kern. or *S. officinale* subsp. *uliginosum* (A. Kern.) Nyman (cf. Jávorka 1925, Domin & Podpěra 1928, Dostál 1958, Pawłowski 1963, Soó 1968, Pawłowski 1972, Dostál 1989). The main diagnostic characters of this species are stems with generally no branches, not or only

shortly decurrent leaves, long hairs along margins and at midribs of the calyx lobes and dark purple campanulate to urceolate corollas. It inhabits permanently wet and waterlogged lands, such as swamps, marshes or banks of water canals and rivers. The distribution of this species is not sufficiently known and detailed study is required. According to the published records, it occurs in south-eastern Slovakia (Eastern Slovak Lowland), south-western Russia, Ukraine, Romania, Hungary, south-eastern Poland, Netherlands and Italy (Gadella *et al.* 1983, Májovský & Hegedüšová 1993, Peruzzi *et al.* 2001).

Material and methods

The distribution of *S. bohemicum* and *S. officinale* is primarily based on the revision of herbarium specimens deposited in the following national herbaria and some local collections (BRNL, BRNM, BRNU, CB, CESK, FMM, GM, HR, CHOM, LIM, LIT, MJ, MMI, MP, MZ, NJM, OL, OLM, OP, OSM, PL, PR, PRC, ROZ, SOKO, SUM, VM, ZMT, herb. muz. Česká Lípa; acronyms follow Hradílek *et al.* 1992) and two private collections (herb. J. Doležal, herb. B. Trávníček). In addition, several records based only on the flow cytometric analyses were also included. These records are marked by „not.“. Altogether, more than 2 100 records were collected. The final distribution maps were supplemented by several reviewed records (not part of the list of localities in Electronic Appendix 7.2.1) previously entered into the Pladias database (*S. bohemicum*: 31 records, *S. officinale*: 1 730 records; Kaplan *et al.* 2016).

The list of used records in Electronic Appendix 7.2.1 follows the methodology of the previous part of this series (Subchapter 7.1). Morphological descriptions of both taxa were compiled on the basis of own investigation and revision of literature (Chapter 4). For the specification of the ecological requirements and phytosociological affinity of both species, the dataset of 1 671 vegetation plots (186 relating to *S. bohemicum* and 1 485 to *S. officinale*) selected from the Czech National Phytosociological Database (Chytrý & Rafajová 2003) was analysed. For *S. bohemicum*, only records from verified localities and those confirmed using the flow cytometry were included. For *S. officinale*, records from southern Bohemia, Moravia and Silesia, where diploids do not occur, were primarily used. The relevés were classified in JUICE 7.0 software (Tichý 2002).

Results and discussion

Symphytum officinale L. – Common comfrey (kostival lékařský)

Symphytum officinale Linnaeus, Sp. Pl. 1: 136 (1753)

Syn.: *Symphytum officinale* var. *purpureum* Pers., Syn. Pl. 1: 161 (1805), nom. inval. – *S. molle* Janka, Termesz. Füz. 1: 29 (1877) – *S. officinale* subsp. *eu-officinale* Domin, Pl. Čechosl. Enum. 175 (1935), nom. inval.

Description

Perennial, roughly hirsute, large, rhizomatous herbs with dense, basal leaf rosettes. Rhizomes vertical, fusiform, not tuberous, blackish and inside whitish. Stems (40–)50–90(–120) cm tall, erect, often hollow, above the lower cauline leaves ca. 6–14 mm wide, broadly winged from decurrent leaf bases and mostly branched from the base of the stem. The wings of the middle cauline leaves in the middle of internodes more than 3 mm (mostly 3–5 mm) wide. Branches at flowering time of the main stem densely leaved and many-flowered. Stems and leaves usually green to dark green, stems often purplish at the base. Leaves alternate, ovate-lanceolate to lanceolate, acute, setaceous. The lower cauline leaves long petiolate, 15.6–21.6 cm long and 5.8–8.4 cm wide. The middle cauline leaves shortly petiolate, ca. 15.4–21.6 cm long and 5.8–8.4 cm wide. The upper leaves almost sessile, ca. 11.2–16 cm long and 3–4.4 cm wide. The uppermost cauline leaves (forming bracts of inflorescence) sessile. Flowers in dense boragoids and double boragoids. Peduncles setaceous, elongated in fruit. Calyces campanulate, 8.6–10.5 mm long (at flowering time), sparsely to densely hispid, deeply divided, enlarged in fruit. Calyx lobes long-lanceolate to narrowly-triangular. Corollas violet, red-violet, blue-violet, rarely pink or white (sometimes striped with combination of two different colours), tubular-infundibuliform, ca. 14.3–16.2 mm long with lower narrowed part of the corolla tube 7.8–9.2 mm long. Anthers included. Connectives with apical appendages, projecting beyond thecae. Styles erect, 15.3–17.5 mm long, only slightly exserted. Nutlets ovoid, black to dark brown, smooth, shiny.

Flowering time: May to September.

$2n = 4x = 48$ [extra fines]. *Symphytum officinale* is a tetraploid (e.g. Gadella & Kliphuis 1967, 1972, Murín & Májovský 1982, Gadella *et al.* 1983). In the Flora of the Czech Republic (cf. Slavík 2000), the chromosome count $2n = 24$ [ČR: 8. Český kras] is also reported. However, the origin of this report is unclear and most probably erroneous.

Variability

Symphytum officinale is very variable species (e.g. height, indumentum, leaf shape, colour of flowers) and several intraspecific taxa have been described. The great variation is in the colour of the corollas (from white to a variety of shades of pink and violet). The most problematic are white-flowered plants similar to *S. bohemicum* (see below). In some populations, plants with longitudinally striped flowers (e.g. white corollas with purple stripes) can be found, which can raise suspicions of the hybrid origin of those plants (cf. Smejkal 1978). Otherwise, these plants do not morphologically differ from typical, purple-flowered individuals of *S. officinale*. Moreover, there is no difference in the genome size (Kobrlová unpubl.). Besides that, identification of plants in mixed populations with *S. asperum* and especially their hybrid *S. ×uplandicum* (see Kobrlová & Hroneš 2017) may also be quite problematic. In contrast to *S. officinale*, *S. ×uplandicum* is more scabrid, leaves are not decurrent, calyces are shorter (only 5–7 mm long) and corollas are often bluish.

Ecology

Symphytum officinale is the most frequent comfrey growing from the lowlands to the montane zone. In comparison with the next species, it is more tolerant to soil humidity and reaction and therefore, it grows on various substrates including loam, clay, gravel or sand. It inhabits wet meadows (often on peaty or fen soils), the banks of rivers, brooks and canals, river arms, alder carrs and alluvial forests. It is also frequently found in ruderal habitats such as roadsides, railways and waste places in villages, ruderal grasslands in settlements, edges of arable fields, scrub fringes and forest paths.

According to the analysis of phytosociological relevés, most records of *S. officinale* are from meadows and mesic pastures (class *Molinio-Arrhenatheretea*, especially alliances *Deschampsion cespitosae*, *Calthion palustris* and *Arrhenatherion elatioris*, 27.5 % of analysed phytosociological relevés) and wetlands (class *Phragmito-Magno-Caricetea*, particularly alliances *Magno-Caricion gracilis* and *Phragmition australis*, 24.2 % of analysed phytosociological relevés). In contrast to *S. bohemicum*, it is also recorded from the forest and scrub vegetation (mainly alliances *Alnion incanae*, *Salicion albae*, *Alnion glutinosae* and *Berberidion vulgaris*, 14.3 % of analysed phytosociological relevés). Besides that, it is also quite abundant in ruderal types of vegetation (30.5 % of analysed phytosociological relevés).

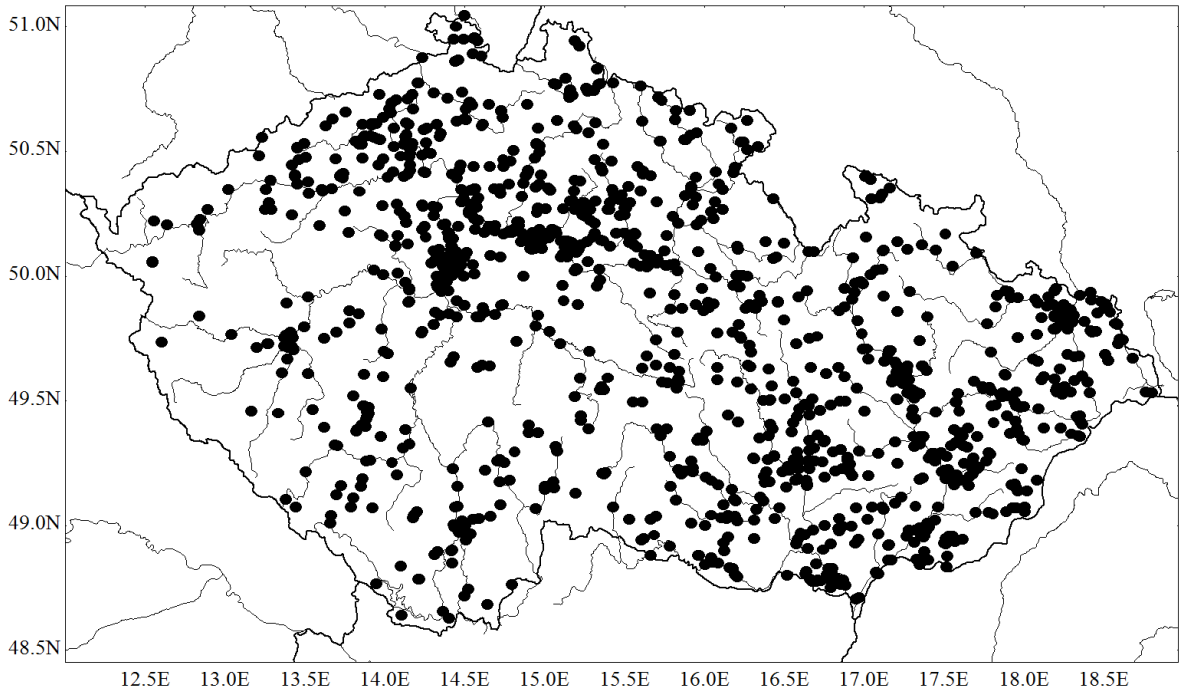

FIGURE 7.2.1. Distribution map of *Symphytum officinale* in the Czech Republic: ● revised herbarium specimens, ○ other records.

Distribution in the Czech Republic

It is scattered to common and grows from the lowlands to the mountains (Fig. 7.2.1). To the higher attitudes (max. 1 240 m a.s.l., Krkonoše Mts.), it is probably introduced along roads and mountain huts (Slavík 2000). In the Czech Republic, *S. officinale* occurs less frequently only in western Bohemia (Fig. 7.2.1), where it is most probably only under-recorded.

Distribution

Symphytum officinale represents the most common species of the genus and it is distributed almost throughout the whole of Europe. It is missing or rarely occurs in the Mediterranean area (almost the whole Iberian Peninsula, south parts of the Italian and Balkan Peninsula), and also in cold areas of northern Europe (Meusel *et al.* 1978, Gadella *et al.* 1983, Hultén & Fries 1986). Moreover, it was also introduced to North America (Gadella 1984), China (Zhu *et al.* 1995) or New Zealand (Hultén & Fries 1986), mostly as green forage for livestock.

***Symphytum bohemicum* F.W. Schmidt – Bohemian comfrey (kostival český)**

Symphytum bohemicum F.W. Schmidt, Fl. Boëm. 3: 13 (1794)

Syn.: *Symphytum officinale* var. *bohemicum* (F.W. Schmidt) Pers., Syn. Pl. 1: 161 (1805) – *S. officinale* subsp. *bohemicum* (F.W. Schmidt) Čelak., Sitzungsber. Königl. Böhm. Ges. Wiss. Prag 1891: 29 (1891) – *S. officinale* var. *ochroleucum* DC., Prodr. 10: 37 (1846) – *S. officinale* var. *flavescens* Opiz in Bercht. et Opiz, Oekon.-Techn. Fl. Böhm. 2/2: 170 (1839), nom. nud.

Description

Perennial, hirsute, slender (in comparison with *S. officinale*), rhizomatous herbs with basal leaf rosettes. Rhizomes vertical, fusiform, not tuberous, blackish and inside whitish. Stems (30–)45–85(–130) cm tall, erect, above the lower cauline leaves ca. 4–10 mm wide, narrowly winged from decurrent leaf bases and mostly branched in the upper part or from the middle of the stem. The wings of the middle cauline leaves in the middle of internodes less than 3 mm (mostly ca. 1 mm) wide. Branches at flowering time of the main stem sparsely leaved and few-flowered. Stems and leaves usually bright green to yellow-green. Leaves alternate, long-lanceolate to lanceolate, acute, softly hairy. The lower cauline leaves long petiolate, ca. 14–25 cm long and 5.1–8.8 cm wide. The middle cauline leaves shortly petiolate, ca. 14.1–21.9 cm long and 5–7.5 cm wide. The upper cauline leaves almost sessile, ca. 11.6–17.5 cm long and 3.2–5.1 cm wide. The uppermost cauline leaves (forming bracts of inflorescence) sessile. Flowers in dense boragoids or double boragoids. Peduncles densely, softly hirsute, elongated in fruit. Calyces campanulate, 6.8–7.6 mm long (at flowering time), sparsely hispid (less rough than *S. officinale*), deeply divided, enlarged in fruit. Calyx lobes long-lanceolate to narrowly-triangular. Corollas initially green-white or slightly yellowish, later almost purely white, tubular-infundibuliform, ca. 12.5–14.2 mm long with lower narrowed part of the corolla tube 6.5–7.5 mm long. Anthers included. Connectives with apical appendages,

projecting beyond thecae. Styles erect, 13.5–15.8 mm long, only slightly exserted. Nutlets ovoid, black to dark brown, smooth, shiny.

Flowering time: May to June or July.

$2n = 2x = 24 + 0–4B$ [ČR: 4b. Labské středohoří (Smejkal 1978); 15b. Hradecké Polabí (Gadella & Kliphuis 1978)]. *Symphytum bohemicum* is a diploid member of the *S. officinale* group. In contrast to *S. officinale*, the occurrence of B chromosomes was repeatedly reported (e.g. Gadella & Kliphuis 1967, 1972, Smejkal 1978, Kamari *et al.* 2001, Peruzzi *et al.* 2001).

Variability

Less variable in comparison with previous species. Only morphological differences between populations are due to different habitats, e.g. plants inhabiting tall-sedge and reed vegetation are taller and slender than those growing in wet meadows. The most similar are white-flowered individuals of *S. officinale*. Howbeit, mixed populations of both species are very rare in the Czech Republic. However, it is necessary to determine more plants from the population, not only one individual. The collections of more than one individual per population are very rare in herbaria, therefore, in some cases the improper determination cannot be ruled out in certain specimens containing only single plant. The most problematic herbarium specimens were those containing only fragments of plants (often only inflorescences). Especially in the case of white-flowered individuals from the areas of sympatric growth of both species, it was not possible to make clear identification and therefore, these records are listed as *S. officinale* agg. (see Electronic Appendix 7.2.1).

Ecology

Symphytum bohemicum is a species typical of wet meadows in lowland floodplains of large rivers, river arms, channels and alluvial pools. It grows on loamy or clayey soils rich in nutrients, which are wet, waterlogged or even flooded in spring.

According to the analysis of phytosociological relevés, most of the records of *S. bohemicum* are from tall-sedge and reed vegetation (61.8 % of analysed phytosociological relevés), namely alliances *Magno-Caricion gracilis*, *Phragmition australis*, *Magno-Caricion elatae* and *Phalaridion arundinaceae*. The next most common vegetation types are lowland floodplain meadows and wet tall-herb meadows (22.6 % of analysed phytosociological relevés). In contrast to *S. officinale*, it rarely inhabits wet forests and scrub vegetation (2.7 % of analysed phytosociological relevés) and only exceptionally ruderal types of vegetation (1.1 % of analysed phytosociological relevés).

Distribution in the Czech Republic

Symphytum bohemicum was described from wet meadows in the Elbe Basin near the town of Mělník (central Bohemia; Schmidt 1794, Kirschner *et al.* 2007). From this locality, only one herbarium specimen collected by F.W. Schmidt is reported (deposited in PRC), which was also selected as a lectotype (Kirschner *et al.* 2007). In the Czech Republic, it occurs only in Bohemia, particularly along the middle and lower stretches of the Labe, Ohře, Metuje and Cidlina rivers, as well as the lower stretches of the Jizera and Vltava rivers, in eastern, central

and northern Bohemia (Fig. 7.2.2). The northern border passes the surroundings of the towns and the cities of Ústí nad Labem, Česká Lípa, Mladá Boleslav, Jičín, Hořice and Opočno. The western border is formed by the surroundings of the towns of Klášterec nad Ohří and Kadaň. The westernmost locality was recorded in the basin of the Ohře river near the town of Kynšperk nad Ohří. The eastern border passes the surroundings of the cities and towns of Pardubice, Hradec Králové and Opočno, with the easternmost localities documented in the basin of the Orlice river near the towns of Kostelec nad Orlicí and Brandýs nad Orlicí. The southern border is partly formed by the stretches of the Labe and Ohře rivers and by the surroundings of the cities and towns of Praha, Kladno and Slaný. This taxon is missing from Moravia and Silesia (Fig. 7.2.2).

Distribution

The distribution of *S. bohemicum* is not sufficiently known (especially in Eastern Europe) and its revision is required. The diploid white-flowered "*S. officinale*" that we consider to be *S. bohemicum* is recorded from eastern England (Stace 2010), the Netherlands, France (Gadella & Kliphuis 1967, 1978), Germany (Basler 1972), southern Poland (Wcisło 1972), south-eastern Slovakia (Murín & Májovský 1982), northern Hungary (cf. Soó 1968, Kobrlová unpubl.), southwestern Slovenia (Jogan *et al.* 2001) and northern Italy (Kamari *et al.* 2001, Kobrlová unpubl.).

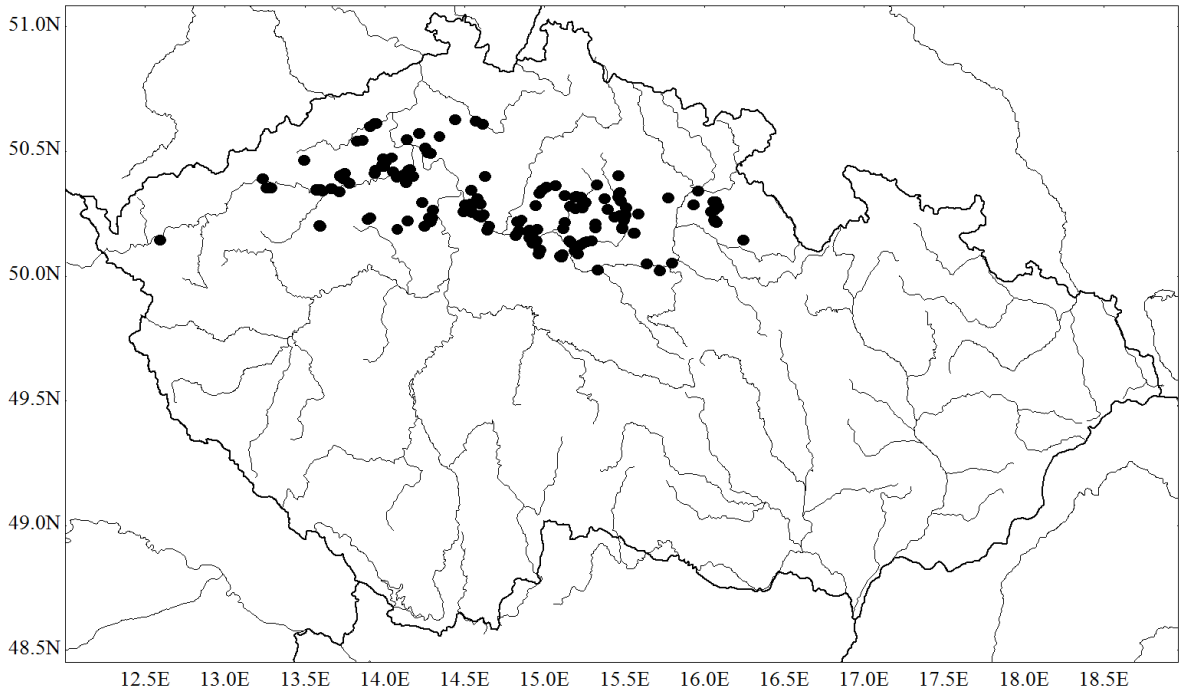

FIGURE 7.2.2. Distribution map of *Symphytum bohemicum* in the Czech Republic: ● revised herbarium specimens, ○ other records.

Subchapter 7.3

Zprávy Čes. Bot. Společ., Praha, 52: 225–248, 2017 225

Rod *Symphytum* L. (kostival) v České republice III. Nepůvodní a pěstované druhy

The genus *Symphytum* L. (Comfrey) in the Czech Republic III. Introduced and cultivated species

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Abstract

The last part of a revision of the genus *Symphytum* in the Czech Republic deals with naturalised and cultivated species. Two naturalised taxa are traditionally recognised in the Czech flora, namely *Symphytum asperum* and *S.* ×*uplandicum*. In addition, the occurrence of three other alien species is discussed: *S. tauricum*, a Crimean species discovered in south-west Bohemia, *S. grandiflorum*, a Caucasian species found in Praha, and *S.* ×*hidcotense*, an artificial hybrid taxon discovered in the vicinity of the town of Ivančice, Moravia. *Symphytum grandiflorum* and *S.* ×*hidcotense* are reported from the Czech Republic for the first time. A detailed morphological description, ecology, distribution of these taxa and the history of their cultivation in our country are presented. In addition, several species cultivated in Central Europe (*S. bulbosum*, *S. caucasicum*, *S. cordatum* and *S. orientale*) are discussed.

Keywords: Boraginaceae, Central Europe, cultivation, distribution, ecology, fodder, introduction, morphology

Taxonomy and nomenclature: Pawłowski (1972), Stace (2010), Danihelka *et al.* (2012)

Introduction

The first two parts of this series were focused on native species of the genus *Symphytum* L. in the Czech Republic, i.e. *Symphytum tuberosum* (Subchapter 7.1) and *S. officinal*e (Subchapter 7.2) complexes. This final part aims to introduce non-native, naturalised and cultivated taxa.

Traditionally, the genus *Symphytum* (comfrey) has been used as a medicinal plant (in Czech, it is also known as svalník, medunice or černé koření; Rystonová 2007). Moreover, the scientific name *Symphytum*, from the Greek "*symphein"* meaning growing together, refers to its use in the treatment of fractures and wound healing (Polívka 1901, Gams 1966, Stearn 1985). The plants contain various active components (alkaloids, phenolic acids, tannins, etc.) and therefore, they are frequently cultivated as medicinal herbs. The rhizomes and leaves are collected to treat bone fractions, swellings, contusions or to reduce joints or muscles pain. In folk medicine, the most popular and used are common comfrey (*S. officinale*), tuberous comfrey (*S. tuberosum*) and Russian comfrey (*S. ×uplandicum*; Kucera *et al.* 2000, Frost *et al.* 2013, Weigend *et al.* 2016).

Besides that, some comfrey species have been cultivated as nectar-bearing and forage plants and fertilisers (e.g. Srb 1958, Ingram 1961, Smejkal 1978, Slavík 2000). At the beginning of the last century, two species of *Symphytum* have been widely cultivated as a lucrative fodder in Europe (including the Czech Republic), namely rough comfrey (*S. asperum*) and Russian comfrey (*S. ×uplandicum*; Srb 1958, Smejkal 1978, Slavík 2000). In the Czech Republic, the first experimental cultivations of *S. asperum* are dated around 1840, but with no satisfactory yield (Srb 1958, Smejkal 1978). During the first half of the 20th century, it was grown mainly as livestock fodder, especially around the cities and towns of Tábor, Sušice, Louny, Praha (Klecany, Bašť, Řež), Kouřim (Zalešany) and Litomyšl (Netřeby; Srb 1958, Smejkal 1978, Dostál 1989). Afterwards, at the end of the 19th and the beginning of the 20th century, *S. ×uplandicum* started to be also frequently cultivated (Smejkal 1978, Slavík 2000). Both of these taxa have escaped from cultivation and currently they can be found locally naturalised (e.g. Smejkal 1978, Dostál 1989, Slavík 2000, Danihelka *et al.* 2012, Kaplan *et al.* 2016).

In addition, some comfrey species are occasionally cultivated as ornamentals in gardens and parks. The majority of the non-native taxa cultivated in the Czech Republic (and most of Europe) originate from the area around the Black Sea, the centre of diversity of this genus (cf. Wickens 1969, Davis 1978, Gadella & Kliphuis 1978). Some of these species escaped from cultivation and became locally established in some European countries. In the Central Europe, following taxa were recorded: *S. bulbosum*, *S. caucasicum*, *S. cordatum*, *S. grandiflorum*, *S. ×hidcotense*, *S. orientale* and *S. tauricum* (Pawłowski 1963, Gams 1966, Smejkal 1978, Schmeil & Fitschen 1988, Fischer *et al.* 2008, Bomble & Schmitz 2013, Kniely 2015, Kaplan *et al.* 2016, BfN 2017).

Material and methods

The distribution of non-native and cultivated species of the genus *Symphytum* in the Czech Republic is solely based on examined herbarium specimens deposited in the following

national herbaria and some local collections (BRNL, BRNU, CB, HR, CHOM, LIM, LIT, MJ, MP, NJM, OL, OLM, PL, PR, PRC, ROZ, herb. B. Trávníček; acronyms follow Hradílek *et al.* 1992). In total, 76 records were collected. The final distribution maps were supplemented by several reviewed records (not part of the list of localities in Electronic Appendix 7.3.1) previously entered into the Pladias database (Kaplan *et al.* 2016). The list of records in Electronic Appendix 7.3.1 follows the methodology of previous parts of this series (Subchapter 7.1, 7.2). Morphological descriptions of species were compiled based on own observation and revision of literature.

Results and discussion

The revision of herbarium specimens deposited in the Czech herbaria revealed the occurrence of six non-native *Symphytum* taxa in the Czech Republic, namely *S. asperum*, *S. ×uplandicum, S. tauricum*, *S. grandiflorum*, *S. ×hidcotense* and *S. cordatum*. The specimen of *S. cordatum* most likely originated from cultivation. In the case of *S. grandiflorum* and *S. ×hidcotense*, it is the first record of their occurrence in our country. Moreover, another cultivated and sporadically escaped *Symphytum* species in the Central Europe are also mentioned (*S. bulbosum, S. caucasicum* and *S. orientale*).

Symphytum asperum Lepech. – Rough comfrey (kostival drsný)

Symphytum asperum Lepechin, Nova Acta Acad. Sci. Imp. Petrop. Hist. Acad. 14: 442 (1805)

Syn.: *Symphytum asperrimum* Donn ex Sims, Bot. Mag. 24: 929 (1806)

Description

Perennial, roughly hirsute, rhizomatous herbs. Rhizomes vertical, fusiform, not tuberous. Stems 60–180 cm tall, erect, branched, with dense, rough, hooked hairs and short bristles. Branches at flowering time of the main stem many-flowered. Leaves alternate, ovate, oblong-ovate to ovate-lanceolate, mostly 2× longer than wide, acute, the lower cauline leaves often with subcordate base, not decurrent, not amplexicaulous, roughly hirsute with hooked hairs along middle leaf vein. The lower cauline leaves long petiolate, the upper cauline leaves shortly petiolate or almost sessile. Flowers in dense boragoids and double boragoids. Calyces 3–5 mm long (at flowering time), divided about 2/3–3/4 of its length. Calyx lobes broadly-lanceolate, obtuse or rounded. Corollas initially pink, later deep blue, campanulate, 11–17 mm long, 3–5× longer than calyx, faucal scales included, lingulate, with dense, long, acute marginal papillae. Connectives without apical appendages. Styles erect, not or only slightly exserted. Nutlets black, curved, reticulate-rugose and minutely verrucose.

Flowering time: May to August.

$2n = 4x = 32$ [extra fines] (e.g. Gadella & Kliphuis 1969, Basler 1972, Gadella *et al.* 1983, Gagnidze *et al.* 2015).

Variability and similar species

In the Czech Republic, the only similar taxon is *S.* ×*uplandicum* which differs by shortly decurrent, semiamplexicaulous, mostly shortly petiolate leaves, absence of hooked hairs along middle leaf vein (plants are less rough), rounded to cuneate, never cordate leaf bases and permanently red-violet to dark purple corollas that are 2–3× longer than calyx (see below). Blue-flowered *S. caucasicum* is sometimes cultivated in parks and gardens across Europe as an ornamental plant. In comparison to *S. asperum*, individuals of *S. caucasicum* are softly hirsute, greyish, with dense basal leaf rosette, short, thick rhizomes and with calyces divided only about 1/3 of its length (Bucknall 1913, Wickens 1969).

Ecology

In its native range, *S. asperum* inhabits the banks of streams and rivulets, tall-forb communities and mountain meadows, spruce forests and forest edges (Popov 1953, Kurtto 1982, Slavík 2000). In the Czech Republic, occurrences of secondary origin, which most likely represent garden escapes, have been reported from ruderal habitats such as road verges, ruderal grasslands in settlements, parks, gardens, fallow lands or wastelands, usually on wet and mineral-rich soils (Slavík 2000).

Distribution in the Czech Republic

In the Czech Republic, *S. asperum* is scattered to rare in Bohemia, mainly throughout south and southwestern Bohemia (especially in the surroundings of the towns of Klatovy, Strakonice and Prachatice), only once recorded (from the botanical garden in Olomouc, see Electronic Appendix 7.3.1) from Moravia and missing from Silesia (Fig. 7.3.1). The earliest record is dated back to the second half of the 19th century (1872 *s. coll.*, PR), but it is not certain whether it originated in culture or in nature. The majority of the records came from the 1970s. However, since 2000 only two specimens have been recorded.

Distribution

Symphytum asperum is native to montane and subalpine zones of the northern Caucasus (Lepechin 1805). The occurrence in northern Iran and Asia Minor is also considered its native distribution (Kurtto 1982). It has been introduced to England around the year 1799, being cultivated as a nectar-bearing and medicinal plant, and later on also as a green forage for livestock (Wade 1958, Smejkal 1978, Kurtto 1982). During the first half of the 19th century, it has been introduced to and become naturalised in many other countries of Europe, being widely used as a forage plant (Pawłowski 1972, Smejkal 1978, Kurtto 1982, Hultén & Fries 1986). At the same time, it has been also introduced to North America (Kurtto 1982, Gadella 1984, Hultén & Fries 1986).

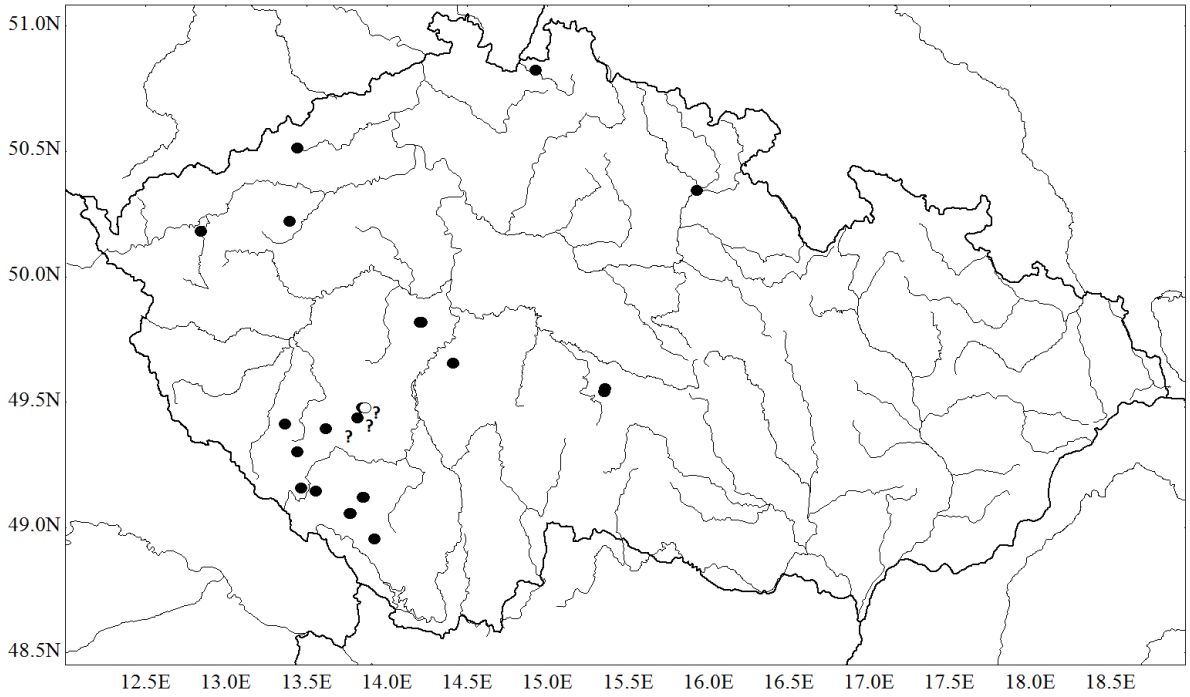

FIGURE 7.3.1. Distribution map of *Symphytum asperum* in the Czech Republic: ● revised herbarium specimens, ○ other records, ? uncertain records.

Symphytum ×*uplandicum* Nyman – Russian comfrey (kostival uplandský; Fig. 7.3.4D)

Symphytum ×*uplandicum* Nyman, Syll. Fl. Eur. 80 (1855)
(*S. asperum* × *S. officinale*)

Description

Perennial, hirsute, rhizomatous herbs. Rhizomes vertical, fusiform, not tuberous. Stems 60–140(–200) cm tall, erect, branched, without hooked hairs. Branches at flowering time of the main stem many-flowered. Leaves alternate, ovate-lanceolate, elliptic-lanceolate to lanceolate, at least 2× longer than wide, acute, with rounded to cuneate, never cordate base, shortly decurrent, semiamplexicaulous, mostly shortly petiolate, only upper cauline leaves sessile, less roughly hirsute, often without hooked hairs along middle leaf veins. Flowers in dense boragoids and double boragoids. Calyces 5–7 mm long (at flowering time), divided about 2/3–4/5 of its length. Calyx lobes ovate, acute. Corollas initially reddish, later red-violet to dark purple, tubular, 12–18 mm long, 2–3× longer than calyx; faucal scales included, narrowly-triangular, rounded at apex, with short marginal papillae. Connectives with short apical appendages. Styles erect, not or only slightly exserted. Nutlets dark brown to black, curved, minutely verrucose, shiny.

Flowering time: May to August.

2*n* = 36, 40, 44 [extra fines] (e.g. Gadella & Kliphuis 1967, 1969, Gadella *et al.* 1983).

Variability and similar species

The determination of this taxon is quite intricate, especially due to its hybrid origin and potential backcrossing, resulting in considerable morphological variability (e.g. indumentum, shape and size of leaves, size and colour of corollas). Therefore, plants can be wrongly identified as one of the parental species (see above) and numerous populations of *S.* ×*uplandicum* had undoubtedly been overlooked until recently. In most of the characters, an intermediate state between parents was observed and documented, e.g. connectives with short apical appendages (*S. officinale* with vs. *S. asperum* without apical appendages; Smejkal 1978). Moreover, different alkaloid compounds were detected in *S. asperum* and *S. officinale*, whose content is combined within *S.* ×*uplandicum* as well (Gadella *et al.* 1983, Huizing *et al.* 1983).

Ecology

In contrast to *S. asperum*, it is more tolerant to soil conditions. It also inhabits dry, poor or moderately nutrients rich soils (Ingram 1961, Skalický 2000) and therefore, it is more widespread (Tutin 1956, Wade 1958, Smejkal 1978), which is also illustrated by its more frequent occurrence in the Czech Republic (see Fig. 7.3.2). It grows in city lawns, parks, roadsides, ditches and the banks of rivers or ponds.

Distribution in the Czech Republic

In the Czech Republic, its presence is documented mainly from Bohemia, while only a few records exist from Moravia and none from Silesia. Most of the records comes from western, southern and central Bohemia, where it was widely cultivated (Srb 1958, Smejkal 1978). Based on the study of Smejkal (1978), the herbarium specimen from Karlovy Vary (Tuhnice) collected in 1848 is considered as the earliest one in the Czech Republic. However, this specimen was later determined as *S. officinale* and moreover, it is dated to 1898, not 1848 (Subchapter 7.2). Based on our revision, the oldest records came from July 1914 (cultivated plants collected in Lukohořany, *s. coll.*, PR) and from 1927 (Plzeň, wild origin, *F. Maloch*, BRNU). Most of the herbarium specimens were collected in the 1970s and 1980s, similarly as those of *S. asperum*. In contrast to *S. asperum*, more records have been recorded in last two decades (see Electronic Appendix 7.3.1).

Distribution

The origin of this hybrid taxon remains unclear. The first records of escaped or accidentally introduced plants dated back to the 1920s and 1930s and were recorded in Western (Great Britain, France) and Northern (Sweden) Europe. Afterwards, *S.* ×*uplandicum* was introduced as a green forage for livestock to other parts of Europe (Bucknall 1913, Gadella 1972, Pawłowski 1972, Smejkal 1978, Gadella *et al.* 1983). Moreover, it has been introduced into North America (Gadella 1984), in some regions has also become naturalised.

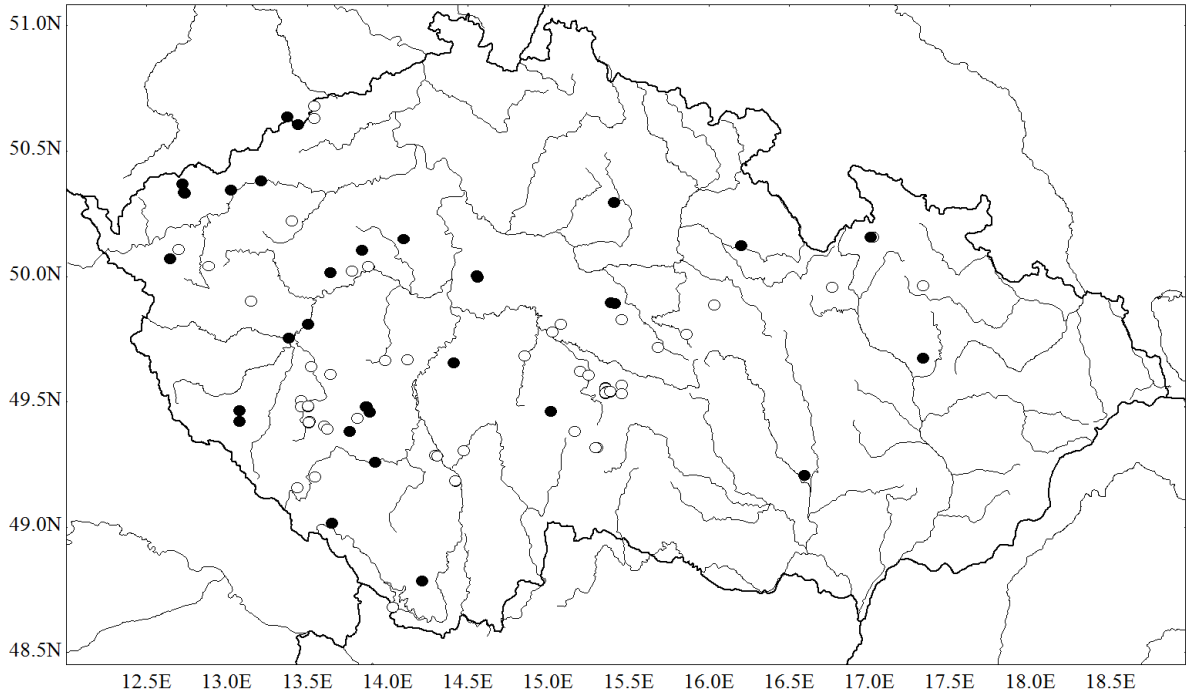

FIGURE 7.3.2. Distribution map of *Symphytum ×uplandicum* in the Czech Republic: ● revised herbarium specimens, ○ other records.

Symphytum tauricum Willd. – Crimean comfrey (kostival krymský)

Symphytum tauricum Willdenow, Neue Schriften Ges. Naturf. Freunde Berlin 2: 121, t. 5, f. 1 (1799)

Description

Perennial, densely hairy, rhizomatous herbs. Rhizomes short, cylindrical, vertical, unbranched, not tuberous. Stems ca. 60 cm tall, erect, branched from the base of the stem, with dense, short hairs and long bristles. Branches at flowering time of the main stem with many flowers. Leaves alternate, ovate to triangular, ca 1.5× longer than wide, acute, with rounded to subcordate base, not decurrent, the middle and lower leaves long petiolate, densely, softly hairy. Flowers in boragoids or double boragoids with 16–20 flowers. Calyces divided almost to base. Calyx lobes lanceolate, obtuse. Corollas white or creamy yellow, faucal scales included, lingulate, rounded at apex, with dense marginal papillae. Connectives without apical appendages. Styles erect, included. Nutlets dark grey, slightly curved, verrucose.

Flowering time: April to June.

$2n$ = ? Two chromosome numbers were published: $2n$ = 18 (Tarnavschi 1948) and $2n$ = 40 (Britton 1951). However, the origin of these counts is unclear and determination of plant material may be wrong (e.g. Tarnavschi published the same chromosome number also for *S. tuberosum* and *S. cordatum*). Therefore, detailed karyological revision is required.

Variability and similar species

It is most similar to *S. tuberosum*, but differs by not tuberous rhizomes, long petiolate, ovate to triangular leaves with rounded to subcordate base, white or creamy yellow corollas and lingulate faucal scales. Besides that, plants can be wrongly identified also as *S. orientale* (fusiform rhizomes, ovate to oblong-ovate obtuse to acute leaves, slightly divided calyces, purely white corollas and faucal scales with sparse marginal papillae; Bucknall 1913, Popov 1953; see below) or *S. grandiflorum* (long, creeping shoots, not or only poorly branched stems, broadly ovate to ovate-lanceolate leaves, yellow-white corollas and lingulate, cordate at apex faucal scales; Pawłowski 1961, Wickens 1969; see below).

Ecology

It inhabits shady, humid forest habitats as well as dry, often sandy places like forest-steppes. In the Czech Republic, it was found on garden waste and road verge.

Distribution in the Czech Republic

In the Czech Republic, the occurrence of this species was first recorded by Smejkal (1978) based on the herbarium specimen collected in the town of Černošice near Praha in 1912 by E. Liebald. On the herbarium sheet one specimen of *S. tauricum* is admixed with three specimens of *S. tuberosum*. Unfortunately, the origin of these plants is unclear, i.e. whether they originated from garden, park or represented an escape from cultivation (cf. Smejkal 1978). The possibility of accidental mixing of plants during the preparation of herbarium specimens (both species are yellow-flowered) also cannot be ruled out. However, in the 1980s, it was repeatedly recorded and collected from the vicinity of the villages Miřetice and Ptákova Lhota in south-western Bohemia (Fig. 7.3.3). Due to a change in management on these sites, this species no longer occurs here (V. Žíla pers. com.).

Distribution

Symphytum tauricum was described based on the plant material originating from Crimea (Willdenow 1799) and it is native to coastal areas around the Black Sea, i.e. south Ukraine, Bulgaria, Romania, southern parts of European Russia and Anatolia (Popov 1953, Dobroczajeva 1957, Guşuleac 1960, Pawłowski 1972, Smejkal 1978, Fedorov 2001). It is quite frequently cultivated as an ornamental plant and sometimes escapes from cultivation. In the Central Europe, a garden escape is reported e.g. from Poland and Germany (Pawłowski 1963, BfN 2017).

***Symphytum grandiflorum* DC. – Creeping comfrey (kostival velkokvětý; Fig. 7.3.4A)**

Symphytum grandiflorum A.P. de Candolle, Prodr. 10: 40 (1846)

Description

Perennial, rhizomatous herbs. Rhizomes stout, with long, creeping shoots. Stems up to 40 cm tall, erect, unbranched or only few-branched (from the base of the stem), rosette leaves absent. Leaves alternate, petiolate, broadly ovate to ovate-lanceolate, obtuse to acute, with cordate base, shortly decurrent, the uppermost ones almost sessile, with dense, short hairs and long, scattered bristles. Flowers in boragoids or double boragoids, few-flowered. Calyces strongly divided, shorter than corolla tube. Calyx lobes linear-lanceolate, obtuse. Corollas initially reddish, later yellow-white, campanulate, faucal scales included, lingulate, cordate at apex, marginal papillae sparse. Connectives without apical appendages. Styles erect, exserted. Nutlets dark grey, slightly curved, minutely verrucose.

Flowering time: April to June.

2*n* = 60 [extra fines] (e.g. Gviniashvili 1972, Jaarsma *et al.* 1990, Gagnidze *et al.* 2015).

Variability and similar species

Two varieties are recognised within this species – var. *grandiflorum* and var. *abchasicum* (Trautv.) Kusn. (Bucknall 1913, Wickens 1969). *Symphytum grandiflorum* var. *abchasicum* differs from the type variety by leaves rounded or gradually attenuated at the base and calyces nearly as long as corolla tube. In the Czech Republic, only nominate variety was observed.

The most similar species are *S. cordatum* (see below) and *S. orientale*. In contrast to *S. orientale*, *S. grandiflorum* is densely, softly hirsute and has branched stems, undulate leaves with rounded or subcordate base, slightly divided calyces and larger, pure white corollas (Bucknall 1913, Wickens 1969).

Ecology

Symphytum grandiflorum grows in the mountain broad-leaved forests and along small streams, up to 1000 m a.s.l. (Popov 1953). In the Czech Republic, it was found in abandoned garden.

Distribution in the Czech Republic

It is only very rarely cultivated in gardens and parks. In the Czech Republic, it was first recorded in Praha-Krč (2016) by Jiří Sádlo (Fig. 7.3.3).

Distribution

Symphytum grandiflorum is native to the Caucasus Mts. (Georgia, Armenia, NE Turkey; Bucknall 1913, Popov 1953, Wickens 1969). In the Central Europe, it is rarely cultivated as an ornamental plant and may escape from cultivation (documented e.g. from Germany, BfN 2017).

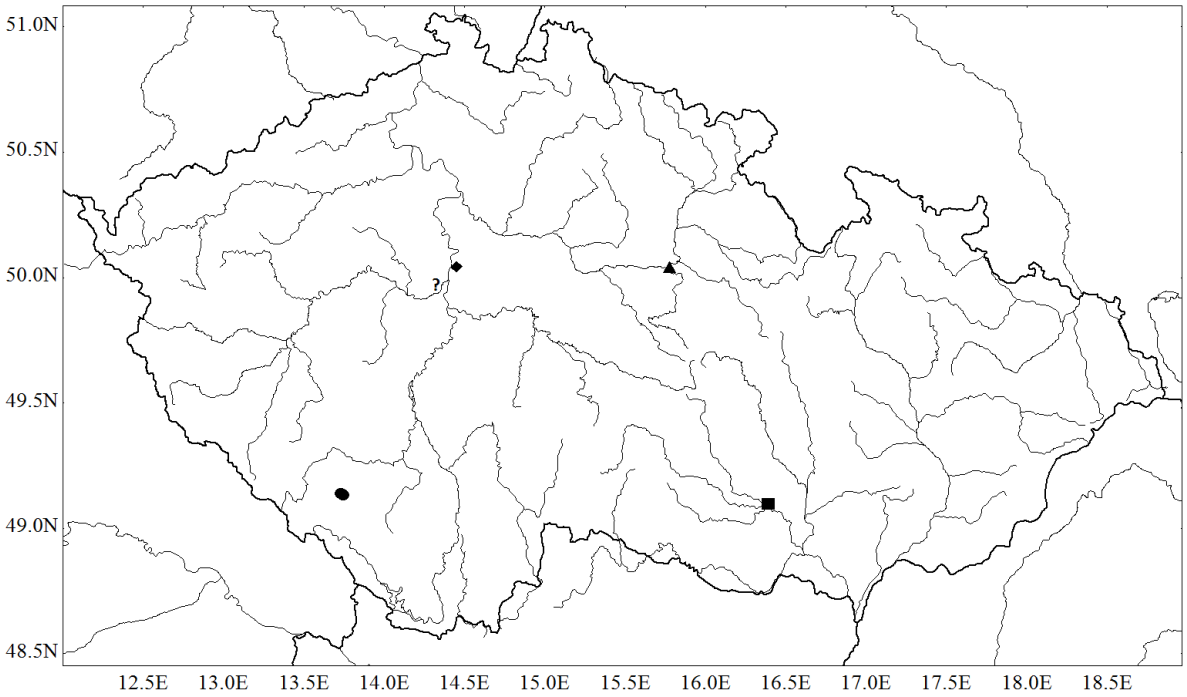

FIGURE 7.3.3. Map of occurrence of *Symphytum cordatum* (▲), *S. grandiflorum* (♦), *S. ×hidcotense* (■) and *S. tauricum* (● revised herbarium specimens, ? uncertain record) in the Czech Republic.

***Symphytum ×hidcotense* P.D. Sell – Hidcote comfrey (kostival trojbarevný; Fig. 7.3.4B)**

Symphytum ×hidcotense P.D. Sell, Fl. Gr. Brit. Ireland 3: 520 (2009)
(*S. grandiflorum* × *S. ×uplandicum*)

Description

Perennial, stout, hirsute, rhizomatous herbs. Rhizomes with long, creeping shoots. Stems up to 50 cm tall, erect, branched, with dense, scattered, short and rarely long bristles. Leaves alternate, ovate to ovate-lanceolate, obtuse to acute, with cordate base, petiolate, not or shortly decurrent. Flowers in dense scorpioid monochasia. Calyces divided about 2/3 of its length, shorter than corolla tube. Calyx lobes narrowly triangular, obtuse. Corollas initially carmine-reddish, later white-blue (corolla tube blue, limb white), tubular, faucal scales included. Connectives without apical appendages. Styles erect, not or only slightly exserted. The morphology of nutlets unknown.

Flowering time: April to June.

2*n* = ca. 52 [extra fines] (Johnson *et al.* 1997).

Variability and similar species

Symphytum ×hidcotense was described from Great Britain as a garden hybrid taxon originating from the crossing of *S. grandiflorum* and most probably *S. ×uplandicum* (Poland

& Clement 2009, Stace 2010). In contrary to both parents (see above), *S.* ×*hidcotense* has bicolour, blue-white corollas. In cultivation, these plants are commonly known as cultivar *Symphytum* 'Hidcote Blue' (Sell & Murrell 2009). However, other cultivars with presumably the same parentage, e.g. those with pink-white flowers (*S.* 'Hidcote Pink'), are documented from cultivation.

Ecology

Plants of this hybrid taxon are cultivated in gardens and parks, occasionally have been recorded as a rare garden escape (Bomble & Schmitz 2013).

Distribution in the Czech Republic

In the Czech Republic, it was first collected near the town of Ivančice in 2016, where it was apparently cultivated for a long time as an ornamental plant and escaped (Fig. 7.3.3).

Distribution

Symphytum ×*hidcotense* is a hybrid taxon described from Great Britain. It is widely cultivated as an ornamental plant in gardens and parks and sometimes escapes, e.g. in Great Britain, Belgium and Germany (Poland & Clement 2009, Stace 2010, Bomble & Schmitz 2013, Verloove & Lambinon 2014).

***Symphytum cordatum* Waldst. et Kit. ex Willd. – Cordate comfrey (kostival srdčitý)**

Symphytum cordatum Waldstein & Kitaibel ex Willdenow, Neue Schriften Ges. Naturf. Freunde Berlin 2: 121 (1799)

Syn.: *Symphytum pannonicum* Pers., Syn. Pl. 1: 161 (1805) – *S. cordifolium* Baumg., Enum. Stirp. Transsilv. 1: 126 (1816)

Description

Perennial, hirsute, rhizomatous herbs. Rhizomes thick, cylindrical, creeping, not tuberous. Stems up to 50 cm tall, erect, simple, in the lower part with erect bristles and appressed, hooked hairs. Basal leaves usually 1–2, long petiolate, large, cordate, acute. Cauline leaves 2–4, petiolate, cordate, the uppermost ones almost sessile, with rounded base, with dense appressed and scarce erect bristles. Flowers in few-flowered boragoids or double boragoids. Calyces campanulate, strongly divided. Calyx lobes narrowly lanceolate, acute. Corollas brightly yellow, tubular, faucal scales included, triangular, marginal papillae dense, long. Connectives without apical appendages. Styles erect, only slightly exserted. Nutlets black, erect, with wide ring, densely verrucose.

Flowering time: April to June.

2*n* = 120 [extra fines] (Wcisło 1972, Gadella & Kliphuis 1978, Murín & Májovský 1982).

Variability and similar species

The most similar species is *S. grandiflorum*, which differs by rhizomes with long, creeping shoots, absence of rosette leaves, broadly ovate to ovate-lanceolate leaves with rounded or subcordate base, yellow-white corollas and faucal scales lingulate, cordate at apex (Pawłowski 1961, Wickens 1969). In the Carpathian Mts., non-flowering, young plants with leaves without typical cordate leaf base can be wrongly identified as *S. tuberosum* (higher ploidy levels, i.e. 8*n* and 12*n*, Kobrlová *et al.* unpubl.). Nevertheless, *S. tuberosum* differs well by tuberous rhizomes, branched stems, broadly ovate to ovate-lanceolate leaves with cuneate base and many-flowered inflorescences.

Ecology

It inhabits shady, moist and nutrient-rich places in submontane and montane areas (Májovský & Hegedüšová 1993). It grows in broad-leaved, mostly beech, less often coniferous forests, and in the valleys of mountain streams or small rivers (Pawłowski 1963, Smejkal 1978, Májovský & Hegedüšová 1993). It is occasionally cultivated as an ornamental plant in gardens and parks, but the garden escapes are very rare.

Distribution in the Czech Republic

It has been rarely cultivated in gardens and parks, which is documented by a herbarium record from the Tyršovy Sady park in the Pardubice city (Fig. 7.3.3).

Distribution

Carpathian endemic taxon with its centre of distribution in the Eastern Carpathian Mts., growing in Poland, Slovakia, Ukraine and in Romania (Dobroczajeva 1957, Guşuleac 1960, Pawłowski 1963, 1972, Májovský & Hegedüšová 1993, Fedorov 2001). In the Central Europe, an escape from cultivation is documented e.g. from Germany (BfN 2017).

***Symphytum bulbosum* K.F. Schimp. – Bulbous comfrey (kostival cibulkatý; Fig. 7.3.4C)**

Symphytum bulbosum K.F. Schimper, Flora 8(1): 17 (1825)

Syn.: *Symphytum zeyheri* K.F. Schimp., Flora 12(2): 418 (1829) – *S. tuberosum* subsp. *bulbosum* (K.F. Schimp.) P. Fourn., Quatre Fl. France 747 (1937)

Description

Perennial, hirsute, rhizomatous herbs. Rhizomes slender, creeping, tuberous, with bulb-like thickenings. Stems up to 50 cm tall, erect, often branched. Leaves petiolate, ovate to elliptic-lanceolate, obtuse to acute, shortly decurrent to subcordate base, the uppermost ones almost sessile, with soft bristles. Flowers in many-flowered boragoids and double boragoids. Calyces divided almost to the base. Calyx lobes lanceolate, acute. Corollas brightly yellow with whitish corolla tubes, small, tubular-campanulate, faucal scales exserted, triangular to

lingulate, protracted at apex, marginal papillae dense. Connectives without apical appendages. Styles erect, exserted. Nutlets black, slightly curved, verrucose.

Flowering time: April to June or July.

2*n* = 48, ca. 72, 84, 96, (104), 120 [extra fines] (Strey 1931, Grau 1971, Gadella & Kliphuis 1978, Johnson *et al.* 1997, Bottega *et al.* 2001, Peruzzi 2003). Likewise, several ploidy levels were detected using flow cytometry (Kobrlová unpubl.).

Variability and similar species

This species is characterised by strongly exserted faucal scales, a unique character of *Symphytum* sect. *Bulbosa* (Pawłowski 1961), and therefore can be easily distinguished from most of the *Symphytum* relatives. The only similar European species is *S. ottomanum*, from the same infrageneric section, which occurs on the Balkan Peninsula and differs by fusiform, not tuberous rhizomes, smaller leaves with more prominent leaf venation and smaller, white corollas (Pawłowski 1972).

Non-flowering plants can be wrongly determined as *S. tuberosum* s. l., which can be easily distinguished based on the different morphology of rhizomes (see Chapter 2, Subchapter 7.1).

Ecology

It prefers moist and shady sites and grows especially in the lowlands. Most frequently, it inhabits floodplain sites, the banks of rivers and canals, along wet forest tracks, as well as ruderal and disturbed habitats, such as road verges, ditches along roads or the banks of fishponds.

Distribution

Symphytum bulbosum represents the Mediterranean floristic element. It is distributed from southern France (incl. Corsica), across south Switzerland, Italy (incl. Sicily), Slovenia, Croatia to Balkan Peninsula, Romania and Turkey (Davis 1978, Stearn 1985, Strid 1991, Cecchi & Selvi 2015). In some Central European countries, mainly in Austria and Germany, it is grown as a vegetable or cultivated as a decorative herb (in the Province of North Rhine cultivated since 1822, Smejkal 1978). In both of the mentioned countries, an escape from the cultivation is known (Schmeil & Fitschen 1988, Fischer *et al.* 2008, Kniely 2015, BfN 2017).

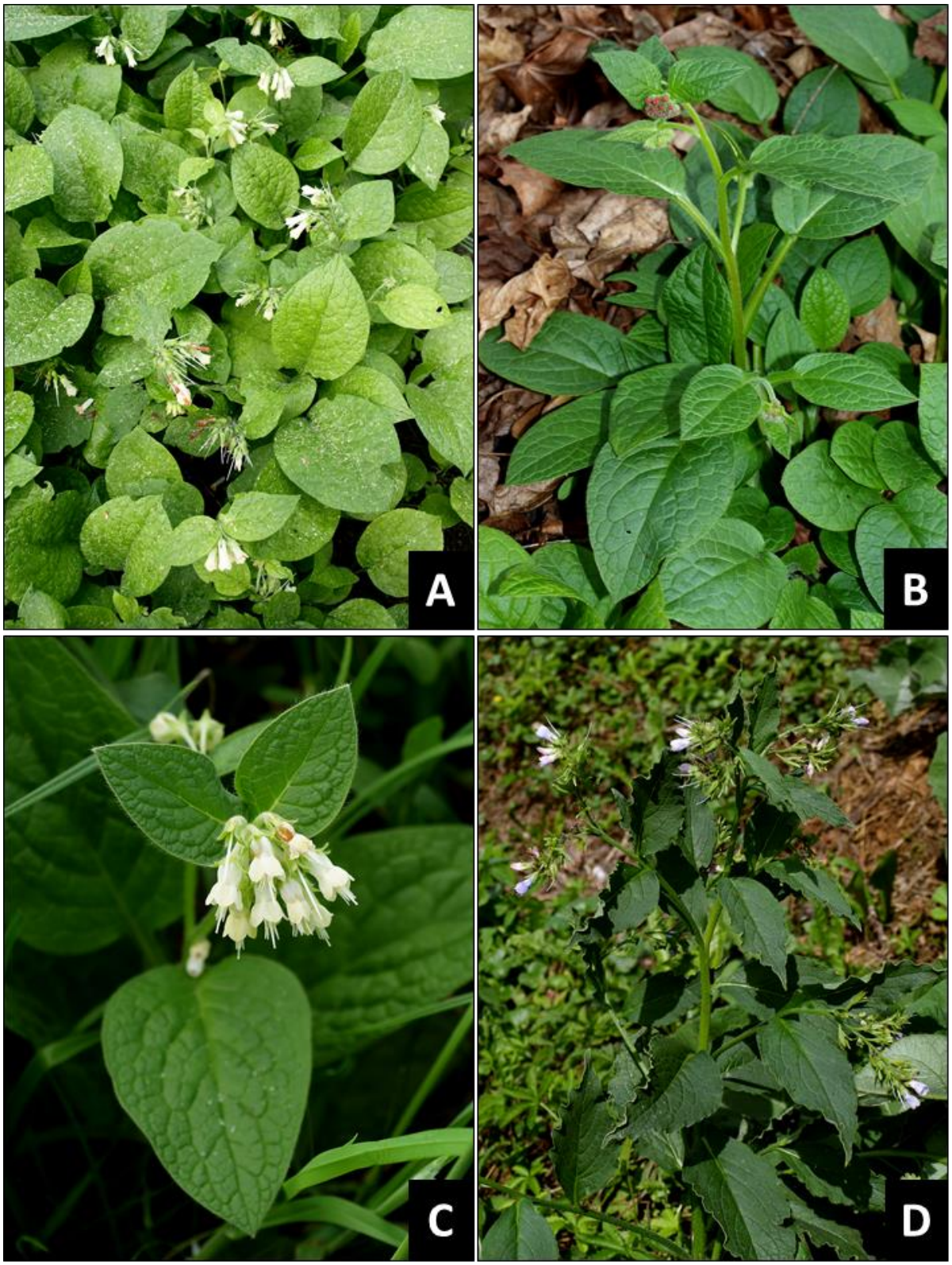

FIGURE 7.3.4. Selected cultivated and naturalised Comfrey species in Central Europe: (**A**) *Symphytum grandiflorum*. (**B**) *S. ×hidcotense*. (**C**) *S. bulbosum.* (**D**) *S. ×uplandicum* (**A** – photo L. Kobrlová, **B, C, D** – photo M. Hroneš).

***Symphytum caucasicum* M. Bieb. – Caucasian comfrey (kostival kavkazský)**

Symphytum caucasicum Marschall von Bieberstein, Fl. Taur.-Caucas. 1: 128 (1808)

Description

Perennial, softly hirsute, greyish, rhizomatous herbs with dense basal leaf rosette. Rhizomes short, thick, fusiform, without tubers. Stems 40–60 cm tall, erect, branched, winged from decurrent leaf bases, softly pubescent. Leaves oblong-ovate to ovate-lanceolate, acute, with truncate to rounded base, softly hairy. Basal cauline leaves petiolate, the upper cauline leaves almost sessile. Flowers in many-flowered boragoids and double boragoids. Calyces divided about 1/3 of its length. Calyx lobes lanceolate, obtuse. Corollas initially pink to reddish, later deep blue, campanulate, faucal scales included, lingulate, obtuse, marginal papillae dense. Connectives without apical appendages. Styles erect, not or shortly exserted. Nutlets black, shining, almost erect, reticulate-rugose and minutely verrucose.

Flowering time: May to June.

2*n* = 24, (36), 48 [extra fines] (Strey 1931, Gviniashvili 1972, Gadella & Kliphuis 1978, Gagnidze *et al.* 2015).

Variability and similar species

The only similar species occurring in the Czech Republic is *S. asperum* (see above).

Ecology

In the Caucasus, it grows in open forests and forest fringes (Popov 1953). The secondary habitats are road verges or edges of paths (Bomble & Schmitz 2013).

Distribution

Symphytum caucasicum, in relation to its scientific name, is distributed in the area of the Caucasus Mts. (Bucknall 1913, Popov 1953, Wickens 1969). As a decorative plant it is commonly cultivated, rarely escapes from cultivation and becomes locally established in some Central European countries, e.g. in Germany (Bomble & Schmitz 2013).

***Symphytum orientale* L. – White comfrey (kostival východní)**

Symphytum orientale Linnaeus, Sp. Pl. 1: 136 (1753)

Description

Perennial, stout, softly hirsute, rhizomatous herbs. Rhizomes fusiform, not tuberous. Stems erect, branched, lateral branches few-flowered. Leaves ovate to oblong-ovate, obtuse to acute, with truncate, rounded or subcordate base, undulate, petiolate, not decurrent, softly hairy. Flowers in many-flowered boragoids and double boragoids (often flowering mainly by lateral branches). Calyces slightly divided. Calyx lobes short, triangular, obtuse to acute. Corollas

purely white, quite large, tubular, faucal scales included, lingulate, marginal papillae sparse. Connectives without apical appendages. Styles erect, usually inserted. Nutlets black, slightly curved, verrucose.

Flowering time: April to June.

2*n* = 32 [extra fines] (e.g. Gadella & Kliphuis 1978, Markova 1983, Markova & Goranova 1995, Bottega *et al.* 2001).

Variability and similar species

In the Czech Republic, the only similar, white or creamy yellow flowered species are *S. tauricum* and *S. grandiflorum* (see above), which are both infrequently cultivated.

Ecology

It inhabits damp, shady places in forests or the banks of rivers or streams (Wickens 1969).

Distribution

Symphytum orientale was described based on the plant material originating from the vicinity of the town of Istanbul (Linnaeus 1753a). Except of Turkey (Wickens 1969), it apparently occurs in the southern part of the European part of Russia and in Ukraine (Popov 1953, Pawłowski 1972, Fedorov 2001). It is frequently cultivated as an ornamental plant, sometimes escapes from cultivation and becomes locally established, e.g. in Poland (Pawłowski 1963). In the Czech Republic, it was cultivated in the former Zahrada Kanálka garden in Praha (Smejkal 1978).

Chapter 8

General discussion

Kobrlová Lucie

What is the diversity of the genus *Symphytum* in the Central Europe?

The field botany and botanical research in the Central Europe, particularly in the Czech Republic, have a long tradition resulting in detailed multivolume monographs of Czech flora and vegetation (Chytrý *et al.* 2017). Recently, this tradition has led to the extensive on-line database of critically revised data on both (Chytrý *et al.* 2021). Therefore, the basis for this kind of study is more than satisfactory, enabling us to build on the previously collected data, including rich herbarium collections (cf. Danihelka *et al.* 2017) and a large set of vegetation relevés (Chytrý & Rafajová 2003), which is also quite unique in comparison with other European countries.

The presented thesis aimed mainly at getting comprehensive insight into the diversity of the genus *Symphytum* in the Central Europe, with a special focus on the Czech Republic. Although with about 40 species recognised (cf. Bucknall 1913, Wickens 1978) it is not a species-rich genus, its taxonomy remains unresolved with many questions to be answered. In general, a common feature of most of the *Symphytum* species is the morphological variability and phenotypic plasticity, which has led to the description of a great number of taxa, having different taxonomic value. In most cases, this variability has questionable or no taxonomical value, e.g. various forms of *S. officinale* based on corolla colour (Fig. 8.1, see e.g. Persoon 1805, Grecescu 1898). Likewise, some taxa were repeatedly described from various parts of Europe under a different name, e.g. *S. tanaicense* vs. *S. uliginosum* (Steven 1851, Kerner 1863, Degen 1930). Unfortunately, detailed morphological descriptions are missing for many taxa and therefore, authors had sometimes adopted their names rather randomly (cf. Wickens 1969, 1978). This approach resulted in a large number of names that are often difficult to interpret, such as a nomenclatural problem of *S. peregrinum* Ledeb. (Ledebour 1820) and its unclear relation to *S. asperum* and/or *S.* ×*uplandicum* (cf. Kuznetsov 1910, Bucknall 1912, Faegri 1931, Tutin 1956, Wade 1958, Gadella *et al.* 1983). However, thanks to the introduction of advanced research tools (classical karyology, modern cytogenetical and molecular markers), taxonomic concepts can be revised and classifications changed accordingly. An example would be the species delimitation of *S. asperum* complex, combining morphological and molecular approach (Kurtto 1982, Tarıkahya & Erik 2010, Özgişi & Tarıkahya-Hacıoğlu 2021) or implementation of flow cytometry to study tangled, polyploid groups as *S. officinale* (Chapter 4) or *S. tuberosum* (Chapter 2, 3) complexes.

Traditionally, five to seven native taxa have been reported from the Central Europe, according to the respective national floras (Pawłowski 1963, Schmeil & Fitschen 1988, Májovský & Hegedüšová 1993, Slavík 2000, Fischer *et al.* 2008). Throughout the whole area, two native species complexes occur, namely *S. officinale* complex and *S. tuberosum* complex, both with different species concepts within above-mentioned floras. Besides them, an endemic Carpathian species *S. cordatum* also occur in the Central Europe (Pawłowski 1963, 1972, Májovský & Hegedüšová 1993). Further records of non-native, naturalised and cultivated taxa vary between each country with the following species being listed: *S. asperum*, *S. bulbosum*, *S. caucasicum*, *S. grandiflorum*, *S.* ×*hidcotense* P.D. Sell, *S. orientale*, *S. tauricum*, *S.* ×*uplandicum* (Pawłowski 1963, Gams 1966, Smejkal 1978, Schmeil & Fitschen 1988, Fischer *et al.* 2008, Bomble & Schmitz 2013, Kniely 2015, Kaplan *et al.* 2016, BfN 2017). The above-mentioned *S. cordatum* is also occasionally cultivated (see below).

FIGURE 8.1. An example of the variation of corolla colour of tetraploid *Symphytum officinale* s. str. in the Central Europe.

How many species occur in the Czech Republic? How are these species distributed?

For the Czech Republic, the first (and at the same time the only one) treatment of the genus *Symphytum* that aimed to be complete and critical was provided by Smejkal (1978). In its scope, this work represents one of the few published revisions of the genus at all (cf. Kuznetsov 1910, Bucknall 1913, Wickens 1969, Gviniashvili 1976, Stearn 1985), although it only focuses on a limited part of the genus range. It provided a detailed revision of distribution patterns of taxa in former Czechoslovakia, solely based on the examination of herbarium and living collections, complemented by some taxonomic remarks, and above all by a detailed identification key. This is quite valuable since only a few such studies have been published even within the whole family (see e.g. Miller 1988, Selvi & Sutorý 2012, Cecchi & Selvi 2015, 2017, Madika & Moteetee 2021, Meudt 2021). According to Smejkal (1978), eight taxa (not counting primary hybrids) have been reported for the studied area. Two of them have exclusively been documented only for Slovakia, namely Carpathian endemic *S. cordatum* and *S. tanaicense*, a member of the *S. officinale* complex only found in Eastern Slovakia (Smejkal 1978, see also Májovský & Hegedüšová 1993). Apart from these, the list of species has included additional three native taxa for both Czech and Slovak flora, i.e. *S. bohemicum*, *S. officinale* (both *S. officinale* agg.) and *S. tuberosum*. The occurrence of the three alien taxa for Czech flora only, i.e. *S. asperum*, *S.* ×*uplandicum*, *S. tauricum*, were further documented.

The last updated checklist of vascular plants of the Czech Republic (Danihelka *et al.* 2012) listed five taxa of the genus *Symphytum*, three native species (*S. tuberosum*, *S. officinale* agg., i.e. *S. officinale* s. str. and *S. bohemicum*) and two naturalised taxa (*S. asperum* and *S.* ×*uplandicum*), which is entirely in line with the modern multivolume

Flora of the Czech Republic (Slavík 2000) as well as with the widely used Key to the flora of the Czech Republic (Kubát *et al.* 2002).

Compared to the latest Czech checklist (Danihelka *et al.* 2012), the recent revision of the genus *Symphytum* presented in this thesis almost doubled the number of species. Specifically, the revision of 3 676 herbarium specimens, including a few flow cytometric records, confirmed the occurrence of nine taxa of the genus *Symphytum* in the Czech Republic (Chapter 7). Unlike the latest inventories of the genus (cf. Slavík 2000, Kubát *et al.* 2002, Danihelka *et al.* 2012), two subspecies of the native *S. tuberosum* complex, i.e. *S. tuberosum* subsp. *tuberosum* and *S. tuberosum* subsp. *angustifolium*, have been distinguished throughout the Central Europe, especially thanks to the implementation of the flow cytometry (Chapter 2). The questionable occurrence of *S. tauricum* has been confirmed and two other non-native taxa (*S. grandiflorum* and *S.* ×*hidcotense*) have been reported from the Czech Republic for the first time (Subchapter 7.3). This revision also significantly expanded the previously known distribution ranges and provided a reliable knowledge about the genus in our country (see also Supplementary File 1). Consequently, this revision has been accepted in the updated version of Key to the flora of the Czech Republic (Chapter 5).

The most common native species is *S. officinale* s. str. (i.e. tetraploid cytotype of *S. officinale* complex; Chapter 4), that is also the most widespread species of the whole genus (Hultén & Fries 1986). In the Czech Republic, it is distributed through the country, being less frequent or under-recorded only in western Bohemia (Subchapter 7.2, Supplementary File 1). From the same species complex, one more taxon is reported from our country, namely *S. bohemicum* (i.e. diploid cytotype; Chapter 4), which is found in calcareous fens in the lowlands along the stretches of rivers in eastern, central and northern Bohemia (Subchapter 7.2, Supplementary File 1). The last of our native species is *S. tuberosum*, with two subspecies accepted in this thesis (Chapter 2), i.e. *S. tuberosum* subsp. *tuberosum* and *S. tuberosum* subsp. *angustifolium*. The more frequent nominate subspecies which occur mainly in southern and central Bohemia and in northern Moravia and Silesia, inhabiting shady, humid sites as the banks of watercourses, alder carrs or alluvial and ravine forests (Subchapter 7.1, Supplementary File 1). In contrast, subsp. *angustifolium* is more thermophilous and it is confined only to central, south and south-eastern Moravia, especially growing in thermophilous broad-leaved forests and semi-dry grasslands (Subchapter 7.1, Supplementary File 1).

Which non-native species have been introduced to the Central Europe, and especially to the Czech Republic?

Most of the *Symphytum* species have spread beyond their native area as ornamental plants, or as nectar-bearing, medical and forage plants (e.g. Srb 1958, Ingram 1961). Generally, a number of Boraginaceae species are commonly grown as ornamentals (e.g. *Omphalodes* spp., *Brunnera macrophylla* (Adams) I.M. Johnst., *Nonea lutea* (Desr.) DC.), with a number of commercial cultivars (e.g. *Myosotis* spp.), or for medicinal purposes (e.g. *Anchusa officinalis* L., *Borago offficinalis*, *Lithospermum officinale* L., *Pulmonaria officinalis* L.). Therefore, some of those taxa are widespread and present on several continents (cf. Johnston 1927, Miller 1988, Verdcourt 1991, Ariza-Espinar 2006), even widely introduced as noxious

weeds (e.g. *Echium* spp., *Amsinckia* spp.; e.g. Pusateri & Blackwell 1979, Parsons & Cuthbertson 2001, Weigend *et al.* 2016). Because of the content of toxic pyrrolizidine alkaloids (PAs, e.g. Frölich *et al.* 2007) and the plant indumentum, the economic importance of the Boraginaceae family as food plants is rather low (Weigend *et al.* 2016; but see Boraginaceae oils, e.g. Guil-Guerrero *et al.* 2003, Mhamdi *et al.* 2009).

The majority of *Symphytum* taxa occurring in the Central Europe are non-native, eventually naturalised and make up an assemblage of species derived from different regions, primarily from Asia Minor and Southwest Asia (cf. Pawłowski 1972). According to the literature available, there are nine taxa recorded for this part of Europe (summarised in Subchapter 7.3).

An insight from the field and herbarium provided in this thesis indicate that six *Symphytum* species have been cultivated in the Czech Republic (*S. tauricum*, *S. grandiflorum*, *S.* ×*hidcotense*, *S. cordatum*), with two of them become locally established (*S. asperum*, *S.* ×*uplandicum*). It is a compilation of species that are also reported as naturalised from neighbouring countries, except for *S. bulbosum*, *S. caucasicum* and *S. orientale* which are not yet known from the Czech Republic. *Symphytum bulbosum* is predominantly a Mediterranean species (esp. distributed in southern France, Italy, Balkan Peninsula and north-eastern Turkey, Stearn 1986, Strid 1991, Cecchi & Selvi 2015). In the Central Europe, it has only been documented as naturalised in Austria (Fischer *et al.* 2008, Kniely 2015) and Germany (Schmeil & Fitschen 1988, BfN 2017), whence it was described (Schimper 1825, lectotype designated by Bottega & Garbari 2003). In addition to its cultivation as an ornamental plant, it is occasionally cultivated as a vegetable (esp. for stolon tubers). *Symphytum caucasicum*, a Caucasian (Kuznetsov 1910, Wickens 1969) blue-flowered species is commonly cultivated as an ornamental in some countries, although the garden escape and the occurrence in the wild are rather rare in Europe (often does not persist, Stace 2010). Recently, it has been reported as a neophyte from Germany (Jäger *et al.* 2007, Bomble & Schmitz 2013). *Symphytum orientale* has been widely cultivated across Europe (formerly allegedly even in Prague, Smejkal 1978), and rarely become naturalised, such as in Poland (Pawłowski 1963). This species is native (endemic?) to western and north-western Turkey (Kuznetsov 1910, Wickens 1969, Kurtto 1982), and by some authors (e.g. Popov 1953, Dobroczajeva 1957, Pawłowski 1972) has also been reported as native from Ukraine, howbeit the specimens collected here seems to be of garden origin (Kurtto 1982).

Only three out of the six above-mentioned taxa have previously been documented from our country (Slavík 2000, Kubát *et al.* 2002, Danihelka *et al.* 2012). The prickly comfrey (*S. asperum*) has been widely cultivated across Europe since the turn of the 19th century (Wade 1958) as forage, nectar-bearing and ornamental plant (e.g. Faegri 1931, Tutin 1956, Kurtto 1982), and was later introduced to North America (Ingram 1961, Gadella 1984) and Japan (Fedorov 2001). As a native species it grows in the Caucasus region, the north-eastern parts of Turkey and the south to the northern parts of Iran (Wickens 1969, 1978, Kurtto 1982). For a similar purpose, maybe even on a larger scale, the hybrid taxon *S.* ×*uplandicum* has become to spread through Europe during the first half of the 20th century (its origin remains unclear, cf. Tutin 1956, Wade 1958). Being much like the relative *S. asperum*, it has often been confused with it, and therefore reports on its distribution and frequency in gardens may be affected by this. This is evidenced for example by the revision of

the genus *Symphytum* in North America, where only a few plants have been correctly identified as *S. asperum* and most plants referred to as *S. asperum* appeared to belong to *S.* ×*uplandicum* or *S. officinale* (Gadella 1984). The occurrence of both of these taxa in the Czech Republic has been critically evaluated, solely based on revised herbarium specimens, both being rare to scattered throughout Bohemia with *S.* ×*uplandicum* having few records also in Moravia (Subchapter 7.3, Supplementary File 1).

The third species is *S. tauricum* (assumed to be native in Crimea, Caucasus and Anatolia, e.g. Kuznetsov 1910, Gviniashvili 1976, Wickens 1978, Fedorov 2001), whose occurrence in our country was at first only discussed (unclear plant origin on the mixed herbarium sheet with *S. tuberosum*, Smejkal 1978). However, *S. tauricum* was repeatedly recorded from south-western Bohemia in the 1980s (Subchapter 7.3, Supplementary File 1), and therefore it has recently been classified and listed as a casual neophyte of our flora (Chapter 5). There is no doubt that *S. tauricum* plants were originally cultivated as ornamentals (quite decorative leaves, i.e. densely hirsute, triangular-cordate, petiolate) and escaped from the cultivation. Herbarium specimens were collected in garden waste and road verge. Likewise, a garden escape and local establishment of this species are reported from Poland and Germany (Pawłowski 1963, BfN 2017).

On the contrary, two taxa (i.e. *S. grandiflorum* and *S.* ×*hidcotense*) have been newly discovered for Czech flora (Subchapter 7.3). The first one, white-flowered and hirsute *S. grandiflorum*, native to Caucasia and Turkey (Wickens 1969), is common in gardens and parks and well naturalised in Western Europe (Stace 2010). As a neophyte species, it is also reported from Germany (BfN 2017). However, with an almost cordate leaf base it can be very easily confused with *S. cordatum* (see below), especially in the Carpathian region (cf. Pawłowski 1972). In our country, it has been found and repeatedly observed in an abandoned garden in Prague (Subchapter 7.3). By some authors, two varieties have been recognised (Bucknall 1913, Wickens 1969), with only nominate variety being observed in the Czech Republic (Subchapter 7.3). The second taxon is an artificial hybrid of a garden origin, *S.* ×*hidcotense* (*S. grandiflorum* × *S.* ×*uplandicum*, Poland & Clement 2009, Stace 2010). The corolla colour polymorphism has been the source of several cultivars (*S.* 'Hidcote Blue', *S.* 'Hidcote Pink', Sell & Murrell 2009), but typical and prevalent is the blue-flowered type. It is quite common in gardens and horticultures, especially in Western Europe (Poland & Clement 2009, Stace 2010, Verloove & Lambinon 2014), and from the Central Europe it has also been reported from Germany (Bomble & Schmitz 2013). A relatively large population has been found in the nature park in south-western Moravia (Subchapter 7.3).

Finally, the species *S. cordatum* is also listed within the presented inventory of the genus in the Czech Republic, but it has only been documented from a public park in the Pardubice city (Subchapter 7.3). Although its escape from cultivation has been reported from Germany (BfN 2017), it is more likely a misidentification of commonly cultivated *S. grandiflorum* due to the rare cultivation of *S. cordatum* (see above, cf. Bucknall 1913, Stace 2010). In the Central Europe, it represents a native taxon of the Slovak (Májovský & Hegedüšová 1993) and Polish Flora (Pawłowski 1963).

Given the common geopolitical history with the adjacent Slovakia (former Czechoslovakia), a rather interesting finding was the difference in the number of non-native species (see Chapter 5, 6). In contrast to the Czech Republic, no alien taxa have been listed

for the Slovak part of the territory according to the detailed inventory of Smejkal (1978), which is also consistent with more recent Slovak botanical studies (e.g. Murín & Májovský 1982, Májovský & Hegedüšová 1993, Marhold & Hindák 1998, Medvecká *et al.* 2012). This is rather surprising since the climate, as well as the human activities in both countries seem to be quite similar (cf. Medvecká *et al.* 2012, Pyšek *et al.* 2012, 2017). In addition, the occurrence of all non-native taxa reported from the Czech Republic have also been documented at least from some other Central European countries (e.g. *S. tauricum*, Pawłowski 1963) and even more, some of them (*S. asperum* and *S. ×uplandicum*) are widely naturalised across whole Europe (cf. Pawłowski 1972). This discrepancy could potentially be related to the less frequent cultivation of alien *Symphytum* taxa in this territory (cf. Smejkal 1978) and therefore, the lower chance of their naturalisation.

Which morphological characters are the most useful for species determination?

As pointed out by Pawłowski (1961), the morphology of flowers, i.e. the character of calyces, corollas, fornices and nutlets (Fig. 1.3, Figs 8.2–8.4), is of key importance in the taxonomy of the genus *Symphytum*. At the same time, the most relevant infrageneric classification of the genus is primarily based on the generative characters (cf. Pawłowski 1961, 1971). The morphology of leaves, plants indumentum and/or the morphology of roots/rhizomes (Fig. 1.3) was also proved to be useful in the identification of particular taxa (e.g. *S. asperum, S. bulbosum, S. cordatum, S. orientale*).

Historically, within the first classification systems of the genus *Symphytum*, the morphology of rhizomes and branching of the stem, together with the morphology of flowers, have been of special interest (Boissier 1879, Kuznetsov 1910, Bucknall 1913). The species concept of Bucknall (1913) has been of great importance and most authors referred to it (cf. Pawłowski 1961, 1971, Wickens 1969, Sandbrink *et al.* 1990, Hacıoğlu & Erik 2011). According to Bucknall (1913), two divisions have been differentiated, one containing plants with branched stems, fusiform rhizomes and dense inflorescence (*Ramosa* Buckn.) and the second containing those with a simple stem, more or less creeping and tuberous rhizomes and sparse inflorescences (*Simplicia* Buckn.). The first division is further divided by the calyx gamosepaly into two subdivisions. Furthermore, within both divisions, several series have been proposed (Bucknall 1913). This system reflected quite well the observed morphological variability of the genus and provided sophisticated identification key including European as well as Asian taxa. However, there are some discrepancies in it in terms of delimitation of some taxa and their taxonomical status, e.g. *S. armeniacum* Buckn. (synonym of *S. asperum*) or *S. zeyheri* Schimp. (synonym of *S. bulbosum*).

Later on, the infrageneric system based mainly on generative characters was proposed by Pawłowski (1961, 1971), including six sections. Recent molecular studies (Sandbrink *et al.* 1990, Hacıoğlu & Erik 2011) have indicated that these above-mentioned morphological classifications are to some extent correlated with molecular markers. However, divisions and subdivisions proposed by Bucknall (1913) were not supported at the molecular level (Hacıoğlu & Erik 2011). Thus, in fact, the genus *Symphytum* seems to be divided into nine sections according to ITS and *trn*L-*trn*F sequences (Hacıoğlu & Erik 2011), combining the

sections recognised by Bucknall (1913) and Pawłowski (1961, 1971). Nevertheless, the complete phylogenetic revision is still required since all previous studies were not comprehensive (mostly lacking the Asian part of the area) and did not focus on understanding the role of hybridization and polyploidy in the evolution of the genus. Both of these processes have been proved to be significant (e.g. Gadella 1972, Chapter 2, 4). Altogether, these findings could be beneficial for the elucidation of taxonomic problems and unravelling the confusion caused by nomenclature chaos (e.g. the mystery of the name *S. peregrinum*).

In most taxa, the generative characters are the most reliable for the species identification, as evidenced by their prevalence in identification keys (e.g. Pawłowski 1972, Wickens 1969, 1978); being even unique for particular sections, such as the sect. *Procopiania* whose representatives have deeply divided corollas and long-exserted stamens, a combination of characters that no other species group has (Fig. 8.4; Pawłowski 1971b, 1972). Among others, this corresponds to the fact that species of this section have previously been recognised as a separate genus *Procopiania* (Riedl 1963, Pawlowski 1971, 1972, Stearn 1985). Traditionally, the size and division of calyx, the ratio of calyx and corolla length and the corolla colour has been used for species identification and infrageneric classification of *Symphytum*. Since the vegetative morphology of many species is very similar, the set of these characters seems to be relevant for the clear delimitation. An example of this would be the morphological investigation of *S. ottomanum*, *S. pseudobulbosum* and *S. orientale* (Kurtto 1985), where the flower morphology is often crucial for their determination (see Fig. 8.4). Similarly, the exsertion of corolla scales represents a specific feature characteristic for sect. *Bulbosa* Kuzn. (Pawłowski 1961), which is simultaneously a key character for distinguishing between *S. bulbosum* (sect. *Bulbosa*; Pawłowski 1961) and *S. tuberosum* s. l. (sect. *Tuberosa* Buckn.), whose vegetative morphology is very similar as their flowers are of the same yellow colour (Fig. 8.2, Kobrlová unpubl.).

From the taxonomical viewpoint, the morphology of corolla scales, i.e. fornices (Figs 8.2, 8.3) appears to be most species-specific. Unfortunately, the examination of fornices might be difficult since their correct characterisation require using of stereomicroscope or a good magnifying glass. Apart from the shape of fornices, a relatively important feature is also the structure of their margins, i.e. distribution and density of papillae along margins (Fig.8.3; Pawłowski 1961). At the same time, corolla scales are also useful for differentiation of the genus *Symphytum* from several related genera of the tribe Boragineae, which are occasionally misidentified as such, as have been observed during herbarium revision (Kobrlová unpubl.). In these genera, the scales are either missing (e.g. *Onosma*, *Nonea*, *Pulmonaria*) or never triangular-lanceolate but shortly trapezoid and shortly pubescent on the entire surface (e.g. *Pentaglottis* Tausch, Weigend *et al.* 2016).

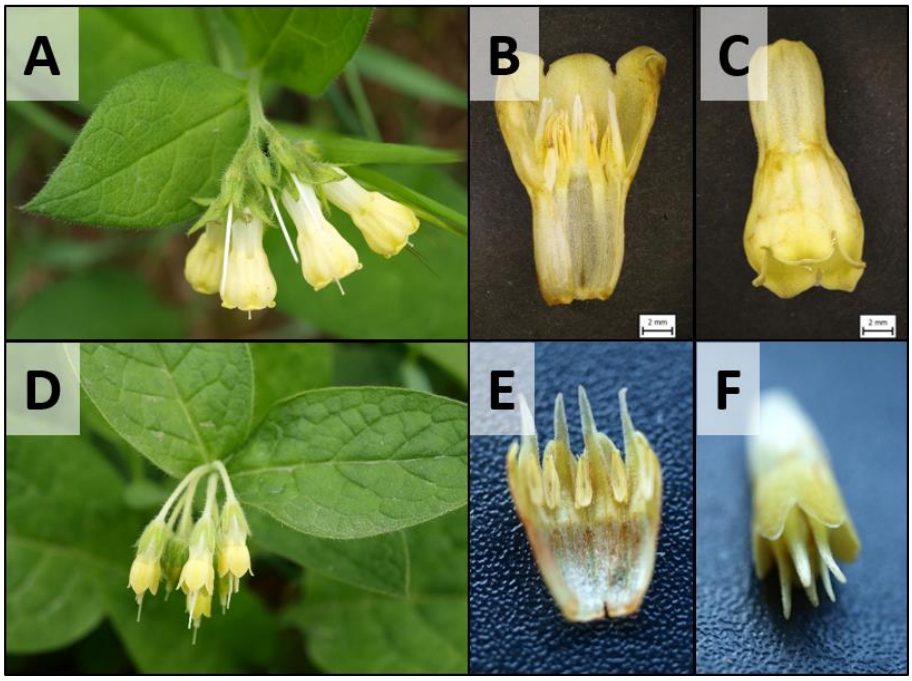

FIGURE 8.2. The flower morphology of (**A–C**) *S. tuberosum* s. str. (sect. *Tuberosa*) and (**D–F**) *S. bulbosum* (sect. *Bulbosa*) with the second species having characteristic exserted corolla scales, the key feature for distinguishing between these two taxa.

The morphology of fruits is another important character, especially the shape and the surface ornamentation (Fig. 1.3). In general, the fruits provide the most important set of characters for the classification of the family Boraginaceae (Weigend *et al.* 2016). They are useful in the systematics of *Anchusa* L. (Selvi & Bigazi 2003) or *Cryptantha* s. l. (Hasenstab-Lehman & Simpson 2012, Mabry *et al.* 2016). To give some *Symphytum* example, the mericarpids (= nutlets) of *S. officinale* complex may be mentioned, since unlike in the other *Symphytum* species, they are smooth and shiny but not verrucose (Fig. 1.3; e.g. Pawłowski 1972). However, fruits are not available in most herbarium collections as botanists primarily collect flowering specimens. At the same time, fruiting plants are sometimes quite rare also in nature (based on own observations of European *Symphytum* species), partly because of the inflorescence nibbling by wild game and in some taxa also possibly due to the prevalence of the vegetative reproduction connected with high ploidy level (e.g. sect. *Tuberosa* and sect. *Bulbosa*). In this respect, I have observed apparent differences between members of various sections, e.g. members of the *S. officinale* complex appear to produce seeds more often compared to *S. tuberosum* relatives, which may also be related to the higher level of polyploidy and the prevalence of vegetative reproduction in *S. tuberosum* complex (Kobrlová unpubl., cf. Herben *et al.* 2017). Alternatively, the fruiting plant material that I examined had, in the vast majority of cases, only one or two nutlets per flower developed into maternity, instead of four 1-seeded mericarpids that are characteristic for the whole family (Weigend *et al.* 2016; eventually two 2-seeded twin-nutlets, an autapomorphic trait of the genus *Cerinthe* L., Selvi *et al.* 2009). Nevertheless, the reduction in the number of nutlets (by abortion or fusion) frequently occurs within the various genera and has been observed for example in

Hormuzakia Gușul., *Rochelia* Rchb. (usually reduction to two mericarps; Edmondson 1978, Bigazzi *et al.* 1999), *Lobostemon* Lehm., *Moritzia* DC. ex Meisn. and *Thaumatocaryon* Baill. (only a single nutlet usually reaches maturity; Weigend *et al.* 2010, 2016, Buys 2011). For the sake of completeness, the mericarpid multiplication (up to 10) is only known from *Trigonotis* Steven ("*Zoelleria*"), without further knowledge of its ontogeny (Weigend *et al.* 2016).

Considering the vegetative features, the most useful characteristics are the shape of the leaf blade (e.g. *S. cordatum* with cordate vs. *S. grandiflorum* with rounded or subcordate leaf base, respectively), whether stems are winged from decurrent leaf bases (e.g. species delimitation in *S. officinale* complex), and the character of rhizomes. In general, the rhizomes of the *Symphytum* species are pleiocorm, and/or rhizomatous or stoloniferous (Weigend *et al.* 2016). The presence of stoloniferous root with distinct stolon tubers (*S. tuberosum*, *S. bulbosum*) is, in fact, very rare within the whole family (Weigend *et al.* 2016). In particular, based on the shape and the position of the stolon tubers, the two above-mentioned taxa can also be reliably distinguished. Unfortunately, the knowledge of the character of rhizomes is weak or none at all in some taxa, especially of those exclusively distributed in Asia (cf. Wickens 1978), since most of the previous morphological studies were focused on European or Eurasian taxa (or those cultivated in Europe; cf. Pawłowski 1972).

The whole family is characterised by the presence of usually well-developed indumentum (with few exceptions, e.g. *Cerinthe*, *Mertensia* Roth.) with various types of trichomes, setae or papillae (e.g. Selvi & Bigazzi 2001, Buys 2005, Weigend *et al.* 2016), and therefore could easily be described as a "*hairy family*". The structural diversity of trichomes may be quite valuable for the systematics of some genera, as in the case of *Onosma* (e.g. Riedl 1978), *Pulmonaria* (e.g. Sauer 1974, Bolliger 1982) and *Anchusa* (Selvi & Bigazzi 1998). The taxonomic relevance of the composition of the plant indumentum seems to be relevant also within the genus *Symphytum*. Take the example of blue-flowered *S. caucasicum* that is softly hirsute, as compared to similar blue-flowered, but scabrid, roughly hirsute *S. asperum*. Moreover, the study of the leaf anatomy of several Boragineae genera has found hooked hairs to be exclusive for *Symphytum*, with different distribution and density among investigated species, suggesting their taxonomic significance (Selvi & Bigazzi 2001). However, within the whole genus *Symphytum* or a group of similar/related species a detailed micromorphological study and description of the hair types are rather unique (but see Kuznetsov 1910, Kurtto 1982, 1985, Tarıkahya & Erik 2010).

Other phenotypic variation (the height of plants, the size of leaves) is apparently the result of the plastic response of individual plants to the environment (particularly light conditions, soil humidity and nutrition, cf. Chapter 2, 4). To some extent, in some species complexes, the observed variability could also be associated with the polyploidy and particular ploidy level (see below). Last but not least, the content of chemical compounds proved to be also quite significant and useful additional marker, as evidenced by some chemotaxonomic studies on the sections *Officinalia* and *Caerulea* (Gadella *et al.* 1983, Huizing *et al.* 1983, Jaarsma *et al.* 1989).

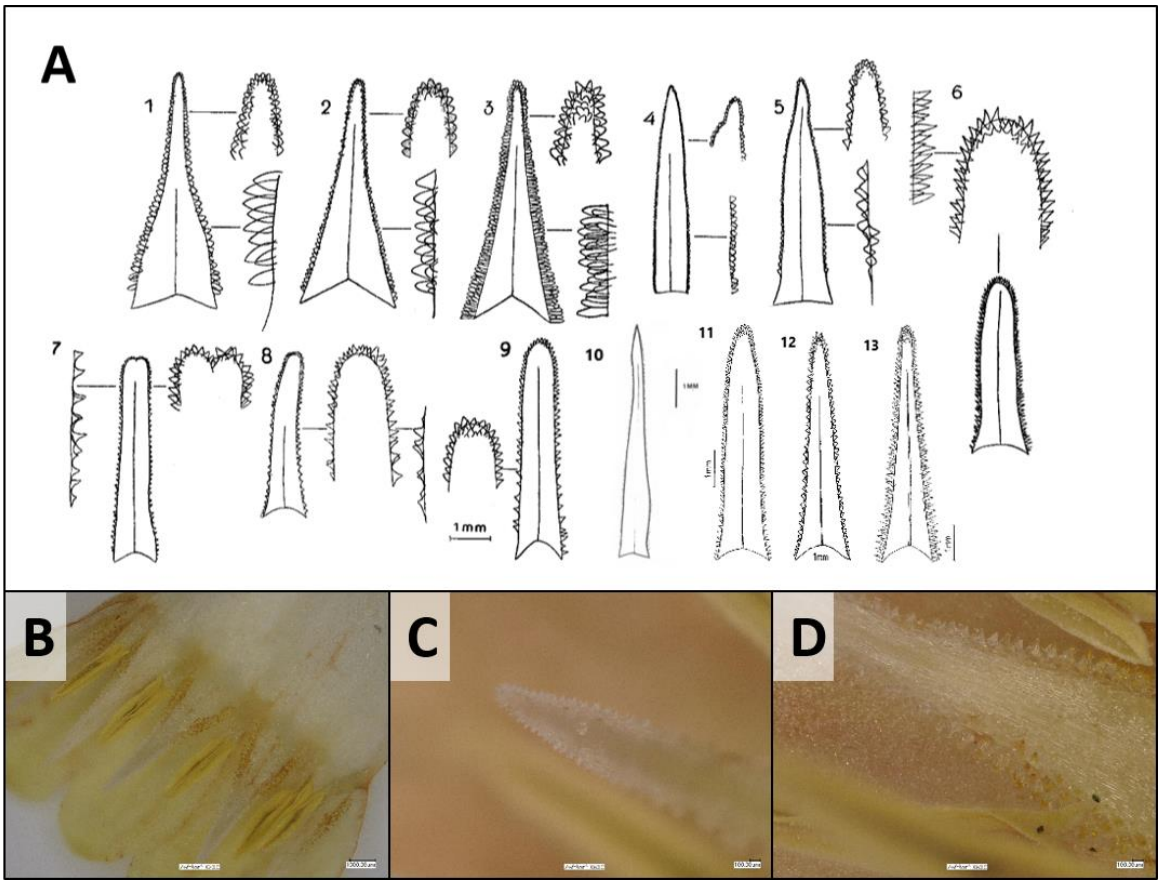

FIGURE 8.3. (**A**) The microanatomy of corolla scales (= faucal scales, fornices) of several European *Symphytum* species (or those reported from Europe). The most peculiar features of the corolla scales useful in species determination are the overall shape, size, the shape of apex and the marginal papillae. 1) *S. officinale*; 2) *S. tuberosum* s. l.; 3) *S. cordatum*; 4) *S. ottomanum*; 5) *S. bulbosum*; 6) *S. asperum*; 7) *S. grandiflorum*; 8) *S. tauricum*; 9) *S. orientale*, all adapted from Pawłowski (1961); 10) *S. pseudobulbosum*, adapted from Kurtto (1985); 11) *S. davisii* s. str.; 12) *S. naxicola* Pawł.; 13) *S. icaricum* Pawł., all adapted from Pawłowski (1971a). (**B**) The faucal scales of the *S. tuberosum* s. l. with a detail of (**C**) the apex and (**D**) papillae along margins.

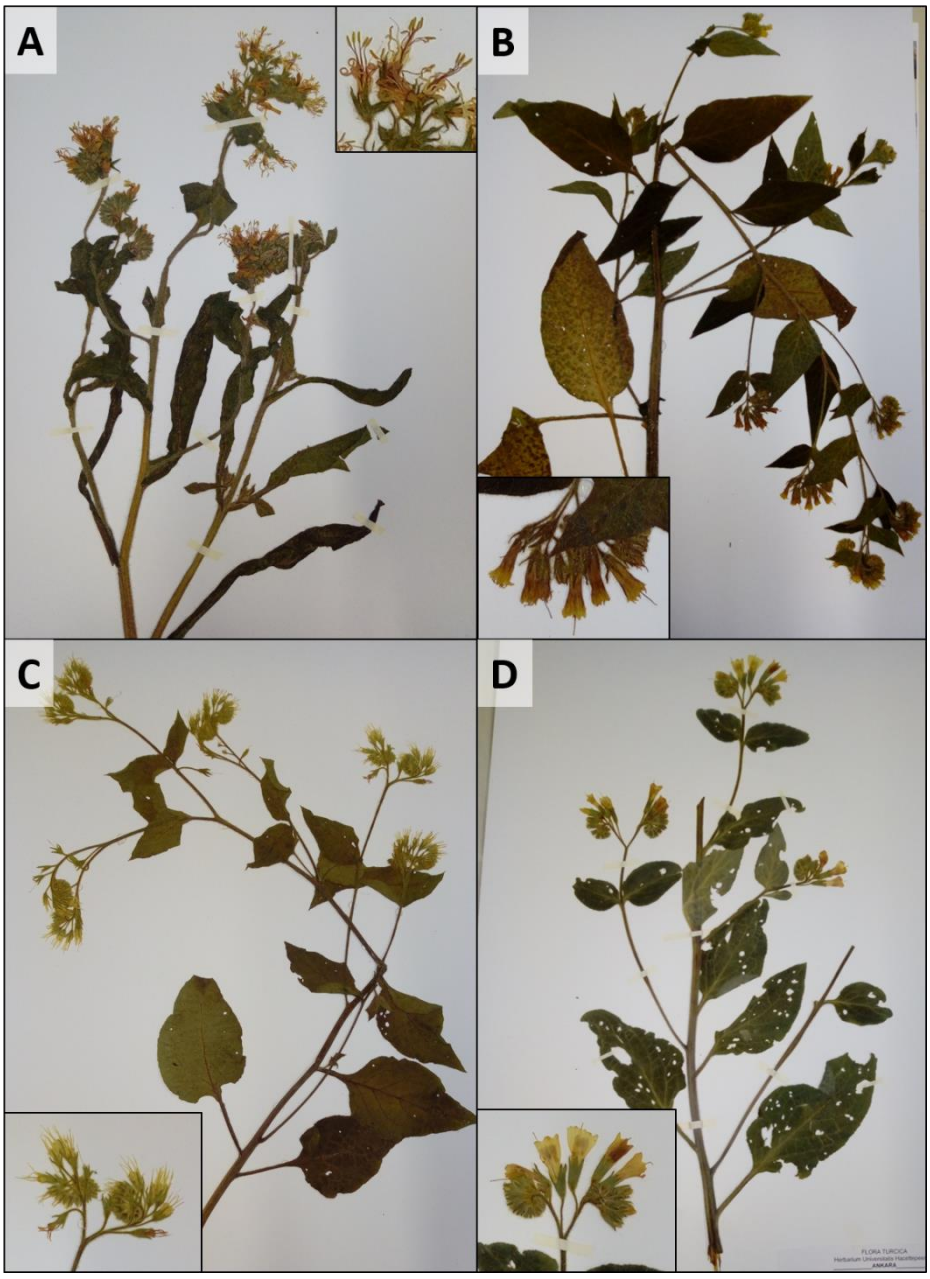

FIGURE 8.4. An example of *Symphytum* representatives with distinct floral morphology, which in some species (**B, C, D**) is an important determinant character. (**A**) *S. circinale* Runemark (sect. *Procopiania*, sections *sensu* Pawłowski 1961, 1971b): well characterised by deeply divided corollas with corolla lobes being spirally contorted above, longer than corolla tube and long-exserted stamens (Pawłowski 1972), an endemic species of East Aegean Islands (Wickens 1969). (**B**) *S. pseudobulbosum* (sect. *Bulbosa*): yellowish corollas with only shortly exserted, lanceolate fornices (Kurtto 1985, Fig. 8.3), an endemic species of the Asiatic side of the Bosporus (Wickens 1969, Kurtto 1985). (**C**) *S. ottomanum* (sect. *Bulbosa*): small, whitish corollas with long exserted, linear-lanceolate fornices gradually narrowed to apex (Kurtto 1985, Fig. 8.3), essentially a Balkan species with disjunct occurrence also in Romania and Turkey (Wickens 1969, Pawłowski 1972, Kurtto 1985). (**D**) *S. orientale* (sect. *Lingulata*): white corollas with inserted, lingulate fornices (Kurtto 1985, Fig. 8.3), most likely endemic to Turkey (Wickens 1969, Kurtto 1985).

How frequent is hybridization among the (Central) European species?

Within the whole family, the hybridization occurs with varying frequency, resulting in both natural and artificial hybrids (e.g. Jepson *et al.* 2012, Kolarčik *et al.* 2014, Meudt 2021). However, the majority of hybrids have been described based on several plants/herbarium sheets, often reported from single population. Therefore, it is hard to consider whether these plants are real hybrids or individuals of extreme morphotype. Nevertheless, several studies have evidenced a significant role of the hybridization, contributing to the evolutionary history of some genera of Boraginaceae, e.g. *Onosma* (Kolarčik *et al.* 2014) and *Pulmonaria* (Meeus *et al.* 2016). Similarly, several hybrids (morphological, unrelated to chromosome numbers) are reported within the genus *Myosotis*. However, experimental crosses of selected wetland taxa resulted in different pattern, where species with different number of chromosomes (and particularly ploidy levels) hybridized rarely or not at all, unlike those of the same chromosome counts (Štěpánková 2000).

From the perspective of plant taxonomy, an understanding of the evolutionary history of a given group represents a crucial step, providing the basis for further research. Unfortunately, studies resolving a phylogeny of the genus *Symphytum* are almost missing and/or incomplete (Sandbrink *et al.* 1990, Hacıoğlu & Erik 2012, Özgişi & Tarıkahya-Hacıoğlu 2021), therefore its evolutionary history and systematics remain fuzzy. Despite this, the role of polyploidization has been proved to be involved in evolution of this genus (e.g. Murín & Májovský 1982), and hybridization has probably also played a significant role (cf. Sandbrink *et al.* 1990).

According to the scanty literature available, several recognised taxa may be of a hybrid origin. Kurtto (1982) suggested that *S. pseudobulbosum* evolved through hybridization event between *S. ottomanum* and *S. orientale*, being intermediate in many morphological characters (see Fig. 8.4; cf. Bucknall 1913, Wickens 1969, Kurtto 1982) and geographically located on the border of the distribution ranges of the putative parents (cf. Kuznetsov 1910, Kurtto 1982). Similarly, Wickens (1969) stated that *S. longisetum* Hub.-Mor. & Wickens (Turkish endemic, Wickens 1978) might be a hybrid between *S. officinale* and *S. brachycalyx* Boiss. (syn. *S. palaestinum* Boiss., Wickens 1969, 1978) and likewise argued for a hybrid origin of *S. longipetiolatum* Wickens (endemic of Northeast Anatolia, Davis 1988) because of the same floral morphology with *S. asperum* agg. (*S. sepulcrale* Boiss. & Bal. or *S. asperum*) and similar vegetative shoots as present in *S. grandiflorum* (Wickens 1969, 1978). However, most of the studies are based on patterns of morphological variation (often on several specimens), so the real evolutionary history of these taxa may be quite different. This is evidenced by the study of Kurtto (1982), who has revisited taxonomically complicated *S. asperum* complex. He has found *S. longipetiolatum* (incl. type specimens) to be morphologically nearly identical to *S. sepulcrale* (Kurtto 1982[2]), which has subsequently been supported using molecular approach (Özgişi & Tarıkahya-Hacıoğlu 2021).

Without any doubt, the best-known and widely naturalised hybrid (see above) is an allopolyploid *S.* ×*uplandicum* (2*n* = 36, 40, e.g. Gadella & Kliphuis 1971, 1973, Basler 1972) formed by the crossing of *S. officinale* s. l. (2*n* = 40, 48; e.g. Gadella & Kliphuis 1967, Wille

[2] As turned out later, based on the nomenclatural priority, the name *S. sylvaticum* should be used for this taxon (Davis 1988, Tarıkahya & Erik 2010).

1998) and *S. asperum* (2*n* = 32; e.g. Basler 1972, Gagnidze *et al.* 2015). However, both of these species are naturally allopatric with a limited zone of overlap in the Northwest Caucasus (Kuznetsov 1910), where they are more or less ecologically isolated (i.e. grow at different altitudes and prefer distinct habitats, Gadella 1972, 1984, Gadella & Kliphuis 1983). Therefore, *S.* ×*uplandicum* most probably arose after the introduction of *S. asperum* to Europe. Many authors are inconsistent in view where it originated (i.e. Western–Great Britain, France or Northern–Sweden, Europe; Nyman 1855, Lindman 1911, Lawrence 1954, Tutin 1956, Wade 1958) and whether it originated in culture or in nature (cf. Bucknall 1913, Faegri 1931, Gadella & Kliphuis 1983). Therefore, the origin of *S.* ×*uplandicum* remains unclear. Moreover, the history of this taxon is tangled thanks to the nomenclature ambiguity with the name/taxon *S. peregrinum* (cf. Faegri 1931), originally described from Talysh Mts., south-eastern Azerbaijan and north-western Iran (Kuznetsov 1910, Bucknall 1913, Gadella & Kliphuis 1983). It has been synonymised with *S.* ×*uplandicum* by some authors (Bucknall 1913, in that case, *S. peregrinum* represents the older name, Ledebour 1820 vs. *S.* ×*uplandicum*, Nyman 1855) or considered to be a local form of *S. asperum* (e.g. Popov 1953), although *S. asperum* is absent from that region (cf. Kurtto 1982). The hypothesis about the conspecifity of European *S.* ×*uplandicum* and plant material from Azerbaijan (i.e. *S. peregrinum*) has been refuted by Gadella and Kliphuis (1983), based on the morphological, chemical, partly cytological and distributional differences. Nevertheless, a detailed revision using population genetics is still required due to the backcrossing of *S.* ×*uplandicum* with both parents (e.g. Wade 1958, Gadella 1972; particularly *S. officinale*, Gadella & Kliphuis 1983).

According to results presented in this thesis, only two hybrids/hybridogenous taxa have been identified in the Czech Republic as part of the revision of herbarium specimens, i.e. *S.* ×*uplandicum* and *S.* ×*hidcotense* (see above). However, the previous inventories also reported occasional occurrence of several other hybrids (except for backcrosses of *S.* ×*uplandicum* with one of its parental species): *S. bohemicum* × *S. officinale* and *S. officinale* × *S. tuberosum* (cf. Slavík 2000, Kubát *et al.* 2002, Danihelka *et al.* 2012). The first one is a hybrid between the members of the *S. officinale* complex, which was formally described as *S.* ×*rakosiense* (Soó) Pénzes (Pénzes 1941). It has been characterised by bicolour white-pink flowers (vertically striped). Such intermediate plants have also been reported from other Central European countries (Slovakia: Smejkal 1978, Májovský & Hegedüšová 1993; Austria: Buch *et al.* 2007; Germany: Bomble 2013), mostly with rare or questionable occurrence. In his inventory, Smejkal (1978) argued for the disputable occurrence of this hybrid in former Czechoslovakia, according to the rare formation of hybrids between diploids and tetraploids within the experimental crossing published in the literature (cf. Basler 1972, Gadella 1972). This predication has been supported by the almost complete absence of triploids within mixed diploid-tetraploid Central European populations detected in this thesis (Chapter 4). Moreover, the corollas of diploid *S. bohemicum* are always yellowish to greenish white, never pure white or different shades of purple (Chapter 4). Based on my own experience, the plants having striped flowers with a combination of two different colours can sometimes be found in some tetraploid populations of *S. officinale* (Fig. 8.1), and therefore, they are just morphotypes of *S. officinale* s. str. The second one is an intersectional hybrid combination of *S. officinale* (sect. *Officinalia*) and *S. tuberosum* (sect. *Tuberosa*), that is

connected with the names *S.* ×*foliosum* Rehmann or *S.* ×*wettsteinii* Sennholz in the literature (Smejkal 1978, Májovský & Hegedüšová 1993, BfN 2017). Nevertheless, for this hybrid combination, the name *S.* ×*foliosum* has priority over *S.* ×*wettsteinii* (Smejkal 1978; according to Pawłowski (1963) with the subspecies *S. tuberosum* subsp. *nodosum*). However, based on the protologue (Rehmann 1868), *S. foliosum* corresponds to plants of *S. tuberosum* s. l. and most probably should be evaluated as a synonym of dodecaploid *S. tuberosum* subsp. *tuberosum* (Kobrlová unpubl.). Likewise, Májovský and Hegedüšová (1993) in the Flora of Slovakia pointed out the similarity with *S. tuberosum* (i.e. creeping rhizomes, yellow flowers), but they have not mentioned any features corresponding with *S. officinale*. In the Czech Republic, only few records of this hybrid have been reported (cf. Smejkal 1978, Slavík 2000). Moreover, *S. officinale* and *S. tuberosum* seem to co-occur at the same localities rather rarely (cf. Supplementary File 1). Therefore, the presence of this hybrid in the Czech Republic is questionable. Similarly, it is not included in the Floras of Germany and Austria (cf. Schmeil & Fitschen 1988, Fischer *et al.* 2008, BfN 2017).

To draw some conclusion from the revision presented in this thesis, the frequency of the hybridization within Central European species appears to be rather low. Explanations for this may be as follows: (1) distinct flowering period (e.g. *S. officinale*: May to September vs. *S. tuberosum* agg.: April to May); (2) different ecology (e.g. *S. officinale* agg.: typically, wet meadows, wetlands vs. *S. tuberosum* agg.: typically, thermophilous or mesophilous broad-leaved forests and semi-dry grasslands; Subchapter 7.1, 7.2); (3) geographical isolation (e.g. *S. cordatum*: Carpathian endemic; *S. tanaicense*: only Eastern Slovakia, Májovský & Hegedüšová 1993); (4) polyploidy and/or different basic chromosome number (e.g. *S. tuberosum* agg.: $n = 8$ vs. *S. cordatum*: $n = 10$ or 12, Chapter 2, Kobrlová unpubl.); (5) prevalence of vegetative reproduction in several groups (e.g. sect. *Tuberosa* and sect. *Bulbosa*, Kobrlová unpubl.). However, hybrid origin (i.e. allopolyploid) of some taxa cannot be ruled out, especially within above-mentioned polyploid complexes (although an autopolyploid origin is rather suspected, cf. Chapter 2, 4), and therefore molecular analyses are more than necessary.

The revision of *Symphytum officinale* and *S. tuberosum* complexes at Central-European landscape

The second part of the thesis focused on a detailed study of *S. officinale* and *S. tuberosum* groups providing a new insight into the direct morphological and ecological consequences of polyploidy, with respect to the taxonomy of both groups. These complexes represent interesting study objects, in which various evolutionary processes (polyploidy, aneuploidy/dysploidy) have taken place, leading to the present-day complex variation. Naturally, these processes are reflected in a number of taxa recognised, with diverse opinions regarding their taxonomic value and circumscriptions. The data presented in this thesis summarise the findings concerning mainly Central-European populations, in connection with a comprehensive inventory of the genus *Symphytum* in our country. In the following sections, the role of polyploidy to distribution, ecology, morphology and taxonomy of both complexes will be shortly discussed.

What is the cytotype diversity of *S. officinale* and *S. tuberosum* complexes? What is the pattern of their distribution in the Central Europe?

Although several authors have reported the variation in chromosome numbers within some Boraginaceae taxa (e.g. *Myosotis* spp., e.g. Štěpánková 1993, 2001, 2006; *Pulmonaria* spp., e.g. Sauer 1975, Bolliger 1982), even more indicating the formation of putative polyploid series (e.g. *Anchusa thessala* Boiss. et Spruner (2*x*, 4*x*, 6*x*), cf. Markova 1983, Markova & Goranova 1995, Bigazi & Selvi 2000; *Cynoglottis chetikiana* s. l. (2*x*, 4*x*, 6*x*), Bigazi & Selvi 2001), almost no attempts have been made to revise this variation in detail and to investigate the origin of this variability and taxonomic implications, if any (but see e.g. Bigazzi & Selvi 2001, 2003, Selvi *et al.* 2009, Kolarčik *et al.* 2014).

According to the literature available, the high variation in chromosome counts is also evident within both *Symphytum* complexes (Chapter 2, 4), suggesting that ancient and/or recent genome multiplication has undoubtedly influenced their evolutionary history (cf. Gadella 1972, Murín & Májovský 1982). At the same time, the whole genus is considered to be karyologically the most variable within Boraginaceae family (cf. Weigend *et al.* 2016). The detailed screening of cytotype distribution on a Central-European scale (more than 2 500 plants from ca. 435 populations have been evaluated in total, Chapter 2, 3, 4) has confirmed the existence of high cytotype diversity and has demonstrated the presence of more than two ploidy levels for each group.

The first studied group represents the *S. officinale* complex with three dominant cytotypes confirmed, diploids (2*x*), tetraploids (4*x*) and hypotetraploids (4*x*-), the last one only reported based on quite a large amount of reliable chromosome counts published so far (Chapter 4). Except of these, two rare triploid (3*x*) plants have also been detected within a single diploid population, most probably originating from the fusion of a reduced and an unreduced diploid gamete (also cf. Mandáková & Münzbergová 2006, Trávníček *et al.* 2010). The presented revision corroborates the omnipresence of tetraploids in the Central-European landscape, whereas diploids and hypotetraploids are more sporadic. The diploid cytotype has a scattered distribution and occurs in several regions in Germany, eastern, central and

northern Bohemia, southern Poland, south-eastern Slovakia and northern Hungary. In contrast, hypotetraploids are documented only from the Eastern Slovak Lowland (cf. Chapter 4).

Despite the high cytotype diversity reported in *S. tuberosum* complex (cf. Chapter 2), almost nothing is known about the geographic pattern of the cytotypes (but see Murín & Májovský 1982). Based on the pilot screening of Central-European populations, the presence of two dominant cytotypes, tetraploids (4*x*) and dodecaploids (12*x*), has been revealed (Chapter 2, 3). Traditionally, it was assumed that there are only dodecaploid populations in most of this region, with tetraploids reported only from Slovakia (Murín & Májovský 1982, Májovský & Hegedüšová 1993). However, results clearly indicate, that tetraploid cytotype also occurs in the Czech Republic and Hungary, where it has never been recorded before. In spite of this, dodecaploids still represent the most frequent cytotype, occurring through the whole area, whereas tetraploids are geographically restricted only to the hilly landscapes at the northern border of the Pannonian Lowlands and the lower parts of the Western Carpathians (Chapter 2). Similar to *S. officinale* complex, the ploidy-level screening has confirmed the occurrence of rare minority cytotypes, but with higher frequency. Specifically, in four tetraploid populations, DNA-hexaploids (~6*x*, note that validation using chromosome counting has not been provided) have been discovered, most probably with an analogous scheme of origin like in diploid–triploid populations of *S. officinale* s. l. (also cf. Marhold *et al.* 2010, Šafářová & Duchoslav 2010, Dančák *et al.* 2012, Koutecký *et al.* 2012). By contrast, there is no simple explanation of the origin for DNA-decaploids (~10*x*) and DNA-tetradecaploids (~14*x*), which have occasionally been found within 17 dodecaploid populations.

Last but not least, given the relatively high variation in the relative DNA content of both *Symphytum* groups (esp. tetraploid *S. officinale*, both dominant cytotypes of *S. tuberosum* s. l.), the presence of aneuploidy/dysploidy (directly confirmed within *S. tuberosum* complex, Chapter 2) or B chromosomes (documented within *S. officinale* complex, e.g. Gadella & Kliphuis 1967, 1970) seems to be the most relevant explanation (cf. Leitch & Leitch 2008). The future studies involving in situ hybridization may provide a considerable insight into the dynamics of these polyploid complexes.

How frequent are mixed-ploidy populations? Which cytotypes participate in their composition?

With the application of flow cytometry, a novel insight into the cytotype diversity and distribution patterns of cytotypes has been enabled. This methodological approach has resulted in a proliferation of cytogeographical studies (e.g. Mráz *et al.* 2008, Trávníček *et al.* 2010, Mandák *et al.* 2015, Rejlová *et al.* 2019, Afonso *et al.* 2021), even allowing to provide an extensive screening of thousands of individuals (e.g. Trávníček *et al.* 2012, Čertner *et al.* 2017, 2022, Duchoslav *et al.* 2020), which would be logistically very difficult (and probably not possible) using classical karyological methods. As a result, it has significantly increased the number of known plant species with two and more different ploidy levels, either within a specific geographic area (e.g. Marhold *et al.* 2010, Godsoe *et al.* 2013, Wefferling *et al.* 2017, Muñoz-Pajares *et al.* 2018) or within the same population (e.g. Duchoslav *et al.* 2010,

Trávníček *et al.* 2011, Čertner *et al.* 2022). Consequently, it contributes to the deeper insight into the mechanisms of cytotype coexistence and polyploid speciation.

The mixed populations of dominant cytotypes within both studied *Symphytum* groups represent a rather rare phenomenon, since the ploidy-level screening revealed only few ploidy-mixed populations (*S. officinale* agg.: three diploid–tetraploid populations, i.e. 1,9%, *S. tuberosum* agg.: four tetraploid–dodecaploid populations, i.e. 1,5%). Moreover, no intermediate cytotype (i.e. *S. officinale* agg.: triploid (3*x*), *S. tuberosum* agg.: octoploid (8*x*)) has been detected in these mixed populations (Chapter 2, 4), so the possibility of gene flow appears to be excluded or limited at present. In the case of *S. officinale* complex (for *S. tuberosum*, there is a lack of information), this is consistent with previously published cytological investigations and experimental crossings (Gadella & Kliphuis 1967, 1969, 1972, Gadella 1972). Therefore, the observed pattern of geographic distribution and spatial arrangement of cytotypes of *S. officinale* and *S. tuberosum* complexes indicate the mosaic regional parapatry (*sensu* Kolář *et al.* 2017).

How strong is the niche differentiation between different cytotypes? Is there a different pattern of niche shift with increasing ploidy level?

The presence of ecological niche differentiation between cytotypes is the subject of study in many polyploid groups (e.g. López-Jurado *et al.* 2019, Castro *et al.* 2020, Decanter *et al.* 2020, Duchoslav *et al.* 2020, Kiedrzyński *et al.* 2021), struggling to understand what shaped their current global and local distribution patterns. Without any doubt, this is a challenging task associated with a large-scale comparative analysis (Glennon *et al.* 2014, Marchant *et al.* 2016), such as niche modelling (Warren *et al.* 2008, 2010) and multivariate analyses of niche variables (Broennimann *et al.* 2012). Thanks to these advanced ecoinformatic approaches, an examination of large-scale cytotypes distributions in association with environmental data is enabled, creating predictions about their niche breadth, niche shifts and/or niche conservation (cf. Treier *et al.* 2009, Theodoridis *et al.* 2013, Glennon *et al.* 2014, Kirchheimer *et al.* 2016).

The intraspecific ecological niche divergence in *S. officinale* complex (diploid–polyploid system) as well as in *S. tuberosum* complex (polyploid system without a diploid progenitor) has been found. In both of these groups, higher ploidy levels (i.e. *S. officinale* agg.: tetraploids, *S. tuberosum* agg.: dodecaploids) have a much wider niche, inhabiting a broader spectrum of habitats and being more tolerant to extreme abiotic factors (esp. temperature, moisture and nutrients), reflecting their wide distribution across a studied area, including the occurrence in colder regions or anthropically disturbed sites (Chapter 2, 4). Such finding is consistent with the general pattern of higher frequencies of polyploids at higher latitudes and/or altitudes (Husband *et al.* 2013, Rice *et al.* 2019), and/or in specific, ecologically more different/challenging, geographical regions (e.g. Arctic Flora, Brochmann *et al.* 2004; the Mediterranean Basin, Marques *et al.* 2017). Likewise, several studies showed a stronger synanthropic affinity of higher ploidy levels in comparison with diploid congeners (e.g. Němečková *et al.* 2019, Rejlová *et al.* 2019, Urfus *et al.* 2021). To some extent, this may also be linked to human activities, especially in tetraploids of *S. officinale* complex which have been commonly cultivated, or influenced by the multiple origins of cytotypes (cf. Karunarathne *et al.* 2018, López-Jurado *et al.* 2019, Duchoslav *et al.* 2020).

An increase of ecological niche breadth of polyploids was demonstrated in both polyploid systems studied. However, environmental analyses on a broader scale within the *S. tuberosum* complex are required, to fully support presented results. In this case, it could be even more interesting. Given the fact that more cytotypes have been identified throughout Europe (Kobrlová unpubl.), it provides the opportunity to compare the variation among polyploids (cf. Decanter *et al.* 2020, Duchoslav *et al.* 2020).

What are the morphological differences between cytotypes of both complexes?

Polyploidy is a common source of taxonomical problems, mediating morphological and physiological shifts in newly formed cytotypes (e.g. Otto & Whitton 2000, Beaulieu *et al.* 2008, Maherali *et al.* 2009, Chansler *et al.* 2016, Ulum *et al.* 2021), and therefore forming complicated, often highly variable polyploid complexes (e.g. Španiel *et al.* 2008, 2011, Padilla-García *et al.* 2017, Rejlová *et al.* 2021). Before the modern genomic era, the morphological similarity within a taxonomic unit and its distinctness from other ones was regarded as the most important criterion in traditional taxonomy (cf. Hörandl 2022). Thus, the occurrence of high morphological variability in polyploid complexes led taxonomists either to the description of number of taxa across the whole range of the group, often without a relation to the ploidy level, or vice versa to the recognition of the whole complex as one highly variable and widespread species. Nowadays, it is recommended to thoroughly examine the effects of polyploidy using multiple approaches (incl. also aspects of ecology, genetics, breeding systems) and, at best, to carry out the detailed revision across the whole distribution range, in relation to the classification of polyploids (especially autopolyploids, Soltis *et al.* 2007). In any case, the clear morphological delimitation of plants is still crucial as it is a key prerequisite for the practical use (field botany, vegetation science, medical and agriculture research).

The results of the morphological analyses presented in this thesis confirm the delimitation of dominant cytotypes of both *Symphytum* groups, with each cytotype forming separated (Chapter 4) or almost separated (Chapter 2, 3) cluster. Specifically, to determine the cytotypes within the studied groups, a set of distinct qualitative and quantitative morphological characters should be used. In the case of *S. officinale* complex (note that only diploids and tetraploids have been morphologically investigated), the cytotypes could be clearly distinguished, especially based on the colour of flowers and plants, the width of the wing below lower and upper leaf, the length/width ratio of the middle leaf lamina, the calyx, corolla, peduncle and style lengths and corolla width (Chapter 4, 5, 6, Subchapter 7.2). In contrast, the higher morphological variability (possibly plasticity) has been found in the second group represented by the *S. tuberosum* complex. Although the dominant cytotypes significantly differ in most of the characters studied, character ranges substantially overlap (see taxonomical consequences below). According to the multivariate morphometric analysis, the most relevant were the following characters: the size/ratio of leaves (esp. the length/width ratio of the middle leaf lamina), the corolla size and the length of the narrow part of the corolla. Of the characters not directly included in measurements, the field observations also

confirm the differentiation of cytotypes in the character of rhizomes (i.e. thickness, fleshiness, the width of tubers; Chapter 2, 5, 6, Subchapter 7.1).

How significant are all of these findings for the taxonomy of both complexes?

To sum up, the presented data support treating main cytotypes of both *Symphytum* complexes (i.e. diploids and tetraploids of *S. officinale*, tetraploids and dodecaploids of *S. tuberosum*) as separate taxa. They have distinct distribution patterns, which mirror their different habitat preferences. This is also evidenced by the almost absent ploidy-mixed populations. Moreover, they seem to be reproductively isolated, although the mating barrier may probably not be complete (this biological aspect was not studied in detail). Last but not least, they are morphologically different and can be distinguished from each other, which is a key information for field botanists and taxonomists. Despite all of this, the different taxonomic concept has been suggested for each group.

Symphytum officinale complex (Fig. 8.5)

In most European floras, only one polymorphic species *S. officinale* is recognised, eventually diploids and tetraploids are considered as mere cytotypes of *S. officinale*, while hypotetraploids are almost exclusively identified as *S. tanaicense* Steven (Chapter 4). Therefore, a special attempt was made to clarify the status of diploids and to support the taxonomic concept of each cytotype as separate taxonomical unit.

In light of the results obtained in this thesis, the clear morphological differentiation and ecological segregation of studied cytotypes are obvious. The apparent presence of hybridisation barriers (Gadella & Kliphuis 1967, 1972) further strengthens the observed pattern. Therefore, both cytotypes should be treated as separate species, i.e. diploids as *S. bohemicum* and tetraploids as *S. officinale* s. str.). According to available data (Májovský & Hegedüšová 1993, Gadella *et al.* 1983, Peruzzi *et al.* 2001), the hypotetraploids seem to represent a well-defined species too (i.e. *S. tanaicense* Steven), having not or only shortly decurrent leaves, dark purple campanulate to urceolate corollas, long hairs along margins and at midribs of the calyx lobes, and inhabiting permanently wet and waterlogged lands. In addition, dysploidy is considered a strong reproductive barrier (Mandáková & Lysák 2018). However, a subsequent study is required, to support the taxonomic value of this species.

Symphytum tuberosum complex (Fig. 8.6)

On the other hand, the *S. tuberosum* complex is more variable and intricate, as evidenced by the high level of polyploidy and considerable morphological variation (Gadella & Kliphuis 1978, Murín & Májovský 1982). This is confirmed by the fact that up to ten species have been described within this complex up to now (cf. Chapter 2). However, only three taxa are, in fact, generally recognised by many recent authors, i.e. a Sicilian endemic *S. gussonei* F. W. Schultz and *S. tuberosum* with western-European subsp. *tuberosum* and central- and eastern-European subsp. *angustifolium*/*nodosum* (cf. Pawłowski 1972, Valdés 2011).

The pilot study of the *S. tuberosum* complex in the Central Europe showed the existence of two taxonomic entities corresponding to tetraploid and dodecaploid ploidy levels, with diverse distribution patterns, habitat preferences and different morphology. However, as the ranges of most morphological characters overlap and habitat requirements are not entirely distinct in some areas, the taxonomic treatment of cytotypes as subspecies has been proposed, i.e. tetraploids as *S. tuberosum* subsp. *angustifolium* and dodecaploids as *S. tuberosum* subsp. *tuberosum* (Chapter 2).

As part of the revision of Central-European populations, the taxonomic identity of the name *S. leonhardtianum* Pugsley (described from the vicinity of Vienna, Austria, Pugsley 1931) has also been investigated. When considering all available evidence, the plants from the locus classicus of *S. leonhardtianum* do not differ substantially from the nominate subspecies. Therefore, the name *S. leonhardtianum* has been proposed as a heterotypic synonym of *S. tuberosum* subsp. *tuberosum* (Chapter 3).

Given the existence of more ploidy levels in Europe (Kobrlová unpubl.), other taxonomical units within *S. tuberosum* are assumed. In this context, the revision through the whole distribution range is necessary (and planned) to fully complete the taxonomic concept of this complex.

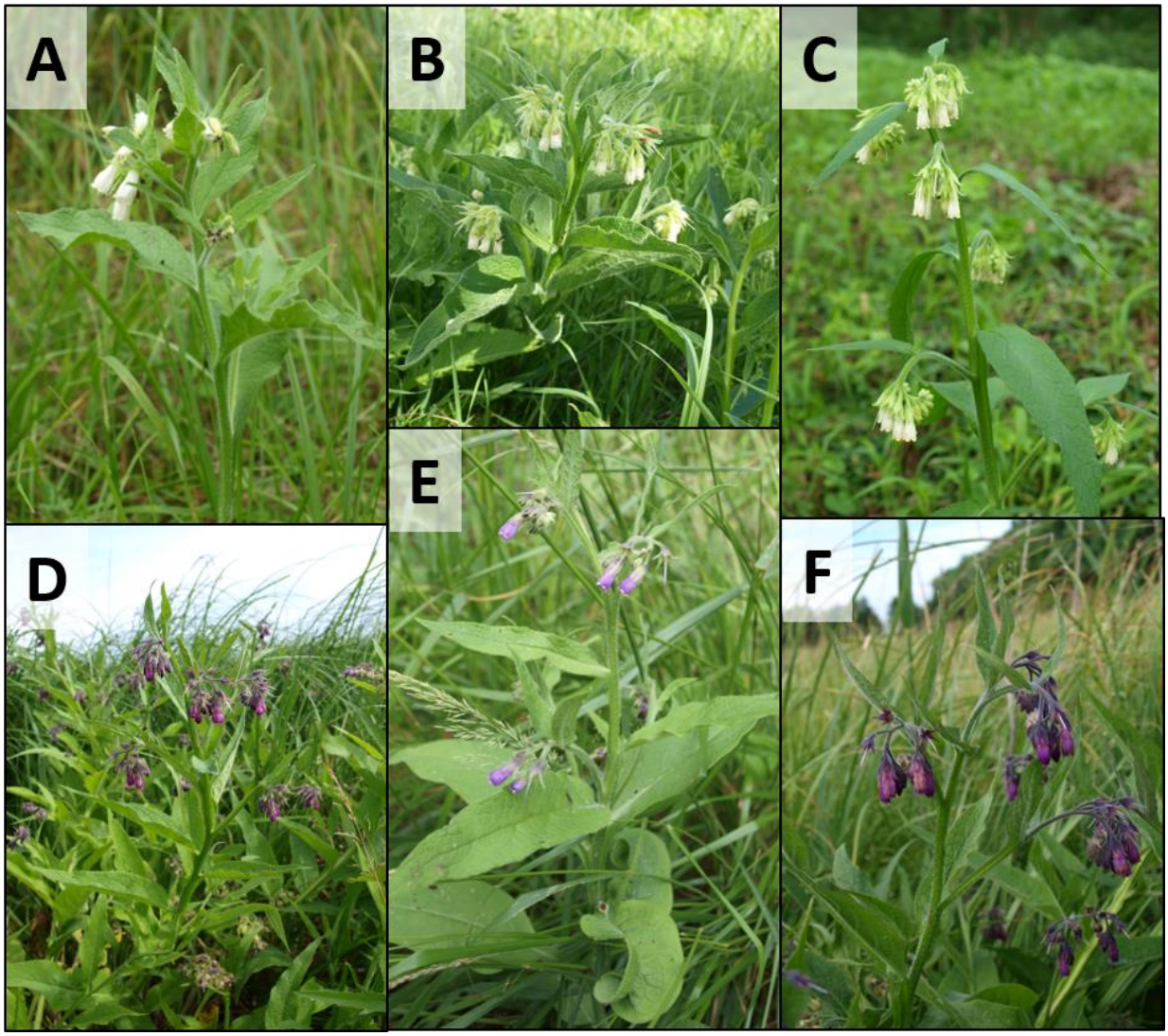

FIGURE 8.5. The morphological variability of the *Symphytum officinale* complex in the Central Europe. (**A–C**) Diploid (2*x*) *S. bohemicum* (**D–F**) Tetraploid (4*x*) *S. officinale*.

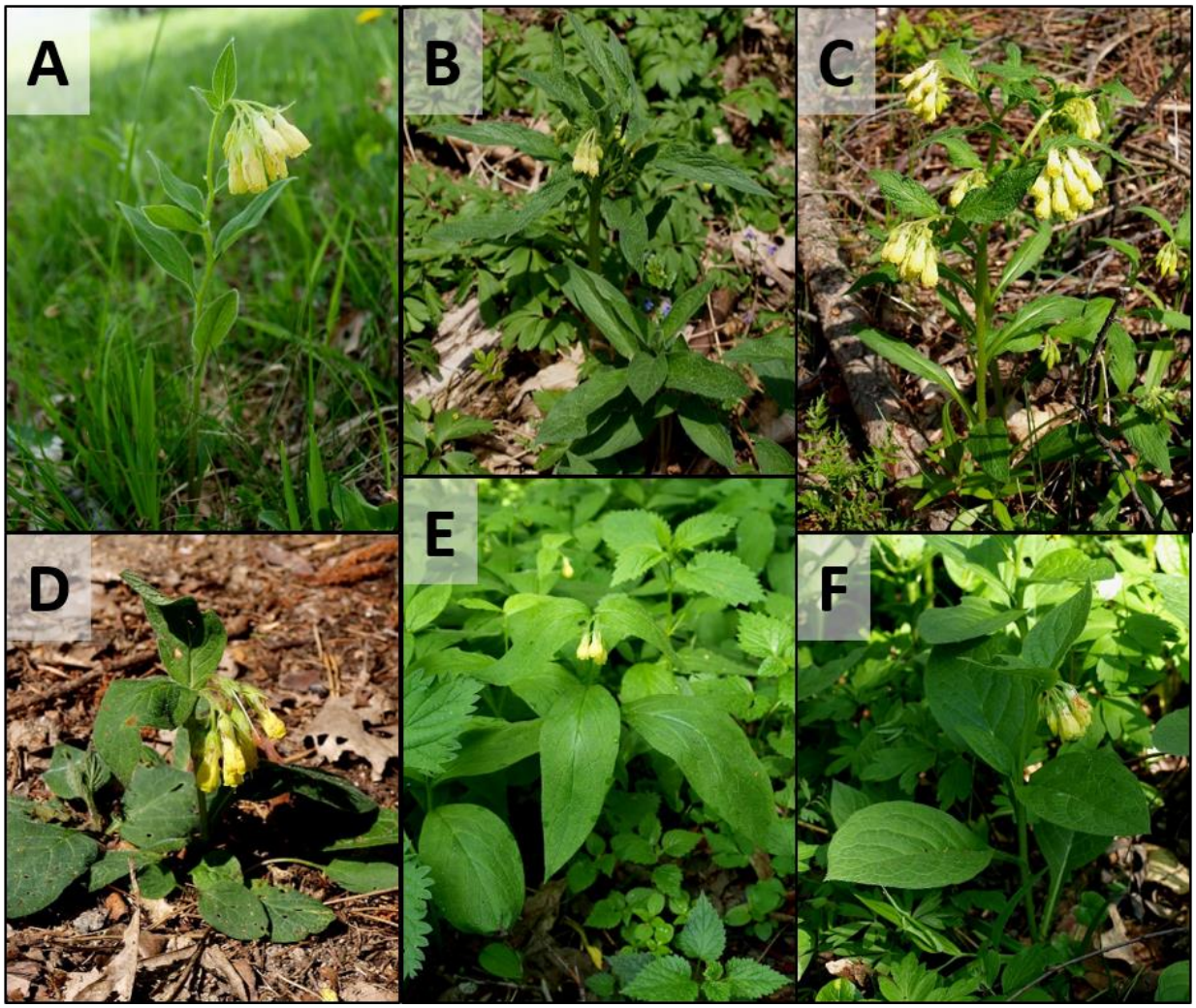

FIGURE 8.6. The morphological variability of the *Symphytum tuberosum* complex in the Central Europe. (**A–C**) Tetraploid (4*x*) *S. tuberosum* subsp. *angustifolium*. (**D–F**) Dodecaploid (12*x*) *S. tuberosum* subsp. *tuberosum*.

Chapter 9

Conclusion and future outlook

Kobrlová Lucie

The presented thesis provided an overview of the genus *Symphytum* in the Central Europe (especially in the Czech Republic) and aimed at getting novel insights into the treatment of this genus in the study area. This revision can serve as a valuable source for further botanical research and particularly for the practical, field botany. Although this thesis brings many novelties about the evolutionary mechanism of Central-European *Symphytum* relatives, there are still many questions to be answered. Further study should primarily focus on molecular analyses to reveal the relationships between cytotypes, to identify their origin (auto- vs allopolyploidy) and to support the proposed taxonomic value of taxa in both groups.

Within the *S. officinale* complex, a detailed investigation of hypotetraploid populations of *S. tanaicense* is required, since its distribution has remained little explored and the morphological differences and ecological requirements have never been studied in detail (cf. Peruzzi et al. 2011). Another step towards the better knowledge of this species group goes to the detailed sampling in Eastern Europe, to expand the currently known distribution ranges of each cytotype, to support results of broader ecological niche breadth of tetraploids and above all to better understand to the evolutionary history of this polyploid complex. However, given the current geopolitical situation, this may be quite a difficult task. In connection with this complex, the process of hybridization is another possible topic for the future research project, which may allow exploring the genomic consequences of alloploid speciation within the genus *Symphytum*.

In the case of *S. tuberosum* complex, the situation is even more interesting, given the fact that more cytotypes have been identified throughout Europe (Kobrlová unpubl.), providing the opportunity to compare variation in several ploidy levels (including high cytotypes). Further research will require sampling across the whole Europe and ploidy level screening combined with molecular analyses. In particular, the discovery of diploid populations would be crucial, since they have not yet been confirmed. Without any doubts, the chromosome counting is another fundamental objective of the future study. This is necessary for the calibration of the flow cytometric data of other ploidy levels. In addition, it could also explain some of the observed variability in genome sizes. The occurrence of aneuploidy in some populations has already been confirmed (see Chapter 2). Therefore, the role of aneuploidy in populations could represent another potential part of this project. The final problem, which should be addressed in future, is the evaluation of the observed variability (morphological, ecological etc.) and the proposal of a taxonomic concept for the whole polyploid complex.

Although many questions have been answered in this thesis, there is still much to learn, discover and explain…

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Supplementary files

SUPPLEMENTARY FILE 1. Distribution of the genus *Symphytum* in the Czech Republic was also compiled and published as a part of the series *Distributions of vascular plants in the Czech Republic*:

KAPLAN, Z., DANIHELKA, J., LEPŠÍ, M., LEPŠÍ, P., EKRT, L., CHRTEK, J., KOCIÁN, J., PRANČL, J., **KOBRLOVÁ, L.**, HRONEŠ, M., ŠULC, V. 2016. Distributions of vascular plants in the Czech Republic. Part 3. Preslia 88: 459–544.

Only maps and comments on the representatives of this genus are given here. The full article is attached in the electronic supplement (Supporting Information_Chapter 7).

Symphytum asperum (Fig. 74)

Symphytum asperum is probably native to the Caucasus and Anatolia or to adjacent regions (Bucknall 1913, Kurtto 1982). It has become widely naturalised all over Europe (Pawłowski 1972, Smejkal 1978, Hultén & Fries 1986), in North America (Gadella 1984) and Japan (Fedorov 2001), mainly as a nectar-bearing and forage plant. The earliest records from the Czech Republic date back to the second half of the 19th century when it was grown mainly as livestock fodder (Smejkal 1978). Since then it has escaped several times and became locally naturalised (Pyšek et al. 2012). The records of *S. asperum* are scattered throughout Bohemia, mainly in the surroundings of the towns of Klatovy, Strakonice and Prachatice. The species was collected in ruderal grasslands in settlements, parks, castle gardens, railway stations, and along roads and railways. In the Czech Republic it was recorded particularly in the 1970s, and there have been only two finds since 2000. The map is based solely on revised herbarium specimens because some literature records may be wrong, based on misidentifications of *S. officinale* or *S. ×uplandicum*.

Symphytum bohemicum (Fig. 75)

Symphytum bohemicum is a diploid member of the *S. officinale* group. It is quite well defined morphologically by its greenish or yellowish white flowers and only shortly decurrent leaves. It was described from central Bohemia by F.W. Schmidt as early as the late 18th century (Kirschner et al. 2007). Further records of the diploid white-flowered "*S. officinale*" that we consider to be *S. bohemicum* are from eastern England, the Netherlands, Germany, southern Poland, south-eastern Slovakia, northern Hungary, southwestern Slovenia and northern Italy (Gadella & Kliphuis 1969, 1972, Májovský & Hegedüšová 1993, Jogan et al. 2001, Stace 2010). Even so, *S. bohemicum* remains neglected in most national floras despite its morphological distinctiveness and strong reproductive isolation from *S. officinale* (Gadella & Kliphuis 1969, 1972). In the Czech Republic *S. bohemicum* is found in calcareous fens in the lowlands along the middle and lower stretches of the Labe, Ohře, Metuje and Cidlina rivers in eastern, central and northern Bohemia. The species is classified as endangered (Grulich 2012).

Symphytum officinale (Fig. 76)

This species is widespread and somewhat difficult taxonomically. Several cytotypes have been reported (e.g. Gadella & Kliphuis 1969, 1972, Gadella 1972), mainly diploids (2n = 24),

hypotetraploids (2n = 40) and tetraploids (2n = 48). In our opinion, only the tetraploid populations should be considered as *S. officinale*, whereas diploid populations correspond to *S. bohemicum* and hypotetraploids to *S. tanaicense* (syn. *S. officinale* subsp. *uliginosum*; Gadella & Kliphuis 1969, Májovský & Hegedüšová 1993). *Symphytum officinale* s. str. is distributed almost throughout the whole of Europe (Hultén & Fries 1986). It was introduced to China (Zhu et al. 1995) and North America (Gadella 1984), mostly as green forage for livestock and due to its use in traditional medicine. It grows on wet meadows, along rivers and in humid ruderal habitats such as damp ditches or road edges. In the Czech Republic it is common from the lowlands to the mountains, being less frequent or under-recorded only in western Bohemia.

Symphytum tauricum (Fig. 77)

Symphytum tauricum is native around the Black Sea, i.e. in southern Ukraine, southern European Russia, Anatolia, Romania and Bulgaria (Smejkal 1978, Wickens 1978, Fedorov 2001). In the Czech Republic the occurrence of *S. tauricum* was first reported by Smejkal (1978), based on a herbarium specimen collected in the town of Černošice near Prague in 1912. On the sheet one specimen of *S. tauricum* is mounted together with three specimens of *S. tuberosum* subsp. *tuberosum*. Unfortunately, it is not clear whether these plants originated from cultivation or not. They also may have been mixed accidentally in herbaria. In the 1980s, *S. tauricum* was repeatedly recorded from the vicinity of villages Miřetice and Ptákova Lhota in south-western Bohemia where it was found on garden waste and road verge. It is not clear if it was only an ephemeral occurrence or if it still grows on any of these localities.

Symphytum tuberosum subsp. *angustifolium* (Fig. 78)

The taxonomy of *Symphytum tuberosum* in central Europe was revised recently by Kobrlová *et al.* (Chapter 2). They showed that two subspecies of *S. tuberosum* occur in the Czech Republic. The taxonomy within this group is quite intricate, especially due to the occurrence of high polyploids and considerable morphological variability. In its broad circumscription, *S. tuberosum* is distributed all over Europe except for Scandinavia, the Netherlands, Belgium, north-western Germany, southernmost Spain and Portugal (Bucknall 1913, Murín & Májovský 1982). *Symphytum tuberosum* subsp. *angustifolium* is tetraploid (2n = 32), and has an obvious affinity to the Pannonian basin (Chapter 2). Until recently, this taxon was known only from northern Hungary and the southern part of Slovakia (Májovský & Hegedüšová 1993, Marhold & Hindák 1998, both as *S. angustifolium*), but it has been omitted from flora accounts of the former country. It was recently discovered in south-eastern Moravia in the Czech Republic and confirmed for northern Hungary (Chapter 2). In comparison with the type subspecies, *S. tuberosum* subsp. *angustifolium* is more thermophilous, occurring mainly in the lowlands. It occurs rarely at higher altitudes, reaching them through warmer valleys. It grows in drier habitats than the type subspecies, such as thermophilous broad-leaved forests and semi-dry grasslands. In the Czech Republic it is confined to the westernmost Carpathians in south-eastern Moravia, mainly to the Bílé Karpaty Mts, Litenčické vrchy hills, Chřiby hills and Ždánický les hills. Its northern distribution limit runs through central Moravia, its western

limit west of the city of Brno. The map is based only on revised herbarium specimens and our own field records as no earlier records exist.

Symphytum tuberosum subsp. *tuberosum* (Fig. 79)

Symphytum tuberosum subsp. *tuberosum* is dodecaploid (2n = 96) and it is the most widespread member of the *S. tuberosum* group (Chapter 2). In central Europe it is found in Austria, Germany (mostly in the south and along the lower stretches of the Elbe river), southern Poland, northern Slovakia, and in southern and western Hungary (Chapter 2). In the Czech Republic *S. tuberosum* subsp. *tuberosum* prefers shady, moist and also nutrient-rich habitats. It inhabits the banks of rivers or streams, forests in deep river valleys, the fringes of wet meadows, alder carrs, and alluvial, ravine and mesophilous forests. It was also recorded from ruderal or disturbed places (e.g. roadsides and abandoned wet meadows) and parks. It occurs mainly in southern and central Bohemia and in northern Moravia and Silesia. The distribution map is based solely on revised herbarium specimens and our own field records.

Symphytum ×*uplandicum* (Fig. 80)

Symphytum ×*uplandicum* is a hybrid with the assumed parentage of *S. officinale* and *S. asperum*. Its origin is unclear but it was first reported from Sweden and Great Britain in the first half the 19th century and afterwards introduced as a forage plant to large parts of western and central Europe (Bucknall 1913, Gadella 1972, Gadella et al. 1983) and also become naturalised in North America (Gadella 1984). It is a quite robust plant with greater biomass production than the parental S. asperum, therefore favoured in cultivation (Smejkal 1978). Since its escape from cultivation, natural backcross hybrids with both parents have been found repeatedly (Gadella&Kliphuis 1969, Gadella 1972). In the Czech Republic *S.* ×*uplandicum* was cultivated mainly in the late 19th and early 20th centuries (Smejkal 1978). It was found mainly in ruderal places, parks, urban grasslands, roadsides or railways. The records of *S.* ×*uplandicum* are scattered throughout Bohemia, while only 5 records exist from Moravia. Some finds are related to the occurrence of *S. asperum* (see Fig. 74) as one of the parental species.

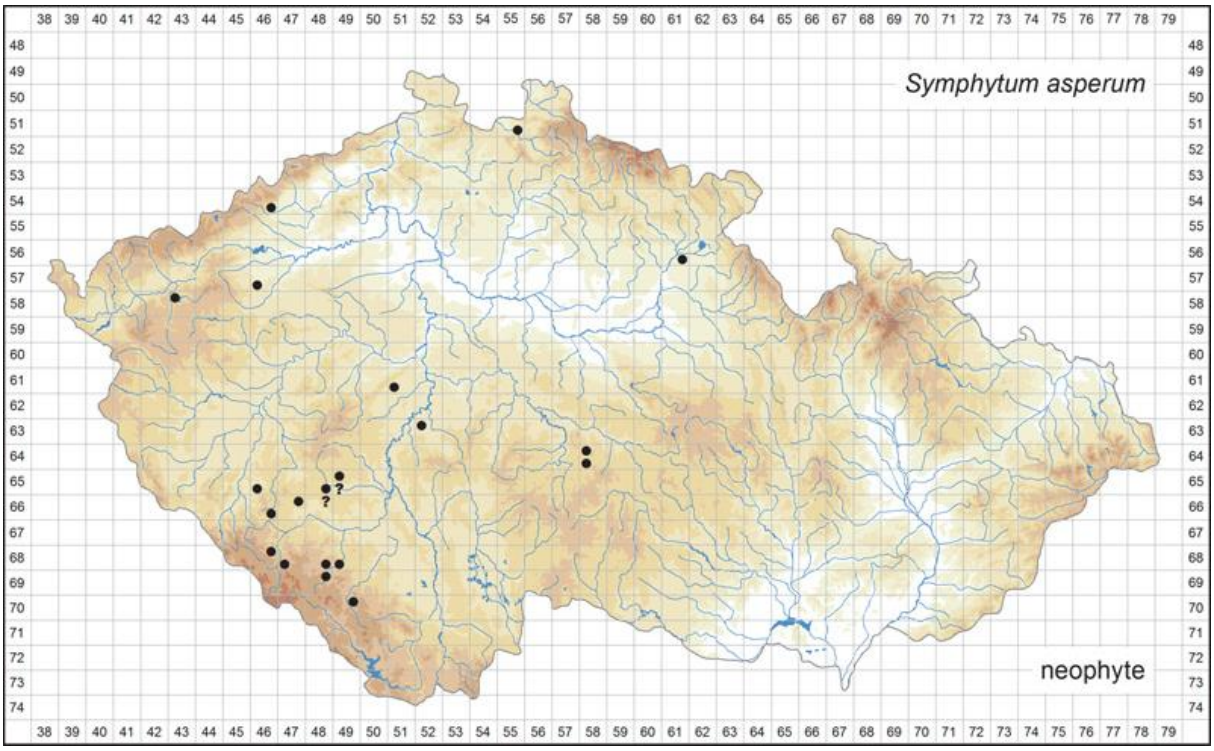

FIGURE 74. Distribution of *Symphytum asperum* in the Czech Republic (20 occupied quadrants). Prepared by Lucie Kobrlová and Michal Hroneš.

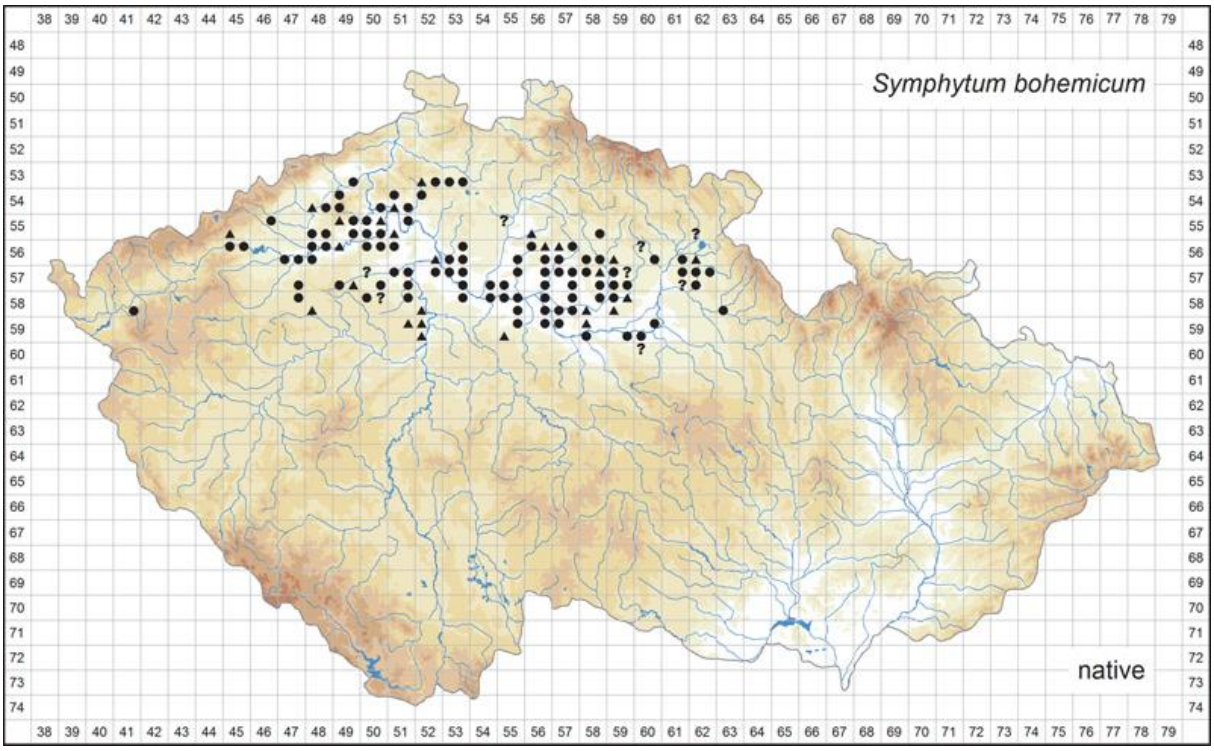

FIGURE 75. Distribution of *Symphytum bohemicum* in the Czech Republic: ● occurrence documented by herbarium specimens (94 quadrants), ▲ occurrence based on other records (26 quadrants). Prepared by Lucie Kobrlová and Michal Hroneš

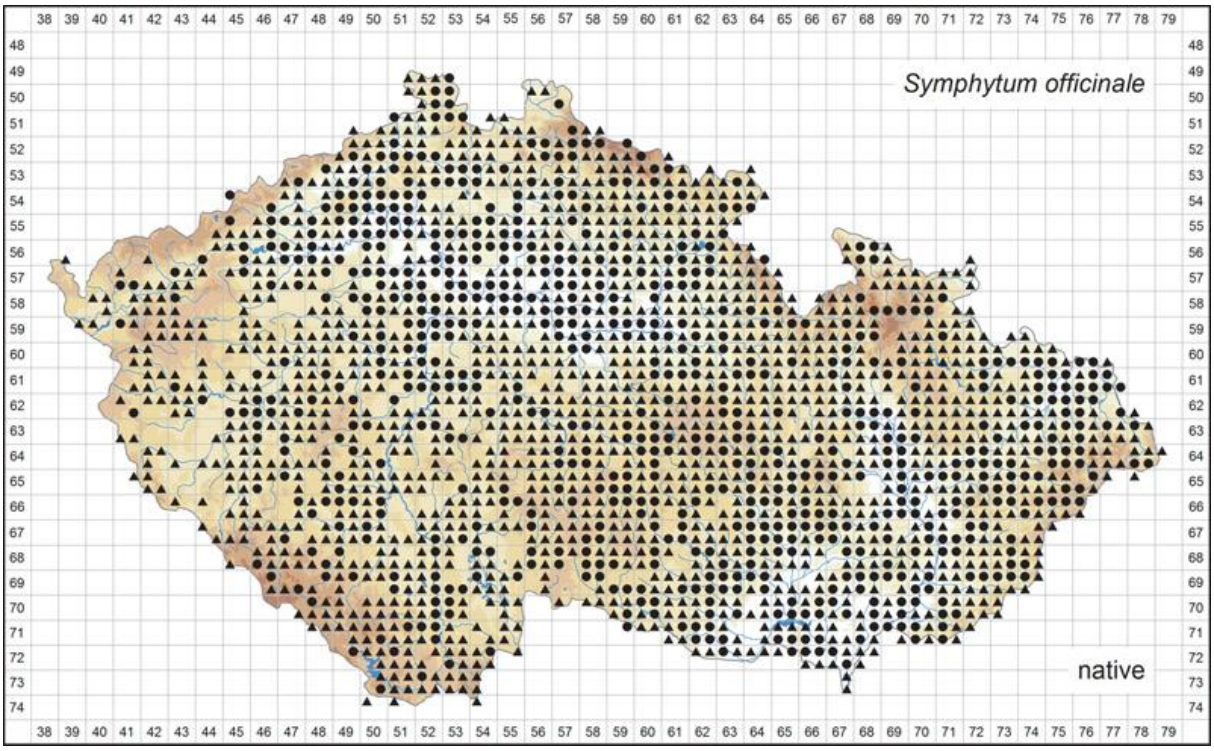

FIGURE 76. Distribution of *Symphytum officinale* in the Czech Republic: ● occurrence documented by herbarium specimens (714 quadrants), ▲ occurrence based on other records (1317 quadrants). Prepared by Lucie Kobrlová and Michal Hroneš.

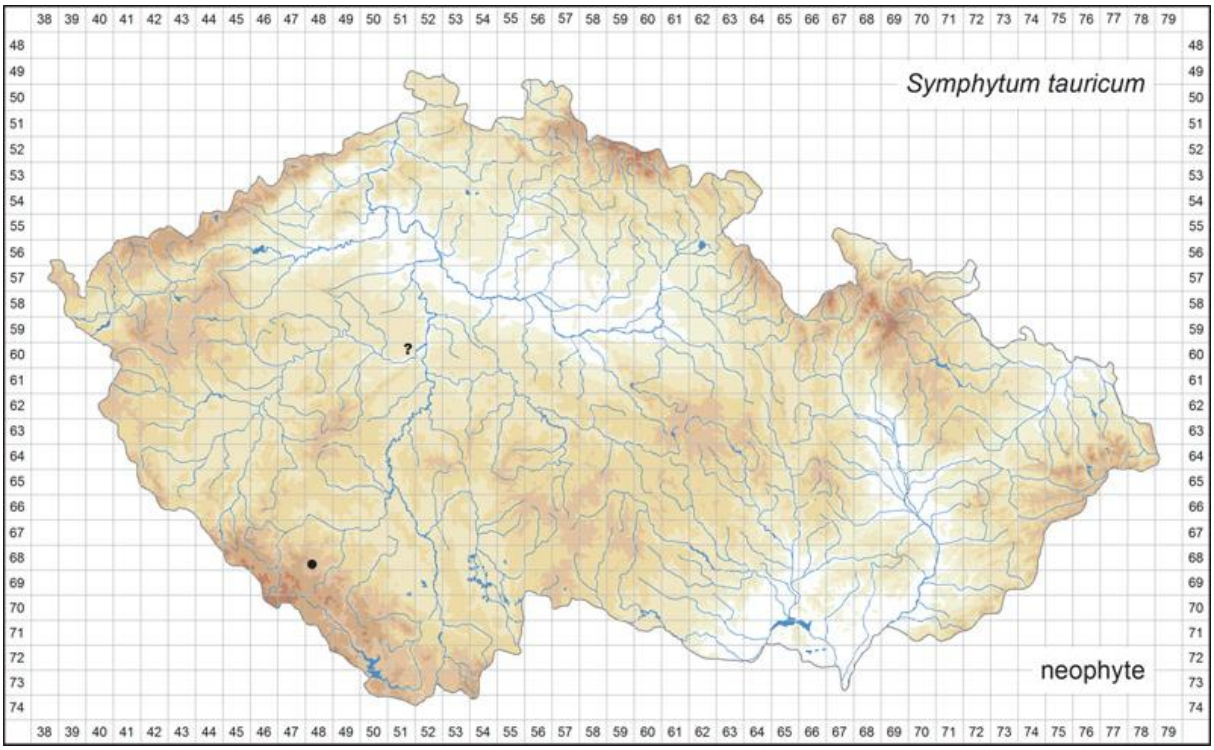

FIGURE 77. Distribution of *Symphytum tauricum* in the Czech Republic (1 occupied quadrant). Prepared by Lucie Kobrlová and Michal Hroneš.

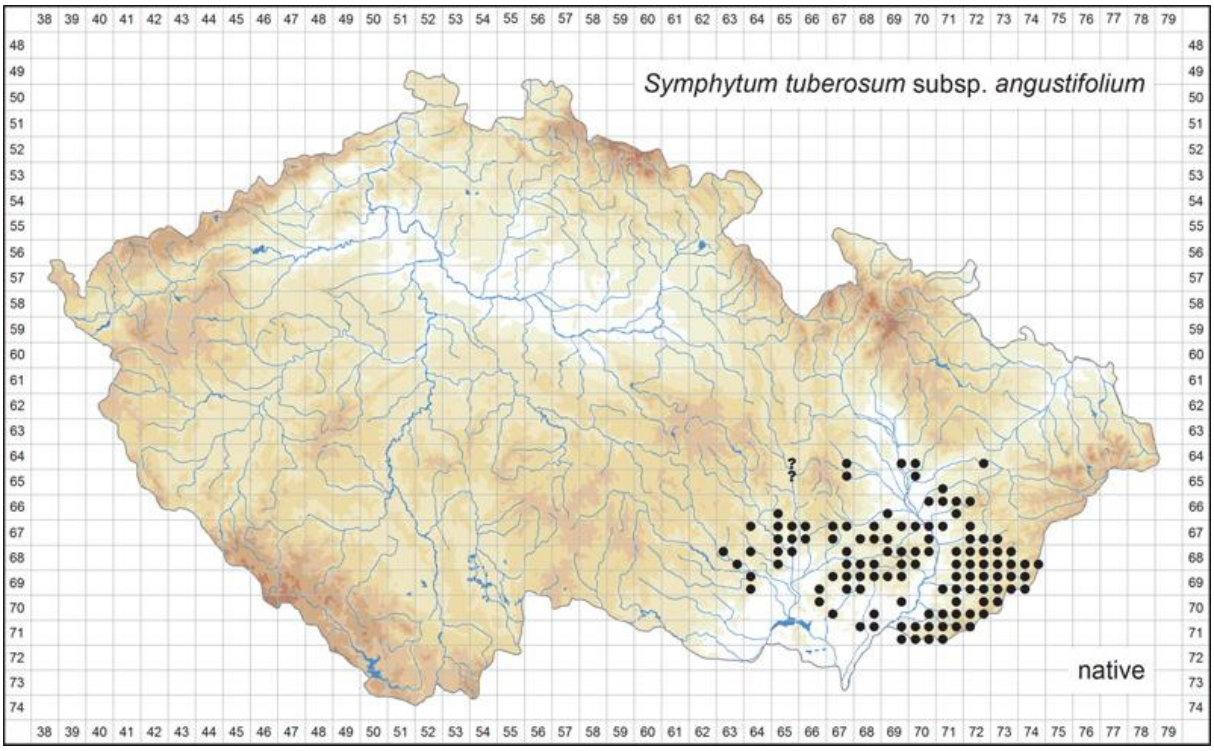

FIGURE 78. Distribution of *Symphytum tuberosum* subsp. *angustifolium* in the Czech Republic (113 occupied quadrants). Prepared by Lucie Kobrlová and Michal Hroneš.

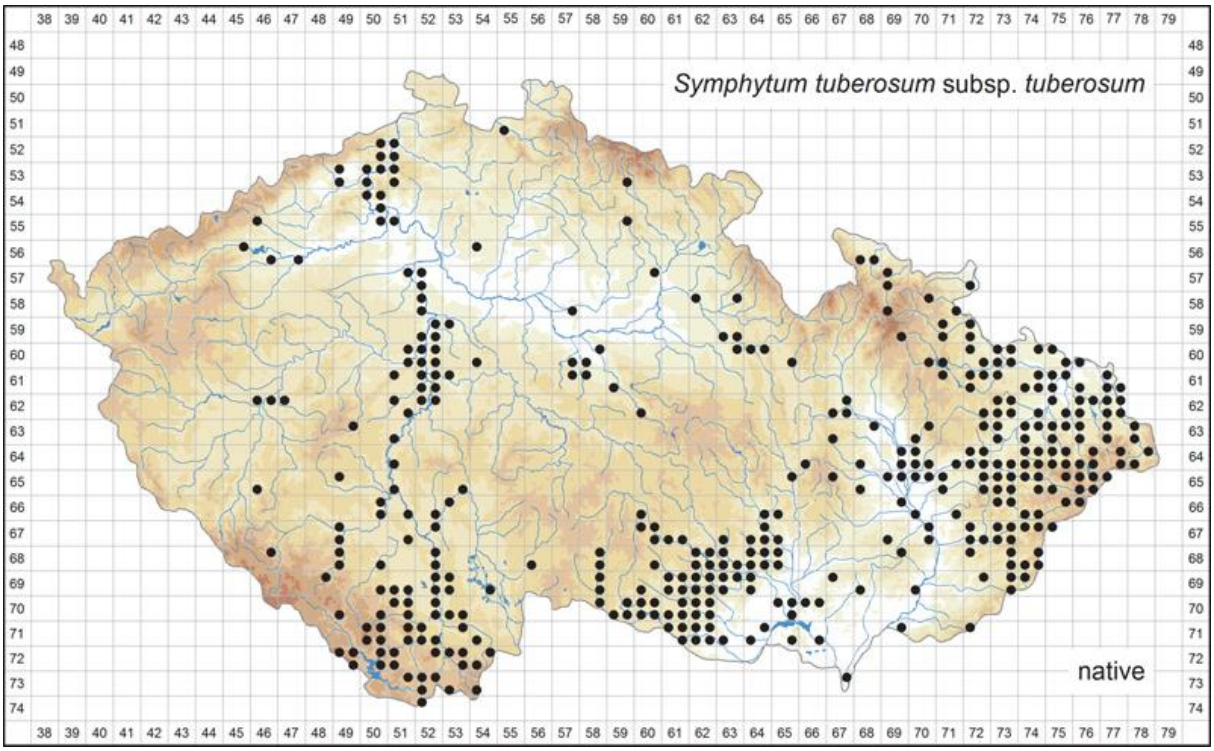

FIGURE 79. Distribution of *Symphytum tuberosum* subsp. *tuberosum* in the Czech Republic (389 occupied quadrants). Prepared by Lucie Kobrlová and Michal Hroneš.

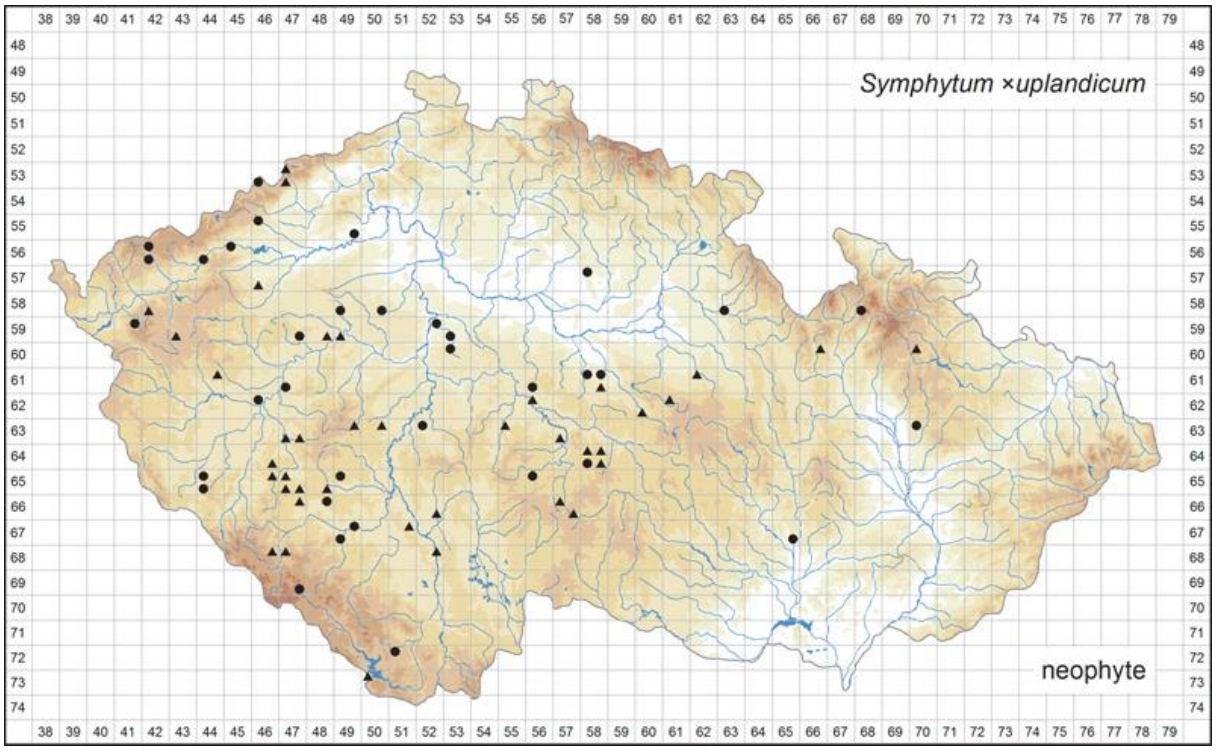

FIGURE 80. Distribution of *Symphytum ×uplandicum* in the Czech Republic: ● occurrence documented by herbarium specimens (35 quadrants), ▲ occurrence based on other records (39 quadrants). Prepared by Lucie Kobrlová and Michal Hroneš.