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Plant systematics as a jigsaw puzzle: from population diversity to species stories

Habilitační práce

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1. Introduction

Plants have fascinated man since time immemorial. They were originally adored for their beauty and used as food and pharmaceutical sources. A need to sort plants according their use and conspicuous characters arose very early, and systematic classification efforts have accompanied botany from its origin. Although systematics is viewed as an old-fashioned science by some, the reality is different. Current plant systematics is not the static classification and labelling of distinguished taxa, as it often used to be hundred years ago. An understanding of evolutionary processes and the reconstruction of plant phylogenies and distributions in the context of environmental changes are the primary goals of current systematics (Stuessy 2009, Simpson 2010, Judd et al. 2015). Traditional taxonomy (the description, identification, nomenclature and classification of organisms) represents an indispensable part of systematics, integrating all available knowledge about the kinds and diversity of plants. Taxonomy facilitates communication about plants (Stuessy 2009) and allows us to study the relationships among them. Systematics understood in this sense means an exciting search for evolutionary stories regarding plants on various temporal and geographical scales (Willis & McElwain 2013). New methods, their quick development, and exponentially growing knowledge coming from very different branches of science provide a huge amount of diverse information, which presents plants as actors in a wonderful story of land colonization followed by their huge diversification. This story is truly fascinating, and modern methods allow us to trace its individual strands into the far past.

1.1 Phylogeny of vascular plants

An integrative approach merging phylogenetic and phylogenomic analyses of recent plants with functional genomics, developmental biology, palaeobotany, palaeogeography, geology, geobiology, palaeoecology and other research branches shows an increasingly sharper image of land plant ancestors (Wodniok et al. 2011, Ruhfel et al. 2014, Wickett et al. 2014, Delwiche & Cooper 2015, Gerrienne et al. 2016, Gitzendanner et al. 2018) and their preadaptation for plant terrestrialization (Delaux et al. 2015, Harrison 2017, Nishiyama et al. 2018) and its timing, progression and impact on environmental conditions (Berner 2006, Rubinstein et al. 2010, Clarke et al. 2011, Meyer-Berthaud et al. 2016, Morris et al. 2018, Barba-Montoya et al. 2018). A key factor in further plant evolution, repeated leaf origin (Tomescu 2009, Harrison & Morris 2018), is elucidated by evolutionary developmental biology in the context of fossil records (Sanders et al. 2007) and ongoing environmental changes after plant colonization (Beerling & Berner 2005, Beerling 2005). Evolutionary developmental biology in conjunction with palaeontology helps to explain many other important evolutionary changes in the plant body (Rothwell et al. 2014). New advances at the molecular level promise to soon shed a new light on the evolution of

the reproductive organs of plants (e.g., seed and flower origin) (Wang, Liu, et al. 2018).

The basic dichotomy within vascular plants, separating a group that includes modern lycophytes from a group composed of all other living vascular plant lineages and named euphyllophytes (with euphylls, i.e., true leaves with marginal or apical meristems and an associated leaf gap in the vascular stele) has been known for many years (Raubeson & Jansen 1992). Similarly, a deep dichotomy in this large group, separating monilophytes (ferns and horsetails) from lignophytes (plants that produce robust wood via a cambium), which include seed plants and spore-bearing fossil progymnosperms, has also been long known and is generally accepted (Kenrick & Crane 1997, Nickrent et al. 2000, Pryer et al. 2001, 2004, Ruhfel et al. 2014). However, the deep phylogeny of both these monophyletic groups of euphyllophytes is still poorly understood, and some relationships (e.g., the position of horsetails, relationships among seed plant groups) remain unresolved despite great progress in recent years (Chaw et al. 1997, 2000, Mathews 2009, Lehtonen 2011, Wu et al. 2013, Xi et al. 2013, Wang & Ran 2014, Wickett et al. 2014, Rothfels et al. 2015, Knie et al. 2015, Lu et al. 2015, PPG I 2016, Li et al. 2017, Wan et al. 2018, Ran et al. 2018).

On the other hand, evolutionary relationships and diversification within recent lineages of the main groups of vascular plants are relatively well known. The phylogeny and determinants of the diversity of modern ferns and fern allies in a framework of changing biotic and abiotic factors have been given special attention (Schuettpelz et al. 2007, Schuettpelz & Pryer 2007, 2009, Rothfels et al. 2012, Field et al. 2016, PPG I 2016, Testo & Sundue 2016). The monophyly of extant gymnosperms (cycads, ginkgos, conifers, gnetophytes), which until recently were considered paraphyletic, has received strong support in recent studies (Chaw et al. 2000, Donoghue & Doyle 2000, Bowe et al. 2000, Ruhfel et al. 2014, Wickett et al. 2014, Gitzendanner et al. 2018). The relatively recent radiation of one of the oldest extant groups, cycads, is well documented (Nagalingum et al. 2011, Condamine et al. 2015, Dorsey et al. 2018). Similarly, a deep dichotomy among the conifers between *Pinaceae* and cupressophytes, comprising the rest of the extant conifers, is generally accepted, but the position of gnetophytes within conifers remains uncertain (Bowe et al. 2000, Hajibabaei et al. 2006, Braukmann et al. 2009, Leslie et al. 2012, Farjon 2018).

Naturally, the dominant extant plant group, angiosperms, has been studied most intensively. Although recent angiosperm phylogeny is known and generally accepted (APG I 1998, APG II 2003, APG III 2009, Soltis et al. 2011, APG IV 2016), the ancestral lineage, origin and explosive early Cretaceous diversification of angiosperms have been a mystery in evolutionary biology since the time of Darwin (Crepet & Niklas 2009, Buggs 2017, Katz 2018). However, the robust phylogenetic framework, the exponentially increasing possible phylogenomic methods, the growing number of

fossil records and palaeoecological information have provided a solid foundation for a number of macroevolutionary analyses seeking to answer questions about the origin and evolution of key angiosperm characteristics, the timing of angiosperm evolution, the co-diversification of angiosperms with other organismal groups during the Cretaceous period, and the environmental determinants of angiosperm diversification (Crepet et al. 2004, 2018, Crane et al. 2004, Heimhofer et al. 2005, Friis et al. 2006, Coiffard et al. 2006, 2007, Bateman et al. 2006, Magallón & Castillo 2009, Berendse & Scheffer 2009, Doyle & Endress 2010, 2014, Bell et al. 2010, Endress 2011, Zeng et al. 2014, 2017, Magallón et al. 2015, Sauquet et al. 2017, 2018, Bukhari et al. 2017, Barba-Montoya et al. 2018). Another intensively studied subject is the diversity of the plant genome (Levin 2002, Wendel et al. 2012, Leitch et al. 2013) and the role of whole-genome duplication (polyploidy) followed by gene loss and diploidization in the phylogeny of vascular plants and especially the importance of polyploidy for the great evolutionary success of angiosperms (Soltis et al. 2009, 2015, 2016, Jiao et al. 2011, Dodsworth et al. 2016, Soltis & Soltis 2016).

Of course, there is a countless number of studies that have investigated the phylogeny, phylogeography, and characteristic evolution of particular angiosperm clades (Wang, Lu, et al. 2009, Wang, Moore, et al. 2009, Hernández-Hernández et al. 2011, Greenberg & Donoghue 2011, Bouchenak-Khelladi et al. 2014, Refulio-Rodriguez & Olmstead 2014, Bouchenak-Khelladi Yanis et al. 2014, Stull et al. 2015, 2018, Hertweck et al. 2015, Lane et al. 2018). However, despite great progress in our understanding of angiosperm diversification, many open questions regarding angiosperm macroevolution remain (Sauquet & Magallón 2018).

1.2 Integrative taxonomy and phylogeography

The stories of vascular plant phylogeny at a gross scale are truly fascinating. However, the origin of extant species and their evolutionary history also offer exciting stories. Tools for disentangling the evolutionary history of recent groups are growing in number and improving exponentially. They include a wide range of methods focused on revealing different aspects of recent and past speciation mechanisms and current variation (Stuessy et al. 2001, Briggs & Walters 2016).

Comparative studies of plant structure and the detection of morphological variation and its description remain the basis of plant diversity and systematic studies (Endress 2002, Stuessy et al. 2003). Morphometrics (a quantitative analysis of form) has undergone a real revolution in recent decades (Rohlf & Marcus 1993, Adams et al. 2013), and many new approaches for classical (Claude 2008, Koutecký 2014) and geometrical morphometrics (Mitteroecker & Gunz 2009, Zelditch et al. 2012, Rohlf 2015) have become recently available. Similarly, new, advanced techniques are now available for comparative anatomy (Endress et al. 2000). Cultivation and hybridization experiments are other basic tools for revealing phenotypic plasticity, ecological requirements, ecological differentiation of populations and species, competitive

relations, adaptation, hybridization ability, progeny fitness, etc. (Koutecký, Štěpánek, et al. 2012, Kolář et al. 2014, Chrtek et al. 2017, Habibi et al. 2018, Hülber et al. 2018).

Methods for cytological and cytogenetic data acquisition have been improved in recent years (Stace 2000). The application of modern cytogenetic methods (FISH/GISH) represents an important current tool for the study of hybridization and polyploidization (Chester et al. 2010). Flow cytometry has become a powerful tool for plant genome diversity research, representing an initial step towards understanding the genetic differentiation of studied groups (Doležel et al. 2007, Suda et al. 2007, Suda & Pyšek 2010, Chumová et al. 2015, Mandák, Krak, et al. 2016). Flow cytometry is often used to characterize the mode of reproduction, another key determinant of variation patterns (Matzk et al. 2000, Matzk 2007, Krahulcova & Rotreklova 2010, Šmarda & Bureš 2010, Ekrt & Koutecký 2016), and to study hybridization (Loureiro et al. 2010, Ekrt et al. 2010, Prančl et al. 2014, 2018, Kaplan et al. 2016).

The number of molecular markers that can be used for the study of genetic variation and speciation processes in plant populations (selection, gene flow, genetic drift, founder and bottleneck effects, hybridization, polyploidization, introgression, etc.) is quickly expanding, as is our understanding of the evolutionary importance of these processes (Soltis & Soltis 2009, The Marie Curie SPECIATION Network 2012, Briggs & Walters 2016, Niklas 2016). Although the analysis of isozymes (multiple forms of enzymes catalysing the same reaction) is still useful for answering some questions (Soltis & Soltis 1989, Koutecký et al. 2011, Lowe & Abbott 2015, Williams et al. 2016), most of the markers used currently in plant systematics analyse DNA composition. Original methods based on the fragment-length polymorphism of total DNA, the polymorphism of fragments from a specific genome region or the direct sequencing of specific genome regions (Soltis et al. 1992, 1998, Weising et al. 2005) have been increasingly replaced by the use of whole-genome markers and whole-genome sequencing using next-generation sequencing (Harrison & Kidner 2011, Hörandl & Appelhans 2015). The exponentially growing amount of molecular data has facilitated the quick improvement of the methods of bioinformatic analysis for phylogenetics and phylogeography reconstruction (Hickerson et al. 2010).

As the current plant diversity and its distributional pattern reflect long-term interactions between evolutionary and historical processes, we need to identify the historical factors affecting the present state. Thus, palaeobotanical and palaeoecological data represent another important piece of the mosaic of the study of the evolutionary history of selected groups. The current changing view of the past vegetation and its changes due to quaternary climate fluctuations forms a solid framework for looking for glacial refugia of target species and reconstructing their postglacial migration (Willis & van Andel 2004, Svenning et al. 2008, Birks & Willis

2008). Good knowledge of ecological requirements then allows environmental niche modelling of present and past distributions (Elith & Leathwick 2009).

Only the integration of the available data allows a complete reconstruction of the evolutionary history of studied groups. This approach, integrating different types of data and methodologies, was recently named integrative taxonomy (Dayrat 2005, Will et al. 2005) An evolution-based taxonomical treatment represents “a happy ending” for the reconstructed story of taxonomically challenging groups. Such results and their comprehensible conversion to floras and identification keys represent important outputs required by the scholarly public (Kaplan et al. in press, Fischer et al. 2008, Martinčič et al. 2010, Jäger 2017). Of course, an integrated approach is also used in phylogeography (Richards et al. 2007), and it is needed to understand the origin of the current genetic diversity within taxonomically simple species, which are interesting from a distribution or conservation point of view (Habel & Assmann 2010). Some recent studies clearly demonstrate that an integrative approach that combines modern tools of plant systematics can reconstruct the complex evolutionary history of challenging groups (Mandák, Vít, et al. 2016, Mandák et al. 2018). The application of a range of phylogeographic methods can track attractive stories of species migration during quaternary climate and vegetation changes (Douda et al. 2014, Havrdová et al. 2015, Mandák, Havrdová, et al. 2016), but the integration of phylogeography and systematic approaches can disentangle taxonomically intricate groups with excellent results (Kolář et al. 2013, 2015, 2016, Mandák, Vít, et al. 2016, Schneeweiss et al. 2017, Vít et al. 2017, Frajman et al. 2018).

Such investigations of the taxonomically intricate groups of Central Europe using an integrative taxonomic approach has been the main goal of the plant systematics group at the University of South Bohemia since its creation. In the following sections, I present our results, which have contributed to the understanding of the evolutionary stories of selected groups of vascular plants.

2. Evolution of the *Knautia arvensis* aggregate in Central Europe

The genus *Knautia* (*Dipsacaceae*) is regarded as one of the taxonomically most challenging European genera. It contains 40–60 species distributed in western Eurasia and north-western Africa, with the highest species diversity in the Alps and on the Balkan Peninsula (Ehrendorfer 1976). Although the taxonomy, and especially the cytotaxonomy, of the genus *Knautia* had been studied previously (Ehrendorfer 1962a, 1962b, 1981, Breton-Sintes 1974, 1975), only recent molecular studies began to disentangle the intricate evolutionary history of the genus (Rešetnik et al. 2014, Frajman et al. 2015, 2016). The widespread occurrence of polyploidy and the high incidence of hybridization seem to play an essential role in the diversification and speciation of this genus. These processes are important, especially in the nearly exclusively perennial and widespread section *Trichera*, with three main ploidy levels (diploids, tetraploids and hexaploids) and a relatively large genome with a base

chromosome number x = 10 (Ehrendorfer 1962a). Although strong intercytotype barriers between diploids and tetraploids have been demonstrated (Ehrendorfer 1962a, Breton-Sintes 1974, 1975), interspecific homoploid hybridization as well as intra- and interspecific hybridization between tetraploids and hexaploids leading to the formation of pentaploids are not rare (Ehrendorfer 1962a, Breton-Sintes 1974, Štěpánek 1982, **Paper 1**).

Knautia arvensis agg. is one of eleven informal groups that were delimited within section *Trichera* on the basis of morphology, distribution patterns and ploidy levels (Ehrendorfer 1962a, 1981). This group is morphologically very heterogeneous, but the leaves are mostly divided. Two species have been traditionally recognized in Central Europe (Ehrendorfer 1976). The exclusively tetraploid (2n=4x=40) *K. kitaibelii* (Schult.) Borbás, with pale yellow flowers, is restricted to the Western Carpathians (Ehrendorfer 1962a, Štěpánek 1982), and *K. arvensis* (L.) J. M. Coult. is an extremely polymorphic species widely distributed throughout Europe. This species includes taxa growing mostly in grasslands and ruderal places, both tetraploids and diploids. Based on morphological and ploidy variation and different ecological requirements, several taxa have been described and distinguished (Ehrendorfer 1962a, 1981, Štěpánek 1983, 1989, Kaplan 1998). The common occurrence of both ploidy levels in Central Europe has been known for a long time as a basic pattern of their distribution (Ehrendorfer 1962b, Štěpánek 1982, 1983, 1997, Kaplan 1998). The main contact zone between diploids and tetraploids is located in the northern margin of the Pannonian basin, with tetraploids occurring northwest of this border and diploids in the southeast direction but also rarely scattered in relict habitats (on serpentine outcrops and in a subalpine glacial cirque) over the tetraploid distribution area. These complex morphological, genetic, ecological and distributional patterns provide an excellent opportunity to study processes generating recent diversity of the group, whose evolutionary history has remained an open question.

A detailed study of cytotype distribution and genome size variation (**Paper 1**) followed by a robust molecular analysis (**Paper 2**) revealed the complex evolutionary history of Central European diploids and tetraploids of *Knautia arvensis* agg., determined by vegetation changes due to quaternary climate fluctuations. The revealed variation in the monoploid genome size and the recognition of primary and secondary contact zones suggest different origins of the grassland diploid populations prevailing in the Pannonian basin and of the relict serpentine and subalpine populations scattered over the tetraploid distribution area (**Paper 1**). The different origins of the two diploid groups were supported by AFLP and cpDNA data, which identified non-relict Pannonian diploids as the most genetically isolated but relatively uniform cluster of Central European populations of *Knautia arvensis* agg. (**Paper 2**). On the other hand, a high level of genetic diversity and among-population differentiation were detected for all relict diploid populations (**Paper 2**). As a member of this relict serpentine and subalpine group, the Western Carpathian *Knautia slovaca* Štěpánek occurs in

limestone relict habitats and was recognized only in 1983 (Štěpánek 1983). The tight relationships among all relict diploid populations from north of the Alps and the distinction of non-relict diploids were also shown in a global study of diploid taxa of the genus *Knautia* (Rešetnik et al. 2014).

This pattern of genetic variation suggests a wide distribution of heliophilous diploid ancestors in the open vegetation of ice-free Central Europe during the late Pleistocene, followed by fragmentation and restriction to serpentine, limestone or subalpine refugia, where mechanisms of allopatric differentiation have taken place (**Paper 2**). The immigration of non-relict diploids and tetraploids into Central Europe as a result of human-induced landscape changes seems to be the next episode in the spatiotemporal history of *Knautia arvensis* agg. in this region. However, the evolutionary history of Central European tetraploids seems to be more complex, including the migration of *ex situ*-originated tetraploids combined with the formation of autotetraploids derived from relict serpentine diploids, followed by the hybridization of the two tetraploid groups (**Paper 2**). The local autopolyploid origin of serpentine tetraploids is supported not only by the genetic proximity of serpentine tetraploids and diploids but also by their higher tolerance to both Mg and Ni stress in serpentine soils compared to their non-serpentine counterparts (**Paper 3**). The identification of independent evolutionary histories giving rise to genetic dissimilarity, differences in monoploid genome size and ecogeographic differentiation sheds new light on the relict Central European population of *Knautia arvensis* agg. and support their independent taxonomic status at the species level (**Paper 4**).

3. Speciation in the genus *Spergularia*

The genus *Spergularia* (*Caryophyllaceae*) offers a different story of speciation in Central Europe. The genus has a nearly cosmopolitan distribution, with diversity centres in South America and the Mediterranean region (Rossbach 1940, Friedrich 1979). *Spergularia* is a difficult genus, with the number of species reported being between 20 and 70 (Friedrich 1979, Dvořák 1990, Bittrich 1993). Most of them are halophytes, often with a wide distribution, and some other species have very restricted distribution ranges. One of them is *Spergularia echinosperma* (Čelak.) Asch. et Graebn., which is considered to be an endemic species with a centre of distribution in southern- and western- Bohemian pond areas (Friedrich 1979, Dvořák 1990). Among the relatively few vascular plant species endemic to Central Europe, *Spergularia echinosperma* represents an entirely unique case, with an ecological link to exposed pond bottoms, whereas other plant species from these habitats usually have larger areas of distribution. This taxon was described many years ago as a subspecies of *Spergularia rubra* (L.) J. Presl et C. Presl (Čelakovský 1881), a common synanthropic species with a large range, and it was raised to species rank shortly thereafter (Ascherson & Graebner 1893). Although it has been generally accepted as a species since this time (Fischer et al. 2008, Jäger 2017), its reliable identification has been a

hard nut to crack because of its morphological similarity to *Spergularia rubra* (Dvořák 1979, 1990). Both species were thought to differ in their ploidy levels, with *Spergularia echinosperma* assumed to be diploid (Dvořák & Dadáková 1984) and *S. rubra* thought to be tetraploid in Central Europe (Dvořák 1990, Wisskirchen & Haeupler 1998). However, only a few chromosome counts were available, and extensive hybridization between the two species was assumed to occur (Dvořák 1989, 1990). Thus, the morphological differentiation of the two species, the ploidy levels of the two species and their possible hybrids, and the actual frequency of hybridization, have remained open questions.

While *Spergularia rubra* seems to be uniform in morphology and ploidy level (tetraploids) in Central Europe, a detailed morphological and cytological study (**Paper 5**) revealed morphological differentiation in *S. echinosperma* corresponding with its two ploidy levels (diploids and tetraploids). The intermediate morphology of the tetraploid *Spergularia echinosperma* specimens between diploid *S. echinosperma* and *S. rubra* plants suggests a hypothesis about the origin of tetraploid *S. echinosperma* by hybridization between the two species. This hybrid was described previously as *S. ×kurkae* (Dvořák 1989), but its mode of origin was not known in regard to the expected different ploidy levels of its parent species (Dvořák 1990). Using cpDNA and specially developed taxon-specific ITS primers, the hybrid origin of *Spergularia ×kurkae* was confirmed (**Paper 6**). Only the diploid *Spergularia echinosperma* was revealed as the maternal species, and heteroploid hybridization via unreduced gametes of *S. echinosperma* seems to be the best explanation of the hybrid origin. Although this event seems to be recent, being triggered in the Middle Ages by human-mediated contact of the two ecologically separated species, *Spergularia ×kurkae* lost the characteristics of recent hybrids. It is completely genetically isolated from the diploid *Spergularia echinosperma*, and ongoing gene flow from *S. ×kurkae* to *S. rubra* is very limited because of their different ecological requirements (**Paper 6**). The view of *S. kurkae* as a stabilized, separate allopolyploid with specific ecology should be incorporated into current floras (Kúr et al. 2017, in press). Additionally, further research revealed noteworthy morphological variation associated with its ecology and distribution at the diploid level (**Paper 7**). The possible adaptive significance of the revealed morphological and genetic differentiation of the diploid populations across their distributional range remains an open question. A set of polymorphic microsatellite loci was developed and is available for future genetic studies (Kúr et al. 2014).

4. Diversity of rhinanthoid *Orobanchaceae*

Hemiparasitic *Orobanchaceae* (root parasites with preserved operating photosynthesis) represent another example of an intricate and taxonomically challenging group of European plants. Traditionally, hemiparasitic *Orobanchaceae* was seen as a part of the family *Scrophulariaceae* (Bentham 1846, 1876, Fisher 2004), but the polyphyly of

Scrophulariaceae s.l. and the redefinition of *Orobanchaceae* as a monophyletic family containing almost exclusively hemiparasitic and holoparasitic (without operating photosynthesis) genera was revealed by molecular systematic studies years ago (Olmstead & Reeves 1995, dePamphilis et al. 1997, Nickrent et al. 1998, Wolfe & dePamphilis 1998, Young et al. 1999, Olmstead et al. 2001, Wolfe et al. 2005, Bennett & Mathews 2006, Tank et al. 2006). The *Orobanchaceae* family in this monophyletic delimitation, with almost cosmopolitan distribution and very diverse life strategies (e.g., different parasitism levels, persistence), ecology and morphology, represents an ideal model group for testing various evolutionary and biogeographical hypotheses (Ree 2005, Tank & Olmstead 2008, 2009, Těšitel et al. 2010, Scheunert et al. 2012, McNeal et al. 2013, Uribe-Convers & Tank 2015). Some non-European hemiparasitic *Orobanchaceae* species, as well as some holoparasitic species, are of great economic importance and cause huge crop losses worldwide and are therefore under intensive study in terms of their biological properties and control possibilities (Parker 2012, Joel et al. 2013).

Thus, the family phylogeny is rather well known, and six monophyletic clades are usually delimited (Bennett & Mathews 2006, McNeal et al. 2013). Central European hemiparasitic genera, originally included in the tribe *Rhinantheae* (Wettstein 1895b, Fisher 2004), belong to two separate clades. The largest hemiparasitic genus, *Pedicularis*, with a Northern Hemisphere distribution, the mostly North American genus *Castilleja*, and other smaller, also mostly North American genera form one clade. The remaining hemiparasitic Central European *Orobanchaceae* genera (*Bartsia*, *Euphrasia*, *Odontites* s.l., *Melampyrum*, *Rhinanthus*, *Tozzia*) belong to the mostly Eurasian clade *Rhinantheae*, whose phylogeny has been studied in detail (*T*ěšitel et al. 2010, Scheunert et al. 2012, Pinto-Carrasco et al. 2017). Due to their many specific biological properties, these hemiparasitic *Orobanchaceae* genera have been a subject of extensive research (Wesselingh & Borg 2005, Štech & Wesselingh 2010, Wesselingh 2016) focused on their parasitism, functional biology (Cameron et al. 2005, 2008, Rümer et al. 2007, Těšitel & Tesařová 2013, Světlíková et al. 2015, 2016, 2018, Těšitel 2016, Matthies 2017, Holá et al. 2017), germination, ontogeny, reproduction and seed dispersal (Kwak 1977, 1979, 1988, Gibson 1993a, 1993b, Kojima & Hori 1994, Svensson & Carlsson 2004, Borg 2005, Průšová et al. 2013, Huang et al. 2016, Liang et al. 2018), population dynamics and association with different types of vegetation (Fibich et al. 2010, 2017, Těšitel et al. 2015), and conservation (Bekker & Kwak 2005, Svensson & Carlsson 2005, Blažek & Lepš 2015, Crichton et al. 2016, Moura et al. 2018). Recently, great attention has been paid to the ability of hemiparasites to suppress host plants and the use of hemiparasitic species for the restoration and conservation of valuable localities degraded by the expansion of competitive clonal plants, such as *Calamagrostis epigejos* or *Carex acuta* (Ameloot et al. 2005, Bullock & Pywell 2005, Hellström et al. 2011, Decleer et al. 2013, Mudrák et

al. 2014, Bao et al. 2015, Demey et al. 2015, Blažek et al. 2016, DiGiovanni et al. 2017, Těšitel et al. 2018).

Therefore, while the intergeneric phylogeny and many issues concerning the biology of hemiparasites have become clearer, there are still multiple open questions about the phylogeny and evolution of some genera and the evolutionary history and taxonomy of many species. The extraordinary morphological variation of mostly annual hemiparasitic genera with a number of species (*Melampyrum*, *Rhinanthus*, *Euphrasia*, *Odontites*) has been the most exciting challenge for plant systematics since the beginnings of systematics research (Wettstein 1896b, Sterneck 1901, Beauverd 1916, Soó 1926, Yeo 1978, Bolliger 1996). These genera possess a very complex pattern of morphological variation formed by some common features. The short lifespan and rapid differentiation of populations due to their high adaptability, phenotypic plasticity and convergent evolution in similar environmental conditions are likely the main factors responsible for their reticulate pattern of morphological variation. Common hybridization (Yeo 1966, Vitek 1982, Ducarme & Wesselingh 2005, 2013, Liebst 2008, Ducarme et al. 2010, Gaudeul et al. 2017), frequent autogamy (Vitek 1988, French et al. 2005) and low dispersal potential (Gibson 1993a, Heinken & Winkler 2009) are supposed to be other factors generating interpopulation variation, blurring species limits and making species identification difficult in many cases. Polyploidization and variability in chromosome number are other speciation mechanisms in some genera (Greilhuber et al. 1984, Koutecký, Tuleu, et al. 2012, Delgado et al. 2015, Wang, Gussarova, et al. 2018).

At the infraspecific level, emphasis has been placed on so-called seasonal variation, a common phenomenon occurring in specific forms in all these genera. Seasonal variation refers to a situation when different populations of one species flower in different periods of the year. The outset of flowering is correlated with many morphological characters, among which the number of internodes, number of intercalary leaves (leaves on the main stem between the uppermost branches and the lowest flowers), and number, position and fertility of branches are the most noticeable, but leaf size and shape and flower size are also not negligible. This phenomenon was studied years ago (Wettstein 1895a, 1900, Sterneck 1901, Ronniger 1911, Soó 1926), and it is known from other plant groups with similar life strategies, e.g. the genus *Gentianella* (Wettstein 1896a, Zopfi 1991, Plenk et al. 2016). Later, comprehensive morphometric studies revealed the pattern, range and nature of this type of variation and showed the ability of annual hemiparasites to quickly and repeatedly form morphologically similar populations under similar types of selection pressure in comparable environmental conditions (Karlsson 1976, Zopfi 1993a, 1995, 1997, 1998a, Štech 2000b, Koutecký, Tuleu, et al. 2012, Pleines et al. 2013). The genetic basis of these seasonal ecotypes was confirmed by cultivation experiments (Zopfi 1993b, 1998b, Štech 2000b). While some seasonal characters are more or less

influenced by environmental conditions, the total number of internodes seems to be the most important, genetically fixed character driving the outset of flowering (Zopfi 1993b, Jonstrup et al. 2016).

Thus, seasonal variation is a noticeable phenomenon in this group, changing the overall plant habit. Such variation is genetically fixed but likely very quickly shaped by environmental conditions (therefore, the differing populations are often called ecotypes), so it forms morphologically convergent forms with polytopic origin. The same ecotypes of different species are often more morphologically similar to each other than to different ecotypes of the same species. This fact makes species identification very difficult in many cases. Moreover, many taxa were described in the past based on seasonal variation as well as on the other types of variation rather schematically, without any knowledge of their biological meaning, genetic variation or evolutionary history (Kerner 1881, Beck 1882, Wołoszczak 1888, Wettstein 1896b, Sterneck 1901, Beauverd 1916, Soó 1926, Smejkal 1963). Consequently, a jumble of unclear taxa based on a traditional, schematic concept is still included in some current regional treatments of this plant group (Martinčič et al. 2010). On the other hand, the complex pattern of variation on different infragenus levels (from the within-population to interspecific level) represents a very suitable model for testing various hypotheses about the population differentiation, speciation mechanisms and evolutionary history of individual taxa. Current systematics and biogeography offer a wide range of tools for answering these questions (Vrancken et al. 2009, 2012, Oja & Talve 2012, Natalis & Wesselingh 2013, Talve et al. 2014, Gaudeul et al. 2017).

4.1 *Melampyrum*

The genus *Melampyrum* represents a basal lineage of the clade *Rhinantheae* (Těšitel et al. 2010, Scheunert et al. 2012) with many unique characteristics (e.g., entire leaves, specific shape of bract teeth, large, smooth and myrmecochorous seeds). Approximately 35 species are distributed in Europe, southeastern Asia and North America, with a centre of diversity on the Balkan Peninsula. The host plants of most of these species are various trees or shrubs, especially from the *Pinaceae*, *Fagaceae*, *Betulaceae* and *Ericaceae* families (Hartl 1974b). Seasonal variation is clearly expressed in many species (Soó 1926, Soó & Webb 1972, Štech 2000b), but with regard to a rather good species delimitation or geographical vicariance of species, it is usually no problem to assign seasonal types to the correct species. However, there are two groups of species (*Melampyrum nemorosum* agg. and *M. sylvaticum* agg.) with unclear species delimitation (Ronniger 1911, 1918, Soó 1926, Jasiewicz 1958) and many described species, some being endemic to Central Europe.

Melampyrum subalpinum A. Kern., a member of *M. nemorosum* agg., represents an excellent example of a polymorphic hemiparasitic annual with a unique fragmented distributional area restricted to Central Europe. The morphological variation in the region at the north-eastern edge of the Alps, from where many taxa have been described (Beck 1882,

Kerner 1882, Wiesbaur 1883), contrasts with the seemingly uniform populations in the fragmented part of the distribution area in eastern and southern Bohemia, western Moravia and western Slovakia. These populations were described as *M. bohemicum* A. Kern. (Kerner 1881), and this concept has been accepted for years (Hadač 1966, Hartl 1974b). Morphometric studies have already recognized the *M. bohemicum* taxon as a part of variation of *M. subalpinum* and suggested an introgression of *M. nemorosum* as one of the sources of morphological variation of *M. subalpinum* in the Vienna Forest (Šípošová & Štech 1997, Štech 2000a, 2006).

Allozyme analyses confirmed the general pattern of lower genetic diversity in the peripheral populations outside the main distribution area on the edge of the Alps and provided the first molecular proof of former hybridization between *M. subalpinum* and *M. nemorosum* in the small area of the Vienna Forest (**Paper 8**). This study also shows high differentiation among the isolated populations and a certain level of selfing within these populations. An artificial pollination experiment supports the possibility of autogamy, which together with small population size may have caused such selfing (**Paper 8**). The long isolation and independent histories of isolated populations and the earlier hybridization of some populations with *M. nemorosum* will be confirmed by an ongoing extensive study of chloroplast and nuclear markers in the whole group (Štech et al. in prep.).

The small range of *M. subalpinum* and its general tendency to form small and isolated populations outside the centre of the distributional area (Reiner 1994, Chlumský & Štech 2011) distinctly contrast with the extensive distributional area of *M. pratense* (Hartl 1974b), which typically forms very large populations over diverse habitats, including those ecologically similar to habitats of *M. subalpinum*. One of the potential reasons for these differences may be the surprisingly different ant-mediated seed removal rates of the two species (**Paper 9**). This study also suggests endozoochory by large mammals as a potential vector for the long-distance migration of some *Melampyrum* species in large boreal regions where the Holocene period was too short for slow dispersal by ants.

The *Melampyrum sylvaticum* group is another example of hemiparasitic annuals with recorded taxa endemic to Central Europe. These taxa, with flowers larger than those of the common boreal and Central European *Melampyrum sylvaticum* L., were described from the Eastern Carpathians. The white flowering *M. saxosum* Baumg. was supposed to be restricted to the Eastern and Southern Carpathians, and an occurrence of yellow flowering *M. herbichii* Woł was also recorded in the Sudetes Mts (Jasiewicz 1958, Štech 2000a). A preliminary study of Central European populations of *Melampyrum sylvaticum* agg. revealed morphological differences between the populations from the Alps and Bohemian Forest and the populations from the Eastern Carpathians (Štech & Drábková 2005). This study showed morphological variation in populations from the Sudetes Mts and Western Carpathians and some similarity of

these populations in terms of RAPD markers with the plants from the Bukovské vrchy Mts, the westernmost part of the Eastern Carpathians.

A morphometric study with more extensive sampling in the Carpathians confirmed the existence of two groups of morphological characters (**Paper 10**). Although the shape of bracts was also used to distinguish between populations from the Carpathians and Sudetes, this character seems to be under strong influence of environmental conditions, such as altitude. On the other hand, most of the flower characters were revealed to be more stable within geographical regions and seem to be less influenced by environmental factors (**Paper 10**).

A subsequent study of the Hercynian and Carpathian populations using molecular tools revealed two evolutionary lineages of *Melampyrum sylvaticum* agg. in this region (**Paper 11**). Although the Hercynian and West Carpathian populations are rather uniform in both their nuclear ITS and chloroplast trnL–trnT DNA regions, populations from the Eastern Carpathians are noticeably more variable in both regions. This pattern likely reflects the different evolutionary histories of *M. sylvaticum* agg. in the two regions. Evidence regarding the presence of a glacial refuge of boreal and temperate species in the Eastern Carpathians is still more convincing (Mráz & Ronikier 2016), and the survival of an *M. sylvaticum* population, which most often parasites the Norway spruce, is probable in this region. On the other hand, the molecular uniformity of the Western Carpathian and Hercynian populations might indicate a recent (Holocene) migration from assumed perialpine refuges. An interesting issue pertains to the contact zone between the two lineages. A discrepancy between the geographic position of the contact zone of the western and eastern cpDNA haplotypes and the nuclear ITS ribotypes likely indicates hybridization and introgression along the main Eastern-Western Carpathian biogeographic boundary. The range and intensity of hybridization and introgression represent open questions for future research. The studied molecular markers also clearly support the low importance of corolla colour and the conspecificity of *Melampyrum herbichii* and *M. saxosum* (**Paper 11**).

4.2 *Euphrasia*

The genus *Euphrasia* is the largest genus of the clade *Rhinantheae*. It comprises more than 350 species distributed throughout temperate regions of the Northern and Southern Hemispheres (Hultén 1976, Gussarova et al. 2008). Apart from Azorean species, all European species are small annuals occurring mainly in grasslands and alpine habitats (Yeo 1968, 1978) and mostly parasitizing herbs and grasses (Hartl 1974a, Metherell & Rumsey 2018). This genus is an extremely complex group and the most taxonomically challenging genus of Europe (Wettstein 1896b, Yeo 1972, 1978). There are many reasons for its complexity. Its relatively young diversification and rapid postglacial/interglacial spread seem to be the key factors for its weak interspecific barriers (Gussarova et al. 2008). Polyploidization and hybridization are likely very important speciation mechanisms in this genus (Yeo 1966, 1968, Karlsson 1976, Vitek 1982, 1986, Ehrendorfer & Vitek

1984, Greilhuber et al. 1984, Liebst 2008, Stone 2012). Species identification is often very difficult because of morphological convergence under similar environmental conditions. Seasonal variation is frequent in this genus, and similar ecotypes of different species are morphologically very similar (Karlsson 1984, Zopfi 1997, 1998b, 1998a, Svensson & Carlsson 2005, Kolseth & Lönn 2005).

Species concepts and the delineation of infraspecific taxa have been a subject of discussion for many years (Karlsson 1976, Vitek 1988). The extreme diversity and wide distribution of the genus make it attractive for solving many evolutionary, biogeographical and identification questions using modern tools of plant systematics. Significant recent advances in our knowledge of genus phylogeny and biogeography are evident (Gussarova et al. 2008, 2012). Great attention has been recently paid to the study of genetic variation in western European species (Kolseth et al. 2005, Kolseth & Lönn 2005, Moura et al. 2018), especially the *Euphrasia* taxa in the United Kingdom (French et al. 2003, 2005, 2008, Wang, Gussarova, et al. 2018), where the study of this genus has a long tradition (Townsend 1897, Pugsley 1930, Yeo 1954, 1956, 1962, 1970, Metherell & Rumsey 2018). However, even advanced molecular methods are not able to successfully solve the complex taxonomy and delimitation of species based on a traditional taxonomical concept (Wang, Gussarova, et al. 2018). Only a thorough, complex and integrative approach may reveal ongoing speciation processes and disentangle the evolutionary history of individual species and populations and may connect a taxonomical view with the biological reality (Liebst 2008, Stone 2012).

Research on the *Euphrasia* genus has an even longer tradition in Central Europe than in the UK (Wettstein 1893, 1896b, 1896c, Metherell & Rumsey 2018). A traditional narrow species concept corresponding to the recent species concept used in Britain has been used in some Central European countries until now (Goliášová & Králik 1997, Smejkal & Dvořáková 2000, Peregrym 2010, Posz 2014). However, a rather wider species concept, which is based on studies particularly on alpine species (Vitek 1982, 1985b, 1985a, 1986, Ehrendorfer & Vitek 1984, Greilhuber et al. 1984), was recently used in Austria and Germany (Vitek 1988, 2008, 2017). An extended integrative approach employing contemporary tools of molecular systematics is obviously needed to shed new light on the complex evolutionary history and relations of Central European populations of *Euphrasia* and to derive a taxonomic treatment of the group from its genetic relationships and evolution.

A recent study focused on a complex group of Central European tetraploid species (**Paper 12**). The aims of this study were to find genetically supported groups among a number of tetraploid species traditionally distinguished in the Czech Republic (Smejkal 1963, 1964, Smejkal & Dvořáková 2000). Microsatellite analysis revealed three well-supported groups (**Paper 12**). Two of them correspond with the dominant European tetraploid species *Euphrasia stricta* J. F. Lehm and *E. nemorosa* (Pers.) Wallr., including the taxa differing from both these species only by a different

indumentum [*E. tatarica* auct., *E. curta* (Fr.) Wettst.]. The third supported group comprises mainly the early-flowering populations morphologically corresponding to plants identified as *E. coerulea* Tausch and *E. slova*ca (Yeo) Holub, which were considered to be endemic to the Western Carpathians or the Sudetes and Western Carpathians, respectively. In this group, the morphological differences in seasonal characters without a genetic difference between the populations from distinct regions (Krkonoše Mts., Beskydy Mts) suggest the quick and recent differentiation of seasonal types and a common evolutionary history of morphologically different populations. Thus, this phenomenon blurs species boundaries, and, consequently, species identification becomes a hard nut to crack. However, the expected extensive hybridization was not proven in the studied populations (**Paper 12**). These results represent an important change in our view of the relationships among the Central European tetraploid *Euphrasia* species, and further integrative study of the group promises to solve evolutionary and taxonomical questions that were previously considered unsolvable.

5. Further perspectives

While the study of the genera *Knautia* and *Spergularia* has concluded in our research group, there are many open questions concerning the genera *Melampyrum* and *Euphrasia*. Ongoing studies focus on the differentiation and phylogeography of *Melampyrum nemorosum* agg. across Europe and the phylogeny of the whole genus. A study of the relationships among the diploid and tetraploid species of *Euphrasia* and hybridization at the tetraploid level is another intended project. Another recently studied taxon is the genus *Calamagrostis* and its Central European relict and endemic species. Members of our group participate in the biodiversity project of mapping of Czech flora and are involved in the ongoing projects on Czech and Slovak identification keys and floras. An international Czech-Bavarian Interreg project focused on the flora of Bohemian forest will be performed during the next three years.

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Appendix 1: Evolution of *Knautia arvensis* agg. in the Central Europe

Paper 1: Kolář F., Štech M., Trávníček P., Rauchová J., Urfus T., Vít P., Kubešová M., & Suda J. (2009): Towards resolving the Knautia arvensis agg. (Dipsacaceae) puzzle: primary and secondary contact zones and ploidy segregation at landscape and microgeographic scales. – Ann. Bot. 103: 963–974.

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Towards resolving the *Knautia arvensis* agg. (Dipsacaceae) puzzle: primary and secondary contact zones and ploidy segregation at landscape and microgeographic scales

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• *Background and Aims* Detailed knowledge of variations in ploidy levels and their geographic distributions is one of the key tasks faced in polyploid research in natural systems. Flow cytometry has greatly facilitated the field of cytogeography by allowing characterization of ploidy levels at both the regional and population scale, and at multiple stages of the life cycle. In the present study, flow cytometry was employed to investigate the patterns and dynamics of ploidy variation in the taxonomically challenging complex *Knautia arvensis* (Dipsacaceae) and some of its allies (*K. dipsacifolia*, *K. slovaca*) in Central Europe.
• *Methods* DNA ploidy levels were estimated by DAPI flow cytometry in 5205 adult plants, 228 seedlings and 400 seeds collected from 292 *Knautia* populations in seven European countries. The flow cytometric data were supplemented with conventional chromosome counts. A subset of 79 accessions was subjected to estimation of the absolute genome size using propidium iodide flow cytometry.
• *Key Results and Conclusions* Five different ploidy levels (from 2*x* to 6*x*) were found, with triploids of *K. arvensis* being recorded for the first time. The species also exhibited variation in the monoploid genome size, corresponding to the types of habitats occupied (grassland diploid populations had larger genome sizes than relict and subalpine diploid populations). Disregarding relict populations, the distribution of 2*x* and 4*x* cytotypes was largely parapatric, with a diffuse secondary contact zone running along the north-west margin of the Pannonian basin. Spatial segregation of the cytotypes was also observed on regional and microgeographic scales. The newly detected sympatric growth of diploids and tetraploids in isolated relict habitats most likely represents the primary zone of cytotype contact. Ploidy level was found to be a major determinant of the strength of inter-cytotype reproductive barriers. While mixed $2x + 4x$ populations virtually lacked the intermediate ploidy level at any ontogenetic stage, pentaploid hybrids were common in $4x + 6x$ populations, despite the cytotypes representing different taxonomic entities.

Key words: Contact zone, cytogeography, flow cytometry, genome size, hybridization, *Knautia arvensis*, ploidy mixture, polyploidy, relict, reproductive isolation, serpentine.

INTRODUCTION

Polyploidy, the presence of more than two complete genomes per cell, has long been recognized as an important force in plant evolution. Recent estimates suggest that about 70 % of angiosperms and up to 95 % of pteridophytes underwent one or more rounds of genome duplication in their evolutionary history (Soltis and Soltis, 1999), and genomic data even assumes near ubiquity of polyploidy (Soltis, 2005). In fact, it has been suggested that at least 2–4 % of all speciation events in angiosperms involve polyploidization (Otto and Whitton, 2000). Because polyploidization potentially confers immediate reproductive isolation, it is widely recognized as the major mode of sympatric speciation in plants (Coyne and Orr, 2004). While different ploidy levels may correspond to different taxa (e.g. Rosenbaumová *et al.*, 2004; see also Soltis *et al.*, 2007), intraspecific ploidy variation is not a rare phenomenon (Lihová *et al.*, 2003; Suda *et al.*, 2007*b*). When several cytotypes occur within the same species, zones of ploidy overlap are often formed. These contact zones are of particular interest to evolutionary biologists because they allow for the study of the mechanisms involved in early stages of polyploid speciation and/or the assessment of selective forces operating in mixed-ploidy populations. Two types of contact zone are recognized according to their evolutionary history (Petit *et al.*, 1999): primary, where a new cytotype originates *in situ*, and secondary, where two formerly allopatric cytotypes meet.

One of the prerequisites for polyploid research in natural systems is knowledge of the geographical distribution of cytotypes or closely related taxa exhibiting ploidy heterogeneity (Hodálová *et al.*, 2007; Suda *et al.*, 2007*b*; Rivero-Guerra, 2008). Distributional data provide useful insights into the evolutionary history of diploid–polyploid groups and can aid in interpretation of phylogenetic relationships and/or experimental results (Baack, 2004, 2005; Baack and Stanton, 2005). In addition, distributional data serve as a foundation for exploring

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the ecological preferences of individual cytotypes and the degree of cytotype interactions (e.g. patterns of mating, competition) and allow for the assessment of the historical development of modern distribution patterns and/or evolutionary forces governing ploidy coexistence (Petit *et al.*, 1999). Thus, the unravelling of variation in ploidy levels and their distributions represents one of the key tasks faced by polyploid research (Favarger, 1984; Perný *et al.*, 2008).

Knautia arvensis agg. (Dipsacaceae) is an intricate polyploid complex of perennial outcrossing herbs inhabiting dry and mesophilous, often human-influenced, grasslands, shrublands, forest margins and open woodlands (Ehrendorfer, 1962*b*; Štěpánek, 1982, 1997). Two species are recognized in Central Europe. While the exclusively tetraploid ($2n = 4x = 40$) *K. kitaibelii* (Schult.) Borbás is restricted to the Western Carpathians (Ehrendorfer, 1962*b*; Štěpánek, 1982), *K. arvensis* (L.) J. M. Coult. *s.s.* is widely distributed throughout Europe. The latter taxon harbours two ploidy levels, usually classified as separate subspecies or varieties [i.e. diploid subsp. *pannonica* (Heuff.) O. Schwarz and tetraploid subsp. *arvensis*] with more-or-less parapatric distribution. Diploids and tetraploids of *K. arvensis* occur in south-eastern and north-western parts of Europe, respectively (Ehrendorfer, 1962*b*); the contact zone runs through Central Europe, and most authors localize it to the northern margin of the Pannonian basin (Ehrendorfer, 1962*b*; Štěpánek, 1982; Kaplan, 1998). In addition, a few diploid populations were discovered in Bohemia and Northern Bavaria in areas otherwise occupied by tetraploids. These populations inhabit relict habitats of serpentine outcrops (sometimes treated as a separate taxon, subsp. *serpentinicola* Smejkal ined.) and a subalpine glacial cirque [= subsp. *pseudolongifolia* (Szabó) O. Schwarz] (Štěpánek, 1982, 1989; Kaplan, 1998). The serpentine localities are rather small (no more than several square kilometres), geographically isolated rocky outcrops with distinct vegetation (usually a mosaic of rocks and open pine forest). Most likely, they have never been covered by compact deciduous forest and are noted for the occurrence of several other relic and/or endemic taxa confined to ultramafic substrates (see, for example, Novák, 1960; Dvořáková, 1988). The subalpine population is restricted to a small outcrop of carbonate rocks in the Krkonoše Mountains (Štěpánek, 1989). Other peculiar diploid populations formerly recognized as *K. arvensis* agg. are known to occur in relict stands (open pine forests on limestone outcrops) in Central–Eastern Slovakia. Based on several unique morphological characters (e.g. type of indumentum and leaf shape), these populations were described as a separate taxon, *K. slovaca* Štěpánek, and assigned to a mainly (sub)Mediterranean group of *K. velutina* agg. (Štěpánek, 1983).

In areas of sympatry, the nominate tetraploid subspecies of *K. arvensis* hybridizes freely with other homoploid species [with both the closely related *K. kitaibelii*, forming the introgressive hybrid *K.* × *posoniensis* Degen, and with the rather distantly related *K. drymeja* Heuffel and 4*x* Carpathian cytotypes of *K. dipsacifolia* (Schrank) Kreutzer]. In fact, the virtual lack of interspecific reproductive barriers at the same ploidy level leads to the high incidence or even predominance of introgressants in some regions (Ehrendorfer, 1964; Breton Sintes, 1974). In addition to homoploid hybridization, some heteroploid crosses between polyploids (e.g. between 4*x* *K. arvensis* and 6*x* cytotypes of *K. dipsacifolia*) have also been documented based on both morphology (Štěpánek, 1997) and chromosome counts (Ehrendorfer, 1962*a*; Štěpánek, 1982). In contrast, there are no records of triploid *Knautia arvensis*, despite the assumed close relationship between putative diploid and tetraploid parents and their occasional sympatric growth (Ehrendorfer, 1962*a*, *b*). The only documented case of interploid $2x \times 4x$ hybridization [involving diploid *K. drymeja* Heuffel var. *tergestina* (Beck) Briq. and tetraploid *K. illyrica* Beck] resulted in a putative tetraploid hybrid (Ehrendorfer 1962*a*). Strong reproductive barriers between 2*x* and 4*x* *Knautia* plants have also been confirmed by several pollination experiments (Breton Sintes, 1974, 1975; Ehrendorfer, 1962*a*).

Although previous karyological studies utilizing conventional chromosome counts have provided a rough picture of the ploidy distribution of *K. arvensis* in Central Europe (Ehrendorfer, 1962*a*, *b*; Štěpánek, 1982; Kaplan, 1998), they have been unable to describe the pattern in sufficient detail due to limited sampling. Therefore, in order to obtain robust and representative results for ploidy variation on various spatial scales, we employed DNA flow cytometry (FCM). This high-throughput technique generates much larger sample sizes that can (1) provide broader geographical information, (2) uncover rare and previously unrecognized cytotypes (e.g. heteroploid crosses), and (3) characterize ploidy variation during different life stages (e.g. seeds, seedlings, and mature plants) (Husband and Schemske, 1998; Suda *et al.*, 2007*a*). In addition, FCM offers the possibility of detecting differences in nuclear DNA content, even in organisms with the same number of chromosomes (e.g. Dimitrova *et al.*, 1999; Mahelka *et al.*, 2005; see Kron *et al.*, 2007, for a review). In many plant groups, genome size has been found to be a useful taxonomic marker that may guide taxonomic decisions and allow for detection of cryptic diversity or incipient speciation (Murray, 2005).

Using FCM, we addressed the following questions. (1) What is the cytotype distribution of *K. arvensis* in Central Europe? What is the structure of the zone of ploidy overlap? Does the cytotype contact correspond to the primary or the secondary zone? (2) How frequent are mixed-ploidy populations? What is the fine-scale distribution of cytotypes in such populations? Do ploidy proportions in mixed populations differ between the various life stages? (3) Is the pattern observed in $2x + 4x$ populations of *K. arvensis* different from the situation present in populations formed by *K. arvensis* (4*x*) and *K. dipsacifolia* (6*x*)? (4) Is there any variation in genome size within the *K. arvensis* agg. complex? If so, is this variation systematically, geographically and/or ecologically structured?

MATERIALS AND METHODS

Field sampling

Plant material was sampled from 2005 to 2008 in the Czech Republic, Slovakia, Austria, Hungary, Poland, Ukraine and Germany. In total, 283 populations of *Knautia arvensis* agg. (i.e. *K. arvensis s.s.*, *K. kitaibelii* and their introgressive hybrid *K.* × *posoniensis*) were analysed. For comparative purposes,

four populations of presumably related *K. slovaca* and five mixed populations consisting of *K. arvensis* and *K. dipsacifolia* were also included in the study. GPS co-ordinates and basic environmental characteristics (e.g. type of habitat, vegetation cover and irradiation) were recorded at each locality. Leaves from approx. ten plants per population were collected, placed in plastic bags and stored at cold temperatures (no more than 1 week) until FCM analyses; to avoid collecting the same genet, the distance between sampled individuals was at least 1 m. The locality details and numbers of plants analysed is available online in the Supplementary Data. Herbarium vouchers are deposited in the CBFS. For mapping the cytotype distribution on a coarse spatial scale, the following literature sources were also considered: Ehrendorfer (1962*a*) – Austria, Hungary; Frey (1969) – Poland; Májovský (1976) – Slovakia; Štěpánek (1982) – the Czech Republic, Slovakia and Poland; and Kaplan (1998) – the Czech Republic.

Three mixed-ploidy populations (see Supplementary Data, available online) were subjected to detailed microspatial screening. The populations were selected to encompass (1) different ploidy combinations ($2x + 4x$ vs. $4x + 6x$); (2) different taxonomic compositions (subspecies of *K. arvensis* vs. mixture of *K. arvensis* and *K. dipsacifolia*); and (3) different habitat types (semi-natural vs. relict habitats). At each locality, initial ploidy screening was performed to locate representative mixed-ploidy plots. Subsequently, every adult individual (both flowering plants and sterile rosettes) in the plot was labelled, its position recorded in a rectangular co-ordinate system, and the DNA ploidy level determined by FCM. Moreover, all seedlings (young individuals with less than three pairs of basal leaves, presumably germinated the same year) and achenes from all fructiferous plants were collected. One additional tetraploid and hexaploid mixed population (Želnava, no. 290; see Supplementary Data) was screened in a different way. Approximately 50 % of the individuals were sampled in *a priori* defined patches (circles of 1 m radius) along a 50-m transect.

Estimation of DNA ploidy levels and genome sizes

DNA ploidy levels (Suda *et al.*, 2006) and genome sizes (C- and Cx-values; Greilhuber *et al.*, 2005) were determined by flow cytometry using Partec PA II and CyFlow instruments (Partec GmbH., Münster, Germany) equipped with a HBO mercury arc lamp and a green (532 nm) solid-state laser, respectively. Sample preparation generally followed the two-step procedure using Otto buffers (Doležel *et al.*, 2007). Intact leaf tissue of analysed *Knautia* plant(s) with an appropriate volume of the internal reference standard (*Pisum sativum* 'Ctirad', Doležel *et al.*, 1998; 2C value set to 8·84 pg following Greilhuber *et al.*, 2007) was chopped with a sharp razor blade in a Petri dish containing 0·5 mL of ice-cold Otto I buffer (0·1 M citric acid, 0·5 % Tween-20; Otto, 1990). The suspension was filtered through a 42-μm nylon mesh and incubated for approx. 30 min at room temperature. The staining solution consisted of 1 mL of Otto II buffer (0·4 M $Na_2HPO_4.12\ H_2O$), β-mercaptoethanol (final concentration of 2 μL mL^{-1}), and a fluorochrome. Propidium iodide (PI) and RNase IIA (both at final concentrations of 50 μL mL^{-1}) were used to determine the genome size in absolute values (pg of DNA), while AT-selective DAPI (4′,6-diamidino-2-phenylindole) at a final concentration of 4 μL mL^{-1} was preferred for the ploidy analyses. Samples were stained for 10 min at room temperature and run on the flow cytometer. Isolated nuclei were excited using either a laser (for PI staining) or a mercury arc lamp (for DAPI staining), and the fluorescence intensity of 3000–5000 particles was recorded. Only histograms with coefficients of variation (CVs) for the G_0/G_1 peak of the analysed *Knautia* sample below 3·0 % were considered. For ploidy screening, pooled samples (up to ten individuals) were often used. Our previous experiments showed that such practice enables reliable detection of a minority cytotype present even at a low proportion (<10 %). Moreover, the lack of endopolyploidy and virtual absence of mitotic activity in the selected leaf tissue guaranteed unbiased DNA ploidy level estimates. Nevertheless, each plant was separately re-analysed if mixed samples were suspected, peaks were asymmetrical, or the CV of the *Knautia* peak exceeded the above-set threshold. In fact, short-term storage of plant tissues in the cold (4 °C) did not negatively influence the quality of analyses, and high-resolution histograms were obtained even after 1 week of storage. More stringent criteria were applied to analyses aimed at genome size determination: (1) peaks for both the sample and the internal standard had to be of approximately the same height; (2) each sample was measured at least three times on different days to minimize potential random instrumental drift; and (3) the between-day variation was defined to not exceed a 2 % threshold; otherwise the most remote value was discarded and the sample was re-analysed. The reliability of FCM measurements (i.e. between-plant differences) was repeatedly confirmed in simultaneous runs of *Knautia* accessions having distinct genome sizes.

DNA ploidy levels in seeds were analysed using the same protocol as for leaf tissues, with the following modifications: (1) only single-seed analyses were performed; and (2) *Bellis perennis* L. (2C = 3·38 pg; Schönswetter *et al.*, 2007) was selected as an internal reference standard. Altogether, 400 seeds with well-developed embryo/endosperm obtained from 103 mother plants were examined. The criteria for seed analyses were more relaxed – the CVs of embryo peaks varied from 2·2 % to 7·3%.

Chromosome counts

To confirm the FCM results, 14 individuals representing different DNA ploidy levels and genome size variants (i.e. ten *K. arvensis* individuals and one individual each of *K. kitaibelii*, *K. slovaca*, *K. dipsacifolia* and a putative hybrid between *K. arvensis* and *K. dipsacifolia*; see Supplementary Data, available online) were subjected to conventional karyological analysis using rapid squash methods. The apical shoot meristems of mature plants were pre-treated with *p*-dichlorbenzene for 3 h, fixed in ice-cold 3 : 1 ethanol : acetic acid for 12–14 h, macerated for 1 min in 1 : 1 ethanol : hydrochloric acid, and stained with lacto-propionic-orcein (Dyer, 1963).

Statistical analyses

Differences in the altitudinal distribution between diploid and tetraploid cytotypes of *K. arvensis* were tested using a

two-tailed *t*-test available in the Statistica package, ver. 7 (StatSoft, 2005; mixed-ploidy populations were excluded from the analysis). The GLM procedure available in SAS 8.1 (SAS Institute, 2000) was used to assess differences in genome sizes (C-values), and Tukey's procedure was applied to compare mean values of particular groups. The spatial segregation of cytotypes in mixed-ploidy plots was analysed using the Mantel test (Manly, 1991) implemented in the zt software (Bonnet and van de Peer, 2002). A correlation coefficient (r_M) was calculated for (1) the matrix of mutual distances among individuals, and (2) the binary matrix of ploidies, and compared to the distribution of coefficients obtained from matrices generated by random rearrangements of the original matrices (999 permutations were performed). Only majority ploidies were considered (i.e. rare odd cytotypes were omitted).

RESULTS

Variation in DNA ploidy levels, chromosome numbers and genome sizes

Five different DNA ploidy levels (from 2*x* to 6*x*) were detected during FCM analyses of 5205 adult individuals from 292 *Knautia* populations (Fig. 1). While *K. arvensis s.s.* showed ploidy heterogeneity ($2x + 4x$ cytotypes), other species were karyologically uniform (i.e. *K. slovaca*, 2*x*; *K. kitaibelii*, 4*x*; and *K. dipsacifolia*, 6*x*). Two minority odd ploidy levels were detected: (1) DNA pentaploids, which corresponded to crosses between 4*x* *K. arvensis* and 6*x* *K. dipsacifolia* (24 plants from four populations); and (2) DNA triploids (two plants from two populations of *K. arvensis* otherwise formed by diploids; see Supplementary Data, available online). In fact, the latter cytotype represents one of the few triploid records for the entire *Knautia* genus and the very first record for *K. arvensis* agg.

Chromosome counts confirmed the estimated ploidy levels and revealed $2n = 2x = 20$ in five accessions of *K. arvensis* and one accession of *K. slovaca*, $2n = 3x \approx 30$ in one accession of *K. arvensis*, and $2n = 4x = 40$ in four accessions of *K. arvensis* and one accession of *K. kitaibelii* (see Supplementary Data). No aneuploidy/accessory chromosomes were recorded. Exact chromosome numbers could not be unambiguously determined in a DNA pentaploid and a DNA hexaploid; nevertheless, more than 40 chromosomes were observed in their somatic cells.

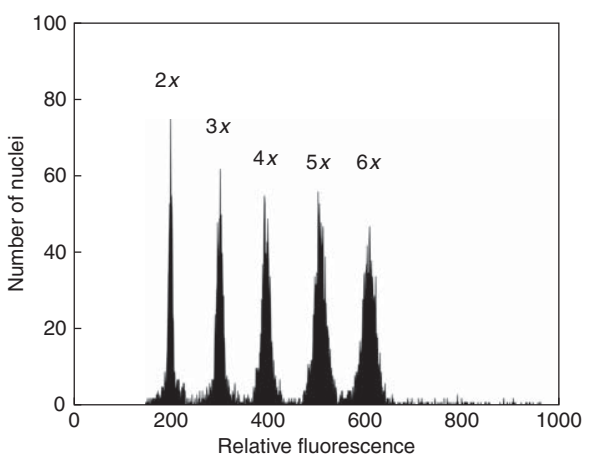

FIG. 1. Fluorescence histogram of propidium iodide-stained nuclei isolated from fresh leaf tissues of diploid, triploid and tetraploid *Knautia arvensis* plants, pentaploid hybrid *K. arvensis* × *K. dipsacifolia*, and hexaploid *K. dipsacifolia*. Nuclei from all samples were isolated, stained and analysed simultaneously.

A subset of *Knautia* accessions was subjected to genome size estimation, including 68 *K. arvensis* individuals, six *K. kitaibelii* individuals and five *K. slovaca* individuals (Table 1; see also Supplementary Data). To assess the genome size variation, the *K. arvensis* accessions were further divided into five groups corresponding to ploidy levels and habitat types. The intra-group variation in genome size was always small (≤4·3 %; Table 1) but genuine, as confirmed by simultaneous analyses of individuals with distinct nuclear DNA amounts (Fig. 2A). No geographical or ecological structuring was observed within the groups (data not shown). Analysis of between-group differences revealed five more-or-less distinct clusters of holoploid genome size ($F = 8879{\cdot}94$, $P < 0{\cdot}0001$; Table 1). The first, most-distant cluster (mean 2C-value = 7·23 pg) corresponded to non-relict diploids of *K. arvensis*, which were widely distributed in the eastern part of the area studied (i.e. subsp. *pannonica*). Geographically vicariant diploids of *K. arvensis* from relict habitats in the western region (serpentine outcrops and a subalpine glacial cirque) formed, together with *K. slovaca*, the second cluster (mean 2C-values = 6·86–6·95 pg). Once again, the divergence in estimated nuclear DNA content between these two groups was confirmed in simultaneous FCM analyses (Fig. 2B). Genome size differentiation at the tetraploid level was less apparent. Despite 2C-values of non-serpentine 4*x* *K. arvensis* (mean = 14·08 pg) differing significantly from those of *K. kitaibelii* (mean = 13·75 pg), both values were quite similar (average difference of only 2·4 %), and the situation was further complicated by relict serpentine populations of *K. arvensis* with mean 2C-values of 13·94 pg.

Cytotype distribution on a coarse spatial scale

The geographic distribution of cytotypes of *K. arvensis* (including introgressants with *K. kitaibelii*) in Central Europe is shown in Fig. 3 (both our FCM results and selected literature records were used in constructing the map). The two majority cytotypes exhibit a clear spatial segregation along a NW–SE gradient. Disregarding the relict populations, diploids were restricted to the SE part of the area investigated, while tetraploids predominated in the NW region. For diploid plants, the centre of distribution was located in Pannonia, from where they extended in several directions, mainly along large river valleys (e.g. the Váh River in Slovakia and the Danube River in Austria). A few scattered diploid populations having distinct genome sizes occurred in relict habitats, such as serpentine outcrops and a glacial subalpine cirque in the Bohemian massif (see inset in Fig. 3). Tetraploids occupied the entire Bohemian massif, the outer ranges of the Western Carpathians, and the northern portions of the Eastern Alps. In the zone of overlap with *K. kitaibelii* (e.g. in the Carpathians and eastern parts of the Bohemian massif), tetraploids were almost exclusively represented by the introgressive hybrid *K.* × *posoniensis*; this taxon can be easily recognized by the intermediate colour of its flower parts (i.e. whitish-to-pale-violet corolla with violet anthers).

TABLE 1. *Genome sizes (C- and Cx-values) obtained for different ploidy levels and ecological types of* K. arvensis *agg. and* K. slovaca

	No. of populations/no. of individuals analysed	2C-value (mean ± s.d.; pg of DNA)*	2C-value range (min–max; pg of DNA)	Variation (%)	Mean Cx-value (pg of DNA)
K. arvensis (2*x*): non-relict	14/23	$7{\cdot}23 \pm 0{\cdot}09^{d}$	7·08–7·37	4·1	3·62
K. arvensis (2*x*): relict serpentine	5/18	$6{\cdot}86 \pm 0{\cdot}09^{e}$	6·72–7·00	4·2	3·43
K. arvensis (2*x*): relict subalpine	1/5	$6{\cdot}95 \pm 0{\cdot}04^{e}$	6·91–7·00	1·3	3·48
K. slovaca (2*x*)	4/5	$6{\cdot}87 \pm 0{\cdot}11^{e}$	6·70–6·96	3·9	3·43
K. arvensis (3*x*): relict serpentine	1/1	$10{\cdot}51^{c}$	–	–	3·50
K. arvensis (4*x*): non-relict	4/11	$14{\cdot}08 \pm 0{\cdot}14^{a}$	13·92–14·34	3·0	3·52
K. arvensis (4*x*): relict serpentine	3/10	$13{\cdot}94 \pm 0{\cdot}18^{ab}$	13·62–14·16	4·0	3·48
K. kitaibelii (4*x*)	4/6	$13{\cdot}75 \pm 0{\cdot}25^{b}$	13·38–13·96	4·3	3·44

* Different letters indicate groups of taxa that are significantly different at $\alpha = 0{\cdot}05$.

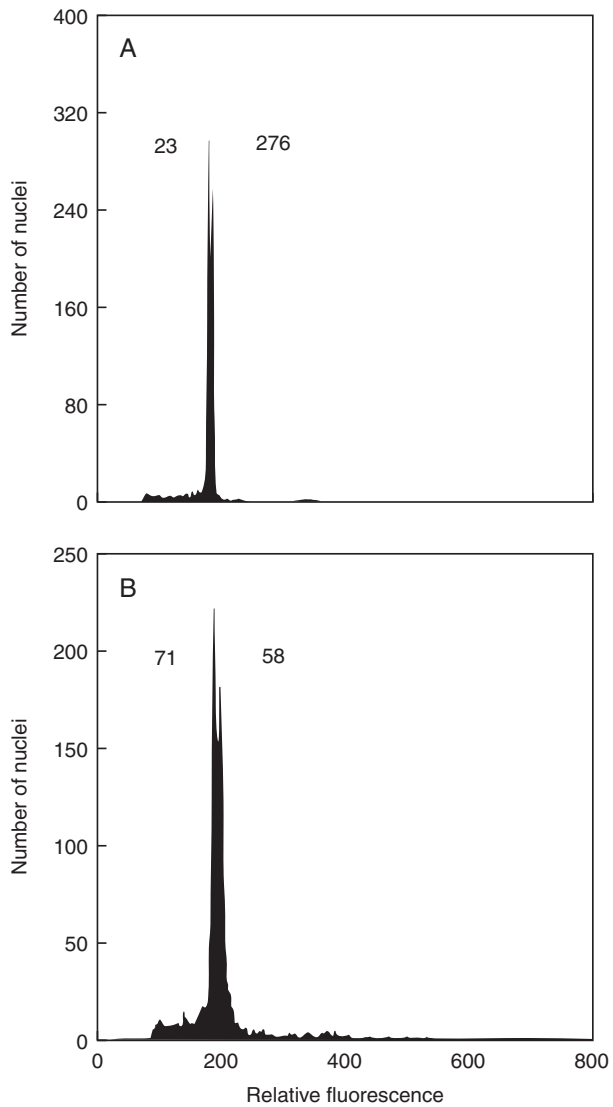

FIG. 2. Flow cytometric histograms documenting variation in genome size between selected diploid accessions of *K. arvensis*. (A) Simultaneous analysis of non-relict populations nos. 23 (2C = 7·21 pg) and 276 (2C = 7·37 pg). (B) Simultaneous analysis of a relict serpentine population, no. 71 (2C = 6·90 pg), and non-relict population, no. 58 (2C = 7·26 pg); see Supplementary Data (available online) for population details. Nuclei from both samples were isolated, stained with DAPI and analysed simultaneously.

Diploids and tetraploids of *K. arvensis* coexist along the NW margin of the Pannonian basin (eastern foothills of the Eastern Alps, eastern part of Lower Austria, South and Central Moravia) and in the Polonian lowlands of north Moravia and south Poland. The contact zone then continues through Slovakia, where the situation is more complex because diploids penetrate along river valleys far into the Carpathian Mountains. Generally, the zone of ploidy contact is quite wide (several dozens of kilometres) and diffuse.

Single-cytotype populations of *K. arvensis* clearly prevailed, even in areas where both ploidies were recorded (Fig. 4A). In the two contact zones where the most representative sampling was performed (south and central Moravia, and north and north-east Austria), elevational differences among the sites of 2*x* and 4*x* cytotypes were tested using a two-tailed *t*-test. Significant differences were found in both cases ($t = -4{\cdot}51$, d.f. = 68, $P < 0{\cdot}0001$ and $t = -2{\cdot}47$, d.f. = 36, $P = 0{\cdot}018$ for the Moravian and Austrian localities, respectively). In each region, diploids generally occupied lower altitudes than their tetraploid counterparts, with an average difference of >100 m (Fig. 5).

Cytotype distribution on fine spatial scales

A detailed study of cytotype distribution at the level of the whole locality and/or selected plots was performed for several populations exhibiting ploidy heterogeneity. Mixed-ploidy populations occurred both in relict serpentine (three populations) and semi-natural grassland (20 populations) habitats. The following types of ploidy mixtures were detected: 2*x* + 4*x* (16 populations), 2*x* + 3*x* (two populations), 4*x* + 6*x* (one population), and 4*x* + 5*x* + 6*x* (four populations). There was a striking difference in ploidy composition between populations formed by diploids + tetraploids vs. tetraploids + hexaploids. All of the former populations lacked the intermediate (3*x*) ploidy level, suggesting the existence of efficient breeding barriers. In contrast, pentaploids of a putative hybrid origin were detected in all but one population comprising 4*x* *K. arvensis* and 6*x* *K. dipsacifolia*, and constituted a considerable proportion of the individuals analysed (3·9–9·5 %).

In Southern and Central Moravia, visual assessment of the spatial distribution of cytotypes in localities was easy due to the differences in flower colour between diploids (violet) and tetraploids (whitish due to the introgression of *K. kitaibelii*).

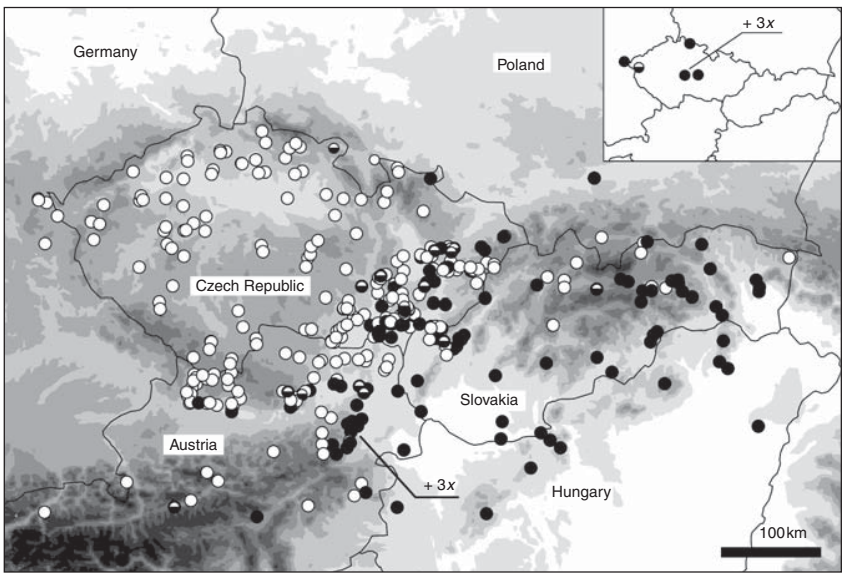

FIG. 3. Distribution of the cytotypes of *Knautia arvensis s.s.* (including an introgressive hybrid *K.* × *posoniensis*) in Central Europe. The overall distribution of cytotypes is shown: note the largely parapatric distribution. The inset shows the distribution of relict populations (these are not depicted in the main figure). Solid circles: diploid; open circles: tetraploid; half-filled circles: mixed $2x + 4x$ population. Two triploids are designated by '3*x*'. The diploid population from west Ukraine (close to Lviv) is not displayed on the map. While the majority of the populations mapped were analysed in the present study, the figure also includes some populations extracted from selected literature sources (see Materials and Methods for details).

All but one population showed clear cytotype segregation, with different ploidy levels growing in more-or-less aggregated patches (at least several metres apart) or even occupying different parts of the same locality. The only exception applied to the locality of Ždánice (no. 267, see Supplementary Data), where a plot with a genuine ploidy mixture was detected and further examined (see below). Cytotype aggregation also occurred in two $2x + 4x$ populations of *K. arvensis* growing in serpentine outcrops (Vlčí hřbet, no. 277, and Planý vrch, no. 278). The majority of the patches analysed (94 % and 92 %, respectively) were cytologically uniform and with prevailing tetraploids. Figure 4B shows the spatial arrangement and ploidy composition of the patches at the latter locality, Planý vrch (a very similar pattern was also found at the other one, Vlčí hřbet; data not shown). Because serpentine tetraploids are phenotypically more similar to coexisting diploids than to their widespread non-serpentine counterparts, we considered this zone as a primary contact.

A microspatial survey was undertaken for selected mixed-ploidy plots in each of the three localities (Ždánice, no. 267, non-serpentine $2x + 4x$; Planý vrch, no. 278, relict serpentine $2x + 4x$; and Horní Planá, no. 288, $4x + 5x + 6x$; see Supplementary Data) to ascertain whether or not the cytotypes were randomly distributed. Figure 6 shows the spatial arrangement of adult individuals. While clear ploidy grouping is apparent in the latter two localities (Fig. 6B, C), the cytotypes seem to be more intermingled in the first locality (Fig. 6A). Nevertheless, the Mantel test strongly supported the non-randomness of ploidy distribution in all plots (Table 2). In addition to the above three plots, the proportions of cytotypes were also assessed along a transect through a mixed population of *K. arvensis* and *K. dipsacifolia* near Želnava (no. 290). The complex distribution patterns of 4*x*, 5*x* and 6*x* ploidies are depicted in Fig. 6D.

Life stage-specific variation in ploidy composition in mixed populations

To acquire a holistic picture of ploidy composition and dynamics in the three mixed populations, data on adult plants were supplemented with ploidy estimates obtained for seedlings and seeds (Tables 2 and 3). For seedlings, both the ploidy variation and distribution pattern clearly reflected the conditions found in adult plants, i.e. seedlings of different ploidy levels were largely restricted to sectors of the plot where cytotype-mixing in adult plants had also been recorded. Interestingly, a few DNA pentaploid seedlings were found at the locality of Horní Planá (no. 288) in a sector primarily occupied by mature tetraploids (see Fig. 6C). In both $2x + 4x$ populations, the proportion of diploids in seedlings was higher than in mature plants. These differences could either reflect differential survival of the two cytotypes or could arise due to a ploidy-specific supply of seeds from outside the experimental plot.

A more complex pattern of ploidy variation emerged when ripe seeds were analysed (Table 3). Notably, a single triploid seed produced by a tetraploid maternal plant was detected in a relict serpentine $2x + 4x$ population in Planý vrch (no. 278). In addition, a pentaploid seed was recorded among a seed set of tetraploid plants from a $4x + 6x$ population in Horní Planá (no. 288). Pentaploid hybrids in the latter population produced mostly 5*x* seeds, although fluorescence intensity corresponding to the tetraploid cytotype was also recorded, along with some putative aneuploids (see Table 3).

DISCUSSION

Cytogeography of Knautia arvensis

The results obtained by ploidy screening of a representative set of *Knautia* samples support previous theories regarding the

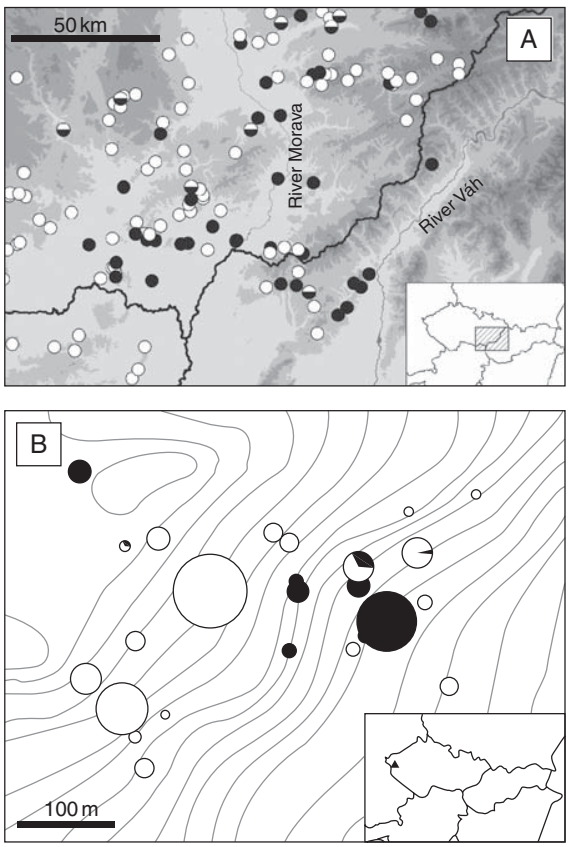

FIG. 4. Distribution of cytotypes of *Knautia arvensis s.s.* (including an introgressive hybrid *K.* × *posoniensis*) on regional and microgeographic scales. (A) Detailed distribution in the zone of ploidy overlap in South and Central Moravia, SW Slovakia and NE Austria. The inset shows the location of the region in within Central Europe. (B) Cytotype composition in the mixed-ploidy relict serpentine population Planý vrch, no. 278 (see Supplementary Data, available online, for population details). Solid circles: diploid; open circles: tetraploid; partially-filled circles: mixed $2x + 4x$ patch. The size of the symbols in (B) corresponds to the number of individuals analysed and the relative proportions of cytotypes are visualized as pie charts. The inset shows the position of the locality within the Czech Republic.

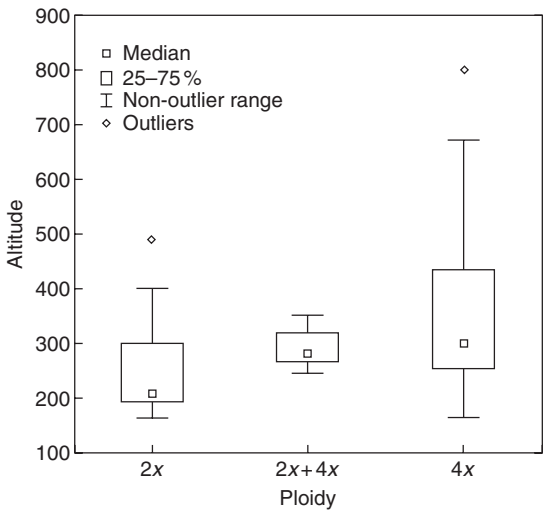

FIG. 5. Box-and-whisker plots demonstrating altitudinal ranges of the diploid, tetraploid and mixed ($2x + 4x$) populations in the contact zone in South and Central Moravia.

parapatric distribution of diploid and tetraploid cytotypes of *K. arvensis s.s.* in Central Europe (Ehrendorfer, 1962*b*; Štěpánek, 1982; Kaplan, 1998). While the centre of the distribution of diploids in Pannonia was also confirmed, marked extensions of the distributional range both to the west (the Danube valley in Upper Austria) and north (Central and North Moravia, and South Poland) were newly detected. Of particular significance is the occurrence of diploids in the southern Polonian lowlands (several localities stretching from North Moravia up to West Ukraine), which indicates the existence of a previously largely unnoticed area (but see Frey, 1969) separated from the Pannonian basin by the mountain ranges of the Western Carpathians (Fig. 3). The Pannonian and Polonian populations seem to be connected via the Moravská brána depression on the western border of the Carpathians. Additional possible connections along the eastern margin of the Carpathians (through Ukraine, Moldova and Romania) remain to be determined. In addition, there are several deep penetrations of diploids into the Western Carpathian mountain systems, mainly along the flat valleys of rivers flowing to Pannonia (e.g. the Bečva and the Váh rivers).

A group of diploid *K. arvensis* plants displaying significantly different genome sizes was found in relict habitats (serpentine outcrops and a subalpine glacial cirque) in the western part of the area, otherwise occupied solely by tetraploids. The same genome size was shared by the Slovakian endemic *K. slovaca*, which also grows exclusively in relict habitats, such as open pine forests on limestone outcrops (Štěpánek, 1983, 1985). These habitat preferences sharply contrast with the semi-natural/semi-ruderal character of stands occupied by the widespread (i.e. Pannonian) diploids and non-serpentine tetraploids. The genome size difference between diploids growing in relict (subsp. *serpentinicola* and subsp. *pseudolongifolia*) vs. semi-natural (subsp. *pannonica*) habitats provides support for the different evolutionary history of both types of plants. It is plausible that relict diploids originated in the early Holocene, and subsequently were forced into serpentine, limestone or alpine refugia by the expanding forest vegetation. Non-relict diploids and tetraploids could have migrated to Central Europe later, perhaps as a consequence of human-induced changes in the landscape, such as deforestation, grazing and/or meadow agriculture (Štěpánek, 1989; Kaplan, 1998).

Although caution should be taken when small differences in genome size are interpreted (Bennett *et al.*, 2008), we are convinced that our results are not negatively influenced by instrumental or methodological errors, including the presence of disturbing secondary metabolites. First, low coefficients of variation were achieved, which are not compatible with the presence of interfering metabolites. It should also be noted that several plants from each group were cultivated in homogeneous conditions (for up to 1 year) before flow analyses. Moreover, between-plant differences remained stable in analyses with either intercalating propidium iodide or AT-selective DAPI (a fluorochrome less sensitive to secondary metabolites). In addition, co-processed samples with different

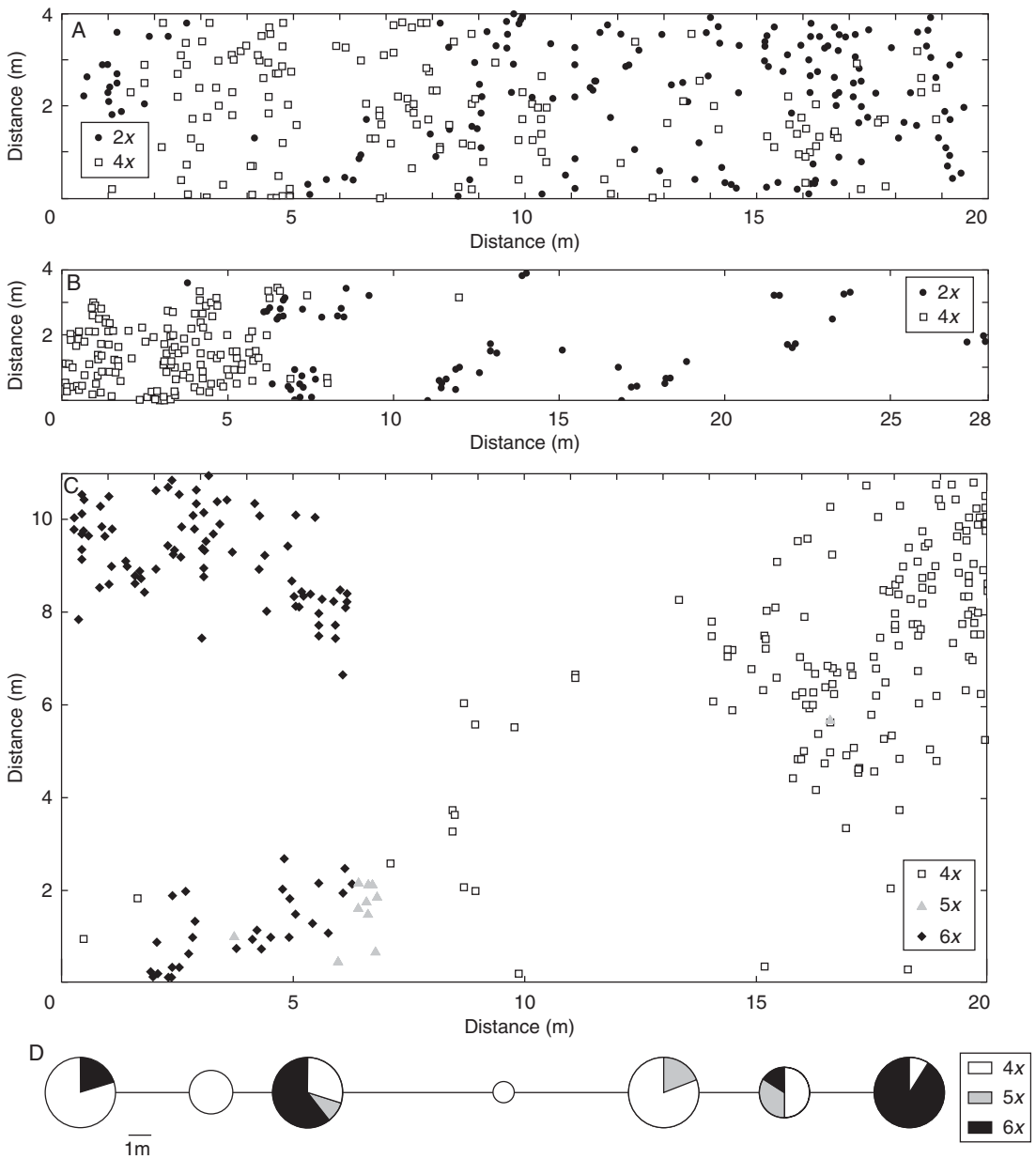

FIG. 6. Detailed distributions of cytotypes in selected mixed-ploidy plots (all adult plants were mapped). (A) Ždánice, no. 267: non-relict $2x + 4x$ population of *K. arvensis*. (B) Planý vrch, no. 278: relict serpentine $2x + 4x$ population of *K. arvensis*. (C) Horní Planá, no. 288: mixed population of $4x$ *K. arvensis* + $6x$ *K. dipsacifolia* (+ $5x$ hybrids). (D) Cytotype composition along a transect through a mixed population of $4x$ *K. arvensis*, $6x$ *K. dipsacifolia* and $5x$ hybrids near Želnava, no. 290. The size of the symbols in (D) corresponds to the number of individuals analysed and the relative proportions of cytotypes are visualised as pie charts. See Supplementary Data (available online) for population details.

genome sizes always gave two distinct peaks, which is considered the most convincing evidence for genuine differences in nuclear DNA content (Greilhuber, 2005).

Primary and secondary contact zones

If more chromosomal races exist within a species, zones of ploidy overlap are often formed (Husband and Schemske, 1998; Weiss *et al.*, 2002; Suda *et al.*, 2007*b*). Primary contact zones differ from secondary contact zones in their evolutionary history (Petit *et al.*, 1999). The former zones arise as a direct consequence of the emergence of a (higher) polyploid within a diploid/lower polyploid population, while the latter results from a secondary contact between previously allopatric cytotypes. The close genetic proximity between coexisting individuals of different ploidy levels characterizes primary zones; in contrast, secondary contact zones are usually composed of distantly related individuals because they combine genetically distinct parental gene pools. While genetic variation is the most robust criterion for discrimination

TABLE 2. *Cytotype composition of adult plants and seedlings in three mixed-ploidy* Knautia *populations subjected to detailed ploidy screening (see Supplementary Data, available online, for population details). The spatial structure of adult individuals was evaluated using the Mantel test.*

Population	Ontogenetic stage	Total number of samples analysed	Proportions of ploidy levels (%)				Mantel statistics	
			2*x*	4*x*	5*x*	6*x*	r_M	*P*-value
Ždánice, no. 267 (non-relict 2*x* + 4*x K. arvensis*)	Adult plants	324	49·7	50·3	–	–	−0·154	0·001
	Seedlings	93	77·4	22·6	–	–		
Planý vrch, no. 278 (relict serpentine 2*x* + 4*x K. arvensis*)	Adult plants	209	31·6	68·4	–	–	−0·519	0·001
	Seedlings	37	48·6	51·4	–	–		
Horní Planá, no. 288 (4*x K. arvensis* + 6*x*	Adult plants	278	–	56·0	4·0	40·0	−0·824*	0·001*
K. dipsacifolia + 5*x* hybrid)	Seedlings	98	–	42·8	3·1	54·1		

* The minority pentaploid cytotype was omitted from the statistical analysis.

TABLE 3. *Ploidy variation in seeds obtained from three mixed-ploidy* Knautia *populations with different cytotype compositions (see Supplementary Data, available online, for population details)*

Population	Ploidy level of mother plants	No. of analysed seeds/no. of mother plants	Numbers and proportions (%) of seeds of different ploidy levels				
			2*x*	3*x*	4*x*	5*x*	6*x*
Ždánice, no. 267 (non-relict *K. arvensis*)	2*x*	80 / 25	80 (100)	–	–	–	–
	4*x*	64 / 20	–	–	64 (100)	–	–
Planý vrch, no. 278 (relict serpentine	2*x*	12 / 4	12 (100)	–	–	–	–
K. arvensis)	4*x*	75 / 21	–	1 (1·3)	74 (98·7)	–	–
Horní Planá, no. 288 (4*x K. arvensis* + 6*x*	4*x*	100 / 17	–	–	99 (99·0)	1 (1·0)	–
K. dipsacifolia + 5*x* hybrid)	5*x*	10 / 1	–	–	1 (10·0)	9 (90·0)*	–
	6*x*	59 / 15	–	–	–	–	59 (100·0)

* Aneuploids are likely to be included due to large variations in fluorescence intensity.

between the two types of contact zones, additional evidence can be obtained from cytological, phenotypic and/or distributional data. Our FCM results support the existence of both primary and secondary contact zones in *K. arvensis*. A distinct geographical pattern in non-relict diploids and tetraploids indicates a secondary contact zone, which stretches from northern Austria through Moravia to southern Poland. An approximate westward continuation of the zone of sympatry is outlined by Ehrendorfer (1962*b*, 1976) and Breton Sintes (1969); the cytotype contact zone runs from SE Austria along the southern flanks of the Alps, and through southern France up to the Pyrenees. The situation in east and SE Europe is, however, unknown and, together with the sparse information available from west Europe, calls for further investigation. In general, the transient zone of *K. arvensis* is diffuse and quite wide (several dozens of kilometres). Similar conditions (mixed populations scattered over large geographical areas) have been observed, for instance, for the grass *Pennisetum* sect. *Brevivalvula*, Poaceae (Renno *et al.*, 1995). Nonetheless, narrow sympatric zones usually seem to prevail in diploid–polyploid plant groups (e.g. van Dijk *et al.*, 1992; Liebenberg *et al.*, 1993; Husband and Schemske, 1998).

Relict *Knautia* populations demonstrate a very different pattern. The coexistence of 2*x* and 4*x* plants in two serpentine populations in Western Bohemia (Vlčí hřbet, no. 277, and Planý vrch, no. 278; see Supplementary Data) suggests the independent autopolyploid origin of serpentine tetraploids from their diploid counterparts, and thus primary cytotype contact. There are a few more plant groups for which both primary and secondary contacts have been suggested, including *Dianthus*, Caryophyllaceae (Weiss *et al.*, 2002) and *Melampodium*, Asteraceae (Stuessy *et al.*, 2004). The hypothesis stating the independent evolutionary history of serpentine *Knautia* populations was first postulated by Kaplan (1998), on the basis of phenotypic variation and ecological observations. The data herein demonstrating novel ploidies and genome sizes support this hypothesis. Initially, a minority of diploid cytotypes was detected in two serpentine localities where only tetraploids had been previously known to exist. Furthermore, the slightly smaller genome size of serpentine tetraploids in comparison with their non-serpentine counterparts may be regarded as additional, supplementary evidence for *in situ* polyploid evolution in relict serpentine habitats. Independent evolution of serpentine populations was also proposed by Westerbergh and Saura (1992) when explaining genetic differences between ruderal and serpentine plants of *Silene dioica* (Caryophyllaceae) in central Sweden. Nevertheless, the possibility of independent colonization of serpentine outcrops by neighbouring grassland tetraploids of *Knautia* can not be rejected at this stage of investigation and molecular data are required to reach a final conclusion. Provided that the independent *in situ* origin of serpentine

tetraploids is confirmed, the *K. arvensis* agg. renders a unique opportunity for a comparative study of patterns and processes in primary vs. secondary diploid–polyploid contact zones.

Spatial segregation of cytotypes in contact zones

Special attention was paid to the distribution of different ploidy levels in contact zones in order to (1) evaluate the level of among-cytotype interactions and (2) assess potential ecological and/or phenological differences among cytotypes. On both the regional and microgeographic scales, the two majority cytotypes (2*x*, 4*x*) were more-or-less spatially segregated. This segregation is well documented by a low number of mixed-ploidy populations (see Figs 3 and 4A). One reason for ploidy segregation at the regional scale seems to be certain cytotype preferences for different altitudes and/or geomorphology of the terrain. Whereas diploids in the contact zone mostly grew in flat lowlands or in valleys of large rivers, tetraploids were more often found in more rolling landscapes. Altitudinal separation of different cytotypes does not seem to be a rare phenomenon in plants exhibiting ploidy heterogeneity, as previously documented, for instance, for *Anthoxanthum alpinum*, Poaceae (Felber-Girard *et al.*, 1996), *Senecio carniolicus*, Asteraceae (Schönswetter *et al.*, 2007) and *Allium przewalskianum*, Alliaceae (Xie-Kui *et al.*, 2008).

The spatial segregation observed at the regional scale was mirrored in selected mixed-ploidy plots within localities. The cytotype distribution observed in the two diploid–tetraploid plots (i.e. non-serpentine Ždánice, no. 267, and serpentine Planý vrch, no. 278) was more-or-less non-random. Because the vegetation cover at both plots was quite homogeneous (F. Kolář *et al.*, pers. obs.), we do not consider microhabitat differentiation as the major driving force behind the spatial segregation. Rather, our non-exclusive explanation takes into account a founder effect and limited distribution capability of *Knautia* seeds. The seeds are dry, rather heavy, and lack any surface structures that facilitate exozoochory. Ants are sometimes discussed as dispersing agents (Ehrendorfer, 1962*a;* Štěpánek, 1997), yet they usually carry seeds for short distances only (Gómez and Espadaler, 1998). A combination of the above-mentioned factors can lead to the formation of single-cytotype patches, even in populations exhibiting ploidy heterogeneity. Indeed, clusters of seedlings were often observed in close proximity to the putative mother plant. An alternative hypothesis assuming that the observed distribution pattern in fact represents a transitional stage in the gradual competitive exclusion of one cytotype can not be ruled out at this stage of investigation.

On the other hand, cytotype sorting along an environmental gradient was observed in the locality of Horní Planá (no. 288) where 4*x K. arvensis* coexisted with 6*x K. dipsacifolia* (plus some 5*x* hybrids). The former species preferred open grassy parts of the plot, while the latter taxon grew mostly in shady parts of the plot close to the forest margin. DNA-pentaploids occupied an ecologically transitional semi-shaded zone of the plot.

Differential reproductive barriers among different cytotypes

In recent years, an increasing number of studies have investigated the role of different pre- and post-zygotic reproductive barriers in the evolutionary dynamics of mixed-ploidy populations (see Husband and Sabara, 2004, and references therein). A cumulative effect of all isolating mechanisms is apparent in the frequency of heteroploid crosses in a population.

In the present study, we observed dramatic differences in the frequency of heteroploid crosses between different cytotype combinations, irrespective of the taxonomic identity of the plant material. Only two triploids were encountered among more than 5400 *Knautia* plants (adults + seedlings) from 292 populations cytotyped in the present study (<0·04 %). They both occurred in otherwise diploid populations (Haidhof bei Baden, no. 262, and a serpentine locality Bernartice, no. 263; see Supplementary Data), suggesting that a fusion of unreduced and reduced gametes is the most plausible mechanism giving rise to triploidy. No triploids have been detected among adult plants or seedlings in mixed 2*x*+ 4*x* populations, despite intensive ploidy screening. A virtual lack of adult triploids in our study is consistent with previous karyological surveys because, within the entire genus, triploidy has been reported only twice: (1) in the artificial interspecific hybrid *K. basaltica* Chassagne & Szabó × *K. arvernensis* (Briq.) Szabó in France (Breton Sitens and Cauderon, 1969), and (2) in a natural population of *K. tatarica* (L.) Szabó in Russia (Plaksina, 1999). Additional support for the evolution of strong reproductive barriers between diploids and polyploids is provided by a series of experimental crosses performed by Ehrendorfer (1962*a*) and Breton Sintes (1974, 1975). Certain ecological segregation of cytotypes in the zone of sympatry (e.g. sorting according to altitude or geomorphology), as observed in our study, may serve as an additional pre-zygotic barrier. Moreover, pollinator foraging behaviour as a consequence of different flower colours in some regions with ploidy mixing (violet in diploids vs. whitish in tetraploids due to the introgression of *K. kitaibelii*) may further contribute to reproductive isolation. However, the mating barrier between 2*x* and 4*x Knautia* cytotypes is not absolute, as revealed by the presence of a triploid seed recorded in a serpentine population exhibiting ploidy heterogeneity (Planý vrch, no. 278). It is therefore possible that interploidy crosses resulting in triploid seeds may occasionally occur in mixed $2x + 4x$ populations; however, the offspring are either non-viable or are later outcompeted due to their reduced fitness. It should also be noted that we cannot at present reject an alternative hybridization scenario involving unidirectional gene flow from 2*x* to 4*x* cytotypes via the formation of unreduced gametes from diploid parents and the production of tetraploid hybrids.

The increase in the genome copy number of *Knautia* seems to be associated with the relaxation of interploidy reproductive barriers. In the present study, we recorded individuals having intermediate ploidy levels in four out of five mixed $4x + 6x$ populations, even though these cytotypes corresponded to different parental species (*K. arvensis* and *K. dipsacifolia*, respectively). In fact, pentaploid crosses were present in all three stages examined (i.e. adult plants, seedlings and seeds). Although both parental species have distinct ecological preferences (Štěpánek, 1997), individuals can coexist at ecotones such as forest margins, where they are highly susceptible to hybridization. Unlike Štěpánek (1997), we are convinced

that pentaploid hybrids are fertile and may further influence the evolutionary dynamics of mixed-ploidy populations. Vigorous and regularly flowering plants that set well-developed seeds were observed in population Horní Planá (no. 288). Pentaploids often seem to form aneuploid seeds, as indicated by the pronounced variation in fluorescence intensity observed for such samples. In addition, gametes having a euploid number of chromosomes may be involved in backcrossing with parental species. The incidence of a presumably tetraploid seed on a 5*x* maternal plant (Table 3) and the discovery of an adult hexaploid with divided leaves (i.e. a characteristic otherwise restricted to tetraploids) in a mixed-ploidy population both support this hypothesis. The observed ploidy-specific reproductive behaviour of *Knautia* (strong mating barriers between 2*x* and 4*x* cytotypes vs. certain compatibility between different polyploids) closely matches the situation reported about five decades ago for *Achillea*, Asteraceae (Schneider, 1958).

Conclusions

In the present study, it has been demonstrated that *Knautia arvensis* is an intriguing taxon that shows variation in both genome copy number and monoploid genome size. Diploid and tetraploid cytotypes exhibit spatial segregation at all the geographical scales examined, including the Central European portion of the distribution range, the zone of ploidy overlap, the mixed-ploidy populations, and the selected mixed-ploidy plots within such populations. Distributional and phenotypic data support the existence of both primary and secondary zones of cytotype contact. Irrespective of taxonomic affinity, ploidy level seems to be the major determinant of the strength of interploidy reproductive isolation. In addition, our study also highlights the importance of involving different ontogenetic stages when assessing evolutionary processes in mixed-ploidy populations. Collectively, *Knautia arvensis* provides a unique model system for studying the evolutionary dynamics of populations exhibiting ploidy heterogeneity, and for examining ecological and genetic circumstances that govern the interactions between different cytotypes in mixed populations.

SUPPLEMENTARY DATA

Supplementary information is available online at www.aob.oxfordjournals.org and provides details of sample localities together with genome size values and chromosome numbers for the *Knautia* accessions analysed.

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Bringing Together Evolution on Serpentine and Polyploidy: Spatiotemporal History of the Diploid-Tetraploid Complex of *Knautia arvensis* (Dipsacaceae)

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Abstract

Polyploidization is one of the leading forces in the evolution of land plants, providing opportunities for instant speciation and rapid gain of evolutionary novelties. Highly selective conditions of serpentine environments act as an important evolutionary trigger that can be involved in various speciation processes. Whereas the significance of both edaphic speciation on serpentine and polyploidy is widely acknowledged in plant evolution, the links between polyploid evolution and serpentine differentiation have not yet been examined. To fill this gap, we investigated the evolutionary history of the perennial herb *Knautia arvensis* (Dipsacaceae), a diploid-tetraploid complex that exhibits an intriguing pattern of eco-geographic differentiation. Using plastid DNA sequencing and AFLP genotyping of 336 previously cytotyped individuals from 40 populations from central Europe, we unravelled the patterns of genetic variation among the cytotypes and the edaphic types. Diploids showed the highest levels of genetic differentiation, likely as a result of long term persistence of several lineages in ecologically distinct refugia and/or independent immigration. Recurrent polyploidization, recorded in one serpentine island, seems to have opened new possibilities for the local serpentine genotype. Unlike diploids, the serpentine tetraploids were able to escape from the serpentine refugium and spread further; this was also attributable to hybridization with the neighbouring non-serpentine tetraploid lineages. The spatiotemporal history of *K. arvensis* allows tracing the interplay of polyploid evolution and ecological divergence on serpentine, resulting in a complex evolutionary pattern. Isolated serpentine outcrops can act as evolutionary capacitors, preserving distinct karyological and genetic diversity. The serpentine lineages, however, may not represent evolutionary 'dead-ends' but rather dynamic systems with a potential to further influence the surrounding populations, e.g., via independent polyplodization and hybridization. The complex eco-geographical pattern together with the incidence of both primary and secondary diploid-tetraploid contact zones makes *K. arvensis* a unique system for addressing general questions of polyploid research.

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Introduction

Serpentine soils, characterized by specific chemical (i.e., low Ca/Mg ratio, high heavy metal content, low nutrient availability) and physical (e.g., drought) properties, strongly influence the plant life that grows on them [1,2]. Although serpentines cover only 1% of dry land surface [3], they are nearly ubiquitous. The worldwide occurrence of serpentine-specific plant endemism highlights the global significance of serpentines in creating and preserving plant diversity. For example, more than 10% of the endemic Californian flora is restricted to serpentines, although serpentine soils make up less than 1% of the state's surface [4].

From an evolutionary point of view, serpentine-rich areas represent 'natural laboratories', allowing researchers to address various evolutionary questions of general significance [1]. The unique features of serpentine soils can shape plant evolution in two main ways [5–7]. Firstly, they can act as a selective factor, picking tolerant genotypes out of mainly non-tolerant gene pools of potential colonizers. Such disruptive selection may result in ecotypic differentiation [8–10] and, provided that reproductive isolation is achieved, it may lead to sympatric or parapatric speciation of serpentine endemics on the border of serpentine area [5,7,11]. Secondly, the exclusion of many non-tolerant species from serpentine sites makes the localities a 'light island', where competitively weak but tolerant species can thrive. During dramatic environmental changes such as the climate fluctuations during the Holocene, non-serpentine populations may become regionally extinct due to massive vegetation shifts such as the postglacial reforestation. The surviving relict serpentine populations could then differentiate by means of allopatric speciation into

separate taxa [12,13]. Considering the island-like distribution of serpentine outcrops [4,6], the spatially isolated populations of a serpentinophyte can ultimately give rise to several local endemics [14]. The evolutionary history becomes even more complicated if the serpentine populations come into secondary contact with their non-serpentine counterparts (e.g., after the progenitor's re-invasion) and hybridize [15].

Serpentines may be viewed as an environmental trigger that can catalyze any evolutionary process [5]. Polyploidy (genome duplication), as a ubiquitous phenomenon in plants [16,17], is generally acknowledged as a leading force in plant sympatric speciation [18]. Amongst other, polyploid taxa can have wider ecological amplitudes in comparison with their diploid counterparts, and this may result in distinct eco-geographic patterns [19–22]. Autopolyploids, i.e., polyploids with all sets of chromosomes derived from the same species, are particularly useful for studying ecological consequences of genome duplication because (i) di- and polyploid cytotypes are genetically very similar, and (ii) recurrent origins of autopolyploids may give rise to several lineages evolving under different selective pressures [23–25]. Despite the wide range of knowledge documented on the individual processes of serpentine and polyploid evolution, virtually no information is available on how these processes act in concert. Two scenarios, how serpentine differentiation interacts with polyploidy, can be invoked: (i) challenging abiotic conditions of serpentine habitats might support their colonization by more plastic polyploids, and (ii) low competitive environment of serpentine outcrops might enable relict survival of diploid lineages. To date, however, the relationships between evolution of serpentinophytes and karyological variation have been studied in a few diploid [26] or polyploid [27] plant groups and the results showed no clear patterns in the distribution of cytological variation and/or serpentine preferences.

The common European herb *Knautia arvensis* (Dipsacaceae) and its closest relatives constitute an intricate diploid-tetraploid complex exhibiting a distinct serpentine vs. non-serpentine habitat differentiation pattern in central Europe [19,28,29] and therefore provide an ideal system for investigations of the concerted action of genome duplication and a serpentine syndrome in plant evolution. Polyploidy, allopatric differentiation, and frequent homoploid hybridization are considered the major forces in the evolution of the complex; their interactive effects resulted in ambiguous species delimitation and fairly provisional taxonomic concepts [28,30]. In contrast to frequent homoploid hybridization, strong reproductive barriers exist between 2× and 4× *Knautia* plants as indicated by the lack of triploid hybrids in sites with cytotype mixtures [31] and both tri- and tetraploid hybrids in artificial crossing experiments [28,30,32].

There are two to three species of *K. arvensis* agg. in central Europe, which show a distinct pattern of geographic, karyological and edaphic differentiation (Fig. 1). In addition to the West Carpathian endemic tetraploid taxon *K. kitaibelii* (Schult.) Borbás, the widespread *K. arvensis* (L.) Coult. s.str. falls into two mostly parapatric cytotypes: diploids ($2n = 2\times = 20$) occurring mainly in the southeastern part of central Europe, and tetraploids ($2n = 4\times = 40$) occupying the northwestern half of the region. These two cytotypes are morphologically very similar and both prefer semiruderal mesophilous grasslands influenced by man [33]. In addition, several spatially isolated diploid populations of *K. arvensis* s.str. have been detected in markedly different habitats such as open pine forests on serpentine outcrops and subalpine grasslands in a glacial cirque [34–36] (Fig. 1). Open pine forests and subalpine communities of central Europe are regarded as classical examples of relict stands (i.e., supporting vegetation similar to that in the early Holocene [37]) that preserve significant plant diversity by providing an environment with low competitive pressure [13,38,39]. Moreover, similar relict habitats are preferred by *K. slovaca* Štěpánek, a diploid endemic taxon of central Slovakia with an unresolved taxonomic position, which was formerly not distinguished from *K. arvensis* s.str. [40] (Fig. 1). Interestingly, *K. arvensis* populations from relict stands and *K. slovaca* share identical genome size, significantly different from widespread semiruderal *K. arvensis* diploids [31]. For the sake of simplicity the two diploid groups with distinct genome size and habitat preferences will be termed 'relict' and 'non-relict' diploids hereafter. Finally, a serpentine tetraploid cytotype occurs in one serpentine area (the Slavkovský les Mts.; see inset in Fig. 1), forming both ploidy-uniform populations and diploid-tetraploid cytotype mixtures. Independent *in situ* autopolyploidization from local relict diploids has been suggested based on very similar morphology and ecological preferences [34], identical monoploid genome size, and co-occurrence of both cytotypes in several populations [31].

We employed two molecular markers that provide complementary information (AFLPs and plastid DNA sequences) to elucidate the evolutionary connection between evolution on serpentine and polyploidization in 40 populations of the *K. arvensis* agg. from Central Europe. This geographic restriction is justified by preliminary sequence and AFLP data (I. Rešetnik, P. Schönswetter & B. Frajman, unpubl.) suggesting that all the relict diploid central European populations of *K. arvensis* are genetically divergent from those elsewhere, e.g. on the Balkan Peninsula. Here, we addressed the following questions: (1) What are the genetic relationships among the species, cytotypes, genome size groups, and edaphic types within central Europe? (2) Is there any genetic differentiation at the diploid level? Do the two diploid groups with distinct genome sizes and divergent habitat preferences (i.e., relict and non-relict diploids) also represent separate genetic lineages? If so, is there any further genetic sub-structuring, e.g., according to geography and/or occupied habitat? (3) Did the serpentine tetraploids originate by recurrent (auto)polyploidization or by colonization of serpentine sites by non-serpentine tetraploids? (4) What are the relationships among serpentine and surrounding non-serpentine tetraploids? Is there indication of hybridization across the borders of serpentine areas?

Materials and Methods

Field Sampling

Plant materials were sampled from 2005 to 2008 in the Czech Republic, Slovakia, Hungary, Austria, Germany, and Ukraine. Because our study aimed at elucidating the evolutionary history of the complex in central Europe, with a particular attention to serpentine populations, the sampling scheme has been adapted to this purpose. Specifically, 34 populations of *K. arvensis* s.s., two populations of both *K. kitaibelii* and *K. slovaca*, and two populations of the introgressive hybrid of *K. arvensis* s.s. and *K. kitaibelii* (determined by morphology according to ref. [33]) were investigated. The resulting set of 40 populations covered the entire taxonomic, morphological and karyological diversity of *K. arvensis* agg. in central Europe. More intense sampling was performed in a serpentine 'archipelago' of the Slakovský les Mts. (western Bohemia), where large ecological and ploidy variation (including mixed-ploidy populations) was detected in our previous study [31]. Diploid and tetraploid subpopulations at two mixed-ploidy sites from this area (P04+ P20 and P05+ P21; see Table 1) were treated as separate populations in all analyses, considering strong inter-ploidy reproductive barriers [30–32]. At each locality information on the habitat type was gathered, accompanied by data from

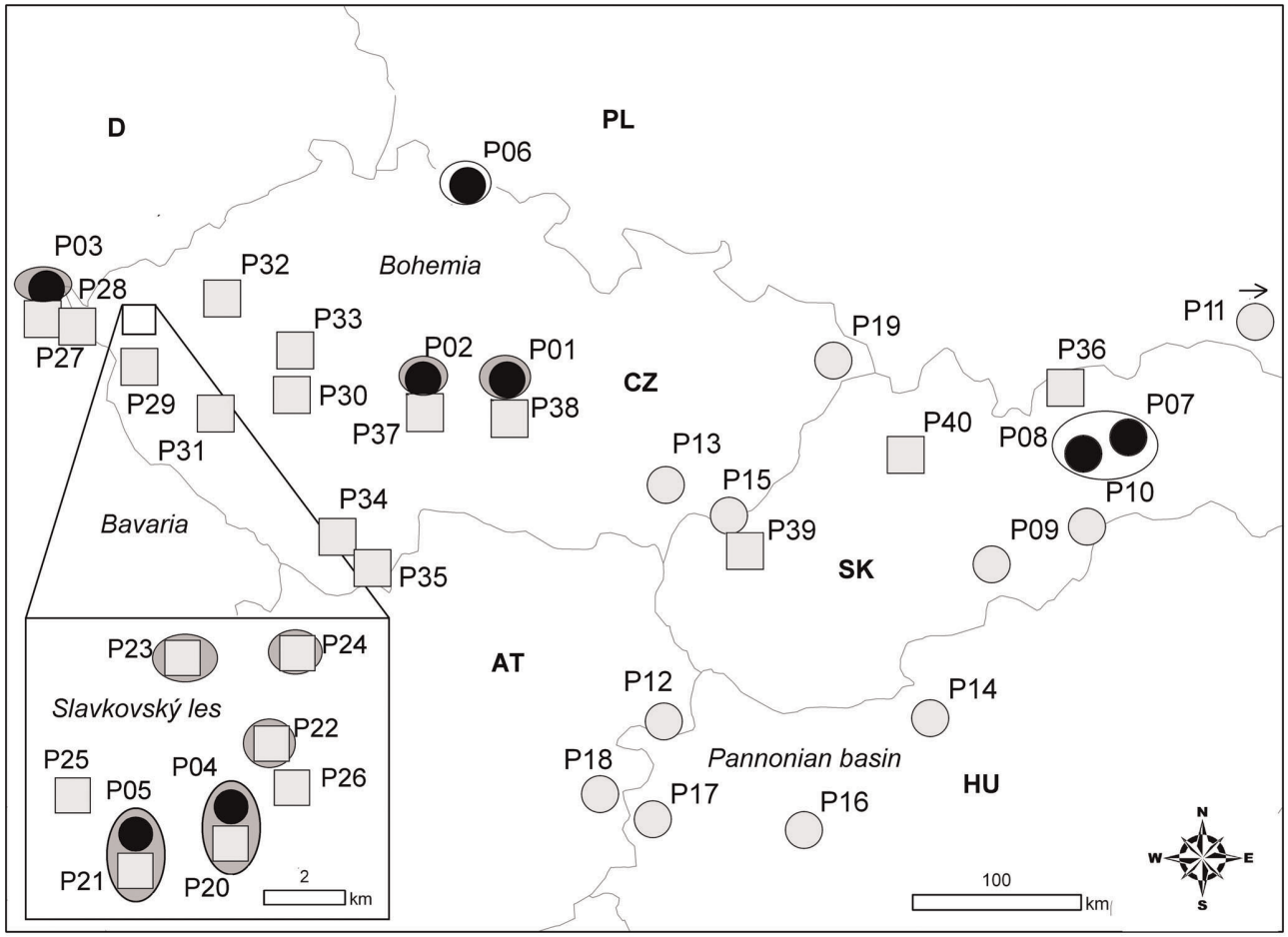

Figure 1. Ploidy level, genome size and habitat differentiation of the examined populations of *Knautia arvensis* agg. Light grey circles – diploids from 'non-relict' genome size group, black circles – diploids from 'relict' genome size group, squares – tetraploids, white ovals – relict limestone habitats (open pine forests or subalpine grasslands), grey ovals – relict serpentine pine forests; the remaining populations inhabit semiruderal grasslands (ploidy levels according to ref. 31). The map covers the region of eastern part of central Europe, the inset displays the situation in the diploid-tetraploid serpentine area in the Slavkovský les Mts.
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geological maps (scale 1:25000; www.geology.cz) and vegetation surveys (e.g., ref. [41]); the status of serpentine sites has been also confirmed by soil analyses (R. Sudová et al., unpubl.). Leaves from approximately ten plants per population were collected and quickly desiccated in silica gel; to avoid collecting same genets, the distance between sampled individuals was at least 1 m. For each individual, flow cytometric results gained in our previous study [31] were available. The species under investigation is neither endangered nor protected and no specific permits were required to collect the plant samples at studied sites. Locality details, ploidy levels, genome size groups, and numbers of analyzed plants are summarized in Table 1. Vouchers have been deposited in the herbarium of the Faculty of Science, University of South Bohemia, České Budějovice (CBFS).

AFLP Amplification and Scoring

Total genomic DNA was extracted using the Invisorb Spin Plant Mini Kit (Invitek) following the manufacturer's instructions. In total, 336 individuals from 40 populations were analyzed for AFLPs using the AFLP Core Reagent Kit I (Invitrogen) and AFLP Pre-Amp Primer Mix I (Invitrogen). Restriction, ligation and pre-amplification followed Rejzková et al. [42], but with the restriction phase extended to five hours. Selective amplification was performed using 2.3 μL of 10 times diluted pre-amplification product as a template, 1 μL of 10× buffer for Ampli Taq Gold (Applied Biosystems), 0.2 mM dNTPs (Fermentas), 0.05 μM of EcoRI-selective fluorescence-labelled primer (Applied Biosystems), 0.25 μM of MseI-selective primer (Applied Biosystems), 0.5 U of Ampli Taq Gold (Applied Biosystems), 0.5 μL of 1.25 mM $MgCl_2$ (Applied Biosystems) and 4.7 μL of ddH_2O (total volume 9.8 μL). Three primer combinations were used for selective amplification: EcoRI-ACA (6-FAM labelled) + MseI-CTG, EcoRI-ACC (NED labelled) + MseI-CTC, and EcoRI-ACG (HEX labelled) + MseI-CTA. The reaction was placed in a Mastercycler ep gradient S thermal cycler (Eppendorf). Reaction conditions were an initial step of 2 min at 94°C, 30 s at 65°C and 2 min at 72°C, followed by eight cycles of 1 s at 94°C, 30 s at 64°C (reduced by 1°C per cycle), 2 min at 72°C, followed by 23 cycles of 1 s at 94°C, 30 s at 56°C, 2 min at 72°C, with a final extension time of 30 min at 60°C. For each sample, 1 μL of each 6-FAM-, NED- and HEX-labelled selective PCR product was pooled and precipitated using an ethanol/sodium acetate precipitation. The precipitate was resuspended in 10 μL deionized formamide and combined with 0.25 μL of GeneScan-ROX-500 size standard (Applied Biosys-

Table 1. Details on the 40 populations of *Knautia arvensis* agg. included in the study.

Code	Locality name [a]	Ploidy level	Habitat [b]	Genome size group [c]	Taxon [d]	N	DW [e]	Nei's gene diversity	FRAG [f]	% POLY [g]	cpDNA sequences [h]	Locality no. [i]
P01	CZ – Staré Ransko	2×	R-S	2×R	*K. arv.*	10	**0.61**	0.165	**93**	41.1	H (2)	71
P02	CZ – Borovsko	2×[+]	R-S	2×R	*K. arv.*	10	**0.56**	0.167	**91**	**44.2**	A (1), F (3)	263
P03	D – Woja	2×	R-S	2×R	*K. arv.*	10	**0.51**	0.137	87	34.9	A (2), B (1), G (1), I (1)	279
P04	CZ – Planý vrch (2×)	2×	R-S	2×R	*K. arv.*	10	0.31	0.141	81	36.4	D (3), L (1)	278
P05	CZ – Vlček (2×)	2×	R-S	2×R	*K. arv.*	4	**0.53**	0.158	83	28.7	–	277
P06	CZ – Krkonoše	2×	R-C	2×R	*K. arv.*	8	0.27	0.169	75	**45.0**	E (4)	72
P07	SK – Branisko	2×	R-L	2×R	*K. slov.*	10	0.29	0.143	76	38.8	A (3)	286
P08	SK – Lesnica	2×	R-L	2×R	*K. slov.*	8	0.38	**0.182**	83	41.9	–	284
P09	SK – Podrečany	2×	N	2×N	*K. arv.*	9	0.37	0.157	75	39.5	A (1)	58
P10	SK – Plešivec	2×	N	2×N	*K. arv.*	11	0.43	0.163	80	43.4	A (2)	61
P11	UA – Lviv	2×	N	2×N	*K. arv.*	5	0.29	0.147	68	29.5	–	70
P12	AT – Apetlon	2×	N	2×N	*K. arv.*	9	0.43	0.162	77	41.1	–	2
P13	CZ – Archlebov	2×	N	2×N	*K. arv.*	8	0.44	**0.175**	74	43.4	A (1)	31
P14	HU – Csobánka	2×	N	2×N	*K. arv.*	9	0.38	0.139	70	35.7	–	50
P15	CZ – Javorník	2×	N	2×N	*K. arv.*	9	0.37	0.173	78	44.2	–	19
P16	HU – Veszprém	2×	N	2×N	*K. arv.*	10	0.46	**0.202**	88	**57.4**	A (1), J (1), M (1)	48
P17	HU – Szombathely	2×	N	2×N	*K. arv.*	10	0.43	**0.198**	**92**	**52.7**	–	49
P18	AT – Bernstein	2×	N	2×N	*K. arv.*	10	0.41	0.135	73	35.7	A (1)	1
P19	CZ – Morávka	2×	N	2×N	*K. arv.*	5	0.43	0.166	72	33.3	–	46
P20	CZ – Planý vrch (4×)	4×	R-S	4×	*K. arv.*	10	0.43	0.121	86	33.3	A (2), D (2), K (1)	278
P21	CZ – Vlček (4×)	4×	R-S	4×	*K. arv.*	9	**0.47**	0.116	**89**	22.9	A (2), K (1)	277
P22	CZ – Pluhův bor	4×	R-S	4×	*K. arv.*	11	0.39	0.132	88	40.3	A (4), B (1)	259
P23	CZ – Křížky	4×	R-S	4×	*K. arv.*	10	0.30	0.111	81	31.8	A (2)	260
P24	CZ – Dominova skalka	4×	R-S	4×	*K. arv.*	9	0.22	0.118	70	31.0	A (3), B (1)	261
P25	CZ – Kladská	4×	N	4×	*K. arv.*	9	0.27	0.110	74	29.5	B (1)	257
P26	CZ – Mnichov	4×	N	4×	*K. arv.*	10	0.32	0.115	82	34.1	A (3)	258
P27	D – Döhlau	4×	N	4×	*K. arv.*	8	0.33	0.166	84	40.3	A (2), H (2)	242
P28	CZ – Libá	4×	N	4×	*K. arv.*	10	0.40	0.121	87	36.4	–	224
P29	CZ – Planá	4×	N	4×	*K. arv.*	10	0.33	0.157	87	41.9	B (2)	221
P30	CZ – Příbram	4×	N	4×	*K. arv.*	7	0.34	0.174	84	41.1	A (2), H (1), I (1)	217
P31	CZ – Přeštice	4×	N	4×	*K. arv.*	9	0.39	0.131	85	35.7	F (2)	215
P32	CZ – Blšany	4×	N	4×	*K. arv.*	10	0.33	0.137	77	36.4	F (2)	225
P33	CZ – Koněprusy	4×	N	4×	*K. arv.*	10	0.26	0.133	78	38.0	–	223
P34	CZ – Křemže	4×	N	4×	*K. arv.*	10	0.41	0.151	88	38.8	A (2)	144
P35	CZ – Benešov n. Černou	4×	N	4×	*K. arv.*	8	0.44	**0.202**	**90**	**50.4**	–	126
P36	SK – Relov	4×	N	4×	*K. arv.*	2	–	0.124	63	12.4	A (3)	256
P37	CZ – Bernartice	4×	N	4×	*K. arv.*×*kit.*	8	0.40	0.142	79	34.9	B (1)	216
P38	CZ – Ždírec n. Doubravou	4×	N	4×	*K. arv.*×*kit.*	8	0.36	0.127	80	31.8	A (1), B (1)	218
P39	SK – Pustá Ves	4×	N	4×	*K. kit.*	2	–	0.147	76	14.7	F (1)	281
P40	SK – Sklabiňa	4×	N	4×	*K. kit.*	1	–	–	51	–	C (1)	283

[a]AT – Austria; CZ – Czech Republic; D – Germany; HU – Hungary; SK – Slovak Republic; UA – Ukraine.
[b]R – relict habitat, i.e., serpentine (R-S) or limestone (R-L) outcrops or a subalpine glacial cirque (R-C); N – non-relict habitat (mostly semi-ruderal mesophilous grassland).
[c]2×R – relict diploid genome size group; 2×N – non-relict diploid genome size group; 4× – tetraploid genome size group according to ref. 31.
[d]*K. arv.* – *Knautia arvensis* s.s.; *K. kit.* – *Knautia kitaibelii*; *K. arv.*×*kit.* – *Knautia arvensis* × *K. kitaibelii*; *K. slov.* – *Knautia slovaca.*
[e]DW = weighted rarity index (only for populations with more than three individuals).
[f]number of fragments.
[g]percentage of fragments exhibiting intrapopulational polymorphism.
[h]list of different cpDNA haplotypes found in the population (numbers of sequenced individuals possessing the particular haplotype in brackets); for details see Fig. 4.
[i]Locality number in ref. 31 where details on geographic location of the localities as well as the results of flow cytometric analyses are provided.
[+]a single triploid individual detected within population P02 was included in the AFLP analysis.

tems). Fragments were resolved on a 3100 Avant Genetic Analyzer and scored with GeneMarker v 1.8 (www.SoftGenetics.com). Thirty-nine samples (12% of all samples) were re-analyzed by repeating the whole AFLP procedure from the extracted DNA onward in order to test reproducibility of the data by estimating the average proportion of correctly replicated bands [43]. Only bands in the range of 100–500 bp, which could be scored unambiguously, were included; those found by comparing replicate runs not to be reproducible were excluded from the analyses. The resulting presence/absence matrix was used in subsequent analyses.

Plastid DNA Sequencing

Plastid DNA haplotype variation was assessed to complement the information given by the mainly nuclear AFLPs. The *pet*N(*ycf6*)–*psb*M region was sequenced for 77 accessions representing all the groups indicated by the AFLP analysis (see Table 1). More thorough haplotype sampling was performed in populations from the Slavkovský les serpentine area (i.e., a region with potentially recurrent polyploidization). PCR amplification with the primers *ycf6*F and *psb*MR of Shaw et al. [44] was carried out in a volume of 20 µl reaction using 5 ng of template DNA, 2 µl of 10× reaction buffer (Sigma), 0.4 µl of 10 mM dNTP mix (Fermentas), 6.25 pmol of each primer and 0.5 U of Jump Start REDTaq DNA Polymerase (Sigma) on a Mastercycler ep gradient S thermal cycler (Eppendorf) with initial denaturation at 94°C for 2 min, 35 cycles of 1 min denaturation at 94°C, 1 min annealing at 55°C and 2 min extension at 72°C, followed by 10 min final extension at 72°C. Amplification products were subsequently purified using the JetQuick PCR Purification Kit (Genomed). Sequencing reactions were performed using BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) according to the manufacturer's instructions using the primers cited above. Purification of sequencing reactions was carried out using an ethanol/sodium acetate precipitation. Products were run on an ABI 3130 Genetic Analyzer (Applied Biosystems).

AFLP Data Analyses

Nei's gene diversity [45] (termed 'genetic diversity' in the following), an estimator of local genetic diversity that can be applied regardless of the ploidy level [46], was computed for each population with the R-script AFLPdat [47]. The same tool was used for the calculation of a rarity index by computing 'frequency-down-weighted marker values' per population (DW) [48]. Only populations with a sample size of more than three individuals were included in the computations. The DW is higher in populations or groups that harbour a high number of rare markers [49]. A two-tailed t-test (calculated using Statistica 8.0) was used for testing the differences in the DW and genetic diversity among particular groups defined by ploidy level and/or genome size.

The genetic structure was inferred using three independent approaches. (1) A non-model-based approach, nonhierarchical K-means clustering [50], was chosen because of the presence of two ploidy levels, and performed using a script of Arrigo et al. [51] in R. This approach has recently been successfully applied in the analysis of genetic structure of the AFLP dataset in polyploid complexes [51,52]. We performed 50,000 independent runs (i.e., starting from random points) for each assumed value of K clusters ranging from 2 to 10. The first run yielding a positive value for the second derivative of the inter-cluster inertia was considered [52]. (2) In the model-based Bayesian clustering approach implemented in STRUCTURE version 2.2 [53,54], the number of clusters was estimated using 10^6 iterations, with a burn-in period of 10^5 iterations under an admixture model with recessive alleles. The number of clusters (K) was used as a prior value; ten replicates for each K were analyzed from K = 1 to K = 10. All analyses using STRUCTURE were carried out at the Bioportal of the University of Oslo (www.bioportal.uio.no). To determine the most likely number of clusters we followed the approach of Evanno et al. [55] implemented in Structure-sum-2009 [47]. After the optimal grouping was determined, each group was analyzed separately under the same settings used for the main analysis. (3) K-means and STRUCTURE clustering results were independently displayed on a principal coordinate analysis (PCoA) computed with the R package ADE-4 [56] based on a Jaccard distance matrix of the AFLP data. Finally, congruence of the two different clustering techniques was compared and tested using a contingency table (calculated in Statistica 8.0) and displayed on a map using ARCGIS 9.3 (ESRI).

The partitioning of genetic variation among the populations, species, cytotypes, and genome size groups was quantified using analyses of molecular variance (AMOVA). AMOVAs were conducted in Arlequin 3.11 [57]. For nested AMOVAs, the populations were divided into: (i) three species (*K. arvensis* s.str., *K. kitaibelii*, and *K. slovaca*); (ii) two ploidy levels (2×, 4×); and (iii) three main groups according to their ploidy level and monoploid genome size that also well correlated with the geographic distribution and habitat preferences (i.e., non-relict diploids, relict diploids, and tetraploids). This approach allowed us to assess the structuring of genetic variation according to both (i) traditional taxonomic concepts, and (ii) the patterns of eco- and cyto-geographical variation, irrespective of taxonomic assignments. In addition, separate AMOVAs were conducted for the mixed-ploidy area in the Slavkovský les Mts. in order to examine the level of differentiation among the diploid and putatively locally originated tetraploid cytotypes.

Plastid DNA Data Analyses

Plastid DNA sequences were edited using Finch TV (Geospiza) and aligned in the MAFFT 6 online application using the default mode [58]. Haplotype networks were constructed using TCS version 1.21 [59], treating gaps as a fifth character state. For this purpose, insertions/deletions longer than 1-bp were treated as single-step events. The sequences together with voucher numbers are available at GenBank (accession no. HM597685-HM597697 for haplotypes A-M).

Results

AFLP Data

The three AFLP primer combinations yielded 129 clear polymorphic fragments (for primary data matrix see Table S2). Based on 39 replicates, the reproducibility of the dataset was 95%. All 336 individuals had different AFLP phenotypes. Genetic diversity (Table 1) varied approximately two-fold, from 0.110 in population P25 (non-relict 4×) to 0.202 in populations P35 (non-relict 4×) and P16 (non-relict 2×). The level of genetic diversity was significantly higher in the diploid than in the tetraploid populations (two-tailed t-test, df = 37, t = 3.65, $p<0.001$, mean values of 0.162 and 0.137 for 2× and 4×, respectively). The rarity index (DW; Table 1) varied by a factor of three, from 0.22 in population P24 (relict 4×) to 0.61 in population P01 (relict 2×). The DW values of the diploid populations were significantly higher than those of the tetraploids (df = 35, t = 2.27, p = 0.030, mean DW of 0.42 and 0.36 for 2× and 4×, respectively). Interestingly, the highest DWs corresponded to four diploid populations from relict serpentine stands (P01, P02, P03, and P05; see Table 1); this was also reflected in the significantly higher DW

values of the serpentine diploids ($df = 35$, $t = 3.89$, $p < 0.001$). Notwithstanding, the group of relict populations as a whole did not have significantly different DW values ($df = 35$, $t = 0.99$, $p = 0.324$).

Nonhierarchical K-means clustering revealed an optimal separation of the dataset into seven groups (the second derivative of the inter cluster inertia was 1.99; Figure S1), mostly reflecting the ploidy level, genome size, and habitat differentiation. Separate clusters were formed by the (i) non-relict diploids (P09–P19), (ii) relict limestone diploids (P07 and P08, corresponding to *K. slovaca*), and (iii) eastern relict serpentine diploids (P01 and P02; see Fig. 2). The remaining western relict serpentine (P03–P05) and subalpine (P06) diploid populations were included in three clusters, which also contained tetraploid *K. arvensis* s.str. (clusters K5, K6, and K7). In addition, one exclusively tetraploid cluster (K4), formed by *K. arvensis* s.str. and *K. kitaibelii* populations, was recognized (Fig. 2). STRUCTURE analysis of the entire data set revealed two main groups comprising (i) non-relict diploids, and (ii) relict diploids + all tetraploids (the highest, 0.99, similarity among runs and the highest delta K; Figures S2A and S2D). Separate STRUCTURE analyses, run for each main group (excluding the two populations P07 and P08 that were highly admixed in the previous STRUCTURE analysis of the entire dataset, Figure S3A), revealed no clear substructure within the non-relict diploids (a decreasing pattern of likelihood together with similarity coefficients below 0.36; Figures S2B and S2E), while the second main group was further divided into seven sub-groups (high, 0.97, similarity among runs and the highest delta K; Figures S2C and S2F). The STRUCTURE groups (Figure S4) were congruent with the K-means clusters (chi-square = 924, $df = 54$, $p < 0.0001$; for details see Table 2). High levels of congruence were achieved at the diploid level; the entirely diploid clusters were fully congruent and only four diploid individuals were assigned to a different STRUCTURE vs. K-means group in the remaining clusters. Several tetraploid individuals were assigned to different clusters in K-means vs. STRUCTURE clustering, what probably reflects generally lower genetic distinctness at the tetraploid level (as was also illustrated by higher genetic admixture of tetraploids, Figure S3B).

The seven K-means clusters were also visible on the PCoA plot (Fig. 3a). The first axis (explaining 24.1% of the total variation) corresponds to the main split in the dataset, i.e., the separation of non-relict diploids (cluster K1) from the remaining samples (all tetraploids + relict diploids). Within the 4×+relict 2 group, the eastern serpentine populations (P01 and P02; cluster K3) and *K. slovaca* (P07 and P08; cluster K2) are well separated from the remaining clusters (Fig. 3b). Results of the STRUCTURE clustering are displayed in Figures S5A and S5B.

AMOVA analyses (Table 3) attributed 37% of the overall genetic variation to the among-population component. In the nested AMOVAs, the variation between the two cytotypes accounted for 18.9% of the overall variation; conversely, species-based grouping explained only 4% of the variation. The highest values of among-population differentiation were found within the relict diploid group (30.5%), whereas the non-relict diploid populations were the least differentiated (14.3%). Interestingly, separate analysis of the mixed-ploidy area of the Slavkovský les Mts. yielded a fairly high (22.9%) inter-population variation while the differentiation between the local 2× and 4× cytotypes was negligible (0.7%).

Plastid DNA Data

Sixteen variable positions (including three coded indels) out of 497 aligned positions were detected. In total, 13 haplotypes were identified within the 77 sequences (Table 1). Half of the accessions belonged to the widespread haplotype A (Fig. 4), regardless of ploidy level, genome size or habitat preference. Globally, AFLP and plastid DNA data sets were not congruent (chi-square = 79.5, $df = 74$, $p = 0.26$; e.g., individuals from all AFLP groups possessed the single central haplotype A, for details see Table S1). Despite this, some interesting insights can be gained from the data. First, derived haplotypes of non-relict diploid populations (cluster K1) were not found in other populations; on the other hand, the relict diploids often shared haplotype with tetraploids (haplotypes B, D, F, H, and I; Fig. 4). Second, the isolated subalpine diploid population P06 from the cluster K6 is exclusively characterized by a 12-bp insertion (haplotype E). Finally, the haplotype D is exclusively shared by diploid and tetraploid individuals from the same mixed-ploidy serpentine population Planý vrch (P04 and P20) from the Slavkovský les Mts. (see Fig. 2 for details on haplotype distribution).

Discussion

In this study, we took advantage of the 'full-factorial' pattern of ploidy variation (diploid vs. tetraploid cytotypes) and edaphic specialization (serpentine vs. non-serpentine populations) in *K. arvensis* agg. from central Europe in order to gain new insight into the evolutionary history of this polyploid plant system and, in particular, to assess how polyploid evolution can be connected with serpentine differentiation. Because of the incongruence between the traditional species delimitation and the inferred genetic structure we will discuss the evolutionary history of the central European populations of *K. arvensis* agg. regardless of their taxonomic assignment.

Differentiation at the Diploid Level

The most pronounced genetic differences within the central European *K. arvensis* agg. were observed at the diploid level. Specifically, the non-relict diploid populations from the Pannonian basin and the Polonian lowlands (P09–P19; cluster K1) formed the most distinct group in the AFLP dataset (Fig. 3a). Moreover, these non-relict diploids also clearly differed in the size of their monoploid genome, i.e., the Cx-value [31]. Variation in genome size is often regarded as an indication of cryptic differentiation or incipient speciation [60–63]. The non-relict diploids can thus be regarded as a very distinct lineage within the central European *K. arvensis* agg.

The remaining diploids (collectively called relict diploids) differ from their non-relict counterparts by smaller genome size [31] and habitat preferences (they mostly grow in open relict pine forests with specific edaphic conditions whereas non-relict diploids grow in anthropogenic semiruderal grasslands). The AFLP markers revealed two distinct genetic clusters within the relict diploids, representing two geographically and ecologically well-characterized lineages (Fig. 3b). One lineage inhabits pine forests on limestone in central Slovakia (cluster K2, corresponding to *K. slovaca*) while the other lineage grows in open pine forests on isolated serpentine outcrops in central Bohemia (cluster K3; Fig. 2). The remaining relict diploid populations (from serpentine outcrops in western Bohemia and a subalpine glacial cirque in eastern Bohemia) contain individuals from three fairly close clusters (K5, K6, and K7; Fig. 3b), all of them containing also tetraploid plants. Furthermore, the relict diploids also exhibited the highest levels of inter-population genetic differentiation (above 30%; Table 3) what is also in line with the high number of identified groups. Collectively, this marked genetic differentiation together with specific habitat requirements may reflect long-term persistence in isolated open island habitats serving as refugia during Holocene

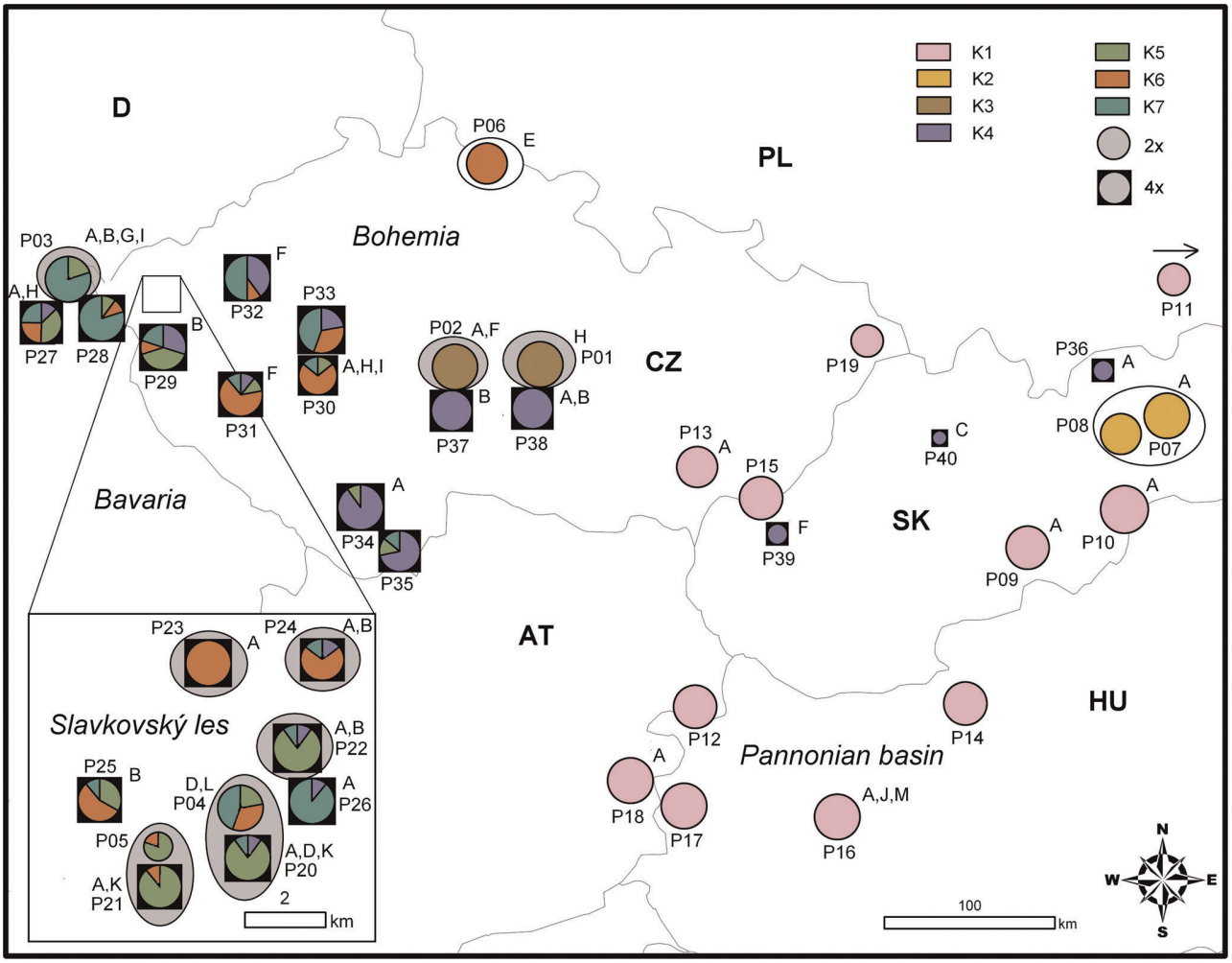

Figure 2. Phylogeographical grouping of 40 analyzed populations of *Knautia arvensis* agg. in central Europe. Grouping is according to the nonhierarchical K-means clustering of AFLP phenotypes. Pie charts represent the proportion of individuals belonging to each of the seven detected groups (K1–K7). The size of the pie chart reflects the sample size. The inset displays the situation in the Slavkovský les serpentine area. White ovals denote populations from relict limestone habitats (open pine forests or subalpine grasslands), grey ovals populations from relict serpentine pine forests. Note the presence of several relict diploid populations in the western part of the area (P03, P04, and P05) with the genetic composition highly similar to the surrounding tetraploids. The distribution of chloroplast haplotypes is indicated (A–M).
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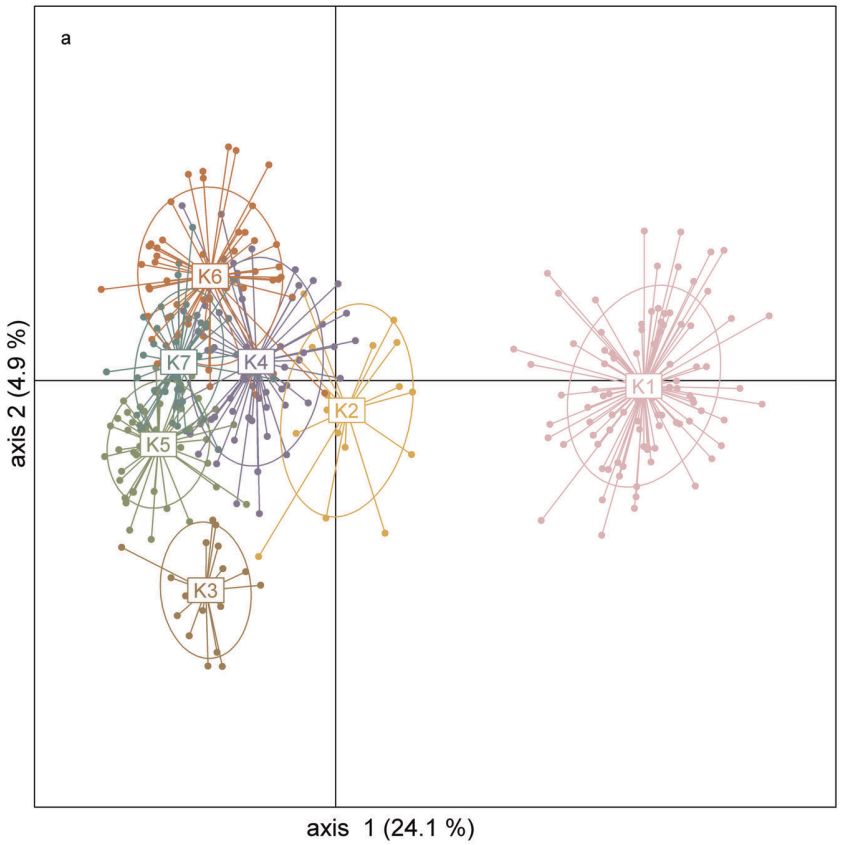
Table 2. Contingency table comparing the clustering results obtained by nonhierarchical K-means and STRUCTURE analyses (numbers of individuals are presented in each field).

	S1	S2	S3	S4	S5	S6	S7	S8	S9	NA
K1	95									
K2		**18**								
K3			**17**							
K4				*31*	*1*	*1*	*6*		*11*	*6*
K5					***45***	*1*				*1*
K6					***3***	*21*	***12***	**8**	***6***	*4*
K7					***44***	*1*			***3***	*1*

Different font styles denote cytotypes with distinct monoploid genome size in the particular field (regular = non-relict diploids only, bold = relict diploids only, italics = tetraploids only, bold italics = relict diploids and tetraploids).
doi:10.1371/journal.pone.0039988.t002

reforestation. For the serpentine populations, long-term persistence is further supported by the accumulation of rare AFLP fragments (significantly higher DW values; Table 1). In addition, despite generally low congruence among AFLP and plastid DNA data (resulting from low discriminative power of the cpDNA data and probably also reflecting the effects of ancestral polymorphism, hybridization and/or recurrent polyploidization), serpentine diploid populations are distinct by their high incidence of rare plastid DNA haplotypes (six out of twelve rare haplotypes; Table 1). A high frequency of rare genetic markers is generally acknowledged as strong evidence for the relict status [48,49,64]. The origin of these relict diploid lineages seems strongly connected to serpentine habitats and is discussed in the section 'Joining edaphic differentiation and polyploid evolution'. The non-exclusive hypothesis of independent immigration from other parts the range such as the Balkan Peninsula (i.e. diversity hotspot of the whole genus, see ref. [65]) is discouraged by phylogenetic data documenting an isolated position of the central European relict


a
K6
K7
K4
K2
K5
K1
K3
axis 2 (4.9 %)
axis 1 (24.1 %)

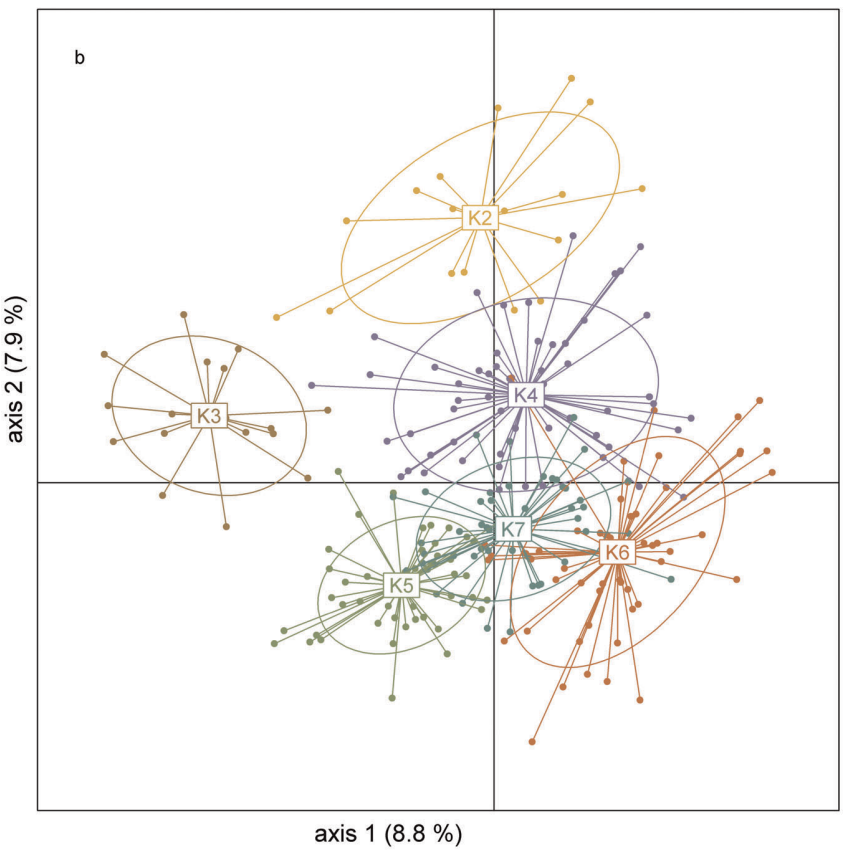

b
K2
K4
K3
K7
K6
K5
axis 2 (7.9 %)
axis 1 (8.8 %)

Figure 3. Principal coordinate analysis based on Jaccard similarity among AFLP multilocus phenotypes of *Knautia arvensis* agg. (a) entire data set; (b) excluding the most divergent group K1 (i.e., non-relict diploids). The different colours represent the groups identified by nonhierarchical K-means clustering (same as in Fig. 2). The centroid of each group and its connection with other points are displayed as well as an ellipse reflecting the variance of the group and the covariance on the axes.
doi:10.1371/journal.pone.0039988.g003

diploids among European diploid *Knautia* (I. Rešetnik, P. Schönswetter & B. Frajman unpubl.).

Recurrent Polyploidization

Recurrent origin is now widely recognized as a frequent component of polyploid evolution that is responsible for the marked diversity of many polyploid complexes [16,66]. Independently formed polyploid lineages can exhibit striking differences in morphology, ecology or genetic profiles, even if originating from the same ancestral source [67,68]. In addition, distinct lineages can meet and hybridize, which further increases variation at the polyploid level [24].

The serpentine 'archipelago' in the Slavkovský les Mts., unlike any other central European relict locality, harbours a tetraploid *Knautia* cytotype. Here, we argue that the serpentine tetraploids were formed independently from their non-serpentine counterparts by independent autopolyploidization from a local diploid cytotype. Close evolutionary relationships between the local serpentine di- and tetraploids have previously been suggested on the basis of phenotypic similarities and habitat preferences [34], as well as cytogeographical patterns and identical monoploid genome size values [31]. Molecular data further support the hypothesis of local auto-polyploid origin of the serpentine tetraploids. Firstly, the diploid populations from the Slavkovský les Mts. grouped together with the surrounding tetraploids (see Fig. 2). Secondly, the AMOVA analysis revealed low differentiation between the co-occurring di- and tetraploids explaining only 0.7% of the total genetic variation in the Slavkovský les Mts. (Table 3). Finally, several di- and tetraploid individuals from the population Planý vrch (P04 and P20) share the same unique 6 bp insertion in their plastid DNA (haplotype D; see Table 1). The alternative hypothesis of strong introgression of the tetraploid genotype into the diploids can be ruled out due to the virtual lack of triploid hybrids [31]. Unidirectional introgression of 2× genotypes into established tetraploids via unreduced gametes alone cannot sufficiently explain such a high genetic similarity between both cytotypes. First, strong inter-ploidy reproductive barriers were indicated by several crossing experiments [28,30,32]. Second, even if the breeding barriers were overcome, vast amounts of viable unreduced gametes would be necessary for dissolving the original 4× genetic pool, which contrasts with the low frequency of

Table 3. Analyses of molecular variance (AMOVA) of AFLP phenotypes of *Knautia arvensis* agg. grouped according to traditionally recognized species, ploidy levels, and cytotypes with distinct monoploid genome size values (according to ref. 31).

	d.f.	% of variation	Fst[a]
A. Complete dataset			
Among all populations	38	37.1	0.371
Within populations	*296*	*62.9*	
Species grouping			
Among species*	2	4.0	0.396
Among populations within species	*34*	*35.6*	
Within populations	*282*	*60.4*	
Ploidy level grouping			
Among all 2× vs. 4×	1	18.9	0.429
Among populations within groups	*37*	*24.0*	
Within populations	*296*	*57.1*	
Genome size grouping			
Among relict 2× vs. non-relict 2× vs. 4×	2	27.5	0.434
Among populations within groups	*36*	*15.9*	
Within populations	*296*	*56.6*	
Among populations of relict 2×	7	30.5	0.305
Within populations	*62*	*69.5*	
Among populations of non-relict 2×	10	14.3	0.143
Within populations	*84*	*85.7*	
Among populations of 4×	21	24.8	0.248
Within populations	*157*	*75.2*	
B. Only Slavkovský les area			
Among all populations in Slavkovský les	8	22.9	0.229
Within populations	*73*	*77.1*	
Among 2× vs. 4× in Slavkovský les	1	0.7	0.233
Among populations within groups	*7*	*22.6*	
Within populations	*73*	*76.7*	

[a]all p-values <0.001.
The two populations of an introgressive hybrid between *K. arvensis* and *K. kitaibelii* (P37, P38) were omitted from this analysis.
doi:10.1371/journal.pone.0039988.t003

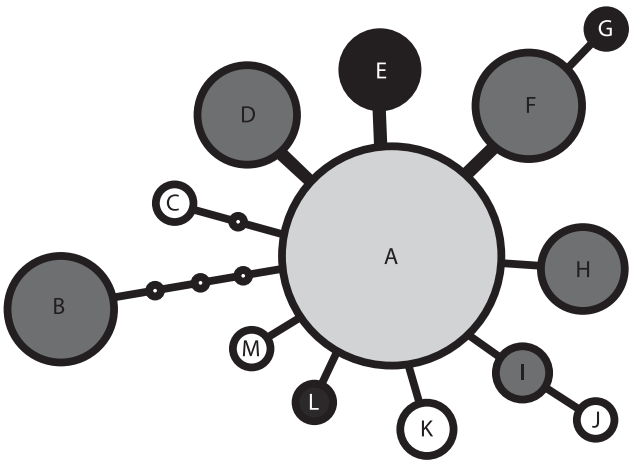


Figure 4. Network of 13 plastid DNA haplotypes found within 77 examined individuals of *Knautia arvensis* agg. The size of the circles is proportional to the number of individuals, while their shading indicates the ploidy level and monoploid genome size of the samples (black – relict 2× only, dark grey – relict 2×+4×, light grey – all 2×+4×, white – unique for a single non-relict 2× – haplotypes J and M – or 4× – haplotypes C and K – population). The double line indicates an insertion-deletion. For more detailed information, see Table 1.
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unreduced gametes formation in general [69,70], and in the *K. arvensis* agg. in particular [31]. Finally, there is no indication of across-ploidy genetic admixture in the other contact zone between the tetraploids and non-relict diploids in the Pannonian basin. To sum up, all lines of evidence such as genetics, cytology, morphology, and ecology point to at least one independent autopolyploidization event, which took place *in situ* in the Slavkovský les Mts., leading to an independent origin of serpentine tetraploids from local relict diploids.

The *K. arvensis* agg. exhibits two strikingly different types of contact zones between cytotypes in central Europe. The ploidy mixtures in the Slavkovský les Mts. arose as a result of *in situ* (auto)polyploidization (i.e., they are composed of almost identical genotypes) and thus fit well into the concept of a primary contact zone [71]. In contrast, ploidy-heterogeneous stands on the borders of other serpentine localities and, in particular, the diffuse contact zone among tetraploids and non-relict diploids in the Pannonian basin [31] represent zones of 2×/4× secondary contact where two distinct gene pools meet (see Fig. 2 and Fig. 3a). There are only a few other plant groups, including *Dianthus* [72,73] and *Melampodium* [74], for which both primary and secondary contacts have been suggested, but these have never been confirmed by molecular markers. According to our knowledge, *K. arvensis* agg. thus represents the first polyploid system for which the incidence of both established primary and secondary contact zones has been supported by molecular evidence.

Joining Edaphic Differentiation and Polyploid Evolution

Serpentines can shape plant evolution either by the selection of tolerant genotypes from the colonizing populations or by providing refugia in island-like serpentine outcrops [6,7]. In the latter case, vegetation shifts caused by climatic changes could cause local extirpation of the non-serpentine populations, while the subsequently isolated populations on serpentine may further evolve by means of allopatric differentiation and local adaptation into new taxa (i.e., the so-called 'depleted species' evolutionary scenario; [4]). The highly differentiated relict diploid populations of *K. arvensis* might fit into this model. Diploid ancestors may have been present in ice-free central Europe during the late Pleistocene as suggested by *Knautia* pollen records from the Allerød interstadial [75,76]. Subsequently, the heliophilous plants were restricted to serpentine, limestone or subalpine refugia by the expanding forest vegetation (see the example of relict *Knautia* serpentine habitat in Fig. 5). As a consequence of spatial isolation and population size fluctuations, mechanisms of allopatric differentiation could have taken place, ultimately leading to the genetic and morphological differentiation currently observed among the relict diploid populations (see Fig. 2; cf. [34,36]). Similar scenarios of speciation in isolated serpentine refugia were also suggested for several central European serpentine endemics – e.g., *Cerastium alsinifolium* [13], *Minuartia smejkalii* [77], and *Potentilla crantzii* subsp. *serpentini* [39]. Irrespective of the relative importance of allopatry vs. potential independent immigration, the highly differentiated diploid lineages within the *K. arvensis* agg. illustrate the significance of Holocene edaphic refugia for preserving rare and distinct genetic diversity.

Regarding the other *Knautia* lineages, i.e., tetraploids and non-relict diploids, it seems plausible that they immigrated into central Europe later as a result of human-induced landscape changes, such as deforestation, grazing, and meadow agriculture [34,36]. This hypothesis corresponds well with the current semi-ruderal habitat preferences of both lineages [33]. Further details on the relationships and evolutionary history of these lineages, however, cannot be inferred without more intensive sampling in other parts of the range of *K. arvensis* agg. A similar scenario of range contraction into serpentine refugia, followed by human-enhanced re-colonization by different genotypes, has been suggested for Scandinavian populations of *Silene dioica* [78].

In addition to the above-discussed 'depleted species-recolonization' scenario, the serpentine *Knautia* populations underwent

Figure 5. Serpentine outcrop covered by open pine forest near Borovsko, central Czech Republic (A). This locality probably served as a Holocene refugium for several rare plant taxa. Morphologically distinct 'relict diploid' cytotype of *Knautia arvensis* (B, population P02 in this study) also occurs at this site.
doi:10.1371/journal.pone.0039988.g005

independent polyploid evolution – a process not yet recorded in the evolution of any other serpentine relict. Moreover, it seems that the genome duplication opened new possibilities for the serpentine lineage. While the serpentine diploids appear to be unable to escape their refugia (probably because of their weak competitive abilities; [79]), the serpentine genotypes seem to have conquered surrounding non-serpentine areas at the tetraploid level (note the significant representation of the 'serpentine' clusters K5, K6, and K7 in adjacent non-serpentine populations; Fig. 2). The better competitive ability and higher phenotypic plasticity of the polyploids might have influenced this spread ([21,80], see [81] for a review). Indeed, wider ecological niches of tetraploids and their ability to survive in less stable human-influenced habitats have been repeatedly documented for the genus *Knautia* [19,82]. The spread of serpentine tetraploid genotypes far beyond serpentine areas could have been enhanced by hybridization with their non-serpentine counterparts (both lineages likely met and hybridized after human-induced deforestation). Strong introgression at the tetraploid level (marked admixture of AFLP groups in tetraploids; Figure S3) seems to be ubiquitous in the genus *Knautia* [28,33,35] and has also been suggested for the Slavkovský les Mts. on the basis of morphology (e.g., non-serpentine tetraploids with 'serpentine-characteristic' reddish corolla colour; [34]). Similar to Californian oaks [15], such 'across-serpentine-border' hybridization might have played a crucial role in creating new genotypes capable of colonizing new sites.

Collectively, the intricate evolutionary history of the *K. arvensis* agg. (Fig. 5) seems to be comparable only with the 'multi-step' evolutionary scenario of the Californian serpentine herb *Streptanthus glandulosus* (Brassicaceae), which underwent habitat restriction, area fragmentation, and subsequent independent evolution in isolated serpentine populations [12,14,83]. Nevertheless, the pronounced role of polyploidy in the whole evolutionary story, both as a background source of differentiation (i.e., concerted edaphic and polyploid speciation) and as a directly acting evolutionary force (i.e., independent genome duplication of serpentine relicts), seems to be a unique evolutionary pathway, firstly documented in the *K. arvensis* agg.

Conclusions

Multifaceted interactions among ecological differentiation and polyploid evolution resulted in a unique evolutionary pattern exemplified by *Knautia arvensis* agg. A wide variety of processes and mechanisms likely took part in the rapid evolution of this complex, including isolation in Holocene refugia, repeated colonization by distinct lineages, hybridization, and recurrent polyploidization. The key role of the serpentine substrate in this scenario arises from its ability to serve as a refugium for particular lineages (in this case, relict diploid lineages). Such lineages could further evolve into distinct types, not only at the homoploid level, but also via independent genome duplication. The recurrently formed polyploids seem to be able to escape from their original refugia, indicating that the serpentine relicts are not evolutionary dead-ends but still have the potential to shape the surrounding populations. Generally, the *K. arvensis* agg. provides a unique system that illustrates the various ways in which the polyploid and serpentine evolution could act together in generating plant diversity. In addition, the genetic data strongly support previous hypotheses regarding the presence of both primary and secondary ploidy contact zones for *K. arvensis* agg., which offers exciting possibilities for addressing general questions about patterns, mechanisms, and dynamics of polyploid evolution.

Supporting Information

Figure S1 Second derivative of the inter cluster inertia of each number of groups (K) as estimated by the nonhierarchial K-means clustering.
(PDF)

Figure S2 Summary of STRUCTURE 2.2 analyses based on AFLP multilocus phenotypes of 360 plants of *Knautia arvensis* agg. Values of ln probability of the data for each number of groups (K) plotted against the K-values and Delta K values).
(PDF)

Figure S3 Cluster membership of individuals estimated by STRUCTURE 2.2. A – analysis of the complete dataset. B – separate STRUCTURE analysis for the relict diploid + tetraploid subgroup (grey in the plot A) resulting in six groups. Population numbers below each plot correspond to Table 1.
(PDF)

Figure S4 Geographical location of 40 analyzed populations of *Knautia arvensis* agg. in central Europe and their phylogeographical grouping according to the STRUCTURE analysis of AFLP phenotypes.
(PDF)

Figure S5 Principal coordinate analysis (PCoA) based on Jaccard similarity among AFLP multilocus phenotypes of *Knautia arvensis* agg. individuals. The different colours represent the groups identified by the STRUCTURE analysis (same as in Fig. S4).
(PDF)

Table S1 Contingency table comparing the pattern in AFLP data (results of the nonhierarchical K-means clustering; clusters K1–K7) and the distribution of chloroplast haplotypes (A-M); numbers of individuals are presented in each field.
(PDF)

Table S2 Primary matrix of the scored AFLP fragments.
(XLS)

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Author Contributions

Conceived and designed the experiments: JS MS. Performed the experiments: FK TF MS ED PT. Analyzed the data: FK TF. Contributed reagents/materials/analysis tools: PS. Wrote the paper: FK JS TF PS.

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REGULAR ARTICLE

Serpentine ecotypic differentiation in a polyploid plant complex: shared tolerance to Mg and Ni stress among di- and tetraploid serpentine populations of *Knautia arvensis* (Dipsacaceae)

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Abstract
Background and aims Serpentine soils impose limits on plant growth and survival and thus provide an ideal model for studying plant adaptation under environmental stress. Despite the increasing amount of data on serpentine ecotypic differentiation, no study has assessed the potential role of polyploidy. We tested for links between polyploidy and the response to serpentine stress in *Knautia arvensis*, a diploid-tetraploid, edaphically differentiated complex.
Methods Variation in growth, biomass yield and tissue Mg and Ni accumulation in response to high Mg and Ni concentrations were experimentally tested using hydroponic cultivation of seedlings from eight populations of different ploidy and edaphic origin.
Results Regardless of ploidy level, serpentine populations exhibited higher tolerance to both Mg and Ni stress than their non-serpentine counterparts, suggesting an adaptive character of these traits in *K. arvensis*. The effect of ploidy was rather weak and confined to a slightly better response of serpentine tetraploids to Mg stress and to higher biomass yields in tetraploids from both soil types.
Conclusions The similar response of diploid and tetraploid serpentine populations to edaphic stress corresponded with their previously described genetic proximity. This suggests that serpentine tolerance might have been transmitted during the local autopolyploid origin of serpentine tetraploids.

Keywords Adaptation · Ca/Mg ratio · Metal tolerance · Nickel · Ploidy level · Serpentine

Abbreviations

AFLP	Amplified fragment length polymorphism
Ca	Calcium

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ICP OES	Inductively coupled plasma optical emission spectrometry
Mg	Magnesium
Ni	Nickel

Introduction

Serpentine soils inflict harsh constraints on plant growth because they are characterized by a low Ca/Mg ratio, increased (even toxic) concentrations of heavy metals (especially Ni, Cr, and Co), deficiency of essential macronutrients and a low water-holding capacity (Proctor and Woodell 1975; Kazakou et al. 2008; Harrison and Rajakaruna 2011). Serpentine plants provide excellent systems for the study of adaptive evolution in plants thanks to facts that the physiological response is well described, that major stressing factors are known and amenable to manipulative experiments and that recurrent evolution of adaptive strategies occurs within single species (Brady et al. 2005). Of the limiting factors imposed by serpentine substrates, low Ca/Mg ratio and high Ni content have gained the most attention because they are considered to be the key factors affecting plant growth and survival in serpentine outcrops worldwide (Proctor 1971; Gabbrielli and Pandolfini 1984; Ghasemi and Ghaderian 2009; O' Dell and Rajakaruna 2011). Elevated levels of heavy metals in soils can affect plants through direct toxicity (resulting in stunting and chlorosis), antagonism with other nutrients, and inhibition of root penetration and growth (Antonovics et al. 1971). High Mg content induces Ca deficiency, leading to cell wall disintegration and localized tissue necrosis (O' Dell and Claassen 2006; O' Dell and Rajakaruna 2011).

Plant species differ in their abilities to evolve tolerance against serpentine stress depending on their genetic resources. They can tolerate edaphic challenges either by a constitutive trait present in all members of a species (that grows both on and off serpentine soils) or by an adaptive mechanism present only in tolerant ecotypes (Kazakou et al. 2008). Available case studies provide ambiguous results in this respect since both adaptive and constitutive patterns of tolerance to low Ca/Mg and/or high Ni have been proven experimentally, depending on the model system studied (e.g., Westerbergh 1994; Boyd and Martens 1998; Nyberg Berglund et al. 2004; Ghasemi and Ghaderian 2009). Despite the growing amount of experimental data, evolutionary mechanisms involved in the adaptive process as well as essential prerequisites facilitating colonization of serpentine sites still remain unclear.

Polyploidy, or whole genome duplication, is widely acknowledged as a leading force in plant evolution (Soltis et al. 2009; Otto and Whitton 2000). The presence of several gene copies within a polyploid genome is considered a possible key factor that widens the ecological niche of a polyploid and enhances its expansion to new environments (Ramsey and Schemske 2002; Ramsey et al. 2008; Parisod et al. 2010). On the other hand, it could be the diploid cytotype which survives in extreme environments such as rocky outcrops, xeric habitats or serpentines, where it finds refugia of suitable conditions (e.g., reduced plant competition) in a dramatically changing landscape (Ehrendorfer 1980; Kolář et al. 2012). Despite the widely-known consequences of polyploidy for various ecological and life-history traits, no study has tested specifically for the associations between polyploidy and serpentine tolerance. Correct evolutionary interpretations of ecological patterns, however, require good knowledge of genetic relationships among ecotypes and cytotypes. Inter-cytotype differences in ecological traits may not only be an effect of polyploidization per se and/or subsequent selection but can also simply reflect different evolutionary histories of the cytotypes under investigation (Ramsey and Schemske 1998; Levin 2002). Unfortunately, the genetic background has often been neglected in experimental studies dealing with ecological differences within polyploid complexes (but see e.g., Ramsey 2011; Mráz et al. 2012).

Knautia arvensis (Dipsacaceae) provides an ideal study system for assessing the role of polyploidy in serpentine tolerance. In Central Europe, it comprises two cytotypes (both occurring on and off serpentine) with well-described patterns of their genetic and habitat differentiation (Štěpánek 1997; Kaplan 1998). Interestingly, habitat differentiation largely correspond to the genetic relationships among populations but only partly reflect ploidy level variation (Kolář et al. 2009; Kolář et al. 2012). In Central Europe, the diploid cytotype (2n=2x=20) splits into two major genetic groups: (i) serpentine diploids inhabiting several spatially isolated serpentine outcrops (plus a single lime-rich stand in a subalpine glacial cirque), where it probably survived unfavourable periods of the Holocene (i.e., periods of large forest expansion that restricted heliophilous plants like *Knautia* to small, isolated refugia such as edaphic islands, Ložek 1973), and (ii) non-serpentine diploids inhabiting a wide range of semi-natural habitats in south-eastern parts of C. Europe. Similarly, at

the tetraploid level (2n=4x=40), a serpentine tetraploid lineage is known to be restricted to a single serpentine area whereas non-serpentine tetraploid populations are widespread in semi-natural grasslands throughout most of C. Europe (except for its SE part). Central-European serpentine diploid populations of *K. arvensis* form a genetically distinct lineage within *Knautia* (I. Rešetnik, P. Schönswetter & B. Frajman, unpubl. results) although certain levels of genetic differentiation have been observed among populations, probably as a result of allopatric differentiation among spatially isolated outcrops (Kolář et al. 2012). Importantly, serpentine tetraploid plants are genetically close to their diploid edaphic counterparts, likely as a result of a local autopolyploid origin from diploids inhabiting the same serpentine area. By contrast, non-serpentine diploids represent the genetically most distinct lineage of the whole *Knautia arvensis* group in Central Europe. Finally, non-serpentine tetraploids are genetically closer to serpentine populations (partly also due to hybridization with serpentine tetraploid plants in the area of immediate contact). Their origin is uncertain, however, as they might also encompass gene pools from other, yet uninvestigated populations (Kaplan 1998; Kolář et al. 2009; Kolář et al. 2012).

The available distributional, cytological, and genetic data suggest a key role of serpentines in the evolution of Central European *Knautia arvensis* populations. However, the patterns of evolution of the tolerance and the ways how different cytotypes and edaphic groups respond to serpentine stress remain unknown. Two major contrasting hypotheses regarding the distribution of serpentine tolerance could be postulated: (1) both serpentine and non-serpentine *K. arvensis* populations exhibit similar response to serpentine stress (i.e., serpentine tolerance is a constitutive trait in *K. arvensis*), (2) populations native to the serpentine stands exhibit higher levels of tolerance than their non-serpentine counterparts (i.e., the adaptive explanation applies). If the second was true, the effect of ploidy level itself would still remain to be evaluated as polyploidization and/or subsequent selection might have significant effects on the tolerance traits in the polyploid (e.g., leading to its higher endurance). In this study, we took *K. arvensis* as a model system of serpentine differentiated diploid-polyploid complex and tested for differentiation of its populations in tolerance to manipulated concentrations of magnesium and nickel (i.e., the two key factors affecting plant growth and survival on serpentines). Detailed knowledge on variation patterns of these eco-physiological traits, complemented by the previously described cytological and genetic background, would allow drawing a novel synthetic view on evolutionary pathways of serpentine tolerance in a ploidy variable plant complex.

Materials and methods

Plant material

Ripe achenes were collected in 2009 from approximately ten plants per each of eight natural populations of *Knautia arvensis* in the Czech Republic and Slovakia. The localities were selected in order to achieve a balanced design of the experiment: two diploid serpentine, two diploid non-serpentine, two tetraploid serpentine and two tetraploid non-serpentine populations. For details on the sampled populations, see Table 1. Data on soil characteristics from the rhizosphere of *Knautia* from the original localities were adopted from a previous study (Doubková et al. 2011). The ploidy level of the plants was confirmed using flow cytometry according to the protocol described in Kolář et al. (2009).

Experimental design and hydroponic cultivation

Achenes were germinated on a moist filter paper over a period of 3 weeks. Vital, undamaged seedlings were then carefully fixed onto a floating plastic disc (14 cm in diameter), maintaining uniform gaps between them. Each disc containing eight plants (one per each population) was placed into a 1.5 L light-impermeable experimental container with a standard nutrient solution described in Huss-Danell (1978) with a slight modification ($Co(NO_3)_2$ was used instead of $CoSO_4$ as the cobalt source). A similar solution has been successfully employed in the assessment of Ni and Mg tolerance in other plant systems, e.g., serpentine vs. non-serpentine ecotypes of *Cerastium alpinum* (Nyberg Berglund et al. 2004). The seedlings were grown in the standard nutrient solution for 11 days prior to the start of the experiment. They were then placed into experimental solutions with manipulated concentrations of Mg^{2+} and Ni^{2+} for the next 22 days ($MgSO_4$ and Ni SO_4 were used as sources of Mg and Ni, respectively; the pH was approx. Seven during the whole experiment). The solutions were changed every

Table 1 Details on investigated populations of *Knautia arvensis*

Substrate type	Ploidy level	Population code[a]	Site	Geographic co-ordinates	Altitude (m asl)	Site description	Ca/Mg ratio[b]	Mg ($mg.kg^{-1}$)[b]	Ni ($mg.kg^{-1}$)[b]
Serpentine	2x	S1	Borovsko (E. Bohemia, CZ)	49°40′57.7″N, 15°07′49.7″E	400	Open pine forest	0.7	2478	543
		S2	Staré Ransko (E. Bohemia, CZ)	49°39′04.9″N, 15°48′57.3″E	640	Coniferous forest margins	0.6	3005	155
	4x	S3	Pluhův Bor (W. Bohemia, CZ)	50°03′01.3″N, 12°46′24.3″E	710	Open pine forest	0.6	1657	170
		S4	Křižky (W. Bohemia, CZ)	50°03′54.2″N, 12°45′03.6″E	790	Rock outcrops, semidry grassland	0.8	1760	264
Non-serpentine	2x	NS1	Tvarožná Lhota (S. Moravia, CZ)	48°51′43.6″N, 17°23′23.3″E	290	Mesophilous meadow	18.3	366	7.1
		NS2	Lajdovci (W. Slovakia, SK)	48°28′29.8″N, 17°38′59.2″E	230	Dry meadow	10.1	521	2.4
	4x	NS3	Aš (W. Bohemia, CZ)	50°13′12.9″N, 12°13′19.2″E	670	Abandoned meadow	6.9	59	2.1
		NS4	Chanovice (SW. Bohemia, CZ)	49°24′39.0″N, 13°43′55.5″E	530	Dry meadow	7.5	267	1.9

[a] Population codes correspond with Doubková et al. (2011, 2012)

[b] Data from Doubková et al. (2011); available Mg and Ni concentrations were determined after 1 M ammonium acetate and 0.005 M DTPA extraction, respectively

3 days for a freshly prepared stock over the entire course of the hydroponic cultivation. The cultivations were performed in a controlled environment growth cabinet at the Faculty of Science, University of South Bohemia, Czech Republic with a cycle of 12 h light and 12 h darkness at the constant temperature of 18 °C with a light supply of approx. 450 $\mu mol.m^{-2}\ s^{-1}$ photons (photosynthetically active radiation).

To test the individual and combined effects of Ni and Mg among *K. arvensis* populations on different soil types (factor 'Substrate') and of different ploidy level (factor 'Ploidy'), we used a mixed-effect full-factorial experimental design. Four experimental treatments were applied: the control (Ni-/Mg-), Ni (Ni+/Mg-), Mg (Ni-/Mg+) and Ni + Mg (Ni+/Mg+). Based on a preliminary cultivation experiment, the concentrations of Ni^{2+} were set to 0 μM (control) and 50 μM, and the concentrations of Mg^{2+} were set to 0.55 mM (control) and 5.5 mM (i.e., Ca/Mg ratio of 2 and 0.2, respectively). Each experimental unit (= plastic container filled with one of the four types of the experimental solutions) consisted of eight seedlings, one seedling per population. Each treatment was replicated eight-times, resulting in the total amount of 32 experimental units and 256 seedlings.

Four characteristics were used as proxies of the plant growth response to different treatments: (i) total root growth, (ii) longest root growth and (iii) lateral root formation (proxies for belowground biomass), and (iv) longest leaf growth (proxy of aboveground biomass). Absolute values of the three root characteristics were acquired using the programme RootArch 1.0 (P. Šmilauer, University of South Bohemia, unpublished) from figures recorded at two time points: (i) at the beginning of the experiment, i.e., before setting the seedlings into the experimental solutions with manipulated elemental concentrations (roots were photographed in order not to harm the experimental plants); and (ii) at the end of the experiment (roots were scanned). Standard camera settings and identical distance from the object were kept when acquiring the photographs. The length of the longest leaf was carefully measured with a ruler again both at the beginning and at the end of the experiment. Growth characteristics were then calculated as differences between their initial and final values for each particular individual. Finally, the plants were harvested, the below- and aboveground organs were separated, dried at 60 °C and the dry biomass was weighted.

Determination of Mg and Ni concentrations in plant tissues

Elemental concentrations of Mg and Ni were quantitatively determined from desiccated leaf tissue (elemental contents in roots could not have been evaluated due to small amounts of the material) using inductively coupled plasma optical emission spectrometry (ICP OES, Hansen et al. 2009). We analysed Mg contents in 119 samples (i.e., approx. half of each population/treatment combination); Ni concentration was estimated only in Ni and Ni + Mg treated samples because of the absence of Ni in the control solution (amounting to a total of 64 samples). The samples were decomposed prior to the analysis. Due to the small amounts of our plant samples (8.4 mg of dry biomass on average), we applied the decomposition method using a multi-tube system and the MWS3+ microwave oven (Berghof, Germany). Dried plant tissue (<10 mg) was inserted into digestion tubes and treated with 2 ml nitric acid under the following conditions: 5 min ramp, 10 min hold on 170 °C. 5 min ramp, hold on 200 °C. The tubes were then filled up to the final volume of 10 ml and subjected to the ICP OES analysis. The elemental analysis of nickel and magnesium was carried out with the sequential, radially viewed ICP OES spectrometer INTEGRA XL 2 (GBC, Dandenong Australia) equipped with the microconcentric nebulizer (400 $\mu l.min^{-1}$) and a glass cyclonic spray chamber (both Glass Expansion, Australia). The analytical lines used were 221.6 nm for Ni and 285.2 nm for Mg. The operation conditions of the ICP OES analysis were the following: sample flow rate 1.5 $mL.min^{-1}$, plasma power 1000 W, plasma, auxiliary and nebulizer gas flow rates 10, 0.6, and 0.65 $L.min^{-1}$, respectively, photomultiplier voltage 600 V for nickel and 350 V for magnesium, view height 6.5 mm, three replicated reading on-peak 1 s, fixed point background correction. Calibration standards containing 10 – 5 – 1 – 0.5 – 0.1 $mg.L^{-1}$ of both nickel and magnesium were used for instrument calibration. The external calibration standards were prepared using commercially available stock standard solutions of Mg and Ni, both containing 1 $g.L^{-1}$ (SCP, Baie D'Urfé, Canada). The limits of detection (concentration equal to three times the standard deviation at the point of the background correction) were 5 $\mu g.L^{-1}$ for nickel and 2 $\mu g.L^{-1}$ for magnesium. Certified reference material (bush twigs and leaves GBW 07602 from the China National Analysis Center for Iron and Steel, Beijing) was used to validate the method.

Statistical analyses

Dependent variables (except for root and shoot biomass yield) were log-transformed in order to improve normality and homoscedascity. As we aimed at identification of the overall differences in serpentine tolerance among plants of different ploidy/edaphic origin, we treated the population of origin as a factor with random effect in all statistical analyses. Differences in biomass yield and in growth of above- and belowground biomass under control conditions (i.e., in the standard nutrient solution) among plants of different edaphic origin and ploidy were tested by an ANOVA analysis with the random factor of original population nested in the interaction between Substrate of origin (serpentine, non-serpentine) and Ploidy (diploid, tetraploid). A more complex ANOVA was used for the evaluation of the differences in growth, biomass yields and Mg tissue accumulation in *Knautia* seedlings in response to elevated concentrations of Mg and Ni. The effects of Substrate of origin, Ploidy level, Mg and Ni treatment and all their interactions were tested in a hierarchical ANOVA where the experimental container (nested in Mg and Ni treatment interaction) and population (nested in Substrate of origin and Ploidy interaction) were treated as random factors. Differences in Ni tissue concentrations were analysed only for plants grown in solutions containing nickel (i.e., treatments Ni and Mg + Ni). Differences among genetic groups were not subjected to statistical testing as the main genetic structure has already been represented by the interaction between Ploidy level and Substrate of origin (see the Introduction and Discussion sections). All analyses were calculated in Statistica 8 (StatSoft, Inc. 2007). Note that Statistica uses Satterthwaite's method of denominator synthesis, which finds the linear combinations of sources of random variation that serve as appropriate error terms for testing the significance of the respective effect of interest—for this reason the complete ANOVA tables, the synthesized error MS and synthesized error degrees of freedom are also presented.

Results

Under controlled conditions, plants of serpentine origin exhibited higher growth of the root system than their non-serpentine counterparts ($F_{1,49}$=8.669, p=0.032). By contrast, no significant differences were detected in either leaf growth or final biomass yields. Individuals of distinct ploidy levels also grew differently under

control conditions. Tetraploid plants exhibited higher root growth ($F_{1,49}$=8.501, p=0.033) as well as higher yields of both belowground and aboveground biomass ($F_{1,56}$=21.13, p=0.010; $F_{1,56}$=13.02, p=0.023 for root and shoot biomass, respectively) and a higher root/shoot biomass ratio ($F_{1,56}$=26.99, p=0.001).

Growth and biomass yield under different concentrations of Mg and Ni

The response of the three root growth characteristics was closely correlated and these will therefore be considered together in the further text (r^2=0.82, p<0.01 and r^2=0.83, p<0.01 for the correlation of the longest root growth and lateral root formation, respectively, with total root growth). Elevated concentrations of Mg and Ni significantly reduced the growth of *Knautia* seedlings in both belowground and aboveground organs (Table 2 and Online Resource 1); high Ni content also reduced root and shoot biomass yields (Table 3 and Online Resource 2). In addition, a significant interaction between Mg and Ni was detected, as high Mg content markedly alleviated the negative effects of Ni on growth and biomass yields of belowground organs (Online Resource 3).

Knautia plants of serpentine and non-serpentine origin responded to Mg and Ni stress in a strikingly different way (Tables 2 and 3). Firstly, elevated concentrations of Mg alone strongly reduced both growth and biomass yields of non-serpentine plants, while serpentine plants remained unaffected (this applied to both below- and aboveground organs; see Figs. 1 and 2). Secondly, under high Ni concentrations, elevated Mg amounts alleviated Ni stress more efficiently in serpentine plants, leading to their higher root growth (but not to higher biomass yields) in comparison with non-serpentine plants (Fig. 1 and Online Resource 4; see also Online Resource 5 displaying the response of individual populations). Finally, the effect of Ni alone was also expressed by a slightly higher root growth of serpentine plants under Ni

Table 2 Effect of manipulated Mg and Ni concentrations, ploidy level and substrate of origin on the growth of *Knautia arvensis* plants in hydroponic cultivation

			Total root growth		Longest root growth		Lateral root formation		Longest leaf growth	
Factor/Interaction	Effect	Effect df	MS	F	MS	F	MS	F	MS	F
Experimental container	*Random*	*28*	***0.11***	***2.01*****	***0.07***	***1.55****	***0.14***	***1.86*****	*0.05*	*0.97*
Population	*Random*	*5*	***0.21***	***3.83*****	***0.32***	***7.31*****	*0.11*	*1.53*	***0.14***	***2.60****
Mg	Fixed	1	**0.52**	**4.66***	**0.36**	**5.24***	**0.86**	**6.31***	**0.79**	**15.5*****
Ni	Fixed	1	**8.48**	**76.36*****	**2.24**	**32.94*****	**9.87**	**72.01*****	**0.37**	**7.32***
Ploidy	Fixed	1	0.14	1.20	0.39	2.59	0.04	0.46	0.04	0.44
Substrate	Fixed	1	0.34	2.96	0.01	0.04	**1.27**	**14.38****	0.03	0.38
Mg*Ni	Fixed	1	**3.86**	**34.77*****	**1.28**	**18.81*****	**6.12**	**44.67*****	**0.48**	**9.55****
Ploidy*Mg	Fixed	1	0.01	0.20	0.02	0.41	0.01	0.09	0.00	0.02
Ploidy*Ni	Fixed	1	0.13	2.34	0.13	3.07	0.11	1.50	0.06	1.09
Substrate*Mg	Fixed	1	**2.00**	**36.19*****	**0.64**	**14.75*****	**2.50**	**33.94*****	**0.43**	**8.14****
Substrate*Ni	Fixed	1	**0.50**	**9.06****	**0.18**	**4.05***	0.21	2.86	0.05	0.90
Ploidy*Substrate	Fixed	1	0.21	1.81	0.62	4.16	0.17	1.87	0.06	0.70
Ploidy*Mg*Ni	Fixed	1	0.01	0.18	0.03	0.61	0.09	1.24	0.00	0.00
Substrate*Mg*Ni	Fixed	1	0.13	2.44	0.08	1.80	0.02	0.33	0.15	2.92
Ploidy*Substrate*Mg	Fixed	1	**0.51**	**9.21****	0.10	2.24	**0.75**	**10.18****	0.08	1.52
Ploidy*Substrate*Ni	Fixed	1	0.07	1.36	0.01	0.22	0.00	0.01	0.00	0.03
Ploidy*Substrate*Mg*Ni	Fixed	1	0.02	0.41	0.00	0.01	0.09	1.18	0.01	0.15
Error		207	0.05		0.04		0.07		0.05	

Statistically significant results are in bold, * p<0.05, ** p<0.01, *** p<0.001. Population identity and experimental container were treated as random factors; the full ANOVA table including Synthetic error MS and df for each tested term (see Materials and Methods for details) is available in Online Resource 1. Dependent variables were log transformed prior to the analysis.

Table 3 Effect of manipulated Mg and Ni concentrations, ploidy level and substrate of origin on biomass yields of *Knautia arvensis* plants harvested at the end of the hydroponic cultivation

			Belowground biomass		Aboveground biomass		Root/shoot ratio	
Factor/Interaction	Effect	Effect df	MS	F	MS	F	MS	F
Experimental container	*Random*	*28*	*0.000006*	***2.48******	*0.000028*	***2.51*****	*0.032142*	*1.05*
Population	*Random*	*5*	*0.000004*	*1.86*	*0.000014*	*1.24*	*0.042261*	*1.37*
Mg	Fixed	1	0.000015	2.60	0.000002	0.06	0.114927	3.54
Ni	Fixed	1	0.000106	**18.24***** ↓Ni+	0.000020	0.71	1.109497	**34.20***** ↓Ni+
Ploidy	Fixed	1	0.000030	**9.59**** ↑4x	0.000057	**4.66*** ↑4x	0.260646	**7.39*** ↑4x
Substrate	Fixed	1	0.000006	1.81	0.000000	0.01	0.129924	3.69
Mg*Ni	Fixed	1	0.000046	**7.97****	0.000011	0.40	1.195312	**36.85*****
Ploidy*Mg	Fixed	1	0.000005	2.32	0.000001	0.07	0.113254	3.67
Ploidy*Ni	Fixed	1	0.000005	2.06	0.000006	0.56	0.001787	0.05
Substrate*Mg	Fixed	1	0.000057	**24.28****	0.000328	**29.40*****	0.174101	**5.64***
Substrate*Ni	Fixed	1	0.000000	0.20	0.000001	0.11	0.005466	0.17
Ploidy*Substrate	Fixed	1	0.000002	0.61	0.000010	0.78	0.015727	0.44
Ploidy*Mg*Ni	Fixed	1	0.000005	2.06	0.000000	0.00	0.003240	0.10
Substrate*Mg*Ni	Fixed	1	0.000000	0.08	0.000015	1.31	0.031809	1.03
Ploidy*Substrate*Mg	Fixed	1	0.000015	**6.45***	0.000061	**5.43***	0.008287	0.26
Ploidy*Substrate*Ni	Fixed	1	0.000000	0.01	0.000001	0.06	0.001190	0.03
Ploidy*Substrate*Mg*Ni	Fixed	1	0.000000	0.05	0.000001	0.10	0.000000	0.00
Error		207	0.000002		0.000011		0.030833	

Statistically significant results are in bold, * $p<0.05$, ** $p<0.01$, *** $p<0.001$. For factors with a significant effect, arrows indicate the direction of change. Population identity and experimental container were treated as random factors; the full ANOVA table including Synthetic error MS and df (see Materials and Methods for details) is available in Online Resource 2. Dependent variables were not transformed

stress (on the contrary, its effect on final biomass yields was not significant; Table 3 and Online Resource 2).

A significant effect of ploidy level on root growth and biomass was manifested by a complex interaction with edaphic origin and Mg treatment (Tables 2 and 3). Generally, the difference between plants of different edaphic origin was more pronounced within the tetraploid cytotype. Under elevated concentrations of Mg, serpentine tetraploids exhibited higher and non-serpentine tetraploids lower root growth than their particular edaphic diploid counterparts (Fig. 1). In the case of biomass yields, the differences among cytotypes were more pronounced in the control (higher yields in non-serpentine vs. serpentine tetraploids but no marked difference among diploids); under high Mg stress, tetraploid populations of both edaphic types yielded relatively more root and shoot biomass than their diploid edaphic counterparts (Online Resource 6). In addition, ploidy level alone had a significant effect on biomass production since tetraploids generally accumulated significantly more below- and aboveground biomass than diploids and also allocated relatively more biomass to the roots (Table 3).

Mg and Ni accumulation in aboveground tissues

Elevated concentrations of Mg and Ni in the experimental solution significantly increased accumulation of both elements in *Knautia* aboveground biomass (Table 4 and Online Resource 7; see also Online Resource 8 for details on the values). Mg concentration in leaf tissues was significantly affected by the interaction between ploidy level and its concentration in the solution (tetraploids accumulated slightly more Mg than diploids in the Mg rich solution; for details see Online Resource 9) but not with the substrate of origin (serpentine vs. non-serpentine). On the contrary, the accumulation of Ni in leaf tissues was significantly affected by the serpentine vs. non-serpentine origin in the interaction with the Mg treatment. Serpentine plants reduced their Ni accumulation when Mg concentrations in the solution were

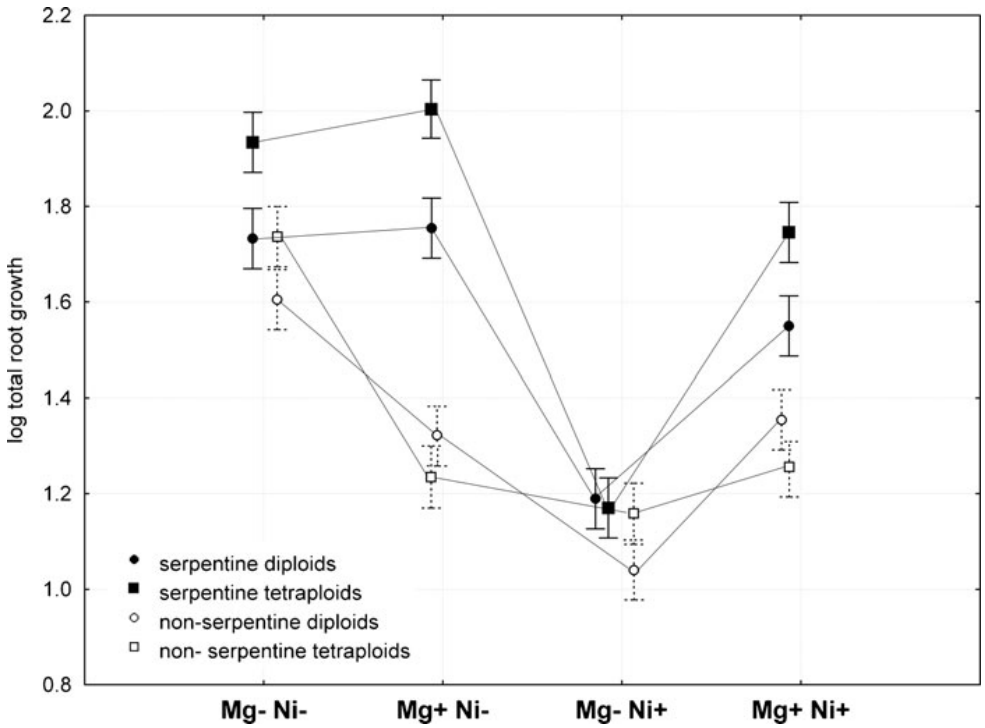

Fig. 1 Different response in total root growth of serpentine and non-serpentine *Knautia arvensis* plants (diploid vs. tetraploid) to the low (−) vs. high (+) concentrations of Mg and the absence (−) vs. presence (+) of Ni in experimental solutions. Symbols and vertical bars denote the mean and standard error of the mean, respectively

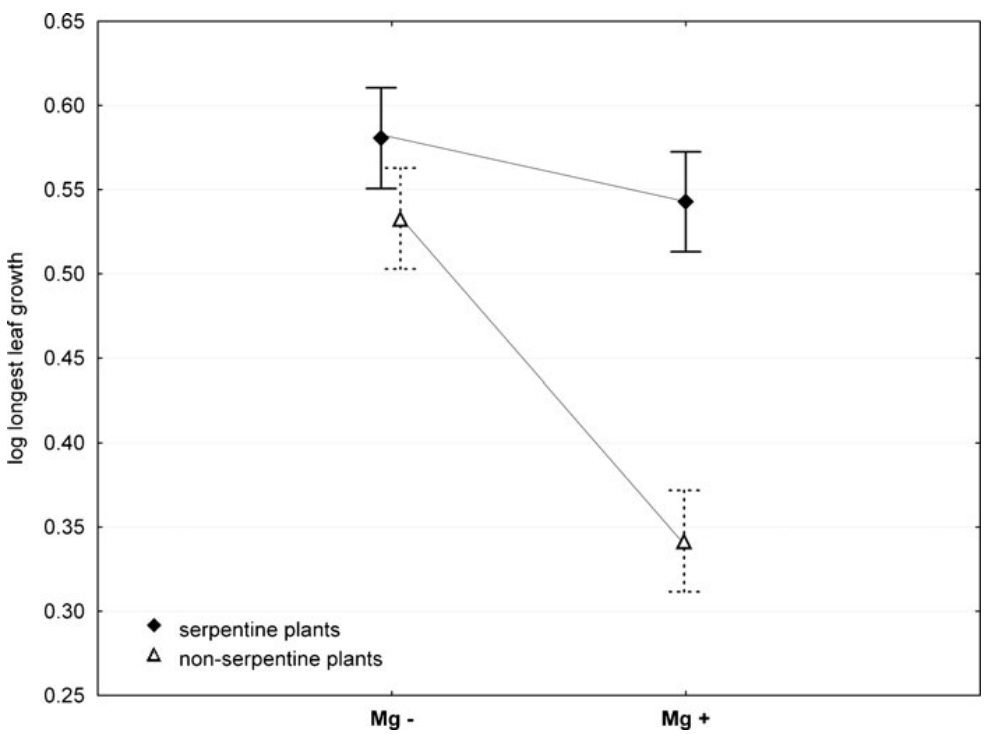

Fig. 2 Different response in the longest leaf growth of serpentine and non-serpentine *Knautia arvensis* plants to the low (−) vs. high (+) concentrations of Mg in experimental solutions. Symbols and vertical bars denote the mean and standard error of the mean, respectively

Table 4 Effect of manipulated Mg and Ni concentrations in an experimental solution, ploidy level and substrate of origin on the concentrations of Mg and Ni in *Knautia arvensis* aboveground biomass. Differences in Ni accumulation were tested only for plants grown in Ni-enriched solutions

		Mg concentration in leaf tissue			Ni concentration in leaf tissue		
Factor/Interaction	Effect	Effect df	MS	F	df	MS	F
Experimental container	*Random*	*12*	*0.05*	*1.60*	*6*	*0.09*	*0.74*
Population	*Random*	*4*	*0.01*	*0.24*	*4*	*0.13*	*1.09*
Mg	Fixed	1	**21**	**629.18*** **	**1**	**1.67**	**19.32****
Ni	Fixed	1	0.08	2.31		–	–
Ploidy	Fixed	1	0	0.08	1	0.01	0.06
Substrate	Fixed	1	0.01	0.17	1	0.82	6.45
Mg*Ni	Fixed	1	0	0.04		–	–
Ploidy*Mg	Fixed	1	**0.2**	**5.96***	1	0.41	3.57
Ploidy*Ni	Fixed	1	0.02	0.49		–	–
Substrate*Mg	Fixed	1	0.05	1.47	**1**	**2.09**	**17.84*****
Substrate*Ni	Fixed	1	**0.22**	**6.45***		–	–
Ploidy*Substrate	Fixed	1	0.03	0.9	1	0.03	0.26
Ploidy*Mg*Ni	Fixed	1	0.01	0.42		–	–
Substrate*Mg*Ni	Fixed	1	0.02	0.64		–	–
Ploidy*Substrate*Mg	Fixed	1	0.02	0.67	1	0.02	0.16
Ploidy*Substrate*Ni	Fixed	1	0.05	1.46		–	–
Ploidy*Substrate*Mg*Ni	Fixed	1	0.02	0.65		–	–
Error		87	0.03		46	0.12	

Statistically significant results are in bold, * $p<0.05$, ** $p<0.01$, *** $p<0.001$. Population identity and experimental container were treated as random factors; the full ANOVA table including Synthetic error MS and df (see Materials and Methods for details) is available in Online Resource 7. Dependent variables were log transformed prior to the analysis

elevated whereas non-serpentine plants exhibited approximately the same levels of Ni in their tissues in both external Mg concentrations (Fig. 3, see also Online Resource 10 for the response of individual populations).

Discussion

Ecotypic response of *K. arvensis* to Mg and Ni stress

Plant adaptation to the stressful conditions of serpentine soils may occur either only in populations experiencing the stress or it can be widespread across all populations of a species. Both diploid and tetraploid cytotype of *Knautia arvensis* occur on and off serpentine, the available distributional data, however, did not allow any conclusion which of the above described scenarios applies to *Knautia*. In the hydroponic cultivation, serpentine and non-serpentine *Knautia arvensis* responded to the low Ca/Mg ratio and high Ni concentration in markedly different ways, suggesting that serpentine tolerance is an adaptive rather than a constitutive trait within this species. Firstly, serpentine plants, unlike their non-serpentine counterparts, did not reduce their growth under elevated concentrations of Mg (Figs. 1 and 2), which were present not only in the solution but also in plant tissues (Online Resource 8, see also Doubková et al. 2012). Tolerance to elevated Mg and/or high Mg requirement has been found to be an almost ubiquitous trait of serpentine-adapted plants that represents an integral part of serpentine tolerance (see Brady et al. 2005; Kazakou et al. 2008 for review). Secondly, Ni toxicity seems to be less harmful to serpentine than to non-serpentine plants when both Mg and Ni are present in elevated concentrations, i.e., in conditions close to those in natural serpentine stands (Fig. 1). A similar effect of certain elements such as Mg and Ca on reduction of Ni toxicity has been experimentally

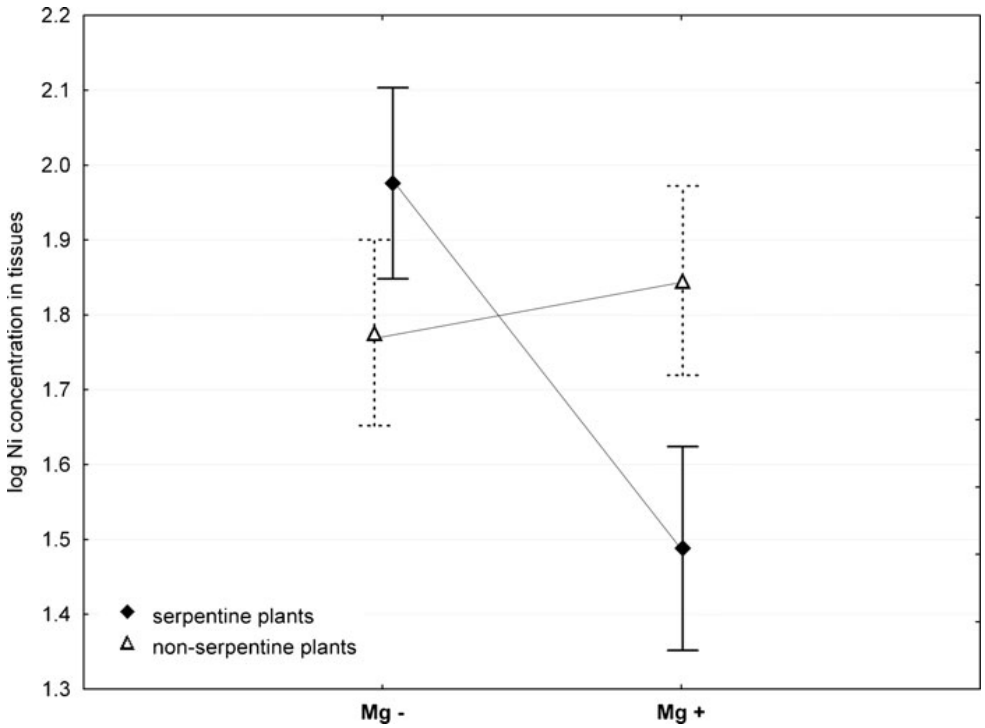

Fig. 3 Different response in the accumulation of Ni in aboveground tissues of serpentine and non-serpentine *Knautia arvensis* plants to the low (−) vs. high (+) concentrations of Mg in experimental solutions. Symbols and vertical bars denote the mean and standard error of the mean, respectively

demonstrated in several other plant species (e.g., *Avena sativa*, Proctor and McGowan 1976; *Alyssum bertolonii*, Gabbrielli and Pandolfini 1984; *Zea mays*, Robertson 1985). The physiological basis of this effect still remains rather unclear, but it possibly reflects a direct interference among both elements during their uptake by roots (Chen et al. 2009). Finally, serpentine plants also exhibited a significant decrease in Ni accumulation in their leaf tissues under elevated Mg, while non-serpentine plants accumulated approximately the same (high) amounts of Ni irrespective of external Mg concentrations (Fig. 3). Regulation of heavy metal uptake (Ni in particular) is often stressed as an important factor in serpentine tolerance, but the opposite—i.e., tolerance to excessive Ni accumulation—has in many cases been taken as evidence of serpentine tolerance (e.g., Ni hyperaccumulator plants such as *Thlaspi goesingense*, Reeves and Bakwer 1984; *T. caerulescens*, Boyd and Martens 1998; *Alyssum bertolonii*, Galardi et al. 2007). Nevertheless, restricted Ni uptake and translocation was also documented in several serpentine-tolerant plant species (e.g., Vergnano et al. 1982; Gabbrielli et al. 1990). To sum up, the consistently better response of the serpentine populations to Mg and Ni stress indicate that the tolerance is an adaptive trait characteristic for serpentine *Knautia arvensis* populations rather than a pre-adaptation (Brady et al. 2005) shared by all members of the complex.

Knautia serpentine populations seem to have evolved a complex mechanism of tolerance against serpentine chemical stress that is based on tolerance to high Mg accumulation and a restriction of Ni uptake. Such a combined response to both Mg and Ni stress has been revealed in several other case studies which examined the effects of both elements (e.g., Gabbrielli and Pandolfini 1984; Nyberg Berglund et al. 2004; Asemaneh et al. 2007). Interestingly, in *Cerastium alpinum,* serpentine populations exhibited considerable variation in the direction of Mg-Ni tolerance, as some populations exhibited a positive effect of Mg on Ni toxicity, while the opposite applied to other populations (well reflecting soil properties at sites of original populations, Nyberg Berglund et al. 2004). By contrast, we have detected a largely congruent pattern of growth response to both Mg and Ni among *Knautia* serpentine populations (see Online Resources 5 and 10), what also corresponds to the rather constant concentrations of both elements in the original soils (Table 1 and Doubková et al. 2011). Our data thus do not indicate any strong local adaptation to Mg and Ni stress within the serpentine *Knautia* ecotype.

Serpentine soils provide a complex set of chemical and physical factors influencing plant life, collectively

summarized under the term 'serpentine syndrome' (Jenny 1980; Brady et al. 2005). However, chemical stress caused by extremely low Ca/Mg ratios and high Ni concentrations is generally perceived as the principal trigger promoting serpentine ecotypic differentiation and adaptation (Brady et al. 2005; Kazakou et al. 2008). Moreover, other important components of the serpentine syndrome such as low levels of macronutrients and drought are probably less important in *Knautia* because its native serpentine soils are rich in nitrogen and organic carbon (in amounts similar to non-serpentine soils, Doubková et al. 2011). Within serpentine areas, *Knautia* plants avoid dry zones with obvious water limitation. Instead, they occupy the forest floor and various depressions where they co-occur with other mesophilous plant species (pers. obs.). Thus, for simplicity, we use the general term 'serpentine tolerance' to describe the different response of serpentine and non-serpentine *Knautia* ecotypes. We are nevertheless aware that other, untested physical factors and/or biotic interactions may still contribute to the tolerance of *Knautia* to the serpentine syndrome. Indeed, the ecotypic differentiation of a whole plant-fungus assemblage in relation to phosphorus uptake has recently been documented among serpentine vs. non-serpentine *Knautia arvensis* populations (the same as those used in our study, Doubková et al. 2012). The specific combination of (i) a serpentine-native arbuscular mycorrhizal fungus strain and (ii) *Knautia* plants represented the most efficient system of phosphorus uptake in serpentine soils. Because the pattern of response was congruent with our results, we can consider mycorrhizal association as another factor contributing to serpentine tolerance in *Knautia*.

Evolutionary background of *K. arvensis* serpentine tolerance

Serpentine ecotypic differentiation ranks among the best documented examples of plant adaptive differentiation, and it has been recorded in various areas around the world (Kruckeberg 1967; Proctor 1971; Ghasemi and Ghaderian 2009). Yet it has never been directly examined in relation to polyploidy. In contrast to classical views on polyploidy associated changes in important plant life-history traits (Levin 2002), polyploidy alone seems to play a rather minor role in the observed differentiation of serpentine compared to non-serpentine *Knautia* ecotypes. A simple effect of ploidy level on tolerance to Mg or Ni stress was not apparent in any of the examined traits in *K. arvensis*. It should nevertheless be noted that our experimental approach was targeted at a single (yet critical) developmental stage (seedlings), so we cannot exclude that some inter-cytotype differences might become pronounced in later stages of the plants' life cycle and/or during reproduction. The effect of polyploidy in *Knautia* seedlings, however, appeared when the serpentine vs. non-serpentine origin was also taken into account. Specifically, serpentine tetraploids exhibited higher growth of their root system under Mg stress than their diploid edaphic counterparts (whereas non-serpentine tetraploids performed even worse than diploids of the same edaphic origin, see Fig. 1). The better growth response to Mg stress as well as overall higher biomass yields detected in serpentine tetraploids of *Knautia* could have contributed to their establishment and spread in the serpentine locality (where they currently prevail over their diploid ancestors, M. Hanzl and F. Kolář, unpubl. results). Still, due to the parapatric distribution of the populations included in our study, we cannot exclude the possibility that the observed inter-cytotype differences might, at least partly, reflect local adaptation of individual populations.

Instead of the effect of polyploidy per se, we argue that the observed pattern of serpentine tolerance (shared tolerance among serpentine di- and tetraploids) more likely reflects the genetic relationships among the populations. As has been evidenced by genome size, AFLP and chloroplast DNA markers evaluated in our previous studies (Kolář et al. 2009; Kolář et al. 2012), *Knautia arvensis* in Central Europe has a distinct genetic structure which only loosely corresponds to ploidy levels but strongly reflects the patterns of edaphic differentiation. While non-serpentine diploids are genetically very distinct and the position of non-serpentine tetraploids is ambiguous, serpentine populations of both ploidy levels are genetically close to each other and probably followed a common evolutionary trajectory (restriction of diploids to edaphic refugia followed by local polyploidization; Štěpánek 1989; Kaplan 1998; Kolář et al. 2012). The shared adaptations to serpentine chemical stress among serpentine diploids and tetraploids, revealed by the present study, correspond with the autopolyploid origin of the serpentine tetraploids within the edaphic refugium (that was detected during our previous investigations, Kolář et al. 2012). We assume that the genes enhancing serpentine tolerance have been probably directly transmitted during the polyploidization process. Alternative explanations such as acquisition of the tolerance through subsequent 2x–4x gene flow can be ruled out based on the existence of strong interploidal reproductive barriers in *Knautia*

(Ehrendorfer 1962; Kolář et al. 2009). In addition, the tetraploids probably originated directly at the edaphic island (Kolář et al. 2012) and thus they should have been serpentine tolerant already in the initial phases of their establishment. Recent serpentine literature documents several evolutionary scenarios describing the acquisition and spread of serpentine tolerance such as gradual selection, catastrophic selection, hybridization and cross-tolerance to other stresses (see Brady et al. 2005; O' Dell and Rajakaruna 2011 for review). Polyploidization, however, represents a novel pathway through which serpentine tolerance could be transmitted during the origin of a new serpentine-tolerant evolutionary unit.

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Paper 4: Kolář F., Kaplan Z., Suda J., & Štech M. (2015): Populations of Knautia in ecologically distinct refugia on the Hercynian massif belong to two endemic species. – Preslia 87: 363–386.

Populations of *Knautia* in ecologically distinct refugia on the Hercynian massif belong to two endemic species

Chrastavce (*Knautia*) z hadcových a subalpínských hercynských refugií představují dva samostatné endemické druhy

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Comprehensive taxonomic studies in which a combination of molecular, cytogenetic, morphological and ecological approaches are used have resulted in remarkable discoveries even in well-known floras. In particular, recognition of new local endemics has important implications for conservation and management of plant diversity. Due to Quaternary climatic oscillations, the vascular flora of the Czech Republic only includes a few endemic taxa, usually microspecies with an apomictic mode of reproduction. Here we re-evaluate the taxonomy of *Knautia arvensis*, an intricate eco-geographically differentiated diploid-polyploid complex, and identify two new sexual species endemic to central Europe, which were previously included in the polymorphic *K. arvensis*. While *K. serpentinicola* Smejkal ex Kolář, Z. Kaplan, J. Suda et Štech is a diploid and tetraploid species restricted to four isolated serpentine areas in the Czech Republic and Germany, diploid *K. pseudolongifolia* (Szabó) Żmuda is known from a single subalpine site in the Krkonoše Mts. Our investigation of 38 populations of *K. arvensis* s. str. and the two newly recognized species sampled across eastern central Europe revealed a distinct yet incomplete (i.e. confounded by phenotypic plasticity) morphological differences between the three species. These results together with available data on cytological (distinct nuclear genome size), genetic (independent evolutionary histories) and ecological (distinct ecological preferences) variation support an independent taxonomic status for the newly described species. Our study highlights the importance of ecologically stable habitats where plant competition is not severe (Holocene refugia) for preserving unique plant diversity. In addition, it demonstrates the value of multi-disciplinary taxonomic research even in botanically well-known areas.

K e y w o r d s: central Europe, endemic species, *Knautia*, multivariate morphometrics, polyploidy, postglacial relict, refugium, serpentine, speciation, taxonomy

Introduction

The rapid developments in molecular, cytogenetic and statistical tools have greatly influenced many research fields, including plant taxonomy and biosystematics. Detailed investigations have resulted in new discoveries and taxonomic re-assessments, even in well-researched floras such as that of the Czech Republic. The last decade has seen an increase in comprehensive studies of groups of plants with locally distributed species (putative endemics or subendemics). These have considerably changed our view of the

ecology (e.g. *Cerastium alsinifolium*: Vít et al. 2014) and distribution (e.g. *Sorbus bohemica*: Lepší et al. 2009; and *S. eximia*: Vít et al. 2012) of some Czech endemics, which resulted in a re-assessment of their taxonomy. While some central-European endemics have lost their species status after careful taxonomic revision (e.g. *Melampyrum bohemicum*: Štech 2006; *Sorbus quernea*: Lepší et al. 2013), new (sub)endemics are still being described, mainly in agamic complexes such as *Rubus* (Trávníček et al. 2005, Lepší & Lepší 2006, 2009, Trávníček & Žíla 2011), *Sorbus* (Lepší et al. 2008, 2009, 2013, Velebil 2012, Vít et al. 2012) and *Taraxacum* (Vašut & Trávníček 2004, Trávníček et al. 2008). In contrast, newly recognized sexual endemics are rare. Out of the 27 endemic taxa with a sexual mode of reproduction currently recognized in the Czech flora (Kaplan 2012), seven were described during the last five decades, namely *Campanula gelida* (Kovanda 1968), *Carex derelicta* (Holub 1960, 1965, Štěpánková 2008), *Dianthus moravicus* (Kovanda 1982), *Euphrasia corcontica* (Smejkal 1963, Dvořáková 1999a), *Minuartia corcontica* (Dvořáková 1999b), *M. smejkalii* (Dvořáková 1988) and *Scilla bifolia* subsp. *rara* (Trávníček et al. 2010). All putative Czech endemics were mainly delimited on the basis of their morphology, occasionally also karyological data, while information on their genetic variation and phylogenetic relationships inferred from molecular markers is largely missing, leaving ample room for taxonomic uncertainties and confusions (see Kaplan 2012). This is very different from the situation in zoology, where new taxa, including endemics in the Czech Republic and adjacent areas, are supported by evidence obtained from many different sources (e.g. Řezáč et al. 2008, Khatib et al. 2014).

In the present study, we investigate the variation of the polymorphic species *Knautia arvensis* (*Dipsacaceae*, or *Caprifoliaceae – Dipsacoideae*) in eastern central Europe as delimited in the Flora of the Czech Republic (Štěpánek 1997). We place special emphasis on the unique diversity preserved at ecologically distinct natural sites such as serpentine outcrops and subalpine glacial cirques that are known to harbour numerous Holocene plant relicts (e.g. Chytrý 2007, Kaplan 2012). *Knautia arvensis* belongs to the highly polymorphic, ploidy-variable and taxonomically challenging *Knautia* section *Trichera* (Schrad. ex Roem. et Schult.) DC., which occurs from western Asia to western Europe, with centres of diversity in the Balkans and Southern Alps (Ehrendorfer 1962, 1981, Rešetnik et al. 2014, Frajman et al. 2015). The evolutionary history of this section is complex, being shaped by several interacting processes. First, different species and/or cytotypes are often eco-geographically differentiated, with diploids usually growing in open less competitive habitats and polyploids at more competitive and/or man-disturbed sites (Ehrendorfer 1962, 1981; but widespread ruderal diploids are known from south-eastern Europe: Rešetnik et al. 2014). Island-like distribution of diploid populations may lead to considerable allopatric divergence potentially resulting in speciation. Divergent diploids may independently undergo genome duplication and the resulting polyploid derivatives frequently hybridize introgressively with other species of the same ploidy level, blurring species boundaries (Ehrendorfer 1962, Breton-Sintes 1974). Finally, great phenotypic plasticity in relation to ecological conditions makes morphology-based taxonomic conclusions uncertain. Interestingly, in contrast to frequent homoploid hybridization, strong reproductive barriers exist between diploid and tetraploid *Knautia* plants (Ehrendorfer 1962, Breton-Sintes 1974, 1975, Kolář et al. 2009, Hanzl et al. 2014).

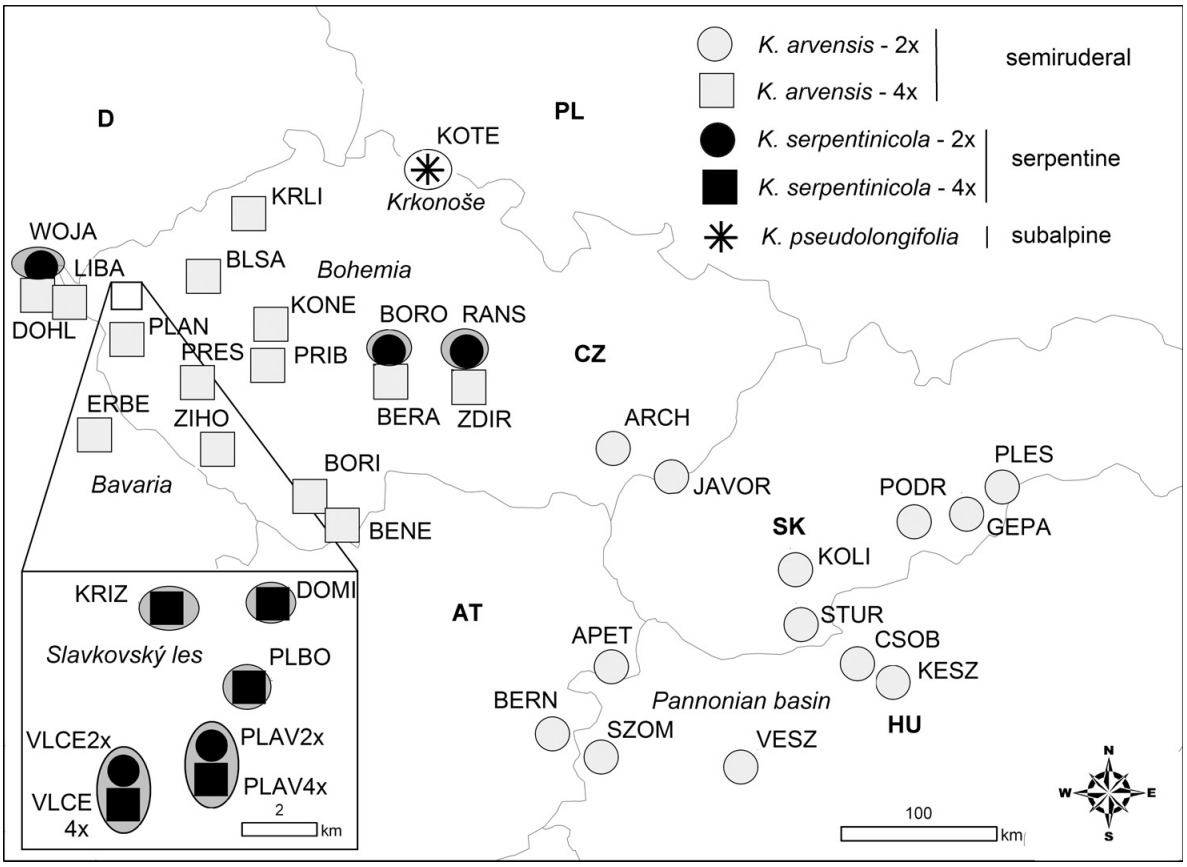

Fig. 1. – Geographic distribution, habitat preferences and ploidy level of the populations of *Knautia arvensis*, *K. serpentinicola* and *K. pseudolongifolia* analysed in this study. Circles – diploid populations, squares – tetraploid populations, white oval – subalpine grasslands, grey ovals – serpentine pine forests; the remaining populations occur in semiruderal grasslands (based on data of Kolář et al. 2009, 2012). The square inset displays the situation in the diploid-tetraploid serpentine area in the Slavkovský les Mts. See Table 1 for population codes.

Four subspecies of *Knautia arvensis* are traditionally recognized in the Czech Republic and adjacent areas, based on phenotypic, ecological, geographic and karyological differences (Štěpánek 1997, Kolář et al. 2012). Widespread diploids [2n = 2x = 20, subsp. *pannonica* (Heuff.) O. Schwarz] occur mainly in the southern part of central Europe, whereas widespread tetraploids (2n = 4x = 40, subsp. *arvensis*) occupy the northern half of the region (Štěpánek 1997, Fischer 2008, Király 2009). Both cytotypes are morphologically very similar and prefer semiruderal mesophilous grasslands (Štěpánek 1997). In addition, there are a few spatially isolated diploid populations in areas otherwise occupied by tetraploids (Fig. 1). These populations inhabit 'Holocene refugia', i.e. sites with a low level of competition where they most likely survived periods of forest expansion during the Holocene (Štěpánek 1989, Kaplan 1998, Kolář et al. 2012). First, a single morphologically distinct population from the subalpine carbonate outcrop in the Kotelní jámy glacial cirque in the Krkonoše Mts has long been recognized as subsp. *pseudolongifolia* (Szabó) O. Schwarz (Štěpánek 1989, Štěpánek & Procházka 1999, Krahulec 2006, Kaplan 2012). Second, three pure diploid populations and one diploid-tetraploid (meta)population occur in open pine forests growing on serpentine outcrops in Bohemia and northern Bavaria, and have been tentatively referred to as subsp. *serpentinicola* nom. inval. (Štěpánek 1997, Kaplan 1998, Danihelka et al. 2012; Fig. 1). Morphological

assessment of five characters of stem leaves and capitula indicate the distinctness of serpentine populations, however, limited sampling preclude robust taxonomic conclusions (Kaplan 1998). Importantly, the isolated diploid serpentine and subalpine populations form a distinct genetic lineage within all European *Knautia* diploids (i.e. the 'Northern Arvensis Group' in Rešetnik et al. 2014). The lineage itself shows a further slight genetic differentiation, most likely reflecting long-term isolation in distinct refugia (Kolář et al. 2012). Finally, serpentine tetraploids occurring in the Slavkovský les Mts (Fig. 1) originated *in situ* from their still present serpentine diploid counterparts and are only distantly related to surrounding non-serpentine tetraploids of *K. arvensis* subsp. *arvensis* (although both tetraploid cytotypes may hybridize; Kaplan 1998, Kolář et al. 2009, 2012).

This article provides a taxonomic evaluation of those central-European populations inhabiting natural habitats with a relict flora that were traditionally assigned to *Knautia arvensis*. We summarize the recently recorded karyological, genetic and ecological evidence and provide a detailed morphological assessment of the group studied. Our synthesis shows that the two lineages from isolated Holocene refugia are sufficiently distinct to merit the status of separate species. *Knautia serpentinicola* and *K. pseudolongifolia* are described at the end of the article, but their names are for the sake of clarity used hereafter.

Materials and methods

Field sampling

Plant material was collected from 2005 to 2008 in Austria, the Czech Republic, Germany, Hungary and Slovakia. Because our study focused on populations inhabiting natural habitats with a relict flora the serpentine 'archipelago' in the Slavkovský les Mts, in western Bohemia was sampled more intensely (Kolář et al. 2009, Hanzl et al. 2014). In total, we sampled 38 populations (Table 1), including (i) 13 populations of 2x *K. arvensis* subsp. *pannonica*, (ii) 14 populations of 4x *K. arvensis* subsp. *arvensis*, (iii) all known serpentine populations of *K. serpentinicola* (five diploid and five tetraploid, the metapopulation from the Slavkovský les Mts was further subdivided) and (iv) the single known subalpine population of *K. pseudolongifolia* in the Krkonoše Mts. Diploid and tetraploid subpopulations at the two mixed-ploidy serpentine sites (Table 1) were treated as separate entities due to strong inter-ploidy reproductive barriers (Breton-Sintes 1974, 1975, Kolář et al. 2009).

At each locality intact stems from 20 individuals on average (range 10–25; three and 35 individuals were collected in two exceptional cases, see Table 1) were sampled together with information on the habitat and geographic coordinates. Due to concerns about the need to conserve these species, we sampled only one fertile stem per individual, leaving basal leaf rosettes and underground parts. We aimed to cover the entire morphological variation present at each locality (except for plants lacking flowering stems or otherwise damaged and/or parasitized). Ploidy levels of all the individuals analysed were taken from Kolář et al. (2009). Genetic data (plastid and AFLP variation) were available for a subset of plants (Table 1; see Kolář et al. 2012). Vouchers are deposited in the herbarium of the Faculty of Science, University of South Bohemia, České Budějovice (CBFS). Isotypes of the newly described *K. serpentinicola* are also deposited in BRNM, BRNU, PR and PRC.

Table 1. – Details of 38 populations of the three *Knautia* species (*K. arvensis*, *K. pseudolongifolia* and *K. serpentinicola*, sorted according to ploidy and population code) subjected to multivariate morphometrics and the proportion of individuals correctly classified in the two modifications of the discriminant analysis. Code 1 – population code as in Kolář et al. (2012); Code 2 – population code as in Kolář et al. (2009), where locality details are provided. * Populations for which the classification success to a particular species is below 50%. Dataset 1 – all 16 characters were analysed in a subset of 675 individuals with divided leaves. Dataset 2 – nine characters were analysed for all 747 individuals (with both divided and undivided leaves). ** coordinates in Kolář et al. (2012) are inaccurate, the correct ones are provided in the description of *K. pseudolongifolia*.

Population code	Code 1	Code 2	Locality name, country	Habitat	Ploidy level	Number of individuals		Correct classification (%)	
						Dataset 1	Dataset 2	Dataset 1	Dataset 2
K. arvensis						501	537	81	75
APET	P12	2	AT – Apetlon	semiruderal	2x	19	20	47*	60
ARCH	P13	31	CZ – Archlebov	semiruderal	2x	20	20	95	100
BERN	P18	1	AT – Bernstein	semiruderal	2x	20	20	75	75
CSOB	P14	50	HU – Csobánka	semiruderal	2x	20	20	80	80
GEPA	–	60	SK – Gemerská Panica	semiruderal	2x	13	18	100	100
JAVOR	P15	19	CZ – Javorník	semiruderal	2x	20	20	100	90
KESZ	–	51	HU – Kesztolc	semiruderal	2x	20	20	75	55
KOLI	–	56	SK – Kolíňany	semiruderal	2x	19	22	79	63
PLES	P10	61	SK – Plešivec	semiruderal	2x	10	20	90	90
PODR	P09	58	SK – Podrečany	semiruderal	2x	13	20	85	65
STUR	–	54	SK – Štúrovo	semiruderal	2x	20	20	80	90
SZOM	P17	49	HU – Szombathely	semiruderal	2x	19	19	95	94
VESZ	P16	48	HU – Veszprém	semiruderal	2x	20	20	100	90
BENE	P35	126	CZ – Benešov n. Černou	semiruderal	4x	20	20	70	80
BERA	P37	216	CZ – Bernartice	semiruderal	4x	19	20	74	50
BLSA	P32	225	CZ – Blšany	semiruderal	4x	19	20	84	70
BORI	P34	144	CZ – Křemže	border-serpentine	4x	18	20	44*	15*
DOHL	P27	242	D – Döhlau	semiruderal	4x	20	20	90	95
ERBE	–	240	D – Erbendorf	semiruderal	4x	20	20	80	70
KONE	P33	223	CZ – Koněprusy	semiruderal	4x	19	20	74	55
KRLI	–	229	CZ – Krásná Lípa	semiruderal	4x	20	20	75	70
LIBA	P28	224	CZ – Libá	semiruderal	4x	19	20	74	55
PLAN	P29	221	CZ – Planá	semiruderal	4x	20	20	90	80
PRES	P31	215	CZ – Přeštice	semiruderal	4x	20	20	80	75
PRIB	P30	217	CZ – Příbram	semiruderal	4x	20	20	85	80
ZDIR	P38	218	CZ – Ždírec n. Doubravou	semiruderal	4x	14	18	79	94
ZIHO	–	181	CZ – Žihobce	semiruderal	4x	20	20	85	75
K. serpentinicola						174	185	87	81
BORO	P02	263	CZ – Borovsko	serpentine	2x	33	35	94	80
PLAV2x	P04	278	CZ – Planý vrch (2x)	serpentine	2x	7	10	100	90
RANS	P01	71	CZ – Staré Ransko	serpentine	2x	20	20	75	70
VLCE2x	P05	277	CZ – Vlček (2x)	serpentine	2x	2	3	100	66
WOJA	P03	279	D – Woja	serpentine	2x	19	19	89	100
DOMI	P24	261	CZ – Dominova skalka	serpentine	4x	20	20	80	80
KRIZ	P23	260	CZ – Křížky	serpentine	4x	20	20	70	60
PLAV	P20	278	CZ – Planý vrch (4x)	serpentine	4x	16	19	94	94
PLBO	P22	259	CZ – Pluhův bor	serpentine	4x	19	20	95	90
VLCE	P21	277	CZ – Vlček (4x)	serpentine	4x	18	19	89	73
K. pseudolongifolia						–	25	–	100
KOTE	P06	72**	CZ – Krkonoše	subalpine	2x	–	25	–	100
Total/average						675	747	82	77

Morphometric analyses

We assessed 16 morphological characters (12 primary and four ratios) of stems, stem leaves and the terminal inflorescence of air-dried herbarium vouchers (Table 2); ploidy level of all individuals investigated was inferred from fresh material prior to desiccation (data published in Kolář et al. 2009). Missing values of the width of the terminal head for three individuals were replaced by population means. Because seven of the 16 characters could only be evaluated on divided or lobate leaves, two datasets were generated and analysed separately. Entire or shallowly-lobed leaves were a feature of nearly all individuals at the subalpine site and also of a few plants in other populations. Dataset 1 included 675 plants with divided leaves and all 16 characters; Dataset 2 included all 747 plants but only nine characters. No pair of characters was highly correlated (Spearman's r always below 0.9). It should be noted that some characters previously used to identify *Knautia* species (e.g. characteristics of daughter rosettes and fruits) could not be statistically evaluated by us due to non-destructive sampling and a single visit to each locality (these characters are nonetheless addressed in the Discussion). Other characters were excluded because of their high environmentally determined plasticity (e.g. the length of involucral bracts, number and length of calyx bristles) as revealed by long-term experimental cultivation (Štěpánek 1979).

Delimitation of taxonomic groups for discriminant analyses was morphology-independent, based on patterns of ploidy and genetic variation (Kolář et al. 2009, 2012) and partly also on habitat-related distribution. Genetically highly divergent diploid populations with distinct monoploid genome size (cluster K1 of Kolář et al. 2012) formed one group (taxonomically corresponding to *K. arvensis* subsp. *pannonica*), while serpentine (*K. serpentinicola*) and subalpine (*K. pseudolongifolia*) diploids formed two additional taxonomic groups. At the tetraploid level, we distinguished two groups reflecting the serpentine/non-serpentine dichotomy (i.e. *K. serpentinicola* vs *K. arvensis* subsp. *arvensis*, respectively) and their independent evolutionary histories and partly also their genetic variation. The genetic structure at the tetraploid level was, however, less pronounced due to introgressive hybridization between different lineages (Kolář et al. 2012).

Data were analysed using a set of R-scripts morphotools 1.1 (Koutecký 2014) in R 3.1.1. Specifically, principal component analysis (PCA) was constructed to visualize main directions of variation of individuals and population means using the prcomp function in package stats. To test differences among the a-priori defined taxonomic groups we applied linear discriminant analysis using the cca function in vegan (Oksanen et al. 2013) and classificatory discriminant analysis using the lda function in MASS (Venables & Ripley 2002). We used four modifications of discriminant analyses: (i) a discrimination of three taxonomic groups regardless of ploidy (i.e. *K. serpentinicola*, *K. pseudolongifolia*, and *K. arvensis*) using only nine characters that were possible to score on plants with undivided leaves, (ii) a discrimination of two taxonomic groups regardless of ploidy using all characters (i.e. *K. serpentinicola* and *K. arvensis*), (iii) a discrimination of *K. serpentinicola* di- and tetraploids, and (iv) a discrimination of *K. arvensis* di- and tetraploids (using all 16 characters for both).

Table 2. – Morphological characters used in statistical analyses and their discrimination power in four separate linear discriminant analyses (LDA). * marginal effect, 0.003 < P < 0.05 (not passing Bonferroni correction), *** marginal effect, P < 0.003 (passing Bonferroni correction), +characters that were possible to score on plants with entire or shallowly lobed leaves. LDA 1 – discrimination of two taxonomic groups: *K. arvensis* and *K. serpentinicola* (Fig. 2B); LDA 2: discrimination of three taxonomic groups: *K. arvensis*, *K. serpentinicola* and *K. pseudolongifolia* (values for the first two canonical axes are shown; Fig. 3B); LDA 3: discrimination of two cytotype groups: 2x *K. arvensis* and 4x *K. arvensis*; LDA 4: discrimination of two cytotype groups: 2x *K. serpentinicola* and 4x *K. serpentinicola*.

Code	Description	LDA 1 axis 1	LDA 2 axis 1	LDA 2 axis 2	LDA 3 axis 1	LDA 4 axis 1
Height+	Plant height	0.69***	0.26***	0.82	–0.19*	0.03
BranchDg+	Degree of branching	0.35***	0.22***	0.42	–0.74***	–0.18
NLeaf+	Number of pairs of stem leaves	0.23***	0.14***	0.26	–0.15*	0.19
StemDiam+	Diameter of the stem 2 cm below the terminal head	0.39***	–0.62***	0.55	0.14*	0.52***
NGlands+	Number of glands on the stem 2–3 cm below the terminal head (semi quantitative)	0.18***	0.12***	0.21	–0.01	–0.10
HeadDiam+	Head diameter (distance between the tips of bracts in the terminal head)	0.06	–0.33***	0.13	0.24***	0.23*
LLength+	Length of the 2nd lowermost stem leaf	0.30***	–0.06***	0.39	–0.13*	–0.23*
LWidth+	Width of the 2nd lowermost stem leaf	0.28***	0.22***	0.29	0.01	–0.19
LatLength	Length of the longest lateral lobe of the 2nd stem leaf	0.31***	–	–	0.05	–0.02
LatWidth	Width of the longest lateral lobe of the 2nd stem leaf	0.45***	–	–	–0.16*	–0.25*
TerLength	Length of the terminal lobe of the 2nd stem leaf	0.15***	–	–	–0.08	–0.14
TerWidth	Width of the terminal lobe of the 2nd stem leaf	0.30***	–	–	–0.16*	–0.21
LL_LW+	Length / width of the 2nd stem leaf	–0.15***	–0.44***	–0.03	–0.12*	0.07
LatL_LatW	Length / width of the longest lateral lobe of the 2nd stem leaf	–0.20***	–	–	0.25***	0.18
TerL_TerW	Length / width of the terminal lobe of the 2nd stem leaf	–0.31***	–	–	0.07	0.11
LL_TerL	Total length / length of the terminal lobe of the 2nd stem leaf	0.11*	–	–	–0.07	–0.08

Results

PCA analysis of populations containing plants with divided leaves, i.e. *K. serpentinicola* and *K. arvensis*, revealed some morphological differentiation between these species (Fig. 2A). However, the phenotypic variation was rather continuous and *K. serpentinicola* largely overlapped *K. arvensis* in the PCA plot of individual plants (Electronic Appendix 1). Slender and less-branched individuals with shorter and narrower stem leaves in the latter species posed considerable problems (see inset in Fig. 2A). Discriminant analysis was highly significant (P = 0.001 with 1000 permutations; Fig. 2B) with 82% successfully classified individuals (Table 1). Plants of *K. arvensis* were slightly less well classified than those of *K. serpentinicola* (81% vs 87%). Most misclassified *K. arvensis* plants originated from populations inhabiting sites with distinct ecological conditions (e.g. pop. APET growing in subhalophilous steppe or pop. BORI at the margin of a serpentine pine forest, both with less than 50% of the individuals correctly classified; Table 1). The following characters contributed most to the discrimination of both taxonomic groups (*K. serpentinicola* plants always have lower character values): plant height, stem diameter, degree of branching, length and width of the second stem leaf, length and width of the longest lateral lobe and length/width ratio of the terminal lobe (Table 2).

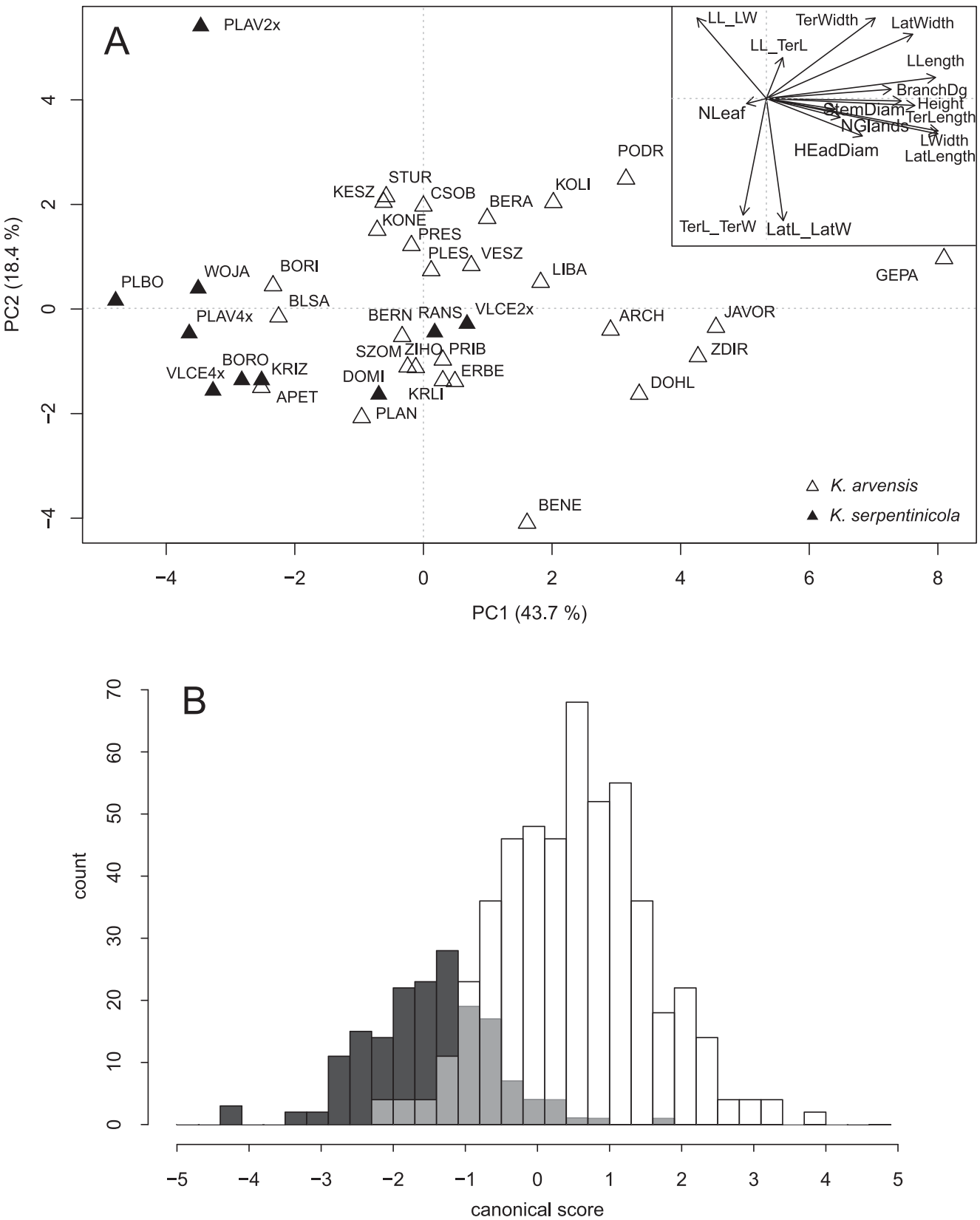

Fig. 2. – Morphological differentiation of *Knautia arvensis* (open triangles) and *K. serpentinicola* (solid triangles). (A) Principal component analysis of 37 populations based on mean values per population (only accessions with divided leaves were included). The inset displays ordination of 16 morphological characters used in the analysis (see Table 2 for code explanations). (B) Scores of individual plants of the two species on the first canonical axis of the linear discriminant analysis using the 16 characters (overlap of both species is indicated by grey colour).

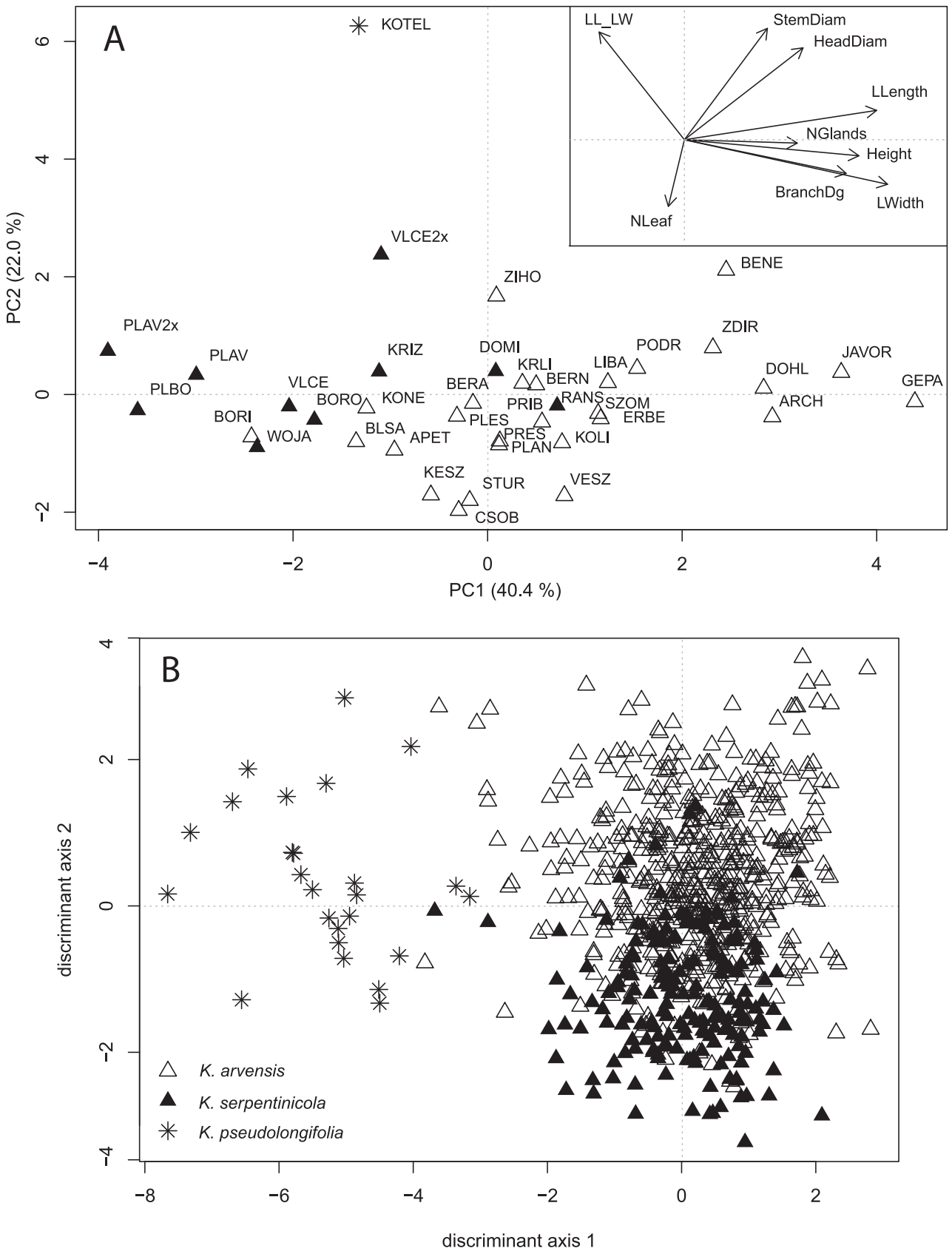

Fig. 3. – Morphological differentiation of *Knautia arvensis* (open triangles), *K. serpentinicola* (solid triangles) and *K. pseudolongifolia* (asterisks). (A) Principal component analysis of all 38 populations. The inset displays ordination of nine morphological characters used in the analysis (see Table 2 for code explanations). (B) Ordination of all 747 individuals along the two canonical axes of the linear discriminant analysis using the nine characters.

The subalpine diploid population of *K. pseudolongifolia* is morphologically distinct and well separated from the other two species (Fig. 3A). Discriminant analysis further confirmed considerable phenotypic differences (P = 0.001 with 1000 permutations; Fig. 3B) and resulted in 100% of the individuals being correctly classified (Table 1). In comparison with other *Knautia* species studied, *K. pseudolongifolia* has generally narrower stem leaves, higher leaf length/width ratio and stouter stems below the terminal inflorescence (Table 2).

Because the above analyses included both diploid and tetraploid individuals, we further investigated potential inter-ploidy differences in each of the ploidy-variable groups separately. Di- and tetraploids were phenotypically similar in both *K. serpentinicola* and *K. arvensis* (Electronic Appendix 2) although discriminant analysis revealed a significant effect of ploidy level (P = 0.001, 1000 permutations in both cases). Classification of individuals according to their ploidy level was largely unsuccessful for *K. serpentinicola* (59% success rate) but moderately successful for *K. arvensis* (72% success rate; see Table 3 for details).

Discussion

In this article we summarize many pieces of evidence that support the independent taxonomic status of two little known sexual lineages of *Knautia* endemic to the Hercynian massif, described here as *K. serpentinicola* and *K. pseudolongifolia*, which were assigned traditionally to polymorphic *K. arvensis*. We argue that the level of genetic, cytological, morphological and ecological differentiation of these central-European endemics is comparable to other currently recognized species in the genus *Knautia* (Ehrendorfer 1976, Rešetnik et al. 2014).

Genetic and cytological evidence

The separate evolutionary position of *K. serpentinicola* and *K. pseudolongifolia* (as against diploid *K. arvensis* subsp. *pannonica*) is indicated by both the nuclear genome size, which is significantly smaller in the two former species (Kolář et al. 2009) and by AFLP markers, which clearly distinguished the lineage containing *K. serpentinicola* and *K. pseudolongifolia* from that of *K. arvensis* subsp. *pannonica* (Kolář et al. 2012). Importantly, the group formed by *K. serpentinicola* and *K. pseudolongifolia* is genetically distinct in both ITS and AFLP analyses of all diploid members of the section *Trichera* (Rešetnik et al. 2014). *Knautia serpentinicola* and *K. pseudolongifolia* are closely related to *K. slovaca* Štěpánek, another local endemic of central Europe occupying somewhat similar habitats in open limestone pine forest (Štěpánek 1983); all three species form a separate lineage within the genus (Rešetnik et al. 2014). *Knautia slovaca*, however, differs from both the newly described species and *K. arvensis* in terms of their AFLP profiles (Kolář et al. 2012), dense and soft indumentum on both stem and leaves, relatively long terminal leaflets of stem leaves and pink to reddish-violet corollas (Štěpánek 1983, 1985). Diploids of *K. serpentinicola* and *K. pseudolongifolia* also differ genetically from the tetraploid *K. arvensis* subsp. *arvensis* (Kolář et al. 2012). In contrast, the 2x and 4x cytotypes of *K. serpentinicola* are genetically very similar, indicating a local autopolyploid origin of the tetraploids (Kolář et al. 2012).

Table 3. – Classification success of diploid and tetraploid individuals analysed separately for *Knautia arvensis* and *K. serpentinicola.*

	N	correct classification (%)
Knautia arvensis		
diploid	233	73
tetraploid	268	71
total	501	72
Knautia serpentinicola		
diploid	81	56
tetraploid	93	62
total	174	59

Reproductive evidence

Both endemic species seem to be well separated reproductively from each other and from other taxa. *Knautia serpentinicola* and *K. pseudolongifolia* are currently isolated by distinct eco-geographical barriers (although we are aware that this does not necessarily imply physiological reproductive barriers; Ehrendorfer 1962, Štěpánek 1979). *Knautia serpentinicola* occasionally meets tetraploid *K. arvensis*, which occurs at the borders of serpentine outcrops. Owing to strong inter-ploidy breeding barriers in the entire section *Trichera* (Ehrendorfer 1962, Breton-Sintes 1974, 1975), including the nearly total absence of triploids (i.e. mediators of possible unidirectional gene flow from tetraploids to diploids; Kolář et al. 2009, Hanzl et al. 2014), this spatial contact poses only a minimal threat to the genetic integrity of the serpentine diploids. In contrast, patterns of genetic and phenotypic variation indicate it is very likely that tetraploid cytotypes of *K. serpentinicola* and *K. arvensis* hybridize in the Slavkovský les Mts. However, the gene flow seems to be unidirectional from serpentine to non-serpentine populations (Kolář et al. 2012), ensuring the integrity of 4x *K. serpentinicola*. Low level of hybridization recorded in stands growing on serpentine may be, at least in part, associated with the reduced fitness of the non-serpentine *K. arvensis* under stressful serpentine conditions (Kolář et al. 2014).

Ecological and distributional evidence

The complex late Pleistocene/Holocene history of the populations investigated has resulted in a distinct spatio-ecological pattern in central Europe (Kaplan 1998, Kolář et al. 2012). While *K. arvensis* typically occupies various semiruderal grasslands and has a rather continuous distribution at low and middle altitudes, the two relict species presumably of late Pleistocene/early Holocene origin occur in spatially isolated, ecologically stable, low-competitive habitats, including open forests on serpentine soil (*K. serpentinicola*) and a carbonate outcrop in a subalpine glacial cirque (*K. pseudolongifolia*). Our cultivation experiments have shown that both cytotypes of *K. serpentinicola* are well adapted to growing in chemically stressful serpentine soils, whereas central-European populations of *K. arvensis* exhibit considerably reduced growth in serpentine conditions (Kolář et al. 2014).

Morphological evidence

Our morphometric analyses using fifteen vegetative and one generative character revealed clear trends in phenotypic variation that correspond with the genetic, cytological and ecological differentiation of the group studied. However, large phenotypic plasticity, particularly of *K. arvensis*, and probable gene flow at the tetraploid level account for the incomplete morphological segregation of the three species investigated.

Knautia pseudolongifolia is morphologically the most distinct taxon and is characterized by prolonged and usually undivided or only shallowly divided stem leaves that are glabrous or sparsely pubescent, large terminal heads born on stout stems and relatively large fruits (our data and that of Štěpánek 1982, 1989, 1997). Thus, these pronounced morphological and ecological differences justify the separation of *K. pseudolongifolia* from the genetically close *K. serpentinicola*.

Despite obvious trends in morphological data, *K. serpentinicola* is only slightly different from the widespread *K. arvensis* as there is considerable overlap in most of the characters examined. Inter-specific differences are usually quantitative and at least some characters seem to be dependent on soil conditions (see below). It should, however, be noted that our sampling intentionally covered the entire morphological variation present in each population and that extreme forms might have blurred boundaries between the species. In addition, we did not formally explore the value of potentially taxonomically informative characters, which are difficult to evaluate statistically or can hardly be assessed on herbarium vouchers, including the overall stature (more slender in *K. serpentinicola*; Štěpánek 1997, our field observations), flower colour (darker reddish-violet in *K. serpentinicola*; Štěpánek 1982, Kaplan 1998), shape of the lower corolla lobe (longer and narrower in *K. serpentinicola*; our field observations) and the frequent production of multiple lateral rosettes in *K. serpentinicola* (Štěpánek 1997). Importantly, several characters used to recognize *K. serpentinicola* (e.g. slender appearance, slender stem, dark flowers, smaller leaves) seem to be (epi)genetically fixed and independent of ecological conditions because they have remained stable in the same individual grown in garden soil over period of 17 years (Z. Kaplan, pers. observation; see Electronic Appendix 3). Thus, long-term common garden experiments combined with reciprocal transplants are needed to understand the interplay of ecological and genetic factors in shaping the phenotype of individual species of *Knautia*.

Knautia plants growing in atypical habitat for a given species (e.g. *K. arvensis* at the borders of serpentine areas and sub-halophilous sites, or *K. serpentinicola* at the margins of forest roads and grasslands on deforested serpentine outcrops) are particularly difficult to determine morphologically because they resemble the other taxon (see Figs. 2, 3). Such phenotypes are most likely manifestations of the great phenotypic plasticity of both species although genetic reasons (i.e. gene flow between serpentine and non-serpentine tetraploids; Kolář et al. 2012) cannot be excluded for certain populations in the Slavkovský les Mts.

Individuals of different ploidy levels within the same species only differ slightly morphologically. This is particularly true for *K. serpentinicola* (classification success below 60%), in which inter-ploidy morphological similarities are likely to reflect close genetic relationships between diploids and their local autotetraploid derivatives. As expected, classification success of different cytotypes of *K. arvensis* (2x subsp. *pannonica* and 4x

subsp. *arvensis*) was higher (~72%), which reflects the deep genetic split between both ploidy levels (Kolář et al. 2012) and their largely parapatric distribution in ecologically distinct regions on the Hercynian massif and in the Pannonian basin (Fig. 1).

Conservation value of the Hercynian Knautia endemics

A combination of intriguing evolutionary history, genetic and karyological distinctness and remarkable ecological and physiological adaptations make *K. serpentinicola* and *K. pseudolongifolia* valuable examples of likely Quaternary speciation and a high conservation priority. Both species are restricted to the Hercynian massif, representing distinct endemic elements in an otherwise endemic-poor Czech flora (Kaplan 2012). Their origin may serve as a textbook example of a complex evolutionary scenario that occurred during a succession of changes in the central-European vegetation during the Holocene, involving spatial isolation in low-competitive Holocene refugia, allopatric differentiation and local polyploidization (Štěpánek 1989, Kaplan 1998, Kolář et al. 2012). The ability of these endemic species to adapt to harsh environments such as subalpine habitats or serpentine outcrops (experimentally proven for *K. serpentinicola*; Kolář et al. 2014) indicates that this group is highly ecologically plastic and highlights the key role of ecological conditions in the origin of these species. Populations of *K. serpentinicola* in the Slavkovský les Mts deserve the particular attention of conservation authorities because they represent a rare case where an edaphically-specialized diploid co-occurs with its auto-tetraploid derivative. Such systems serve as unique 'natural laboratories' offering possibilities for further research on general mechanisms of polyploid origin and establishment (Hanzl et al. 2014). This fact together with indications that the tetraploid genotype can spread beyond the borders of its original refugium imply that serpentine relicts are not evolutionary dead-ends but still have the potential to shape the surrounding biota (Kolář et al. 2012).

All of the (meta)populations of *K. serpentinicola* known are sufficiently large (reaching several hundreds to thousands of individuals), occur in stable habitats and regularly set fertile seeds (although vegetative reproduction via lateral rosettes seems to be frequent; Hanzl et al. 2014). This species would qualify as endangered (EN following IUCN) and in category C2 (see Grulich 2012) in the Czech Republic and critically endangered (CR) in Bavaria, Germany because only the smallest population (Woja) occurs there. *Knautia serpentinicola* should be brought under legislative protection in both countries. The high inter-population genetic differentiation (Kolář et al. 2012) suggests that optimally populations in all the disjunct areas should be protected in order to preserve the major part of this species' genetic diversity. Actually, this is already the case because significant parts of all *K. serpentinicola* populations lie within small-scale protected areas. It is desirable to consider the ecological requirements of this species in the management plans for these sites. Dramatic and large-scale changes in forest management (e.g. establishing dense spruce plantations or the destruction of patches of heliophilous vegetation) represent the greatest potential threat to *K. serpentinicola.*

In contrast, there is a maximum of only 100–200 plants of *K. pseudolongifolia* (authors' pers. obs.), restricted to a small part of a single glacial cirque (Štěpánek 1989). Although there seems to be no immediate threat to this species, we consider the category C1 (critically endangered, CR following IUCN), suggested in previous red lists (Holub & Procházka 2000, Grulich 2012), appropriate given the rarity of *K. pseudolongifolia*.

Descriptions of the two newly recognized species

Values of quantitative characters in the following morphological description are expressed as (minimum–) 5 percentile–95 percentile (–maximum); sizes of the stomatal guard cells, pollen grains and fruits are those cited by Štěpánek (1997).

Knautia serpentinicola Smejkal ex Kolář, Z. Kaplan, J. Suda et Štech, **spec. nova**

T y p e: Czech Republic, Středočeský kraj, Bernartice: pine forest next to the highway bridge; alt. 400 m a.s.l.; lat: 49°41'17.1N", long 15°06'19.8"E; 26.6.2006; leg. F. Kolář & M. Štech (holotype: CBFS, No. 5310, Fig. 4; isotypes: BRNM, No. 414224; BRNU, No. 634135; PR, No. 843328; PRC, No. 455079).

D e s c r i p t i o n: Perennial herbs with a sympodial rhizome, forming numerous basal leaf rosettes. Flowering stems erect, slender, unbranched or sparingly branched, (14–) 24–61 (–89) cm high, (0.6–) 0.8–1.2 (–1.4) mm in diameter below the terminal head, with (1–) 2–6 (–7) pairs of opposite leaves. Indumentum consisting of numerous eglandular hairs and soft bristles, occasionally with glandular hairs below the inflorescence. Middle stem leaves sessile, lanceolate to oval, (3.2–) 4.3–12.1 (–17.7) cm × (1.0–) 1.4–6.9 (–8.3) cm, (1.4–) 1.6–3.9 (–6.2) times longer than wide, usually pinnatifid to pinnatisect with 1–2 (–4) lateral lobes on each side, rarely undivided, bristly hairy. The largest lateral lobe (6–) 10–36 (–48) mm long and (1.2–) 1.7–5.3 (–9.3) mm wide; terminal lobe (12–) 20–64 (–83) mm long and (1.5–) 2.9–12.7 (–21.7) mm wide, accounting for 2/5–1/2 of total leaf length. Stomatal guard cells on the adaxial surface of stem leaves (25–) 28–37 (–43) μm long. Terminal head relatively small, (10–) 15–27 (–36) mm in diameter (measured as the distance between the tips of involucral bracts), with rounded, bristly hairy base. Outer involucral bracts narrowly lanceolate to obovate. Flowers hermaphrodite, bilaterally symmetric, tetramerous, scentless. Calyx synsepalous, with a cup-shaped tube and several terminal bristles, hairy, shed at fruiting. Corolla sympetalous, violet to dark reddish-violet, with a short tube and four unequal lobes, the lower lobe elongated. Stamens four, adnate to corolla tube, protruding out of the flowers; pollen grains (80–) 84–94 (–105) μm in diameter. Ovary inferior, bicarpellate, style protruding from the corolla tube, stigma bilobed. Achenes elliptic, weakly compressed laterally, (3.5–) 4.1–4.7 (–5.4) mm long, hairy, greenish to dark brown, with persistent white fleshy pedicel (elaiosome). Flowers July – September. 2n = 20 (all regions), 40 (the Slavkovský les Mts). Figs 4, 6A.

D i a g n o s i s: *Knautia serpentinicola* resembles slender forms of *K. arvensis*, however, it differs in having numerous lateral rosettes, unbranched or sparingly branched flowering stems, which are more slender below the inflorescences. In addition, *K. serpentinicola* has shorter and narrower middle stem leaves, with less developed lobes. The colour of petals is somewhat darker than in *K. arvensis*.

Distribution, population size and genetic structure

Knautia serpentinicola was first recognized by Miroslav Smejkal who tentatively ("in schedis") identified morphologically distinct serpentine plants from the Ranský Babylon hill as *K. arvensis* subsp. *serpentinicola* in 1967 and 1972 (vouchers deposited in BRNU). The likely independent taxonomic status of serpentine populations was then further discussed in a karyological study of *Knautia* in the former Czechoslovakia (Štěpánek

Herbarium of the Faculty of Science, University of South Bohemia (CBFS) No. 5310

Knautia arvensis subsp. serpentinicola Smejkal ined.

Czechia, Středočeský kraj

Bernartice: pine forest next to the highway bridge

Coord. (WGS84): lat.: +49.6881 , long.: +15.1055
Altitude: 400 m a. s. l. Mapping grid cell: 6356b
Phytogeogr. region: 41. Střední Povltaví

Date: 26.6.2006 Legit: Kolář Filip & Štech Milan

Note: DNA-2x (FCM), plant 23

HOLOTYPE of

Knautia serpentinicola Smejkal ex Kolář, Z. Kaplan, J. Suda et Štech

Fig. 4. – Holotype of *Knautia serpentinicola* deposited in CBFS.

1982) and also reflected (although still within *K. arvensis*) in the Flora of the Czech Republic using a provisional name (Štěpánek 1997).

The species occurs in four spatially isolated serpentine areas on the Hercynian massif, three of which are located in the Czech Republic and one in northern Bavaria, Germany. The largest population of the diploid cytotype, consisting of several thousands of individuals, grows in the eastern part of central Bohemia, in the serpentine area of Dolnokralovické hadce north and north-west of Bernartice village in the valley of the Želivka river (locus classicus). This population occupies nearly the entire serpentine body (population borders are located approx. at 49°41'17"N, 15°05'54"E; 49°41'00"N, 15°06'49"E; 49°41'00"N, 15°08'02"E; and 49°41'24"N, 15°06'40"E; all GPS coordinates for the Czech Republic taken from www.mapy.cz). Most plants occur in pine forests on the flat central area but some occur on the rocky serpentine slopes, avoiding only the most exposed and driest sites. The second diploid population occupies serpentine outcrops on the Ranský Babylon hill, south of the town Ždírec nad Doubravou in the Vysočina region. Here, *K. serpentinicola* occurs in open grassy patches along a paved forest road (approx. between 49°40'06"N, 15°49'46"E and 49°39'05"N, 15°49'02"E) from where it extends into forests on serpentine bedrock. The population size is nearly 1,000 individuals, although we cannot exclude the possibility that additional patches not recorded by us exist at this site. The last purely diploid population inhabits a small serpentine outcrop Wojaleite east of Wurlitz, in the Hof region in northern Bavaria. Plants occur in pine forest both in the flatland above serpentine rocks in the northern part of this site (50°15'14"N, 11°58'28"E) and, more often, in the southern part of the serpentine area (50°15'07"N, 11°58'21"E; coordinates taken from maps.google.com). The total population size was estimated to be several hundreds of individuals. Finally, diploid and tetraploid cytotypes co-occur in a large serpentine area at Mnichovské hadce in the Slavkovský les Mts, in the Karlovarský region. Diploids are less abundant (a few thousand individuals) and confined to several patches on the southern slopes of the Vlčí hřbet massif, between Vlček and Planý vrch hills (approx. between 50°01'47"N, 12°44'08"E and 50°02'17"N, 12°46'01"E). Interestingly, diploid plants almost exclusively occur at permanently forested sites and avoid new forest plantations (Hanzl et al. 2014). In contrast, tetraploids are much more widespread and abundant there (many thousands of individuals); they occupy extensive forested areas on serpentine bedrock between Vlčí hřbet hill (50°01'41"N, 12°43'25"E) and Pluhův bor (50°03'33"N, 12°47'15"E) and also occur on isolated rocky outcrops at Dominova skalka (50°04'17"N, 12°47'09"E) and Křížky (50°03'58"N, 12°44'55"E). For details on the distribution of cytotypes in the Slavkovský les Mts see Hanzl et al. (2014). It is likely that serpentine tetraploids hybridize with tetraploid *K. arvensis* subsp. *arvensis*. The extent of hybridization is currently unknown but considered in a separate study (M. Čertner et al., unpubl.). The altitudinal range of this species is from 380 m a.s.l. (Dolnokralovické hadce) to 883 m a.s.l. (top of Vlčí hřbet hill in the Slavkovský les Mts).

This species seems to be endemic to the above-listed serpentine sites on the Hercynian massif. No other populations were found during a thorough search of other serpentine outcrops and ecologically similar relict non-serpentine sites (e.g. open pine and oak-pine forests in rocky river valleys) (Kaplan 1998, Kolář et al. 2009). Although all known populations of *K. serpentinicola* occur at serpentine sites, this species is not an obligate serpentinophyte and the plants also thrive when planted in non-serpentine garden soil (F. Kolář and Z. Kaplan, pers. observ.).

Some peculiar forms of *Knautia,* with a tetraploid number of chromosomes, were previously recorded from the serpentine area at Křemžské hadce in southern Bohemia (Kaplan 1998). However, following a detailed survey they turned out to be 4x *K. arvensis*, which occasionally spread out from surrounding non-serpentine habitats and a distinct hexaploid species *K. dipsacifolia* (Kolář et al. 2009, 2012).

The two diploid populations in central Bohemia (Dolnokralovické hadce and Ranský Babylon) are genetically distinct from their western-Bohemian and Bavarian counterparts. Intra-population genetic diversity is relatively high and very comparable to central-European populations of the widespread *K. arvensis*. Most of these diploid populations have a high proportion of rare genetic markers, most likely reflecting their long-term spatial isolation (Kolář et al. 2012).

Ecology

Knautia serpentinicola occurs exclusively on serpentine outcrops, mostly in open pine forests of the *Dicrano-Pinion sylvestris* alliance (assoc. *Asplenio cuneifolii-Pinetum sylvestris* and *Vaccinio myrtilli-Pinetum sylvestris*; the nomenclature follows Chytrý 2013) and in different types of secondary forests with dominant pine and spruce (however, it never grows in dense spruce or pine plantations). At Dolnokralovické hadce, *K. serpentinicola* occasionally occurs in relict pine forests with *Sesleria* of the *Erico carneae-Pinion* alliance (*Thlaspio montani-Pinetum sylvestris*). It prefers open and slightly moister patches in forests, forest clearings, old forest roads and/or grassy roadsides. Tetraploids have a broader ecological niche and also occur in secondary grasslands and heathlands on serpentine bedrock. *Knautia serpentinicola* often co-occurs with several other heliophilous species, which are probably also relicts from the last glaciation and/or early Holocene, including *Cerastium alsinifolium*, *Erica carnea*, *Polygala chamaebuxus* and *Thesium alpinum* in the Slavkovský les Mts, *Armeria vulgaris*, *Dianthus carthusianorum*, *Minuartia smejkalii*, *Potentilla crantzii*, *Sesleria caerulea* and *Thlaspi montanum* at Dolnokralovické hadce, and *Armeria vulgaris*, *Dianthus gratianopolitanus*, *Festuca pallens* and *Saxifraga rosacea* at Wojaleite.

Knautia pseudolongifolia (Szabó) Żmuda, Bull. Acad. Sci. Cracov., sci. natur., 1916: 171, 1917.

Syn.: *Knautia arvensis* var. *pseudolongifolia* Szabó, Math. Termeszettud. Közlem. 31: 244, 1911; *K. arvensis* subsp. *pseudolongifolia* (Szabó) O. Schwarz, Mitt. Thüring. Bot. Ges. 1: 118, 1949; *Trichera arvensis* subsp. *pseudolongifolia* (Szabó) Holub, Preslia 51: 282, 1979.

Lectotype (designated here): [Czech Republic:] Flora des Riesengebirges, Kl. Kessel an der Kesselkoppe, d. 28. Juli 1889, E. Fiek (WRSL; Fig. 5).

Note on typification: Two collections (syntypes) used for the description of *K. arvensis* var. *pseudolongifolia* by Szabó (1911) are mentioned in the protologue: "Riesengebirge: in cacumine Kesselkoppe (Engler!, Fiek!)". None of these syntypes were located in BP where the main herbarium of Szabó is kept. When working on his monograph of *Knautia*, Szabó also used specimens borrowed from museums in Berlin and Wrocław (see Szabó 1911: p. 7). Collections of H. G. A. Engler were preserved in B but were destroyed during World War II. Collections of E. Fiek are preserved in WRSL and one specimen (Fig. 5) that matches both the type citation and the description given in the protologue was found there. This is apparently the only extant specimen that meets the definition of the Code for original materials and is thus designated as the lectotype.

D e s c r i p t i o n: Perennial herbaceous plants with sympodial rhizomes, forming several basal leaf rosettes. Flowering stem erect, relatively robust, usually unbranched (rarely sparingly branched), (18–) 28–58 (–64) cm high, 1.4–2.0 (–2.2) mm in diameter below the terminal head, with 3–6 pairs of stem leaves. Indumentum consisting of numerous eglandular hairs and soft bristles, occasionally with glandular hairs below the inflorescence. Middle stem leaves sessile, narrowly lanceolate to lanceolate, (7.1–) 7.5–13.1 (–14.8) cm × (1.3–) 1.4–3.9 (–5.4) cm, (2.4–) 2.8–7.3 (–7.5) times longer than wide, usually undivided, serrate, rarely pinnately-lobed to pinnately-parted with 2–6 usually unequal lobes on each side, scarcely bristly hairy to glabrous, relatively thick. Terminal lobe (if present) short, accounting for 1/3–2/5 of total leaf length. Stomatal guard cells on the adaxial surface of stem leaves (28–) 31–37 (–40) μm long. Terminal head relatively large, (22–) 23–33 (–35) mm in diameter (measured as the distance between the tips of involucral bracts), with rounded bristly hairy inflorescence base. Outer involucral bracts lanceolate to ovate. Flowers hermaphrodite, bilaterally symmetric, tetramerous, scentless. Calyx synsepalous, with a cup-shaped tube and several terminal bristles, hairy, shed at fruiting. Corolla sympetalous, pink to pinkish-violet, with a short tube and four unequal lobes. Stamens four, adnate to corolla tube, protruding out of the flowers; pollen grains (88–) 90–100 (–108) μm in diameter. Ovary inferior, bicarpellate, style protruding from the corolla tube, stigma bilobed. Achenes elliptic, weakly compressed laterally, (4.8–) 5.2–5.7 (–5.8) mm long, hairy, greenish to dark brown, with persistent white fleshy pedicel (elaiosome). Flowers August – September. 2n = 20. Figs 5, 6C.

D i a g n o s i s: *Knautia pseudolongifolia* differs from both *K. arvensis* and *K. serpentinicola* in having narrow and usually undivided or only shallowly-lobed stem leaves, which are scarcely pubescent to almost glabrous. In addition, flower heads of *K. pseudolongifolia* are larger, stem below the terminal inflorescence is stouter and ripe fruits are larger.

Distribution, population size and genetic structure

Knautia pseudolongifolia was described at the rank of a variety at the beginning of the 20th century (Szabó 1911) and recently usually recognized as a subspecies of *K. arvensis* (Štěpánek 1989, 1997). This species is known from a single population confined to the ridge between Malá and Velká Kotelní jáma glacial cirques in the Krkonoše Mts, ~1320–1390 m a.s.l. (50°45'06"N, 15°31'57"E). The population comprises 100–200 individuals at maximum, most of which occur in an area of approx. 30 × 30 m (Štěpánek 1989). Several plants were recently found by us (FK) ca 150 m away at 50°45'10", 15°31'57". Genetic diversity is relatively high despite the long-term isolation and small number of individuals (Kolář et al. 2012).

Ecology

This species grows in neutral soils on carbonate outcrops in subalpine grassland (Šourek 1969). *Knautia pseudolongifolia* usually occurs in relatively basiphilous and moist subalpine grassland communities of the *Agrostion alpinae* alliance (assoc. *Saxifrago oppositifoliae-Festucetum versicoloris*; the nomenclature follows Chytrý 2007; Fig. 6D), occasionally occurring in surrounding dwarf shrub vegetation with dominant *Calluna*

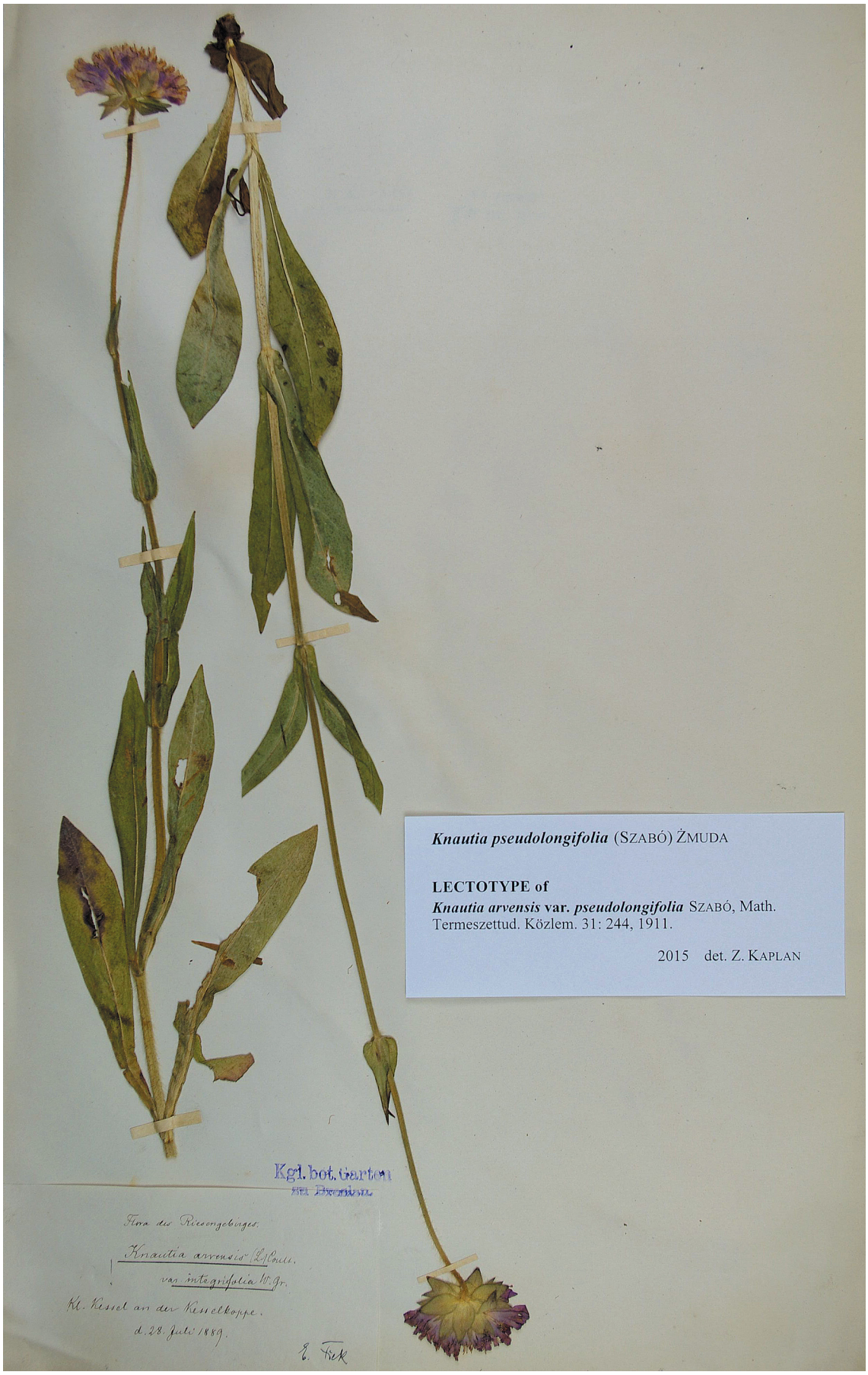

Fig. 5. – Lectotype of *Knautia pseudolongifolia* deposited in WRSL.

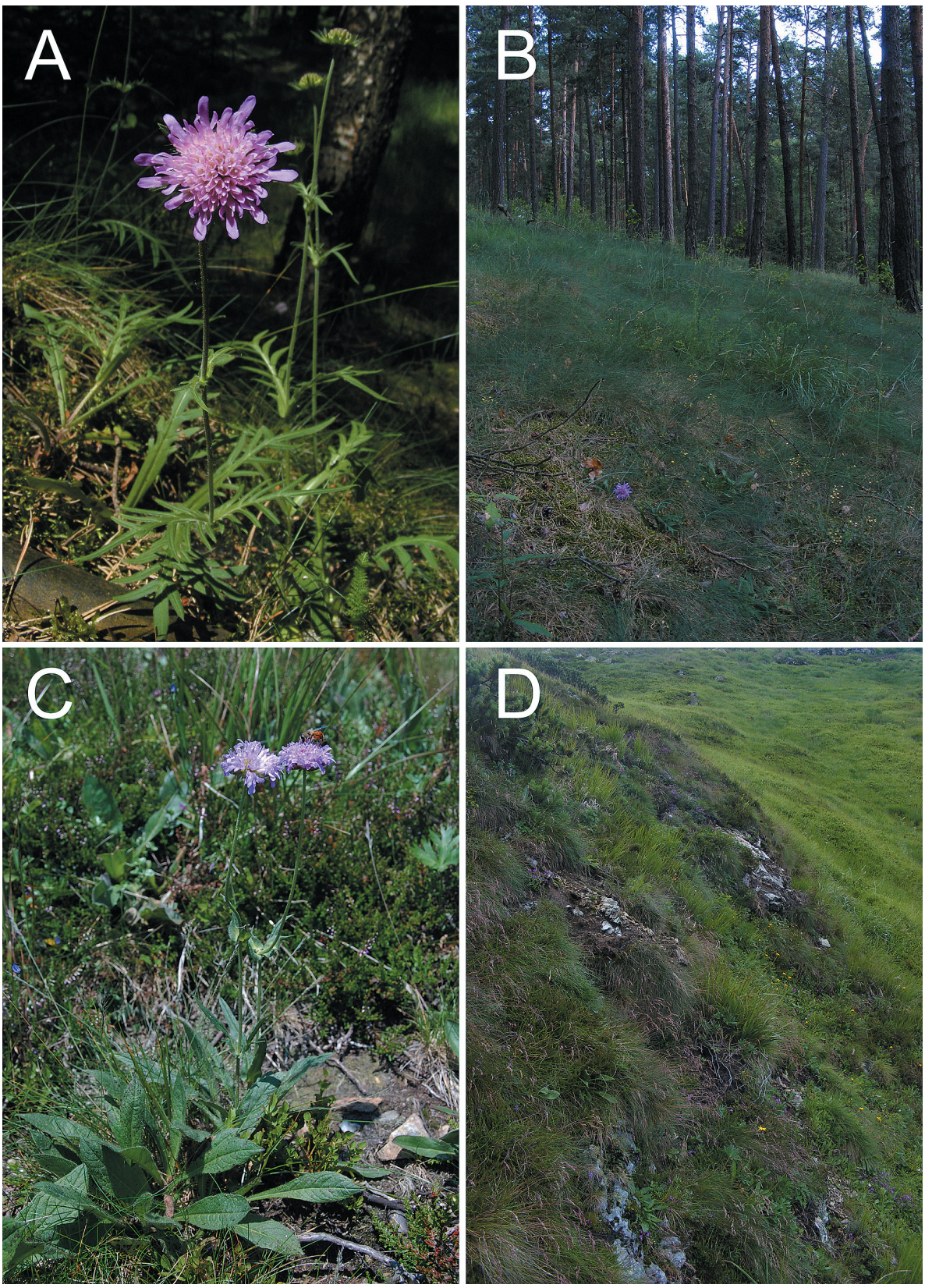

Fig. 6. – General appearance and typical habitat of *Knautia serpentinicola* (A and B) and *K. pseudolongifolia* (C and D) (photographs F. Kolář and J. Suda).

vulgaris (Štěpánek 1989). This species is allogamous, however, only 5–15 % of the plants flower each year, suggesting a significant role of vegetative reproduction (Štěpánek 1989).

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Souhrn

Taxonomické studie založené na syntéze morfologických, cytogenetických, molekulárních a ekologických přístupů stále přinášejí nové poznatky i v relativně dobře prozkoumaných územích. Mezi nejvýznamnější patří objevy nových lokálních endemitů, které mají přímý praktický dopad pro ochranu biodiverzity. Flóra cévnatých rostlin v České republice je v důsledku čtvrtohorních klimatických změn velmi chudá na endemity. Většinu českých endemitů navíc představují málo morfologicky diferencované, apomikticky se rozmnožující druhy (mikrospecie). Náš příspěvek představuje v tomto ohledu výjimku a shrnuje informace o dvou nových sexuálních endemických druzích, dosud nesprávně zahrnovaných do ekogeograficky diferencovaného a ploidně variabilního druhu chrastavce rolního (*Knautia arvensis*). Morfometrická analýza 38 populací *K. arvensis* a obou nově vylišených druhů, pocházejících z východní části střední Evropy, odhalila zjevné, byť neúplné mezidruhové morfologické odlišnosti (rozdíly patrně stírá velká fenotypová plasticita). Tyto výsledky spolu s již publikovanými cytologickými (odlišná velikost jaderného genomu), genetickými (jiná evoluční historie) a ekologickými daty (výrazně rozdílné stanovištní nároky) podporují nezávislý druhový statut hadcových a vysokohorských populací Hercynského masivu. Chrastavec hadcový (*Knautia serpentinicola*) zahrnuje diploidní a tetraploidní populace vázané na čtyři hadcové oblasti, konkrétně Dolnokralovické hadce, okolí Starého Ranska a Slavkovský les v České republice a Wojaleite v severním Bavorsku. Od *K. arvensis* se uvedený druh liší především gracilnějším vzrůstem, celoroční přítomností postranních listových růžic, užšími lodyžními listy i jejich úkrojky a tmavší červenofialovou barvou květů. Vyskytuje se výhradně v otevřených borech na hadcových substrátech, na stanovištích s malou konkurencí ostatních rostlin. Chrastavec krkonošský (*K. pseudolongifolia*) naproti tomu osidluje subalpínské trávníky na jediné lokalitě v Kotelních jamách v Krkonoších. Od ostatních druhů agregátu se odlišuje nedělenými nebo jen nejvýše laločnatými a výrazně protaženými lodyžními listy, velkými terminálními strbouly a delšími plody. *Knautia serpentinicola* i *K. pseudolongifolia* představují vzácné doklady postglaciální evoluce květeny ve střední Evropě a ukazují na významnou úlohu holocenních ekologicky podmíněných refugií v uchování vzácné biodiverzity. Náznaky dalšího rozrůzňování v rámci téže ploidie v geograficky izolovaných oblastech (alopatrická diferenciace) i vznik nové tetraploidní linie chrastavce hadcového (která se zřejmě šíří i za hranice původního hadcového refugia prostřednictvím hybridizace) dokládají nečekaně velký evoluční potenciál těchto reliktů. Evolučně-historický význam obou druhů spolu s jejich maloplošným výskytem ukazuje na nutnost cílené ochrany, především v případě dosud ochranářsky přehlížené *K. serpentinicola*. V neposlední řadě naše studie ukazuje na významný přínos komplexních taxonomických studií pro poznání biodiverzity i v již zdánlivě dobře prozkoumaných oblastech.

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Appendix 2: Speciation in the genus *Spergularia*

Paper 5: Kúr P., Štech M., Koutecký P., & Trávníček P. (2012): Morphological and cytological variation in Spergularia echinosperma and S. rubra, and notes on potential hybridization of these two species. – Preslia 84: 905–924.

Morphological and cytological variation in *Spergularia echinosperma* and *S. rubra*, and notes on potential hybridization of these two species

Morfologická a cytologická variabilita druhů *Spergularia echinosperma* a *S. rubra* s ohledem na jejich potenciální hybridizaci

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Morphological and cytological variation in *Spergularia echinosperma* and *S. rubra* and the possibility of these two species hybridizing were investigated. The plant material was collected mainly in the western- and southern-Bohemian pond basins where *S. echinosperma* is most abundant. Using flow cytometry, we found diploid and tetraploid cytotypes among plants morphologically identified as *S. echinosperma* and only tetraploid *S. rubra*. The two tetraploid cytotypes differed significantly in genome size. Both the diploid and tetraploid *S. echinosperma* and *S. rubra* also differed morphologically. The most important identification characters were stipule length together with stipule length/width ratio, seed colour, seed size and testa verrucosity. Although the morphological data suggest that tetraploid *S. echinosperma* may be a hybrid between diploid *S. echinosperma* and *S. rubra*, its genome size was significantly greater than that of a simulated allotetraploid. Since an increase in genome size following allopolyploidization is an improbable event, it is possible that other pathways were involved in the formation of tetraploid *S. echinosperma*. The nomenclature of *S. echinosperma* was also studied. Lectotypification of the name with a plant morphologically corresponding to the diploid cytotype is proposed. The morphological analysis also indicates that the holotype of *S.* ×*kurkae*, which was described as a putative hybrid between *S. echinosperma* × *S. rubra*, corresponds to tetraploid *S. echinosperma*.

K e y w o r d s: allopolyploidy, classification trees, discriminant analysis, flow cytometry, genome size, inter-ploidy hybridization, morphometric analysis, *Spergularia*

Introduction

There are relatively few vascular plants endemic to central Europe, especially when apomictic microspecies of genera such as *Taraxacum*, *Hieracium*, *Sorbus* and *Rubus* are not considered. One of the long-recognized central European endemics is *Spergularia echinosperma* (Čelak.) Asch. et Graebn. (*Caryophyllaceae*). It is confined to the sandy bottoms of mesotrophic freshwater reservoirs (usually fishponds) that are periodically exposed or sandy banks of large rivers. The center of its distribution is located in the southern- and western-Bohemian pond areas (Friedrich 1979, Dvořák 1990). Recently this species and many other plants inhabiting the exposed bottoms of ponds have declined in abundance due to intensification of fishpond management (Šumberová et al. 2005, 2006).

Spergularia echinosperma was described by Čelakovský (1881) as a subspecies of *S. rubra* (L.) J. Presl et C. Presl. Later, Ascherson & Graebner (1893) raised *S. echinosperma* to specific rank, which is generally accepted (e.g. Friedrich 1979, Monnier & Ratter 1993, Jäger & Werner 2002, Fischer et al. 2008). The main characters cited by Čelakovský (1881) for distinguishing *S. echinosperma* and *S. rubra* were seed colour and testa surface (black bristly seeds vs slightly verrucose brown seeds) and shape of stipules (short and widely triangular vs long and narrowly triangular). Other characters were introduced by Dvořák (1979, 1990), including leaf shape, flower pedicel length and capsule length. *Spergularia rubra* also differs from *S. echinosperma* in its ecology as it is a nearly cosmopolitan species occupying mainly human-affected habitats such as road margins or sandy paths (Friedrich 1979, Dvořák 1990).

Spergularia echinosperma and *S. rubra* are also supposed to differ in their ploidy levels, but few chromosome counts are available. *Spergularia rubra* is reported to be tetraploid (2n = 4x = 36) in central Europe (Dvořák 1990, Wisskirchen & Haeupler 1998), although there are also records of diploid and hexaploid plants of *S. rubra* from southern Europe (Ratter 1964, Fernandes & Leitao 1971). For *S. echinosperma*, only one chromosome count exists, which is diploid (2n = 2x = 18; Dvořák & Dadáková 1984).

Jage (1974) and Dvořák (1979) report the occurrence of more distinct morphotypes within *S. echinosperma*. Later, results of a more detailed study were published as a part of the *Spergularia* treatment for the Flora of the Czech Republic (Dvořák 1990). This author revealed the existence of *S. echinosperma* populations with morphological characters typical of *S. rubra* (especially seed colour and length of stipules and fruit pedicels), which he ultimately explained by inter-specific hybridization. He supposed that hybridization leads to the formation of a primary tetraploid hybrid, which he described as *S.* ×*kurkae* F. Dvořák (Dvořák 1989), accompanied by further gene introgression from *S. rubra* to *S. echinosperma*. However, the assumed tetraploid state of *S.* ×*kurkae* was documented only by a single chromosome count (Dvořák 1989), as in the case of *S. echinosperma*. With such limited data, Dvořák (1990) could not credibly infer the cytotype structure of the populations and morphotypes of the plants he studied.

The current state of knowledge of the central-European endemic *S. echinosperma* is fairly fragmentary. The chromosome numbers supporting the ploidy level difference between *S. echinosperma* and *S. rubra* and their putative hybrid *S.* ×*kurkae* are especially sparse and the morphological delimitation of *S.* ×*kurkae* and several reported morphotypes within *S.* ×*kurkae* (Dvořák 1989, 1990) are rather vague. It is obvious that *S. rubra* and *S. echinosperma* need to be revised based on an extensive screening of their morphological and cytotype variation. Therefore, we have addressed the following questions: (i) What is the cytotype structure of populations of *S. echinosperma* and *S. rubra*? (ii) What is the extent of the morphological variation and differences between particular cytotypes/species? (iii) Does the data on the morphology and genome size support the existence of hybrids between *S. echinosperma* and *S. rubra*?

Materials and methods

Plants

Five hundred and fifteen plants were collected from 27 populations of *Spergularia echinosperma* and *S. rubra* for the morphometric and flow-cytometric analyses during the years 2008 and 2009. They were collected predominantly in the southern part of Bohemia in the center of *S. echinosperma* distribution (see Appendix 1 for the exact localities and acronyms of the populations used in the text). Only mature plants with ripe capsules were collected. The numbers of plants per population ranged from 15 to 24. The only exception was the Cakov population (*S. rubra*), which consisted of only three plants. However, they occurred in a habitat atypical of *S. rubra* (an exposed pond bottom) and were therefore included in the analyses. Voucher specimens are deposited in the herbarium CBFS.

In addition, the type specimens of *S.* ×*kurkae* and *S. echinosperma* were included in the morphometric analyses. The holotype of *S.* ×*kurkae* (Czech Republic, southern Bohemia, Záblatí: southern shore of the Záblatský rybník fishpond, 425 m a.s.l.; approximate coordinates: 49°06'00"N, 14°40'00"E; 27. 6. 1942 leg. R. Kurka, CB 36098) consists of only one plant. There are two syntypes of *S. echinosperma* (Czech Republic, southern Bohemia, Protivín: at the Švarcenberský rybník fishpond near the village, 380 m a.s.l.; approximate coordinates: 49°12'28"N, 14°14'04"E; 08.1876 and 4. 9. 1880 leg. F. Čelakovský, PR 374981 and PR 374982, respectively). There are four plants on the former sheet, all of which were used for the morphometric measurements. There are eight plants on the latter sheet, of which only four are suitable for measuring morphological characters.

Cytological analyses

Flow cytometry was employed for estimating the genome size (relative fluorescence intensity) and DNA ploidy level (sensu Suda et al. 2006) of all the plants collected. We used the simplified two-step procedure of nuclear isolation and staining (Otto 1990) modified for plant tissues following the protocol of Doležel et al. (2007). Fresh leaves together with an appropriate amount of the internal standard were chopped using a razor blade in a Petri dish containing 0.5 ml ice-cold Otto I buffer (0.1 M citric acid, 0.5% v/v Tween 20). *Glycine max* 'Polanka' was used as the internal standard (2C = 2.50 pg, Doležel et al. 1994). The suspension was filtered through a 42 nylon mesh and after five minute incubation at room temperature 1 ml of staining solution containing Otto II buffer (0.4 M $Na_2HPO_4 \cdot 12\ H_2O$), fluorochrome 4',6-diamidino-2-phenylindole (DAPI; 4 μg/ml) and β-mercaptoethanol (2 μl/ml) was added. The staining took 1–2 min at room temperature. The samples were run on a Partec PA II flow cytometer (Partec GmbH, Münster, Germany) equipped with a mercury arc lamp. Fluorescence intensity of 5000 particles was recorded and the sample/standard ratio of fluorescent intensities and coefficients of variation (CV) of the peaks were calculated. Only analyses with coefficients of variation below 5% were accepted. Due to the low quality of the histograms and presence of endopolyploidy, each individual of *S. echinosperma* was analysed separately. For *S. rubra*, it was possible to use pooled samples of up to 5 individuals. Only analyses enabling precise estimation of the relative fluorescence were used for statistical comparisons of the genome size (150 samples with 237 plants), while the poor quality samples were used only for assessing the ploidy level.

The same method, but with the fluorochrome propidium iodide (PI) together with RNaseIIa (both at a final concentration of 50 μg/ml) replacing DAPI in the staining solution, was used for estimating the genome size of an additional set of plants. Since the PI fluorochrome intercalates evenly between the DNA base-pairs, it can be used to assess the total content of DNA in mass units (Doležel et al. 2007). *Lycopersicon esculentum* 'Stupické polní rané' (2C = 1.96 pg, Doležel et al. 1992) was used as the internal standard. The samples were run on a Partec CyFlow SL flow cytometer (Partec GmbH, Münster, Germany) equipped with a 532 nm (green) diode-pumped solid-state laser (100 mW output). Plants grown from seeds in a growth chamber from three populations per species/cytotype were analysed (Appendix 1). Three plants from each population were used for the analysis; each plant was repeatedly measured on three different days. Relatively high coefficients of variation of up to 6.4% were accepted if the repeated measurements resulted in a consistent genome size. If the difference between individual measurements of one individual exceeded 2%, additional measurements were performed and the most outlying measurement was discarded.

To confirm the FCM results, chromosome counts were carried out on three plants of each species and cytotype (populations Malobor, Smrzov, and StHlina) using a rapid squash method. The apical root meristems of germinated seedlings were pre-treated with a saturated water solution of p-dichlorobenzene (3 h, room temperature), fixed in a 3:1 mixture of 96% ethanol and glacial acetic acid overnight at 4°C, macerated in 1:1 mixture of 96% ethanol and hydrochloric acid for 1 minute, and stained with lacto-propionic orceine. The chromosomes were counted using a light microscope at a magnification of 1000×.

Morphometry

In total, 13 quantitative and 6 derived ratio characters were used for the analyses (Table 1). Diagnostic characters reported by Dvořák (1979, 1990) and other important characters based on our field experience were included. The seed colour of the sampled plants, one of the important characters for traditional species delimitation used by Czech authors (Dostál 1989, Dvořák 1990, Hrouda 2002), was also recorded. However, as the colour was difficult to score, it was not used in the statistical analyses. Unfortunately, it was not possible to include floral characters since flowers were not present on plants with ripe seeds. Three randomly selected leaves, stipules and capsules from one of the primary stems were measured and the average values used. One seed was collected from three randomly chosen capsules from the lower part of the main inflorescence. Seed dimensions and papilla length were measured on light microscope photographs (40× magnification) using tpsDig 2.12 (Rohlf 2008). Papilla shape (PapRat) was expressed as the ratio of the width of the upper part (head, usually broad in *S. echinosperma*) and that of the lower part of the papilla (neck; Fig. 1). Papillae without a head wider than its neck were assigned the value 1. The density of papillae (PapNum) was expressed as the number of papillae visible on one quarter of a seed's circumference (Fig. 1).

The data were processed by multivariate statistical analyses. Characters that deviated most from a normal distribution in each of the pre-defined groups were log-transformed (Table 1).

Table 1. – Morphological characters used in the morphometric analyses and summary of their values for *Spergularia rubra* (249 individuals), *S. echinosperma* tetraploids (184 individuals) and *S. echinosperma* diploids (61 individuals). The numbers denote (minimum–) 10th percentile/**mean**/90th percentile (–maximum). Characters log-transformed prior to the CDA analysis are marked with an asterisk.

Acronym	Character [units]	*S. echinosperma* diploid	*S. echinosperma* tetraploid	*S. rubra*
CapsLeng*	capsule length [mm]	(1.9–)2.5/**3.0**/3.5 (–4.1)	(2.6–)3.0/**3.6**/4.3 (–5.5)	(2.3–)3.0/**3.5**/4.0 (–4.6)
FrPedLen*	length of the fruit pedicel adjacent to the capsule [mm]	(1.7–)4.2/**6.0**/8.1 (–12.5)	(2.0–)4.1/**6.9**/10.5 (–23.7)	(1.5–)2.3/**3.6**/5.6 (–8.4)
InterLen*	length of the internode adjacent to the measured leaf [mm]	(5.9–)8.5/**12.0**/15.8 (–26.8)	(2.2–)4.8/**11.1**/19.0 (–33.5)	(1.3–)2.8/**7.7**/16.3 (–28.4)
Int-Leaf*	internode length/leaf length ratio	(0.78–)0.94/**1.35**/1.78 (–2.49)	(0.37–)0.65/**1.03**/1.52 (–2.77)	(0.24–)0.54/**0.98**/1.46 (–2.86)
LeafLeng*	leaf length [mm]	(4.1–)5.6/**9.4**/14.6 (–18.8)	(2.5–)5.5/**11.0**/17.9 (–24.7)	(3.0–)4.5/**7.7**/14.1 (–24.8)
LeafRat*	leaf length/width ratio	(9.8–)13.3/**20.5**/30.2 (–45.3)	(5.0–)11.3/**18.9**/28.3 (–49.4)	(6.6–)9.2/**13.8**/20.0 (–32.5)
LeafWidt*	leaf width [mm]	(0.3–)0.3/**0.5**/0.6 (–0.7)	(0.3–)0.4/**0.6**/0.8 (–1.1)	(0.2–)0.4/**0.6**/0.8 (–1.2)
LengSeed*	seed length [µm] (Fig. 1)	(394–)409/**451**/484 (–514)	(439–)479/**535**/586 (–642)	(415–)469/**517**/567 (–636)
PapHei*	papilla height [µm] (Fig. 1)	(16–)18/**20**/23 (–26)	(16–)21/**25**/29 (–35)	(12–)15/**18**/21 (–25)
PapNum	number of papillae on one quarter of the seed circumference (papillae density)	(10–)12/**15**/17 (–20)	(7–)8/**11**/14 (–16)	(3–)5/**7**/9 (–12)
PapRat*	ratio of the papilla upper part (“head”) width and papilla lower part (“neck”) width (papilla shape)	(1.04–)1.13/**1.29**/1.48 (–1.78)	(1.09–)1.23/**1.49**/1.84 (–2.25)	(1.00–)1.03/**1.15**/1.29 (–1.44)
Ped-Cap*	pedicel/capsule length ratio	(0.68–)1.41/**2.02**/2.69 (–3.68)	(0.70–)1.26/**1.90**/2.89 (–5.39)	(0.40–)0.65/**1.02**/1.52 (–2.37)
PlHeight*	height of the longest stem [cm]	(3–)3/**6**/10 (–12)	(1–)2/**7**/12 (–23)	(3–)6/**10**/16 (–31)
SeedCol	seed color	black	black	brown
SeedRat*	seed length/width ratio	(1.22–)1.25/**1.35**/1.44 (–1.55)	(1.17–)1.25/**1.33**/1.41 (–1.57)	(1.10–)1.23/**1.30**/1.39 (–1.54)
StemsNum*	number of stems	(1–)1/**4**/9 (–19)	(1–)1/**5**/9 (–19)	(2–)5/**18**/34 (–63)
StpLt*	stipule length [mm]	(1.0–)1.1/**1.4**/1.6 (–1.8)	(1.3–)1.7/**2.2**/2.8 (–4.0)	(2.1–)2.9/**3.5**/4.3 (–4.9)
StpRT*	stipule length/width ratio	(0.48–)0.67/**0.86**/1.16 (–2.0)	(0.75–)0.98/**1.31**/1.68 (–2.09)	(1.43–)1.81/**2.34**/2.85 (–4.04)
StpWd	stipule width [mm]	(0.7–)1.3/**1.7**/2 (–2.3)	(0.8–)1.4/**1.7**/2.1 (–2.5)	(1.0–)1.3/**1.6**/1.9 (–2.4)
WidtSeed*	seed width [µm] (Fig. 1)	(267–)310/**337**/373 (–405)	(283–)362/**405**/452 (–491)	(315–)361/**401**/451 (–501)

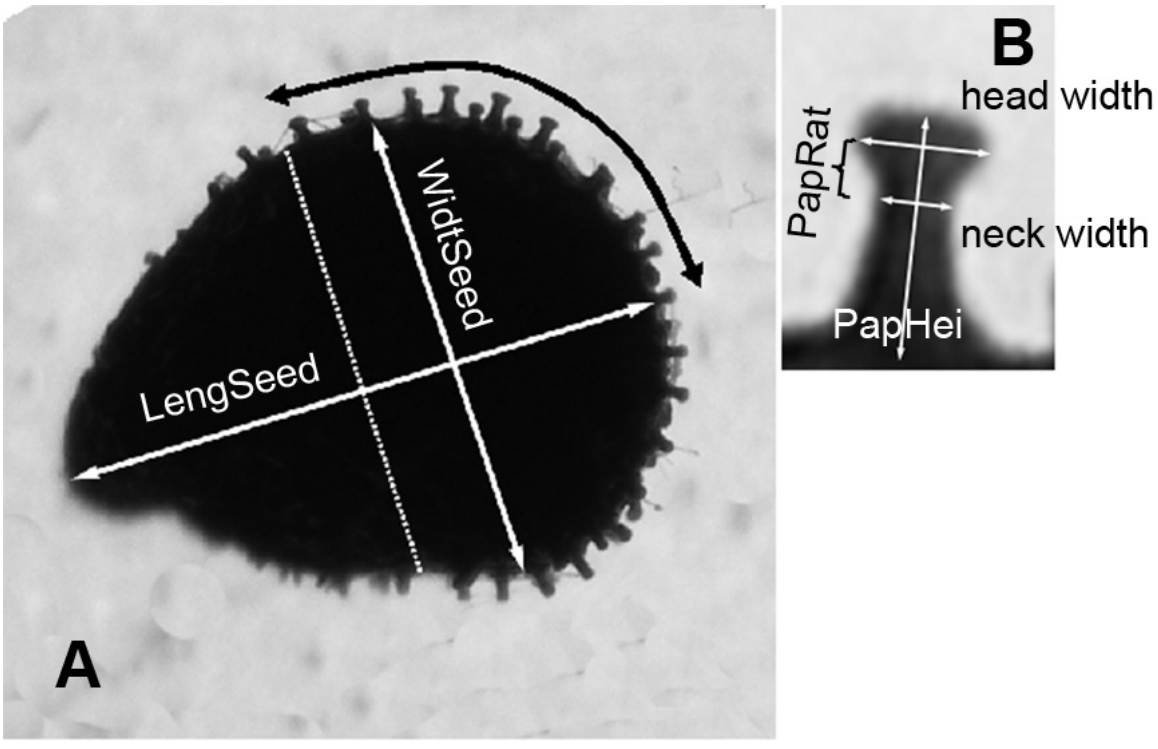

Fig. 1. – Characters measured on the seeds (A) and surface papillae (B). The black curved line specifies the part of the seed circumference where the density of papillae was determined. The longitudinal border of this part is a plane halving the vector of maximal seed length and perpendicular to it (indicated by a dotted line). The character PapRat was computed by dividing the width of the papilla head by the width of the neck.

The plants were divided into two groups based on seed colour, black vs brown, corresponding to the species *S. echinosperma* and *S. rubra*, respectively. The black-seeded plants were additionally divided into two groups based on the flow cytometry data (see Results). One population (Veselsky), however, could not be unambiguously assigned to either of the groups since its seeds were dark brown rather than black or brown. Therefore, it was excluded from the analyses. To find out which characters significantly separated the groups, canonical discriminant analysis (CDA) with forward selection of characters was applied. The type specimens and plants from the Veselsky population were projected to the ordination space as passive samples. The threshold significance level was set to $\alpha = 0.05$ and a Monte-Carlo permutation test (999 permutations) used. The analysis was carried out in CANOCO for Windows 4.5 (ter Braak & Šmilauer 2002). The predictive ability of the selected characters was subsequently tested by classificatory discriminant analysis based on the posterior group membership probabilities in the statistical package R 2.11.0 (R Development Core Team 2010). Cross-validation using each population as a leave-out unit was used (the lda function from the MASS package). The herbarium specimens and plants from the Veselsky population were classified using classification rules based on the other populations with known ploidy levels. The percentage of misclassified samples in each group served as a measure of the predictive ability.

We also reanalysed the data by classification trees that represent a non-parametric alternative to the classificatory discriminant analysis. The essential difference between these two methods is that classification trees, instead of using all characters together, create

a hierarchical classification based on univariate splits that can then be visualized as an easily interpretable tree diagram (Breiman et al. 1984). Although this approach has not been widely used in plant taxonomy, it is suitable for analyzing taxonomic data (e.g. Joly & Bruneau 2007, Depypere et al. 2009). We used the function rpart (package rpart) implemented in the R statistical package (R Development Core Team 2010). The minimum split parameter (minsplit) was set to 1 and the initial complexity parameter (cp) to 0.001. A cross-validation using the populations as the leave-out subsamples was used to assess the optimal tree complexity, instead of random subsamples as implemented in the original method (Venables & Ripley 2002). The resulting tree was selected on the basis of the 1-SE rule (Venables & Ripley 2002).

Results

Cytological analysis

Two groups with different genome sizes were discovered among black seeded plants morphologically determined as *Spergularia echinosperma*. Because the chromosomes are very small (typically < 1 μm) we were able only to roughly estimate the number of chromosomes. However, this was sufficient to identify one cytotype as diploid (2n = ca 18) and the other as tetraploid (2n = ca 36) (hereafter referred to as "diploid *S. echinosperma*" and "tetraploid *S. echinosperma*"). Only diploids were found at three localities and only tetraploids at nine localities, and at two localities there was a mixture in which diploids were in the minority (frequencies of 5% and 30% in the Cky and Driten populations, respectively). Only tetraploids were recorded in the populations of *S. rubra*.

The tetraploid cytotype of *S. echinosperma* has a larger genome than tetraploid *S. rubra*. The mean difference was 7.8% using DAPI staining and 8.3% using PI staining (Fig. 2, Table 2). The monoploid (1Cx) genome size of diploid *S. echinosperma* is larger by 5.3% (DAPI staining) or 3.2% (PI staining) than that of tetraploid *S. echinosperma* (Fig. 2, Table 2). We were able to demonstrate these differences in the genome sizes of the three cytotypes using simultaneous flow cytometry analysis (Fig. 3). The mean somatic (2C) genome sizes based on PI staining and converted into mass of DNA is 0.63 pg for diploid *S. echinosperma*, 1.22 pg for tetraploid *S. echinosperma* and 1.12 pg for *S. rubra*. The genome sizes of the plants from the Veselsky population fall within the range of tetraploid *S. echinosperma*. In *S. rubra* (population Luznice) we found one individual that had a genome size that was 2.5% smaller (PI staining).

Morphometry

Marginal effects of all characters in the CDA were highly significant ($P < 0.001$). Forward selection identified 12 characters that contributed most to the separation of the groups (Table 3, Fig. 4). Both *Spergularia rubra* and the cytotypes of *S. echinosperma* were clearly differentiated from each other. The tetraploid *S. echinosperma* was morphologically intermediate between the diploid cytotype and *S. rubra*. Plants from the Veselsky population, assigned to tetraploid *S. echinosperma* based on genome size, were markedly closer to *S. rubra* (Fig. 4). The position of the plants of the Cakov population, which were collected from the exposed bottom of a pond, was at the edge of the morphological

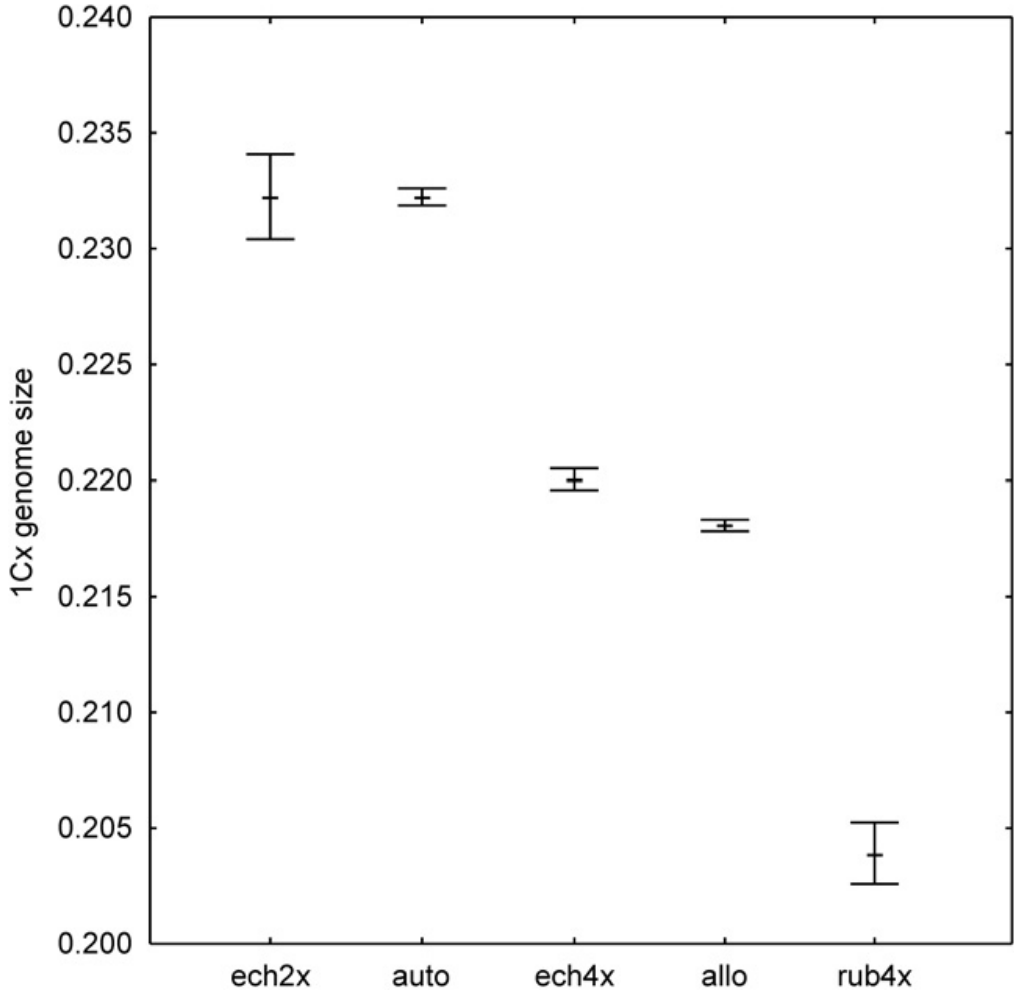

Fig. 2. – Box-and-whisker plot of the equivalents of the 1Cx values calculated from the genome sizes based on DAPI staining for diploid *Spergularia echinosperma* (ech2x), tetraploid *S. echinosperma* (ech4x), *S. rubra* (rub4x), a hypothetical *S. echinosperma-S. rubra* allopolyploid (allo) and hypothetical *S. echinosperma* autopolyploid (auto), expressed in terms of a ratio with the 1C value of the standard *Glycine max*.

Table 2. – Summary of the genome sizes of the *Spergularia echinosperma* cytotypes, *S. rubra*, and simulated auto- and allopolyploids based on DAPI staining (expressed as the ratio to the 1C value of the standard *Glycine max*) and PI staining (expressed in picograms of DNA). 2C – somatic genome size; 1Cx – monoploid g. s.; N – number of samples; SE – standard error of mean.

Taxon	PI staining			DAPI staining		
	N	Mean 2C±SE	Mean 1Cx±SE	N	Mean 2C±SE	Mean 1Cx±SE
S. echinosperma 2x	9	0.627±0.001	0.314±0.001	21	0.464±0.002	0.232±0.001
S. echinosperma 4x	9	1.217±0.002	0.304±0.001	92	0.880±0.001	0.220±0.001
S. rubra 4x	8	1.124±0.001	0.281±0.001	16	0.815±0.002	0.203±0.001
S. rubra outlier	1	1.097	0.274		–	
Hypothetical allopolyploid	72	1.190±0.001	0.297±0.001	336	0.872±0.001	0.218±0.001
Hypothetical autopolyploid	45	1.255±0.001	0.314±0.001	231	0.929±0.001	0.232±0.001

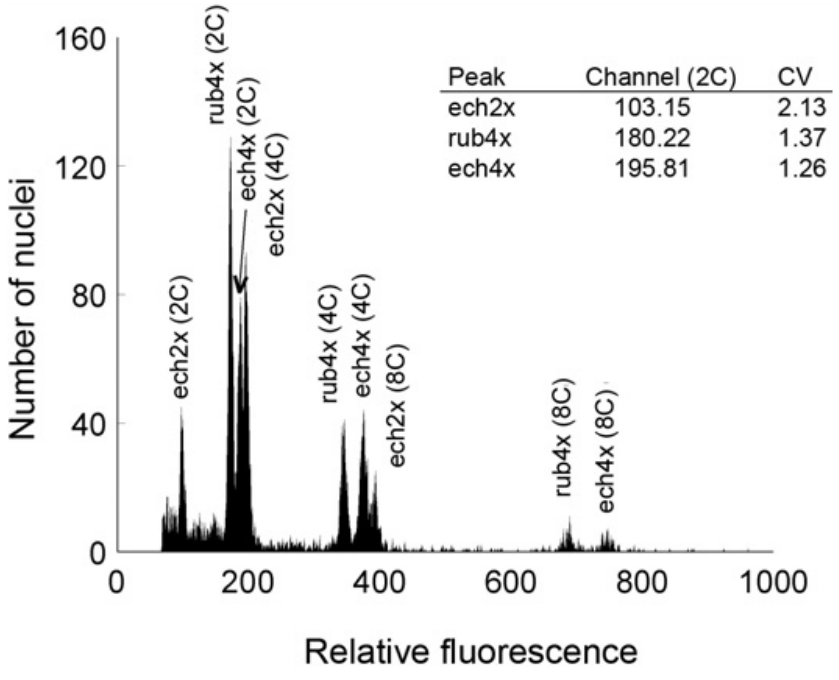

Fig. 3. – Histogram of relative fluorescence of DAPI-stained nuclei of the diploid *Spergularia echinosperma* (ech2x), tetraploid *S. echinosperma* (ech4x) and tetraploid *S. rubra* (rub4x) corroborating the differences in the genome sizes of these three taxa. The genus *Spergularia* displays considerable endopolyploidy with three detectable peaks for a single plant corresponding to 2C, 4C, and 8C DNA content. This allows direct comparison of diploids (4C peak) and tetraploids (2C peaks).

variability of *S. rubra* (not shown), but they did not deviate significantly from the rest of the group either in morphology or genome size.

The best predictors for all the three groups were stipule length (StpLt) and the stipule length/width ratio (StpRT). As they are correlated, only marginal effects of both characters were significant, while inclusion of one character made the conditional effect of the other insignificant. Density of papillae (PapNum) could also be used to discriminate between the three groups. Number of stems (StemsNum) and plant height (PlHeight) proved to be an effective way of discriminating mainly between *S. rubra* and both *S. echinosperma* cytotypes. Seed dimensions (LengSeed and WidtSeed) and capsule length (CapsLeng) differed between the diploids and both tetraploids. Finally, papilla height (PapHei), papilla shape (PapRat), fruit pedicel length (FrPedLen), leaf length (LeafLeng), and stipule width (StpWd) best differentiated tetraploid *S. echinosperma* from the other two groups. Values of all the quantitative characters measured are summarized in Table 1.

The predictive ability of the 12 characters selected was tested using classificatory discriminant analysis. All individuals of *S. rubra* and all but one individual of the diploid *S. echinosperma* were correctly classified. The one misclassified sample was mistaken for the tetraploid *S. echinosperma*. In the tetraploid *S. echinosperma*, the number of misclassifications was higher with three individuals erroneously classified as diploids and one individual as *S. rubra*. The overall percentage misclassified was very low, 1.0% (Table 4).

Only 82.2% of individuals from the Veselsky population were correctly classified, whereas it was 97.8% in all the other populations of tetraploid *S. echinosperma* (Table 4). The discriminant analysis assigned all the misclassified individuals from the Veselsky population to *S. rubra*.

Table 3 – Morphological characters of *Spergularia echinosperma* and *S. rubra* tested in the forward selection with their conditional and marginal effects and their correlations with axes of the canonical discriminant analysis (CorE scores). λ_A – eigenvalue representing the conditional effect of each character (when added to the already selected characters); λ_1 – eigenvalue representing the marginal effect of each character (when it is the only predictor in the model).

Character	Conditional effects			CorE scores		Marginal effects		
	λ_A	F	p	Axis 1	Axis 2	λ_1	F	P
StpLt	0.801	328.8	0.001	–0.8825	0.1497	0.801	328.8	0.001
PapHei	0.413	257.7	0.001	0.5455	0.4967	0.544	183.9	0.001
LengSeed	0.069	47.1	0.001	–0.2011	0.5418	0.334	98.6	0.001
PapNum	0.065	48.4	0.001	0.7830	–0.0389	0.654	239.0	0.001
FrPedLen	0.059	48.6	0.001	0.6068	0.2463	0.429	134.2	0.001
PlHeight	0.063	57.6	0.001	–0.3720	–0.1118	0.151	40.1	0.001
PapRat	0.019	17.7	0.001	0.5631	0.4206	0.494	161.3	0.001
StpWd	0.011	10.4	0.004	0.1834	0.1455	0.061	15.4	0.001
LeafLeng	0.010	9.4	0.001	0.3151	0.1786	0.131	34.5	0.001
WidtSeed	0.009	8.5	0.002	–0.3150	0.4765	0.326	95.9	0.001
StemsNum	0.006	6.1	0.009	–0.6597	–0.1339	0.453	144.1	0.001
CapsLeng	0.005	4.8	0.028	–0.2036	0.3382	0.156	41.5	0.001
InterLen	0.003	n.s.		–		0.158	42.1	0.001
Int-Leaf	0.003	n.s.		–		0.092	23.7	0.001
LeafRat	0.003	n.s.		–		0.186	50.4	0.001
SeedRat	0.003	n.s.		–		0.065	16.5	0.001
LeafWidt	0.002	n.s.		–		0.052	13.0	0.001
StpRT	0.002	n.s.		–		0.785	317.8	0.001
Ped-Cap	0.001	n.s.		–		0.487	158.5	0.001

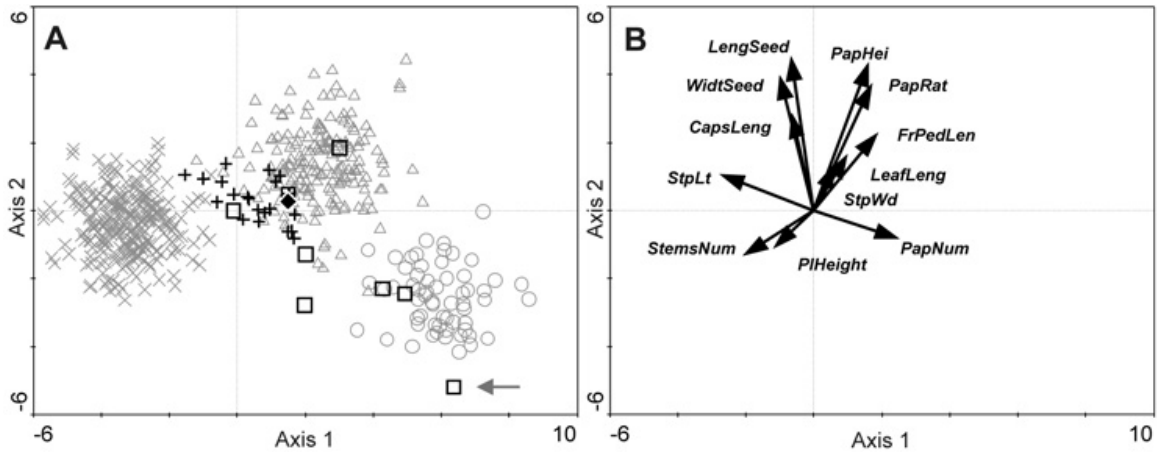

Fig. 4. – Results of CDA of individuals (A) and characters selected by forward selection (B). *Spergularia echinosperma* diploids: grey circles; *S. echinosperma* tetraploids: grey triangles; *S. rubra* tetraploids: grey X-crosses; population Veselsky: black crosses; *S. echinosperma* syntypes: black squares; *S.* ×*kurkae* holotype: black diamond. The arrow denotes the proposed lectotype of *S. echinosperma*. The two canonical axes extract 46.1% and 30.3% of the total variation among the groups.

Table 4. – Summary of the classification matrices of diploid *Spergularia echinosperma* (ech2x), tetraploid *S. echinosperma* (ech4x) and *S. rubra* (rub4x) resulting from the classificatory discriminant and classification tree analyses.

Classificatory discriminant analysis				Classification trees			
observed	ech2x	ech4x	rub4x	observed	ech2x	ech4x	rub4x
predicted				predicted			
ech2x	60 (98.4%)	3 (1.6%)	0 (0%)	ech2x	61 (100%)	5 (2.7%)	0 (0%)
ech4x	1 (1.6%)	180 (97.8%)	0 (0%)	ech4x	0 (0%)	175 (95.1%)	4 (1.6%)
rub4x	0 (0%)	1 (0.6%)	249 (100%)	rub4x	0 (0%)	4 (2.2%)	245 (98.4%)

Table 5. – Posterior probabilities of classification for the *Spergularia ×kurkae* holotype (CB) and *S. echinosperma* syntypes (PR; the proposed lectotype marked as "lt") obtained from the classificatory discriminant analysis (ech2x – diploid *Spergularia echinosperma*, ech4x – tetraploid *S. echinosperma*, rub4x – *S. rubra*).

Specimen	Posterior probability for		
	ech2x	ech4x	rub4x
CB-36098	3.43×10^{-6}	0.99	3.31×10^{-5}
PR-374981 / 1	1.46×10^{-6}	0.99	4.58×10^{-10}
PR-374981 / 2	2.39×10^{-8}	0.69	0.30
PR-374981 / 3	1.37×10^{-6}	0.99	2.40×10^{-5}
PR-374981 / 4 (lt)	0.99	1.06×10^{-11}	5.27×10^{-24}
PR-374982 / 1	0.99	1.08×10^{-03}	5.07×10^{-13}
PR-374982 / 2	7.69×10^{-3}	0.99	2.54×10^{-5}
PR-374982 / 3	0.67	0.32	1.31×10^{-4}
PR-374982 / 4	0.99	6.34×10^{-5}	9.64×10^{-16}

The *S. ×kurkae* holotype was classified as tetraploid *S. echinosperma* with a nearly 100% probability (Table 5). Each of the *S. echinosperma* syntypes contained a mixture of plants classified as either diploid or tetraploid *S. echinosperma* (Table 5).

The final classification tree selected had 7 terminal nodes (complexity parameter cp = 0.011). It confirmed the high discrimination power of the two characters describing stipules, StpRT and StpLt, which distinguished both the cytotypes of *S. echinosperma* and between *S. echinosperma* and *S. rubra*. Other characters were used to discriminate the two cytotypes within *S. echinosperma*, seed length (LengSeed) and density of papillae (PapNum) and for distinguishing between tetraploid *S. echinosperma* and *S. rubra* the number of stems (StemsNum) together with density of papillae (PapNum) (Fig. 5). The overall predictive power of this model was slightly lower than that of the discriminant analysis (error rate 2.6%; Table 4). All individuals of diploid *S. echinosperma* were classified correctly. Within the tetraploid *S. echinosperma*, five individuals were erroneously classified as diploids and four as *S. rubra*. There was also a higher percentage of misclassification among *S. rubra* plants, four of which were incorrectly classified as tetraploid *S. echinosperma* (Table 4).

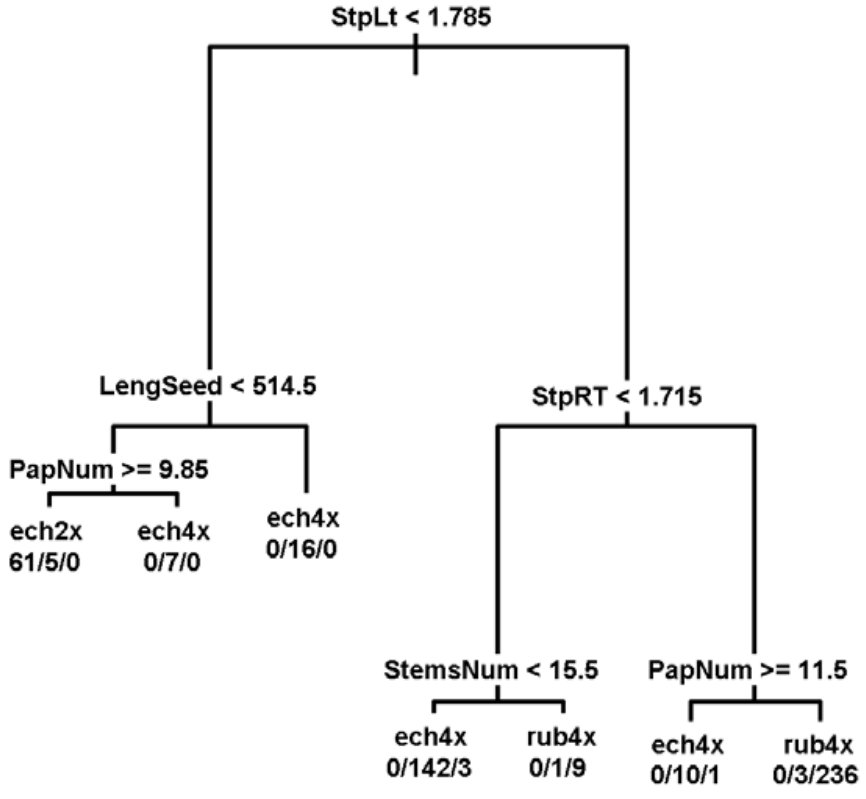

Fig. 5. – Classification tree of individuals of diploid *Spergularia echinosperma* (ech2x), tetraploid *S. echinosperma* (ech4x) and *S. rubra* (rub4x). If a character value matches the classification rule, the determination continues to the left branch, otherwise to the right branch. Lengths of the branches correspond to the relative discriminatory powers of the respective rules. The group names at the terminal nodes indicate the predicted classification of a particular node, whereas the numbers separated by slashes indicate actual membership of samples classified to a particular node (ech2x/ech4x/rub4x).

Discussion

Ploidy levels and morphology

We found three different entities in the populations of *Spergularia echinosperma* and *S. rubra* studied. All the populations collected from outside of the exposed bottoms of ponds and one exceptional population growing on the exposed bottom of the Čakov fishpond belonged to the tetraploid cytotype of *S. rubra*. No other cytotypes were found within this species, which confirms the uniformity of *S. rubra* in central Europe (Friedrich 1979, Dvořák 1990, Wisskirchen & Haeupler 1998, Marhold et al. 2007). The occurrence of one individual with a slightly smaller genome can be most probably attributed to aneuploidy, although this was not confirmed by a chromosome count.

A diploid and a tetraploid cytotype were recorded in the other populations growing on the exposed bottoms of ponds that were identified as *S. echinosperma*. The morphometric analysis showed that the tetraploid *S. echinosperma* cytotype was significantly different from the diploid cytotype and also from *S. rubra*. The best morphological characters for discriminating between diploid *S. echinosperma*, tetraploid *S. echinosperma* and *S. rubra* were those of stipules and seeds (Fig. 4, Fig. 5, Table 3). Stipule length and stipule length/width ratio of all three entities differed (Table 1). However, the latter was more

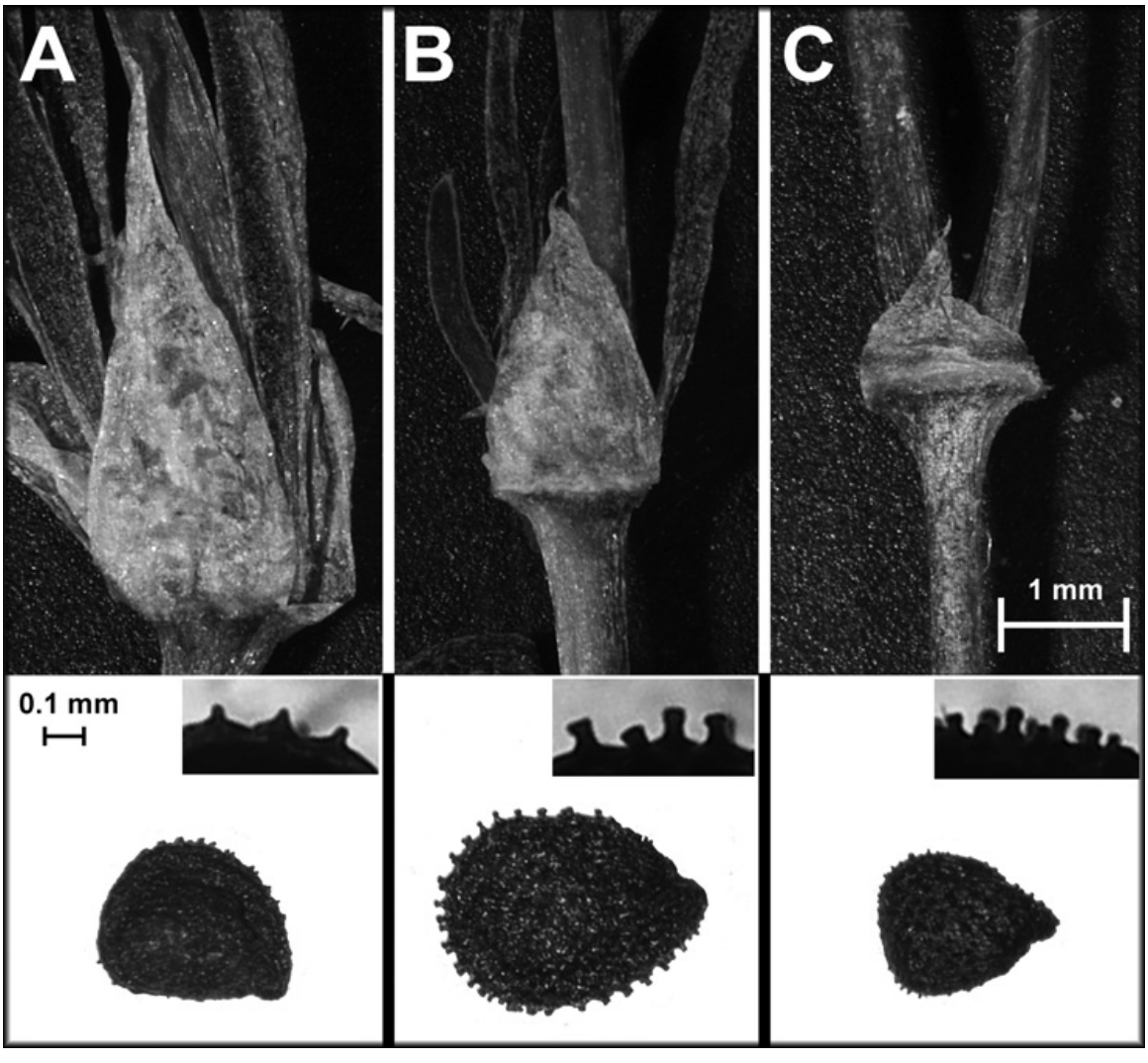

Fig. 6. – Typical stipules and seeds of *Spergularia rubra* (A), tetraploid *S. echinosperma* (B) and diploid *S. echinosperma* (C).

useful for field determination as it can be easily assessed visually. The stipules of diploid *S. echinosperma* are shorter than wide, those of tetraploid *S. echinosperma* as long as or up to 1.7× longer than wide and those of *S. rubra* more than 1.7× longer than wide (Fig. 6). Based on this single character, we were able to classify correctly 87.6% of our samples.

The seed colour is mentioned as the character that can be used to discriminate between *S. echinosperma* and *S. rubra* in the original description of *S. echinosperma* (Čelakovský 1881) and is used by some (e.g. Dostál 1989, Dvořák 1990, Hrouda 2002) but not all authors (e.g. Friedrich 1979, Monnier & Ratter 1993, Jäger & Werner 2002, Fischer et al. 2008). Our analyses confirmed that seed colour can be reliably used to discriminate between *S. echinosperma* (both cytotypes) with black seeds and *S. rubra* with brown seeds.

Other relatively reliable characters, which were less useful in the field, were seed size and testa structure. In accordance with the original description (Čelakovský 1881) and other authors (Friedrich 1979, Dvořák 1990) the seeds of *S. rubra* differ from those of *S. echinosperma* in having a low density of surface papillae, which are also considerably smaller. In addition, the *S. echinosperma* cytotypes strongly differed from each other in seed morphology. The diploids displayed significantly smaller and more densely verrucose

seeds with a lower density of papillae and less pronounced papilla heads than the tetraploids (Fig. 6). Based on the results of the morphometric analyses, we compiled the following determination key for the taxa/cytotypes:

1a Seeds brown, sparsely verrucose (5–9 papillae per 1/4 of the seed circumference); stipules at least 1.7× longer than wide, at least 2.9 mm long; plants usually with more than 5 stems ..... ***S. rubra***
1b Seeds black, densely verrucose (8–17 papillae per 1/4 of the seed circumference); stipules less than 1.7× longer than wide, less than 2.8 mm long; plants usually with fewer than 9 stems 2
2a Stipules shorter than wide, less than 1.6 mm long; seeds less than 0.48 mm long, density of papillae 12–17 per 1/4 of the seed circumference ..... ***S. echinosperma*, diploid cytotype**
2b Stipules longer than wide, more than 1.7 mm long; seeds more than 0.48 mm long, density of papillae 8–14 per 1/4 of the seed circumference ..... ***S. echinosperma*, tetraploid cytotype**

Genome size

The genome sizes of the taxa studied are the first published for the genus *Spergularia*. Their genomes are quite small, which is a common feature of the *Caryophyllaceae* (Bennett & Leitch 2010). The genome of the diploid *S. echinosperma* (2C = 0.63 pg) is even smaller than the smallest genome reported in this family so far (2C = 0.84 pg for *Polycarpaea carnosa* C. Sm. ex Buch; Bennett & Leitch 2010).

Origin of the tetraploid cytotype of Spergularia echinosperma

The tetraploid cytotype of *S. echinosperma* was morphologically intermediate between the diploid cytotype of *S. echinosperma* and (tetraploid) *S. rubra* suggesting hybrid origin. To test the hypothesis of allopolyploid origin of tetraploid *S. echinosperma*, we modelled the genome sizes of the hypothetical allopolyploids by combining two chromosome sets from each of the diploid *S. echinosperma* individuals (an unreduced gamete) with two chromosome sets from each of the *S. rubra* individuals (a reduced gamete) in our dataset (Fig. 2, Table 2). We used the data obtained from both the DAPI and PI staining. The mean genome size of the simulated allopolyploids was lower than the mean genome size of tetraploid *S. echinosperma* by 0.9% based on the DAPI and 2.2% on the PI staining. The difference was tested using a Mann-Whitney U-test in Statistica 8 (StatSoft 1998) and was significant for both the DAPI ($U = 8441$; $P < 0.001$) and PI ($U = 0$; $P < 0.001$) staining. This difference challenges the allopolyploid pathway, because it needs to assume an increase in genome size after polyploidization, which is rarely recorded (Dhillon et al. 1983, Jakob et al. 2004, Leitch et al. 2008) compared to the ubiquitous decrease in genome size.

We are aware that one-step hybridization through unreduced gametes of the diploid is not the only possibility. However, we think it is the most likely scenario. Angiosperms commonly produce unreduced gametes and this is viewed as the primary source of neopolyploid formation, especially in diploid-tetraploid crosses (Ramsey & Schemske 1998). For *Spergularia* it is reported that a few tetraploid seeds were produced by a cross between *S. maritima* (All.) Chiov. (♀, diploid) and *S. rupicola* Lebel ex Le Jolis (♂, tetraploid) (Ratter, 1976). The alternative pathway of allotetraploid formation involves an intermediate stage of (at least partly) fertile triploid progeny formed by fusion of normally developed gametes of the parental species ("triploid bridge"). These triploids can produce tetraploid offspring by selfing or backcrossing to one of the parental taxa (Bretagnolle & Thompson 1995). Though rare, this pathway of polyploid formation can be significant in

diploid-tetraploid hybridization (e.g. Vardi & Zohary 1967, Anamthawat-Jónsson & Thorsson 2003, Aagaard et al. 2005, Lo et al. 2010). In *Spergularia*, nearly all triploid offspring of various diploid-tetraploid crosses are sterile and the fertility of seeds from triploid plants is very low (0.1–0.2%) (Ratter 1976). This together with the absence of triploids in wild populations (both our data and in the literature) makes the triploid bridge pathway highly improbable.

As an alternative to allopolyploidization we also investigated the possibility that tetraploid *S. echinosperma* could be an autopolyploid derived from the diploid cytotype. We modelled the genome sizes of hypothetical autopolyploids by adding the genome sizes of each pair of *S. echinosperma* diploids in our dataset and also by doubling the genome size of each of the diploids (simulating autogamy) (Fig. 2, Table 2). The mean genome size of the hypothetical autopolyploid was greater by 5.4% based on DAPI and 3.1% based on PI staining than that of tetraploid *S. echinosperma*. There was no overlap in the genome sizes of the simulated autopolyploids and tetraploid *S. echinosperma* based on either of the methods of staining. However, this difference is relatively small and could be simply attributed to genome downsizing, which is a common phenomenon in polyploids (Leitch & Bennet 2004). Thus, it is not possible to exclude this pathway of autopolyploid formation based on the available data. The intermediate morphology of tetraploid *S. echinosperma* could result from subsequent homoploid hybridization with *S. rubra*. On the other hand, our morphometric data indicate that tetraploid *S. echinosperma* is morphologically quite homogenous and homoploid hybridization with *S. rubra* is not frequent (only the Veselsky population was conspicuously intermediate between tetraploid *S. echinosperma* and *S. rubra*).

Taxonomy and nomenclature

The tetraploid cytotype of *S. echinosperma* was more or less intermediate between diploid *S. echinosperma* and *S. rubra*. Morphological intermediacy between the "pure" *S. echinosperma* and *S. rubra* is also the attribute of the assumed hybrid *S.* ×*kurkae* according to Dvořák (1990). Indeed, discriminant analyses placed the *S.* ×*kurkae* holotype among the *S. echinosperma* tetraploids (Fig. 4, Table 5). Therefore, we conclude it was this tetraploid cytotype that Dvořák (1989) described as *S.* ×*kurkae* F. Dvořák. It is also obvious that Dvořák (1990) intended to apply the name *S. echinosperma* to the diploid cytotype. He published the diploid chromosome count as the only one for *S. echinosperma* (Dvořák & Dadáková 1984, Dvořák 1990). He even annotated, but never published, a lectotype of the name *Spergularia rubra* subsp. *echinosperma* (Fig. 7) that corresponds well with the diploids based on our results (Fig. 4, Table 5), although the original material of this name is heterogeneous and comprises both diploids and tetraploids. We therefore propose lectotypification of this name in the sense of the diploids in the present paper and we propose the same individual as F. Dvořák as the lectotype (Fig. 7).

Dvořák (1990) also reported the existence of several distinct morphotypes within *S.* ×*kurkae*. In our study, the three entities we identified were quite homogenous except for one population of tetraploid *S. echinosperma* (Veselsky) that was markedly shifted towards *S. rubra* (Fig. 4). This morphotype corresponds to one of the morphotypes described by Dvořák (1990) from the area of the Českomoravská vrchovina Highlands, characterized by the dark brown colour of its seeds and elongated stipules. Taxonomic status of this morphotype is unknown; however, its origin as a cross between tetraploid *S. echinosperma* and *S. rubra* is possible.

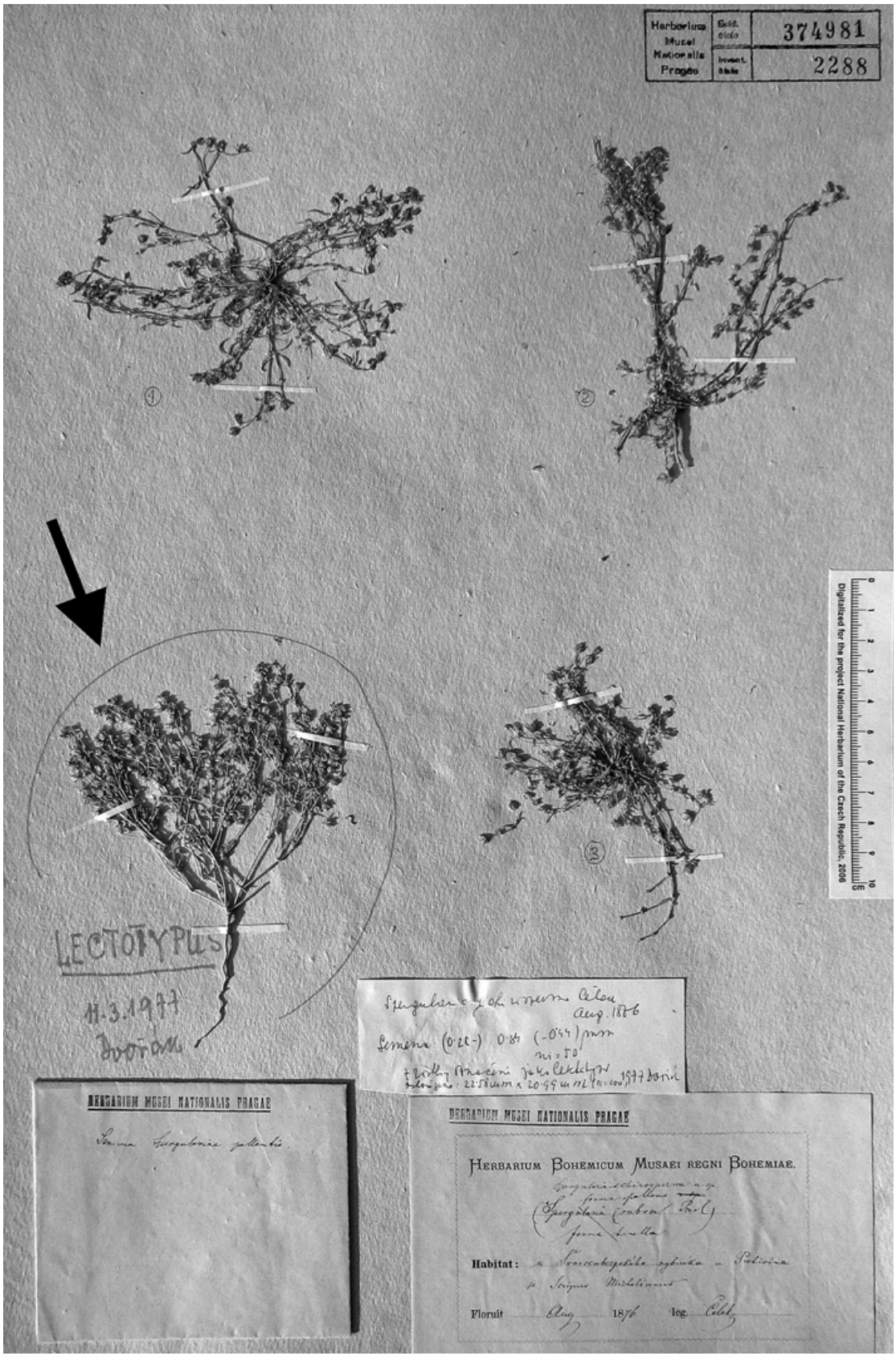

Fig. 7. – The proposed lectotype for the name *Spergularia echinosperma* (Čelak.) Asch. et Graebn., PR 374981, marked by the arrow. The text on the label reads: "*Spergularia echinosperma* n. sp. forma *pallens*, u Švarcenberského rybníka u Protivína se *Scirpus Michelianus*, Aug 1876 leg. Čelak.".

Based on current data it is not possible to designate the definitive taxonomic treatment of tetraploid *S. echinosperma*. Although its hybrid origin is strongly suggested by the morphological data, the discrepancy between the expected and observed genomes size needs further investigation. It is also unknown whether tetraploid *S. echinosperma* represents an ecologically and/or geographically well-separated entity, which would indicate it is a separate species, but this will need more extensive sampling. For now, therefore, we do not propose treating the tetraploid cytotype of *S. echinosperma* as a separate taxon.

Nomenclature of *S. echinosperma*:

Spergularia echinosperma (Čelak.) Asch. et Graebn. in Ber. Deutsch. Bot. Ges. 11: 516, 1893.
≡ *Spergularia rubra* [subsp.] b. *echinosperma* Čelak. in Prodr. Fl. Böhmen 4: 867, 1881.

Lectotype (**designated here**): "*Spergularia echinosperma* n. sp. forma *pallens*, u Švarcenberského rybníka u Protivína se *Scirpus Michelianus*, Aug 1876 leg. Čelak.", PR 374981, left bottom individual (marked by the arrow in Fig. 7); the lectotype belongs to the diploid cytotype.

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Souhrn

V předložené práci jsme se zabývali studiem morfologické a cytologické variability druhů *Spergularia echinosperma* a *S. rubra*. Analyzovali jsme rostliny z celkem 27 populací zejména z jižních a západních Čech, kde je druh *S. echinosperma* nejhojnější. Navíc jsme do morfometrických analýz zahrnuli typové položky druhu *S. echinosperma* a údajného kříženec mezi *S. echinosperma* a *S. rubra*, popsaného jako *S.* ×*kurkae*. Cytometrická měření odhalila existenci dvou různých cytotypů – diploidního a tetraploidního – mezi rostlinami morfologicky odpovídajícími druhu *S. echinosperma*. U druhu *S. rubra* byl detekován jen tetraploidní cytotyp, jenž se velikostí genomu lišil od tetraploidního cytotypu *S. echinosperma*. Velikost genomu byla stanovena na 2C = 0,63 pg pro diploidy *S. echinosperma*, 2C = 1,22 pro tetraploidy *S. echinosperma* a 2C = 1,12 pg pro *S. rubra*. Všechny tři cytotypy se od sebe rovněž signifikantně lišily morfologicky. Tetraploidní cytotyp *S. echinosperma* byl nápadně intermediární mezi diploidním cytotypem a *S. rubra*. Nejdůležitějšími diskriminačními znaky jsou délka a poměr délky a šířky palistů, dále pak barva a velikost semen a rovněž také velikost a hustota jejich povrchových papil. Na základě studia morfologických znaků byl sestaven klíč na determinaci jednotlivých cytotypů:

1a Semena hnědá, řídce bradavčitá (hustota 5–9 papil na 1/4 obvodu semene); palisty alespoň 1,7× delší než široké, alespoň 2,9 mm dlouhé; rostliny obvykle s více než 5 lodyhami .....***S. rubra***

1b Semena černá, hustěji bradavčitá (hustota 8–17 papil na 1/4 obvodu semene); palisty méně než 1,7× delší než široké, kratší než 2,8 mm; rostliny obvykle s méně než 9 lodyhami 2

2a Palisty kratší než široké, kratší než 1,6 mm; semena kratší než 0,48 mm, hustota povrchových papil 12–17 na 1/4 obvodu semene .....***S. echinosperma*, diploidní cytotyp**

2b Palisty delší než široké, delší než 1,7 mm; semena delší než 0,48 mm, hustota povrchových papil 8–14 na 1/4 obvodu semene .....***S. echinosperma*, tetraploidní cytotyp**

Morfologická analýza dále potvrdila totožnost holotypu *S.* ×*kurkae* s tetraploidním cytotypem *S. echinosperma*. Dvě existující typové položky druhu *S. echinosperma* obsahují jak diploidy tak tetraploidy tohoto druhu. Vzhledem k příslušnosti jména *S.* ×*kurkae* k tetraploidnímu cytotypu proto navrhujeme lektotypifikaci jména *S. rubra* subsp. *echinosperma* Čelak. ve smyslu diploidního cytotypu. Ačkoli morfologická data svědčí o hybridním původu tetraploidního cytotypu *S. echinosperma*, velikost genomu tetraploida je signifikantně vyšší ve srovnání s hypotetickým hybridem mezi diploidy *S. echinosperma* a tetraploidy *S. rubra*, a nelze tedy vyloučit i další způsoby vzniku tetraploidů (např. autotetraploidní vznik a následná hybridizace s druhem *S. rubra*). Vzhledem k dosud nejasnému původu tetraploidního cytotypu *S. echinosperma* a nedostatku údajů o jeho ekologii a rozšíření prozatím nenavrhujeme jeho rozlišování jako samostatného taxonu.

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Appendix 1. – List of the localities of the *Spergularia echinosperma* and *S. rubra* populations used in this study together with their cytotype compositions detected by flow cytometry. Populations marked by an asterisk are those from which plants used for the measurements of the genome size using PI staining originated. The geographic coordinates are presented in WGS 84 format. ▶▶▶

Label	Locality	Latitute	Longitude	Altitude (m a.s.l.)	Number of plants	Species and cytotype
Cakov	S Bohemia, Čakov: bare bottom of the Beranov pond	48°58'51.8"N	14°19'11.5"E	420	3	*S. rubra* 4x
Cerna	Českomoravská vrchovina highlands, Černá: field path 1.7 km NW of the village	49°26'00.9"N	15°50'41.7"E	560	20	*S. rubra* 4x
Cky	SW Bohemia, Lažany: bare bottom of the Cky pond	49°21'06.9"N	13°53'28.9"E	490	20	*S. echinosperma* 4x + 2x
DolNovos	S Bohemia, Novosedly: bare bottom of the Dolní rybník pond	49°05'24.9"N	14°16'51.3"E	390	21	*S. echinosperma* 4x
Driten*	S Bohemia, Dříteň: bare bottom of the Kočínský rybník pond	49°08'56.1"N	14°21'15.0"E	460	20	*S. echinosperma* 4x + 2x
Havlic	S Bohemia, České Budějovice, Havlíčkova kolonie: lawn in a city park	48°57'43.2"N	14°28'40.8"E	400	20	*S. rubra* 4x
HorMez	Českomoravská vrchovina highlands, Horní Meziříčko: grassy playground in the village	49°09'19.0"N	15°14'29.7"E	580	19	*S. rubra* 4x
HorNovos*	S Bohemia, Novosedly: bare bottom of the Horní rybník pond	49°05'21.5"N	14°16'24.6"E	400	21	*S. echinosperma* 4x
Hurka	SW Bohemia, Záboří: bare bottom of the Hůrka pond	49°22'23.0"N	13°50'44.3"E	530	19	*S. echinosperma* 2x
Jensov*	S Bohemia, Písek: bare bottom of the Jenšovský rybník pond	49°19'35.8"N	14°06'35.0"E	400	15	*S. echinosperma* 2x
Klec*	S Bohemia, Klec: lawn in the village	49°05'49.5"N	14°44'56.6"E	420	20	*S. rubra* 4x
Knizeci	S Bohemia, Pištín: bare bottom of the Knížecí rybník pond	49°03'01.9"N	14°19'02.6"E	400	20	*S. echinosperma* 4x
Koclirov	S Bohemia, Smržov: bare bottom of the Koclířov pond	49°04'05.3"N	14°41'42.1"E	430	20	*S. echinosperma* 4x
Kozcin	SW Bohemia, Pačejov: bare bottom of the Kozčínský rybník pond	49°24'10.1"N	13°37'19.6"E	510	17	*S. echinosperma* 4x
Lhota	SW Bohemia, Horažďovická Lhota: bare bottom of the Lhota pond	49°21'30.0"N	13°40'38.6"E	470	17	*S. echinosperma* 4x
Luznice*	S Bohemia, Lužnice: road margin in the village	49°03'46.0"N	14°45'37.5"E	420	24	*S. rubra* 4x
Maj	S Bohemia, České Budějovice, Máj: sandy playground	48°59'20.2"N	14°26'08.5"E	400	20	*S. rubra* 4x
Malobor*	SW Bohemia, Sedlice: bare bottom of the Malobor pond	49°22'00.4"N	13°58'32.0"E	460	20	*S. echinosperma* 2x
Pecihradek	W Bohemia, Plzeň, Pecihrádek: field margin	49°46'06.5"N	13°24'57.0"E	330	22	*S. rubra* 4x
Pisek	S Bohemia, Písek: edge of a quarry 3 km E of the town	49°19'00.9"N	14°11'16.1"E	590	21	*S. rubra* 4x
Pracejov	SW Bohemia, Katovice: bare bottom of the Pracejovický rybník pond	49°15'18.7"N	13°50'42.0"E	420	20	*S. echinosperma* 4x
Smrzov*	S Bohemia, Smržov: bare bottom of the Vydýmač u Smržova pond	49°04'44.4"N	14°40'47.5"E	440	15	*S. echinosperma* 4x
StHlina*	S Bohemia, Stará Hlína: road margin in the village	49°02'31.9"N	14°48'36.5"E	430	20	*S. rubra* 4x
Strmilov	Českomoravská vrchovina highlands, Strmilov: crevices in square paving in the village	49°09'32.8"N	15°12'07.0"E	560	20	*S. rubra* 4x
Veselsky	Českomoravská vrchovina highlands, Nové Veselí: bare bottom of the Veselský rybník pond	49°31'17.2"N	15°54'15.2"E	560	21	*S. echinosperma* 4x
Vlkov	S Bohemia, Vlkov: sandy field margin 1.2 km NNW of the village	49°09'36.9"N	14°42'57.0"E	420	20	*S. rubra* 4x
Zavlekov	W Bohemia, Zavlekov: lawn in the village	49°20'20.5"N	13°29'36.2"E	570	20	*S. rubra* 4x

Paper 6: Kúr P., Košnar J., Koutecký P., Tremetsberger K., & Štech M. (2016): Origin of Spergularia ×kurkae, a hybrid between the rare endemic S. echinosperma and its widespread congener S. rubra. – Preslia 88: 391–407.

Origin of *Spergularia ×kurkae*, a hybrid between the rare endemic *S. echinosperma* and its widespread congener *S. rubra*

Původ *Spergularia ×kurkae*, křížence mezi vzácným endemickým druhem *S. echinosperma* a široce rozšířeným *S. rubra*

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Kúr P., Košnar J., Koutecký P., Tremetsberger K. & Štech M. (2016): Origin of *Spergularia ×kurkae*, a hybrid between the rare endemic *S. echinosperma* and its widespread congener *S. rubra*. – Preslia 88: 391–407.

The origin of *Spergularia ×kurkae*, a presumed tetraploid hybrid between the diploid central-European endemic *S. echinosperma* and its widespread tetraploid congener *S. rubra*, was investigated by sequencing the nrDNA ITS region and cpDNA *rpo*C1 intron. *Spergularia echinosperma* and *S. rubra* differed markedly in their ITS sequences. The presence of both sequences within the genome of *S. ×kurkae* confirmed its hybrid origin and parentage; cpDNA sequences identified *S. echinosperma* as the sole maternal parent. Because both parental ITS homeologs were clearly visible in the sequences of almost all of the *S. ×kurkae* individuals, we conclude that this taxon is of a relatively young age. We hypothesize that *S. ×kurkae* might have evolved as a result of human-mediated introduction of *S. rubra* into fishponds. Cross-amplification of species-specific ITS primers revealed high levels of intra-individual ITS polymorphisms in *S. echinosperma* and *S. rubra*. Our results suggest ongoing gene flow from *S. ×kurkae* to *S. rubra*. In contrast, no evidence of gene flow from *S. ×kurkae* or *S. rubra* to *S. echinosperma* was found, providing, despite concerns, no support for the threat of the genetic assimilation of *S. echinosperma*. Our current data also support the view of *S. kurkae* as a stabilized, separate allopolyploid species.

K e y w o r d s: endemism, hybridization, introgression, repeat-specific amplification, *Spergularia*

Introduction

Interspecific hybridization is assumed to be a major force driving the evolution of vascular plants (Rieseberg et al. 1993, Ellstrand et al. 1996, Prentis et al. 2007, Soltis & Soltis 2009, Soltis 2013). It is estimated that as many as 50% of angiosperms may be of hybrid origin (Arnold 1997). The most significant trigger for hybridization has been the effect of humans on ecosystems. Creation of new habitats and the introduction of allochthonous species have led to the formation of an unprecedented number of hybrid zones (Levin et al. 1996, Rhymer & Simberloff 1996, Arnold 1997, Rieseberg 1997, Ellstrand & Schierenbeck 2000, Soltis & Soltis 2009). Whereas hybrid zones are of undeniable

importance for the study of evolutionary processes, they can also pose a threat to endangered species in the form of genetic assimilation by widespread congeners (Levin et al. 1996, Wolf et al. 2001, Prentis et al. 2007).

Gene flow is usually limited to species of the same ploidy level (Chapman & Abbott 2010). Heteroploid hybridization is hampered by the production of sterile odd-ploidy offspring and typically occurs only in higher-ploidy taxa (Schneider 1958, Brochmann et al. 1992, Kolář et al. 2009, Hülber et al. 2015). However, there are an increasing number of documented cases of gene flow between diploids and tetraploids (e.g. Neuffer et al. 1999, Bleeker & Matthies 2005, Thórsson et al. 2010, Jørgensen et al. 2011, Koutecký et al. 2011, Moraes et al. 2013). The most important mechanism of inter-ploidy gene flow appears to be the fusion of reduced (n) and unreduced (2n) gametes (Ramsey & Schemske 1998, Soltis et al. 2004).

Spergularia echinosperma (Čelak.) Asch. et Graebn. is one of the few species of vascular plants that is endemic to central Europe and does not occur in high mountains (Friedrich 1979, Dvořák 1990, Kúr et al. 2012). It is confined to the vegetation of annual wetland herbaceous plants (class *Isoëto-Nanojuncetea*) that are mainly recorded growing in the dried out bottoms of freshwater reservoirs that are periodically drained. The primary habitats of *S. echinosperma* are alluvial pools and sandy banks of rivers (Friedrich 1979, Dvořák 1990). Unfortunately, most of these habitats have been destroyed by the channelling of rivers. *Spergularia echinosperma* also occurs in secondary habitats, especially the bottoms of drained fishponds, where it has also been threatened by the intensification of fishpond management over the last century (Popiela 2005, Šumberová et al. 2005, 2006, Šumberová 2011).

Spergularia echinosperma is morphologically similar to *S. rubra* (L.) J. Presl et C. Presl, a nearly cosmopolitan weedy species that mainly occurs in disturbed habitats such as sandy fields, roadsides and waste ground (Dvořák 1979, Friedrich 1979, Monnier & Ratter 1993, Hartman & Rabeler 2005). *Spergularia rubra* also sometimes grows in the same habitats as *S. echinosperma*, e.g. river banks and drained bottoms of ponds, where the two species have the same ecological niche and mixed populations are occasionally found (P. Kúr et al., unpubl.). These species differ in their ploidy levels, with *S. echinosperma* diploid (2n = 2x = 18; Dvořák & Dadáková 1984) and *S. rubra* tetraploid (2n = 4x = 36; Dvořák 1990, Wisskirchen & Haeupler 1998); there are records of other ploidy levels in countries other than those in central Europe (Ratter 1964, Fernandes & Leitao 1971).

Morphological observations have led some authors to conclude that *S. echinosperma* and *S. rubra* hybridize (Jage 1974, Dvořák 1989, 1990), and the hybrid was formally described as *S.* ×*kurkae* F. Dvořák (Dvořák 1989). The formal description is supplemented with a chromosome count, which is tetraploid (2n = 36). A more detailed study by Kúr et al. (2012) reports tetraploid populations that match the description of *S.* ×*kurkae*. These populations are clearly morphologically intermediate between *S. echinosperma* and *S. rubra*, supporting their hybrid origin. However, their genome size deviates significantly from the genome size of a modelled allotetraploid hybrid *S. echinosperma* × *S. rubra*. Dvořák (1990) also assumes introgressive hybridization between *S.* ×*kurkae* and *S. echinosperma*. However, Kúr et al. (2012) reports no morphological indications of gene flow between these taxa. Rather, they detect possible hybridization at the tetraploid level between *S.* ×*kurkae* and *S. rubra*. Therefore, in the current study, we investigate the hybridization of the two *Spergularia* species using molecular methods.

To accomplish our objective, we sequenced the biparentally inherited internal transcribed spacer (ITS) of nuclear ribosomal DNA and the maternally inherited *rpo*C1 intron of chloroplast DNA (cpDNA). The ITS region was chosen because it is a multicopy marker that often retains intra-individual polymorphism in hybrid taxa, thus allowing clear inferences about their parentage (Sang et al. 1995, Koch 2003). To evaluate interspecific gene flow, we also used repeat-specific amplification of the ITS region. This is a very sensitive method that enables the detection of minority sequence variants and is suitable for inferring hybridization and reconstructing phylogeny (Rauscher et al. 2002, 2004, Soltis et al. 2008, Laureto & Barkman 2011). The cpDNA was used to indicate the direction of hybridization (i.e. to identify the maternal species). The combined use of these two markers allowed us to answer the following two questions: (i) What is the parentage of tetraploid *S. ×kurkae*? (ii) Does introgression among *S. ×kurkae*, *S. echinosperma* and *S. rubra* occur?

Materials and methods

Plant sampling

A total of 516 plants from 91 populations of *Spergularia echinosperma*, *S. rubra* and *S. ×kurkae* (1–20 individuals per population and taxon) in the Czech Republic and Germany were sampled between the years 2008 and 2012 (Fig. 1; see Electronic Appendix 1 for the exact localities and acronyms of the populations used in the text). Voucher specimens were deposited in the herbarium CBFS. Additionally, the holotype of *S. ×kurkae* (deposited in the herbarium CB) was used for DNA sequencing.

Flow cytometry and chromosome counting

To confirm the determination of the plants analysed, flow cytometry was used to estimate the genome size and DNA ploidy level (sensu Suda et al. 2006) of all the plants collected. We followed the protocol presented in Kúr et al. (2012).

We calibrated the flow cytometric measurements using the chromosome counts of all three taxa. One plant from each of the populations Siglovec (*S. rubra*), Kojatín (*S. echinosperma*) and Nový Dářko (*S. ×kurkae*) were used. The apical root meristems of germinated seedlings were pre-treated with a saturated water solution of p-dichlorobenzene (3 h, room temperature) and fixed in a 3:1 mixture of 96% ethanol and glacial acetic acid overnight at 4 °C. Chromosome counts were made after digestion using enzymes and squashing as described by Schwarzacher & Heslop-Harrison (2000) and Schönswetter et al. (2007). The fixed material was maintained in citrate buffer (pH = 4.8; freshly prepared by mixing 4 ml of 0.1 M citric acid monohydrate $C_6H_8O_7 \cdot H_2O$ and 6 ml of 0.1 M trisodium citrate dihydrate $C_6H_5O_7Na_3 \cdot 2\ H_2O$, and diluting 10× with distilled water) for 20 min, transferred to an enzyme mixture containing 1% (w/v) cellulase Onozuka (Serva, Heidelberg, Germany), 0.4% (w/v) cytohelicase (Sigma-Aldrich, Vienna, Austria) and 0.4% (w/v) pectolyase (Sigma-Aldrich) in citrate buffer (pH = 4.8, pre-warmed at 37 °C) and incubated for 30 min at 37 °C. Next, the loose root material was washed in citrate buffer for a minimum of 30 min and transferred to a drop of 60% acetic acid on a microscopic slide. The material was then dissected using entomological needles under a stereomicroscope,

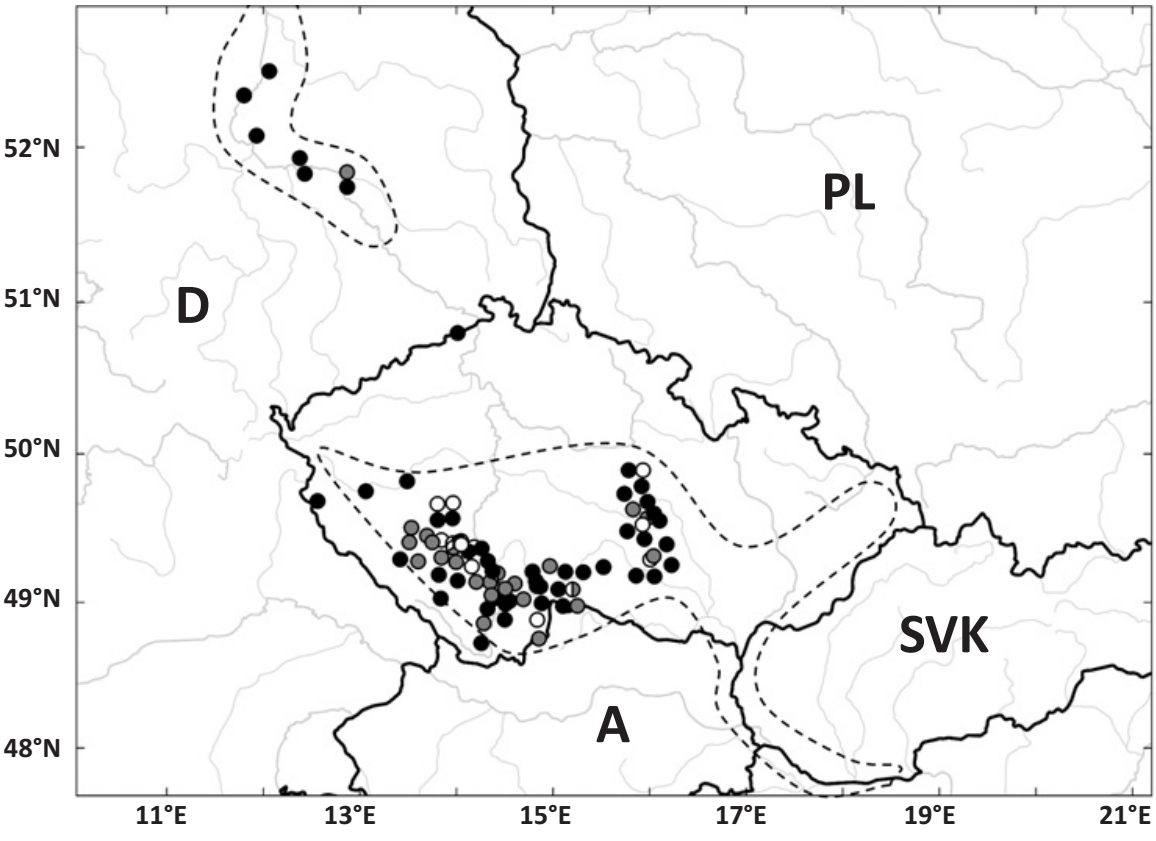

Fig. 1. – Distributions of the populations of *Spergularia echinosperma* (white), *S.* ×*kurkae* (grey) and *S. rubra* (black) studied. The dashed lines denote the distribution of *S. echinosperma* (compiled from the review of herbarium specimens; P. Kúr et al., unpubl.). The distribution of *S. rubra* is not mapped, as this species is widespread throughout the study area.

covered with a cover slip and squashed. Preparations were frozen on a cooling plate, air dried after cover slip removal, and stored at –20 °C until required. After application of 9 µl of Vectashield mounting medium (Vector Laboratories, Burlingame, CA, USA) with 2 µg/ml DAPI to the dry preparations, the preparations were screened for well-spread mitotic metaphases under a Zeiss Axio Imager.M2 epifluorescence microscope equipped with an Axiocam HRm camera. Images were acquired using Zeiss AxioVision SE64 software (Carl Zeiss Meditec AG, Oberkochen, Germany).

DNA extraction

Parts of plants (typically a whole lateral branch) were silica-dried and processed using the rapid DNA extraction method of Werner et al. (2002). A small amount of plant material was ground in 30 µl of 0.5 M NaOH and centrifuged. The supernatant was diluted 1:10 with 100 mM Tris-HCl buffer (pH = 8.3). In order to obtain DNA isolates of high quality, DNA from plants that were only available as herbarium specimens was extracted using the Invisorb Spin Plant Mini Kit (Invitek, Germany) following the manufacturer's protocol.

DNA sequencing and cloning

To amplify the ITS region, the ITS4i and ITS5i PCR primers (Roalson & Friar 2000) were used with the following cycling program: 94 °C for 2 min; 35 cycles of 94 °C for 30 s, 50 °C for 1 min, 72 °C for 1 min; and final elongation at 72 °C for 10 min. The *rpo*C1 intron was amplified using the ANU cp033-L and ANU cp034-R primers (Ebert & Peakall 2009) with the following cycling program: 94 °C for 2 min; 12 cycles of 94 °C for 30 s, 66–51 °C (gradually reduced by 3 °C every second cycle) for 30 s, 72 °C for 45 s; 33 cycles of 94 °C for 30 s, 47 °C for 30 s and 72 °C for 45 s; and final elongation at 72 °C for 10 min. The PCR reactions were carried out with 2.5 µl of Plain PP Master Mix (Top-Bio, Czech Republic), 2 mM $MgCl_2$, 0.2 mM of each dNTP, 0.3 µM of each primer, and 0.4 µl of the template DNA in a final reaction volume of 5 µl. The PCR products were sequenced using the ITS5i and ANU_cp033-L primers on an ABI PRISM 3130xl Genetic Analyzer (Laboratory of Genomics, Biology Centre of the Academy of Sciences of the Czech Republic, České Budějovice). The resulting electropherograms were inspected in Finch TV 1.4 (Geospiza, USA).

All but seven samples of *S. ×kurkae* showed multiple additive peaks in the direct ITS sequences, indicating intra-individual variation in the sequences. To separate the particular ITS molecules, PCR products were cloned into competent *Escherichia coli* DH5 alpha cells using the pGEM-T Easy Vector System (Promega, USA) according to the manufacturer's instructions except that only one quarter of the recommended volumes for the reactions were used. Because the pattern of nucleotide additivity was the same for all of the samples, only one sample was chosen for cloning (population Cky). Five clones were sequenced to investigate the potential presence of different ITS copies.

Repeat-specific amplification

As the sequencing revealed two distinct ITS ribotypes, one specific to *S. echinosperma* and the other to *S. rubra* (see Results), we designed two sets of taxon-specific ITS primers using Primer3 (Koressaar & Remm 2007). Two primer pairs each consisted of a universal forward primer targeting both ribotypes (SIf, 5'-TCGTAACAAGGTTTCCGTAGGTG-3') in the 18S rRNA region and a reverse primer specific for *S. echinosperma* (EIr, 5'-CTCTAACGGGCGGGCG-3') or *S. rubra* (RIr, 5'-CTCTGGAAACGGGGCGG-3') ribotype, which targeted a variable site in the middle of the ITS1 region, producing a short partial fragment of the ITS1 region c. 100 bp long. The other two primer pairs each had a forward primer specific for *S. echinosperma* (EIf, 5'-TTGGTGCGTCCGCTCTAAC-3'; located 9 bp downstream of EIr) or *S. rubra* (RIf, 5'-CGCCCGCTCTGGAAAC-3'; located 7 bp downstream of RIr) and the universal reverse ITS4i primer, producing a long fragment ~ 500 bp long that included partial ITS1, complete 5.8S and complete ITS2 regions (Fig. 2). The *echinosperma*-specific amplification was done using 260 individuals of *S. rubra*, and the *rubra*-specific amplification was done using 58 individuals of *S. echinosperma*. In addition, the *rubra*-specific amplification was tested in the seven above-mentioned individuals of *S. ×kurkae* that had only the *S. echinosperma* homeolog visible on the direct sequences (see Electronic Appendix 1). The PCR program was 95 °C for 3 min; 35/45 cycles of 95 °C for 30 s, primer-pair specific annealing temperature for 60 s, and 72 °C for 60 s; and 72 °C for 10 min. The annealing temperatures were 69 °C for the EIf/ITS4i primer pair, 67 °C for the RIf/ITS4i primer pair, and 64 °C for the SIf/EIr

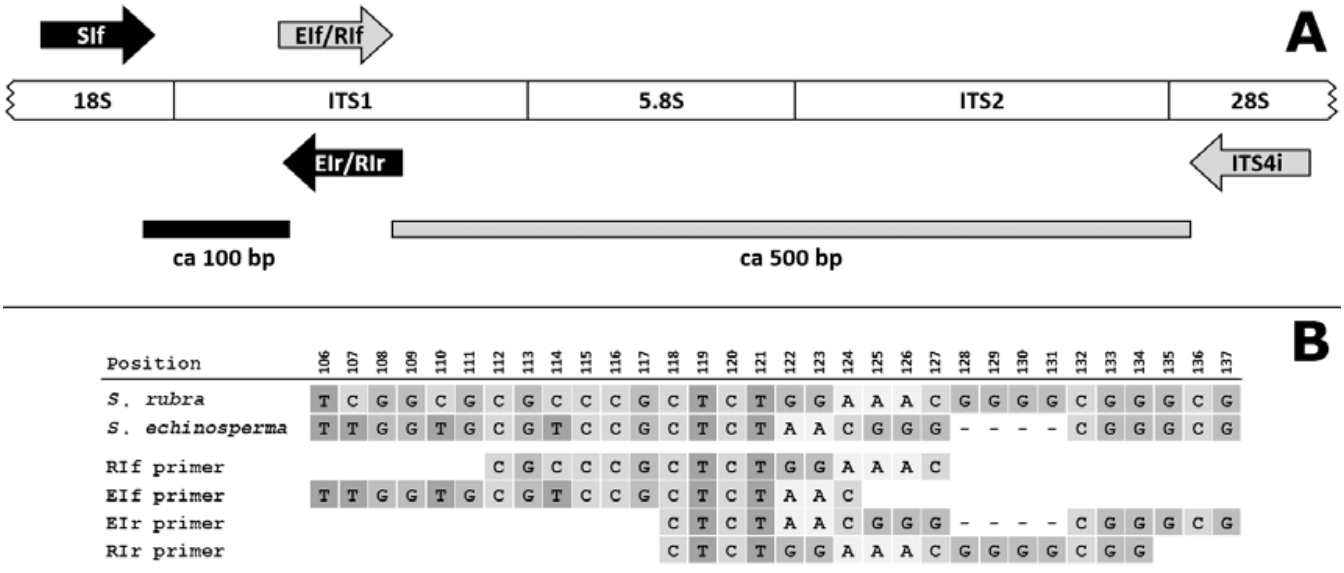

Fig. 2. – (A) Scheme of the primers used in the repeat-specific amplification of *Spergularia echinosperma* and *S. rubra*, with approximate lengths of the PCR products. (B) Variable site in the ITS sequences of *S. echinosperma* and *S. rubra* targeted by the repeat-specific PCR primers.

and SIf/RIr primer pairs. Each PCR was run in two replicates consisting of 35 and 45 cycles. The PCR products were visualized on a 1.5% (w/v) agarose gel.

The percentages of individuals with positive amplification were calculated for each population of *S. echinosperma* and *S. rubra* tested, with at least 3 individuals analysed for each primer pair. Populations with significantly higher amplification rates were detected using Grubbs' test for outlier detection in R 3.2.0 (R Development Core Team 2015; grubbs.test function from the outliers package, ver. 0.14; Komsta 2011).

In addition, the products of the repeat-specific amplification were sequenced in a subset of the samples (17 in EIf/ITS4i, 19 in SIf/EIr, 6 in RIf/ITS4i and 13 in SIf/RIr; Table 1). PCR products showing multiple peaks in the direct sequences were cloned. The resulting sequences were aligned manually in BioEdit 7.2.0 (Hall 1999) and compared with the sequences obtained by direct sequencing. A ribotype network was constructed in TCS 1.21 (Clement et al. 2000) with a 90% connection limit and gaps included as the 5th state. Because of the problems with the RIf/ITS4i sequencing of the longer fragment in *S. echinosperma*, the network was computed only for the sequences of the short fragments (i.e. SIf/EIr and SIf/RIr amplifications).

PCR-RFLP

Only two distinct *rpo*C1 haplotypes were found among all of the samples sequenced, one being specific to *S. echinosperma* and the other to *S. rubra* (see Results). The *rubra*-haplotype possessed a restriction site for the enzyme PdmI (XmnI) at the 569th position of the alignment where the *echinosperma*-haplotype had a one-base substitution preventing this enzyme from cutting. Therefore, a PCR-RFLP protocol for fast identification of particular *rpo*C1 haplotypes was developed. The reactions were as follows: 1.8 µl of the PCR

Table 1. – Ribotypes recorded using repeat-specific PCR amplification in *Spergularia echinosperma* (E) and *S. rubra* (R), and percentages of individuals with positive amplification per population (only populations with more than two individuals tested are considered). Ribotypes EIr0 and RIr0 match the sequences obtained from the direct sequencing of *S. echinosperma* and *S. rubra*, respectively. Values in bold indicate populations with a significantly higher amplification rate for a particular PCR replicate (Grubbs' outlier test; $\alpha = 0.05$).

Population	Individual	Taxon	Haplotypes	Percentage of positive amplification of alternative ribotypes [%]			
				Long fragment (EIf/RIf)		Short fragment (EIr/RIr)	
				35 cycles	45 cycles	35 cycles	45 cycles
Babák	1	E	RIr0, 1	–	–	–	–
	2		RIr1, 4, 8				
Dříteň	1	E	–	0	50	0	0
Hůrka	1	E	–	0	20	0	0
Chvalovec	3	E	RIr1	0	20	0	14
	4		RIr1				
Hoděmyšl	1	E	RIr3	**17**	33	0	40
Jenšov	1	E	RIr0, 9	–	–	–	–
	2		RIr1, 6, 7				
Malobor	1	E	RIr1, 4, 5	0	0	0	0
	2		RIr0, 1				
Mlýnhor	1	E	–	0	0	0	0
Pařezný	1	E	RIr1, 2	0	0	0	0
	2		RIr1				
Podhůrský	1	E	–	0	50	0	17
Skopec	1	E	RIr1	0	33	0	33
Švihov	1	E	–	0	17	0	50
Vosecký	1	E	RIr3	0	0	0	25
Beranov	1	R	EIf0	0	**67**	33	67
	2		EIf0				
	3		EIf0				
	4		EIr0				
Beranov-road	1	R	EIr0, 1	22	11	0	29
Bleddin-road	1	R	–	17	50	0	17
Bohdalov	1	R	–	0	17	0	40
Březejc	1	R	–	0	33	0	0
Černá	1	R	–	0	0	0	40
Dvořák	1	R	–	0	0	0	25
Hoděmysl-road	1	R	–	17	0	0	50
HorMez	1	R	–	0	0	20	0
Chvalovec	1	R	EIf0	**71**	71	67	83
	2		EIr0, EIf0				
Chvalovec-road	1	R	EIf0	**94**	94	35	31
	2		EIr0, EIf0				
Dessau	1	R	EIf0	17	17	17	33
	2		EIr0				
Dobev	1	R	EIr0	17	0	33	17
	2		EIr0, EIf0				
Domburg	1	R	EIf0	0	25	0	0
Grieben	1	R	EIr0	0	17	33	17
Heinrichsberg	1	R	EIf0	**33**	17	0	40
Januš	1	R	EIf1	–	–	–	–
Klec	1	R	–	0	0	0	0
Klieken	1	R	EIf0	**33**	17	17	40
	2		EIr0				
Konračský-road	1	R	–	0	0	0	17

Population	Individual	Taxon	Haplotypes	Percentage of positive amplification of alternative ribotypes [%]			
				Long fragment (EIf/RIf)		Short fragment (EIr/RIr)	
				35 cycles	45 cycles	35 cycles	45 cycles
KrLes	1	R	EIf0	14	29	0	29
Lužnice	1	R	–	0	0	0	0
Máj	1	R	–	0	0	0	0
Mlýnhor-road	1	R	–	0	0	0	33
Mříč	1	R	EIr0	0	0	17	67
Mýto	1	R	–	0	0	0	0
Nový Dářko-road	1	R	–	0	0	0	33
Pecihrádek	1	R	–	0	0	20	0
Písek	1	R	–	0	0	0	40
Pláňava	1	R	–	0	0	0	60
Pobočenský	1	R	EIr0	0	0	25	0
Polom	1	R	EIr0	0	33	33	60
Ptáčov	1	R	EIr0	0	17	17	33
Rožmitál	1	R	–	0	0	0	0
Siglovec	1	R	EIf0	17	50	0	20
	2		EIf2				
Skopec-road	1	R	EIr0	0	50	33	67
Slavkovický	1	R	EIr0, EIf0	20	20	20	20
St Hlína	1	R	–	0	20	0	0
Strmilov	1	R	–	0	0	25	0
Švihov-road	1	R	EIr0	0	33	17	33
Telč Štěpnice	1	R	EIr0	0	17	17	50
Vlkov	1	R	–	0	0	0	0
Vosecký-road	1	R	–	0	11	0	11
Vrbinec	1	R	EIr0	0	0	17	50
Waidhaus	1	R	–	0	20	0	0
Zavlekov	1	R	EIr0	0	0	40	20

product was added to a mixture containing 1.35 U of PdmI enzyme (Fermentas, Lithuania), 0.27 µl of 10× Tango buffer (Fermentas) and 1.98 µl of sterile H_2O. The mixture was incubated at 37 °C for 6 h and the entire reaction volume was analysed electrophoretically on a 1.5% (w/v) agarose gel. The specificity of this method was tested using 27 samples of *S. echinosperma* and 60 samples of *S. rubra*, the identities of which were confirmed by direct sequencing.

Results

Chromosome counts and ploidy levels

Three different cytotypes were found, corresponding to diploid *S. echinosperma*, tetraploid *S.* ×*kurkae* and tetraploid *S. rubra*. All three taxa also differed in monoploid genome size (Fig. 3). An exception was one individual of *S.* ×*kurkae* (population Gbelinek), which had a monoploid genome size significantly larger than that of the other *S.* ×*kurkae* individuals. Chromosome counts confirmed 2n = 36 for *S. rubra* and *S.* ×*kurkae* and 2n = 18 for *S. echinosperma* (Fig. 4).

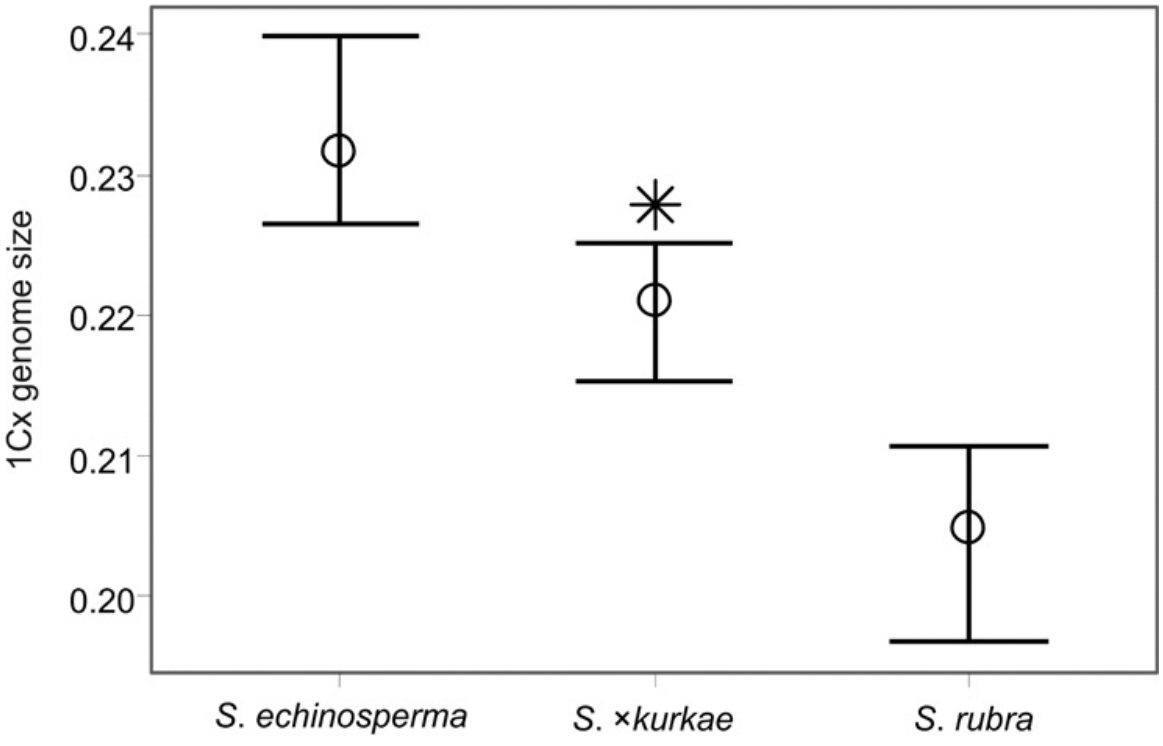

Fig. 3. – Range plot of the equivalents of the 1Cx values calculated from the genome sizes based on DAPI staining for *Spergularia echinosperma*, *S.* ×*kurkae*, and *S. rubra* expressed in terms of a ratio with the 1C value of the internal standard *Glycine max*. Midpoint = median; error bar = min–max. The *S.* ×*kurkae* outlier is marked with an asterisk.

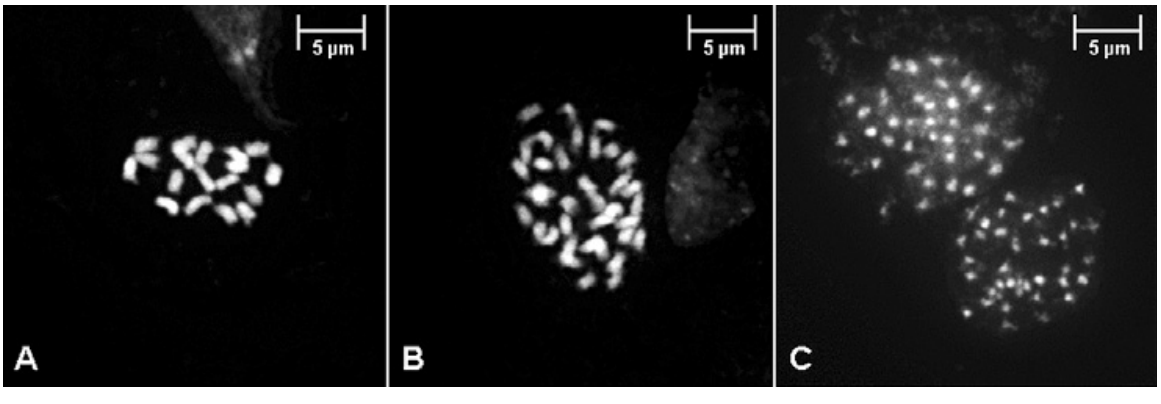

Fig. 4. – Mitotic chromosome spreads of *Spergularia echinosperma* (A; 2n = 18), *S.* ×*kurkae* (B; 2n = 36) and *S. rubra* (C; 2n = 36).

Direct sequencing and cloning

Direct sequencing of the ITS region of 112 plants resulted in two distinct ribotypes that were specific for *S. echinosperma* and *S. rubra* (GenBank acc. no. KU662347–KU662348). The alignment had a length of 674 bp and contained 16 substitutions and 4 indels (Electronic Appendix 2). Cloning of *S.* ×*kurkae* resulted in two ITS sequences that were identical with the ribotypes of *S. echinosperma* and *S. rubra*. The pattern of nucleotide

additivity of these two ribotypes was recorded in the direct sequences of 39 individuals of *S.* ×*kurkae*, including the holotype. Seven individuals were exceptions, in which only the peaks of *S. echinosperma* were observable (populations: Špitálský – 3 individuals, Chvalovec, Bleddin, Kozcin, Veselský – 1 individual each).

Only two different *rpo*C1 haplotypes were found among all of the 95 plants sequenced: one unique for *S. rubra* and the other unique for *S. echinosperma* and *S.* ×*kurkae* (GenBank acc. no. KU671397–KU671398). The alignment had a length of 802 bp and contained two substitutions and one indel (Electronic Appendix 3). The PdmI assay consistently produced two fragments for *S. rubra* (200 and 600 bp), whereas no digestion was detectable for *S. echinosperma*. We confirmed the presence of the *S. echinosperma* haplotype in all 164 individuals of *S.* ×*kurkae* (Electronic Appendix 1). The attempts to amplify the *rpo*C1 intron in the holotype of *S.* ×*kurkae* failed.

Repeat-specific amplification

Both sets of the *rubra*-specific ITS primers amplified positively in five out of the seven individuals of *S.* ×*kurkae* for which the *S. rubra* ribotype was not visible in the direct sequences (populations Bleddin, Chvalovec, Kozcin, Špitálský, and Veselský). The other two samples (population Špitálský) did not show any positive amplification.

The *echinosperma*-specific ITS primers amplified positively in 18% of the 267 individuals of *S. rubra*, averaged over both PCR replicates. The rate of positive amplification was distributed unequally among the *S. rubra* populations, with only a few highly amplifying populations (Beranov, Chvalovec, Chvalovec-road, Heinrichsberg, and Klieken; Table 1). The *rubra*-specific primers amplified positively in 10% of the 64 individuals of *S. echinosperma*, averaged over both PCR replicates. The rates of positive amplification were distributed randomly among the populations of *S. echinosperma*, and there were no populations with consistently higher amplification rates (Table 1).

A subset of the PCR products of the *echinosperma*-specific amplification in *S. rubra* (32 individuals) and the *rubra*-specific amplification in *S. echinosperma* (13 individuals) was sequenced. The longer *echinosperma*-specific EIf/ITS4i products from *S. rubra* were found in all but two of the individuals to be identical with the ITS sequence of *S. echinosperma* (ribotype EIf0). The two *S. rubra* individuals produced sequences differing from this ribotype by a single substitution (ribotypes EIf1–EIf2; Tables 1 and 2). Sequencing of the longer *rubra*-specific RIf/ITS4i products from *S. echinosperma* was unsuccessful due to a very weak signal.

The shorter *echinosperma*-specific SIf/EIr products from *S. rubra* were in nearly all of the individuals identical with the ITS ribotype of *S. echinosperma* (ribotype EIr0). Additionally, one individual displayed intra-individual sequence variation and contained another unique sequence differing from the EIr0 ribotype in one substitution (ribotype EIr1; Tables 1 and 3). The shorter *rubra*-specific SIf/RIr products from *S. echinosperma* resulted in 10 different *rubra*-like ribotypes (RIr0–RIr9), which were clearly separated from the *echinosperma*-like ribotypes (EIr) in the TCS network (Fig. 5). The separation between the RIr and EIr groups of ribotypes was distinct, with at least three hypothetical missing haplotypes. Importantly, the ribotype matching the ITS sequence of *S. rubra* (RIr0) was very rare in *S. echinosperma*, being detected in only three individuals (Tables 1 and 3). The remaining *rubra*-like ribotypes (RIr1–9) found in 13 individuals of *S. echinosperma*

were derived from the RIr0 ribotype of *S. rubra* and differed by 1–4 substitutions. There was no clear pattern in the geographic distribution of the different ribotypes (Electronic Appendix 4).

Table 2. – Ribotypes recorded using repeat-specific amplification by EIf/ITS4i primers in *Spergularia rubra* and their comparison with the ITS sequence of *S. rubra*. Only variable sites are shown. The position numbers correspond to the alignment of the whole ITS region (Electronic Appendix 2).

Haplotype/position	131	171	190	202	205	208	235	306	488
S. rubra	G	A	C	C	T	T	C	A	C
EIf0 (= *S. echinosperma*)	–	A	T	T	C	C	T	A	T
EIf1	G	G	T	T	C	C	T	A	T
EIf2	G	A	T	T	C	C	T	C	T

Table 3. – Ribotypes recorded using repeat-specific amplification by SIf/EIr primers in *Spergularia rubra* (haplotypes EIr0-EIr1) and SIf/RIr primers in *S. echinosperma* (haplotypes RIr0-RIr9). Only variable sites are shown. The position numbers correspond to the alignment of the whole ITS region (Electronic Appendix 2).

Haplotype/position	73	74	77	78	79	80	87	95	107	110	112	114	116
EIr0 (= *S. echinosperma*)	G	G	C	G	C	C	–	T	T	T	C	T	C
EIr1	A	G	C	G	C	C	–	T	T	T	C	T	C
RIr0 (= *S. rubra*)	G	G	C	G	C	C	C	C	C	C	C	C	C
RIr1	G	G	A	G	C	C	T	C	T	T	C	C	C
RIr2	G	G	C	G	C	C	C	C	C	C	A	C	C
RIr3	G	G	C	G	C	C	C	C	C	T	C	C	C
RIr4	G	G	C	G	C	T	C	C	C	C	C	C	C
RIr5	G	G	C	A	C	C	C	C	T	T	C	C	C
RIr6	G	G	C	G	T	C	C	C	C	C	C	C	C
RIr7	G	T	C	G	C	C	C	C	C	C	C	C	C
RIr8	G	G	C	G	C	C	C	C	C	C	C	T	C
RIr9	G	G	C	G	C	C	C	C	C	C	C	C	A

Discussion

Origin of Spergularia ×kurkae

The chromosome counts and flow cytometric measurements confirmed previous reports of the ploidy levels for all the taxa studied. Only one individual of *S. ×kurkae* displayed an exceptionally high genome size and may be an aneuploid. The concurrent presence of the ITS ribotypes from *S. echinosperma* and *S. rubra* in nearly all of the individuals of *S. ×kurkae* (including the holotype) convincingly demonstrates the hybrid origin of *S. ×kurkae*. This finding is in accordance with the morphological evidence of Kúr et al. (2012).

The chloroplast DNA revealed that *S. echinosperma* was the maternal progenitor of *S. ×kurkae* in all cases. No triploids were found among the populations of *S. echinosperma* and *S. rubra* in either this study or in that of Kúr et al. (2012), indicating that triploids do not play a role in the evolution of this group. It is therefore likely that the formation of *S. ×kurkae* was a one-step process that involved unreduced gametes of *S. echinosperma*.

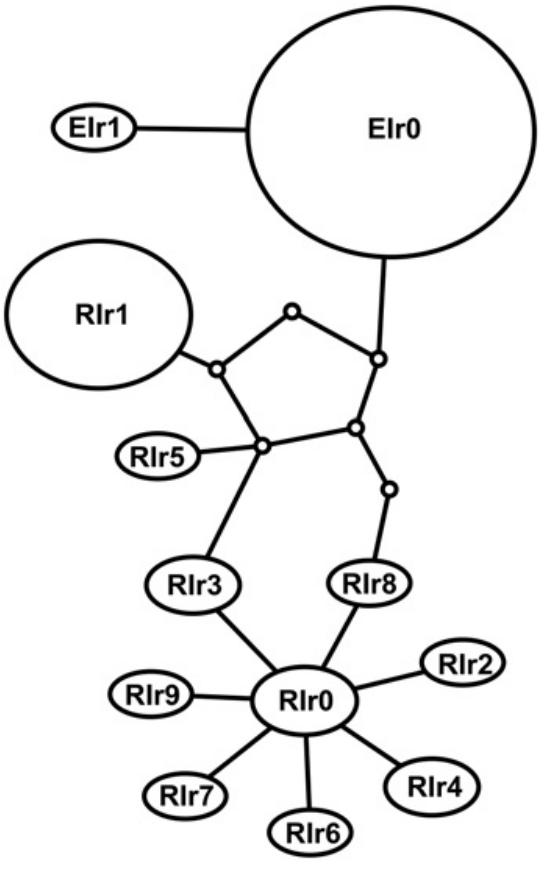

Fig. 5. – Ribotype network based on the sequences obtained from repeat-specific amplification with SIf/EIr and SIf/RIr primers. The size of the ovals indicate the relative frequencies of particular ribotypes in our data. The empty circles represent hypothetical missing haplotypes.

The incomplete concerted evolution in *S.* ×*kurkae* (i.e. lacking homogenization of divergent rDNA copies; Zimmer et al. 1980, Hillis et al. 1991, Elder & Turner 1995), an annual species with a short generation time, indicates its young age (cf. Sang et al. 1995, O'Kane et al. 1996, Koch 2003). We hypothesize that *S.* ×*kurkae* formed after *S. echinosperma* and *S. rubra* came into contact as a result of human-mediated introduction of *S. rubra* into fishponds (e.g. possibly due to grazing of summer-drained fishponds and the sowing of cereals and other culture plants; Šumberová 2003). In Bohemia, such introductions might be associated with fish farming, which began in the 11th century and was most intensive in the 15th and 16th centuries (Šumberová et al. 2006). Before the advent of fish farming, it was likely that the contact between *S. echinosperma* and *S. rubra* was limited as these species were likely ecologically separated at that time based on their contemporary primary habitats along the river banks of the Elbe in Germany (U. Amarell, pers. comm.). Additional insight into the origin of *S.* ×*kurkae* is expected to be provided by an ongoing study based on microsatellite markers (Kúr et al. 2014).

Interspecific gene flow

As the origin of the tetraploid *S. rubra* is unknown, the high incidence of the *S. echinosperma* ITS ribotype in some populations of *S. rubra* might be explained in two

ways. First, *S. rubra* might be of allopolyploid origin, with one parental genome from *S. echinosperma* or a species closely related to *S. echinosperma*. In this scenario, the observed intra-individual ITS variation within *S. rubra* would represent the remains of the *S. echinosperma*-like ancestor retained within *S. rubra*. This pathway could also explain the discrepancy between the recorded genome size of *S. ×kurkae* and that predicted by combining the genome sizes of its parents (Kúr et al. 2012). If *S. rubra* acted as a segmental allopolyploid, it might produce gametes with a higher genome size than half of that of the *S. rubra* somatic genome size, leading to the apparent genome upsizing in *S. ×kurkae*.

Alternatively, the *S. echinosperma* ITS variants within *S. rubra* might be the result of ongoing gene flow between *S. ×kurkae* and *S. rubra*. Gene introgression between these two taxa was previously suggested by the existence of morphologically intermediate plants (Kúr et al. 2012). Importantly, in the present study, all five populations of *S. rubra* that had significantly high rates of amplification of the *echinosperma*-specific ITS primers (Table 1) were located near present (Beranov, Chvalovec, Chvalovec-road) or historical (Heinrichsberg, Klieken) localities of *S. echinosperma* or *S. ×kurkae*. We consider these findings as a good indicator of interspecific gene flow. However, the two hypotheses are not mutually exclusive, and both processes might be involved.

In contrast, we found no reliable evidence of gene flow from *S. ×kurkae* to *S. echinosperma* as the *S. rubra* ITS ribotype (RIr0) was almost never present within *S. echinosperma*. The several *rubra*-like ribotypes found in *S. echinosperma* (Table 1) were more divergent and are likely a result of an ancestral polymorphism retained within *S. echinosperma*. If there was recent gene flow from *S. rubra* to *S. echinosperma*, we would expect the frequent occurrence of the RIr0 ribotype in *S. echinosperma.*

Therefore, our results conflict with Dvořák (1990), who argues that there is a constant gene flow from *S. rubra* to *S. echinosperma*. We conclude that *S. echinosperma* is not currently threatened by genetic assimilation. However, even if the ploidy barrier protects this species from assimilation, it might still be threatened by ecological competition from *S. ×kurkae* (demographic swamping; Levin et al. 1996), as the latter has a higher fitness than *S. echinosperma* in terms of higher seed set and more rapid growth (P. Kúr, unpubl.). For example, in *Typha ×glauca* (Huisman et al. 2012) and *Spartina anglica* (Begon et al. 1991, Ennos & Sheffield 2009), hybrids successfully outcompeted the parental species. Further studies are needed to reliably assess the risks that *S. ×kurkae* poses to the rare endemic *S. echinosperma*.

Status of Spergularia ×kurkae

Since the description of *Spergularia ×kurkae* by Dvořák (1989), this taxon has not been listed in any of the central-European floras or checklists (Fischer et al. 2008, Jäger 2011, Danihelka et al. 2012, Goliašová 2012) except for the Flora of the Czech Republic (Dvořák 1990). Our current data, however, support *S. ×kurkae* as an independent taxon that mostly occurs in the absence of the parental species, which is consistent with its distinct morphological separation (Kúr et al. 2012). Although there are some indications of ongoing hybridization between *S. ×kurkae* and *S. rubra*, it appears to be rare, with little effect on the boundary between the taxa. In addition, according to preliminary data on germination ecology (P. Kúr, unpublished), the percentage germination recorded for

S. ×*kurkae* is similar to that of *S. echinosperma* and it does not suffer from reduced fertility. We therefore propose that *S. kurkae*, originally described as a primary hybrid, be treated as a separate allopolyploid species, in a similar way to *Bolboschoenus laticarpus* Marhold et al. (Marhold et al. 2004), *Galeopsis tetrahit* L. (Bendiksby et al. 2011) and *Veronica hederifolia* L. (Albach et al. 2008).

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Souhrn

Cílem předkládané práce bylo studium původu taxonu *Spergularia* ×*kurkae*, předpokládaného křížence diploidního středoevropského endemita *S. echinosperma* a široce rozšířeného tetraploidního druhu *S. rubra*. Celkem bylo analyzováno 516 rostlin z 91 populací z území České republiky a Německa, a to včetně typové položky jména *S.* ×*kurkae*. Použitými metodami bylo sekvenování jaderného ITS regionu a chloroplastového *rpo*C1 genu. *Spergularia echinosperma* a *S. rubra* se výrazně lišily ve svých ITS sekvencích. Oba ITS ribotypy se rovněž vyskytovaly pohromadě v genomu téměř všech jedinců *S.* ×*kurkae*, což přesvědčivě dokazuje hybridní původ tohoto taxonu. Chloroplastová DNA rovněž prokázala, že ve všech případech byl mateřským rodičem druh *S. echinosperma*. U téměř všech jedinců *S.* ×*kurkae* byly oba ITS ribotypy zřetelně patrné na přímých sekvencích, což ukazuje na neúplnou homogenizaci ribozomální DNA (incomplete concerted evolution) a naznačuje, že *S.* ×*kurkae* je pravděpodobně relativně mladým taxonem. Je možné, že se taxon *S.* ×*kurkae* vyvinul následkem člověkem zapříčiněné introdukce druhu *S. rubra* na obnažená dna rybníků, pravděpodobně ve spojitosti s rozvojem rybníkářství v Čechách. Reciproká amplifikace druhově specifických ITS primerů rovněž naznačila možnost genového toku na tetraploidní úrovni mezi *S.* ×*kurkae* a *S. rubra*, který je ale poměrně vzácný. Naproti tomu nebyly nalezeny důkazy o ohrožení *S. echinosperma* genetickou erozí a genovým tokem od *S. rubra*. Na základě spojení těchto výsledků a předchozích studií (morfologické rozdíly, samostatný výskyt nezávislý na rodičovských druzích) doporučujeme klasifikovat *S. kurkae* jako samostatný allopolyploidní druh.

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Taxonomy and evolutionary diversification of the Central European endemic *Spergularia echinosperma* (Caryophyllaceae)

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Abstract

The patterns of morphological variation and distribution of the rare Central European endemic *Spergularia echinosperma* were investigated. Morphometric analyses revealed the existence of two distinct morphotypes differing each other mainly in seed color, which is either brown or black. Other differences are in density of the seed surface papillae, height and shape of the papillae, seed width, pedicel/capsule and internode/leaf ratios, and leaf length. A geographic separation of the morphotypes also exists. The black-seeded morphotype occurred nearly exclusively in drained fishponds in the south-western part of the Czech Republic, the brown-seeded morphotype was found in drained fishponds in the eastern part of the Czech Republic and in alluvial pools and river deposits of the Elbe River (Germany). We hypothesize that the black-seeded morphotype may have been indigenous in former natural lakes which were widespread in the SW-Czech Republic and they were frequently transformed into fishponds. The brown-seeded morphotype may have its origin in river alluvia of the Elbe (Germany) and possibly also of other rivers in the Czech Republic. Since the two morphotypes are morphologically and geographically well separated, we propose to describe the brown-seeded morphotype as a new subspecies for science, *S. echinosperma* subsp. *albensis subsp. nov*.

Keywords: discriminant analysis, endemism, hierarchical classification, morphometrics, *Spergularia*

Introduction

Vascular plants, which are endemic to Central Europe outside high mountains, are few. Among them is *Spergularia echinosperma* (Čelakovský 1881: 867) Ascherson & Graebner (1893: 517) (see e.g., Čelakovský 1881, Friedrich 1979, Dvořák 1990) which is a species confined to the threatened vegetation of annual wetland herbs bound to exposed bottoms of freshwater reservoirs (phytosociological class Isoëto-Nano-Juncetea Br.-Bl. et Tüxen ex Br.-Bl. *et al.* 1952). *S. echinosperma* has two main centers of distribution: Germany where it grows in alluvial pools and river banks along the Elbe (Friedrich 1979, Jäger 2011, Brück *et al.* 2012), and the Czech Republic where it grows exclusively in secondary habitats, especially drained fishponds (Friedrich 1979, Dvořák 1990, Kaplan *et al.* 2016). It also marginally occurs in Austria (Fischer *et al.* 2008), and Slovakia (Dvořák 1979, Goliašová 2012).

Spergularia echinosperma has been long considered a taxonomically critical species (Jage 1974, Dvořák 1990, Suda *et al.* 2007) due to its morphological similarity to a widespread congener *S. rubra* (Linnaeus 1753: 423) Presl & Presl (1819: 94), and the two species were frequently confused. Recent studies by Kúr *et al.* (2012, 2016) demonstrated that although an interspecific hybrid between *S. echinosperma* and *S. rubra* exists [it is named *S. kurkae* Dvořák (1989: 320)], it is a stable hybrid species reproductively isolated from the parents. It was also showed that all the three species are morphologically well delimited.

The main morphological characters discriminating *S. echinosperma* from the other species refer to the seeds (Kúr *et al.* 2012). However, there has been contradictory information in the literature as for the actual values of some seed characters in *S. echinosperma*. The most conspicuous is the difference in the indicated seed color. Czech authors describe *S. echinosperma* as displaying black seeds (Dostál 1989, Dvořák 1990, Kúr *et al.* 2012), including the

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description of this taxon by Čelakovský (1881). German plants, on the other hand, are characterized by (dark) brown seed color (Jage 1974, Friedrich 1979). Our study of the herbarium material of *S. echinosperma* indeed confirmed the presence of two groups differing in seed color.

The taxonomic value of this character in *S. echinosperma* remains unknown. In the genus *Spergularia*, seed characters are usually important from the taxonomical point of view (see e.g., Monnier & Ratter 1993, Hartman & Rabeler 2005). It is therefore possible the varying seed morphology in *S. echinosperma* may reflect an unrevealed taxonomic structure of this Central European endemic.

The aims of the present study are to investigate correlations of seed color with other morphological characters and to map the distribution of particular morphotypes. Specifically, we asked the following questions: (1) what is the pattern of morphological variation in *S. echinosperma*? (2) Is the morphological variation correlated with different environmental conditions and/or geographical regions?

Materials and Methods

Plant material from 21 herbaria (B, BRNM, BRNU, CB, CBFS, DR, GAT, HAL, JE, LIM, LIT, MJ, MNVD, OLM, OP, PL, PR, PRA, PRC, STU, and ZMT; acronyms according to Thiers 2016+) and 7 personal herbaria, not listed in the *Index Herbariorum* (H. Jage, J. Komárek, J. Zámečník, L. Čech, P. Kúr, R. Paulič, Z. Kaplan), from the Czech Republic and Germany was revised. The habitat types derive from the herbarium labels.

A subset of 114 plants from 15 populations were used for the morphometric analyses [1–19 individuals per population (see Table 1 for the exact localities and acronyms of the populations used in the text)]. Only mature plants with ripe capsules were used.

TABLE 1. List of the populations of *Spergularia echinosperma* used for the morphometric analyses. The geographic coordinates are based on the WGS84 datum.

Label	Locality	Lat.	Long.	Number of plants	Date	Coll.
Bleddin	Distr. Wittenberg, Bleddin: oxbow lake called "Bleddiner Riß", exposed margin	51.79411	12.79542	7	26.6.1982	H. Jage
Gallin	Distr. Wittenberg, Gallin: scour upstream the ferry in the village, the right bank of the Elbe	51.83680	12.75619	4	11.9.1967	H. Jage
Hodemysl	Dist. Příbram, Hoděmyšl: bare bottom of the Velký hoděmyšlský fishpond	49.61600	13.87864	7	21.6.2011	P. Kúr
Hrachoviste	Dist. Jindřichův Hradec, Hrachoviště: bare bottom of the Hrachovišťský fishpond	48.92864	14.76408	10	26.6.2011	P. Kúr
Kojatin	Dist. Třebíč, Kojatín: bare bottom of the Kojatínský fishpond	49.24172	16.00847	6	6.6.2011	P. Kúr
KWurf	Distr. Roßlau, Klieken: oxbow lake called "Kurzer Wurf" WSW of the town	51.88031	12.32558	7	9.9.1989	H. Jage
Malobor	Dist. Strakonice, Sedlice: bare bottom of the Malobor pond	49.36678	13.97556	7	25.6.2008	P. Kúr
Mlynhor	Dist. Strakonice, Drahonice: bare bottom of the Mlýnský horní fishpond	49.19467	14.08694	7	25.6.2011	P. Kúr
Parezny	Dist. Žďár Nad Sázavou, Bohdalov: bare bottom of the Pařezný fishpond	49.47744	15.85317	1	4.6.2011	P. Kúr
Pratau	Distr. Wittenberg, Pratau: oxbow lake N of the town, E of the F2 road (near the "Bude 100")	51.85115	12.64539	7	7.10.1963	H. Jage
Priesitz	Distr. Wittenberg, Priesitz: the Old Elbe ca 1 km NE of the town; sandy-muddy margin of the oxbow lake	51.70727	12.83715	7	12.10.1971	H. Jage
Skopec	Dist. Písek, Nová Ves u Protivína: bare bottom of the Skopec fishpond	49.23108	14.25231	10	25.6.2011	P. Kúr
Svihov	Dist. Chrudim, Švihov: bare bottom of the Švihov fishpond	49.84264	15.85931	10	5.6.2011	P. Kúr
Tangermunde	Distr. Stendal, Tangermünde: right bank of the Elbe opposite the town, under the road bridge.	52.56491	11.98564	5	14.10.1963	H. Jage
Terlicko	Dist. Karviná, Těrlicko: exposed margin of the Těrlicko water reservoir	49.74336	18.49515	19	25.10.2012	H. Jage

TABLE 2. List of the morphological characters used in the morphometric analyses and summary of their values for the black-seeded (41 individuals) and brown-seeded (73 individuals) morphotypes. The numbers denote (minimum–)10th percentile/**mean**/90th percentile(–maximum). Characters log-transformed prior to the multivariate analyses are marked with an asterisk.

Acronym	Character [units]	Seed color group	
		brown	black
Cap-K	capsule-sepal length ratio	(1.00–)1.13/**1.26**/1.56(–1.41)	(1.07–)1.13/**1.21**/1.39(–1.33)
CapsLeng	capsule length [mm]	(2.4–)3.0/**3.2**/4.1(–3.5)	(2.6–)2.8/**3.1**/3.7(–3.5)
FrPedLen*	length of the fruit pedicel adjacent to the capsule [mm]	(1.7–)2.4/**4.1**/9.1(–6.7)	(3.8–)4.1/**5.6**/11.1(–7.5)
InterLen*	length of the internode adjacent to the measured leaf [mm]	(3.33–)6.47/**11.43**/21.27(–16.83)	(5.43–)7.90/**11.94**/25.07(–18.97)
Int-Leaf*	internode length/leaf length ratio	(0.43–)0.77/**1.02**/1.65(–1.34)	(0.72–)0.91/**1.39**/3.42(–1.87)
KLength*	sepal length [mm]	(2.1–)2.3/**2.6**/3.4(–2.8)	(2.1–)2.3/**2.6**/3.2(–3.0)
LeafLeng*	leaf length [mm]	(5.3–)6.4/**11.5**/21.0(–17.6)	(5.0–)5.7/**9.1**/15.6(–12.7)
LeafRat*	leaf length/width ratio	(10.4–)15.6/**22.8**/38.5(–32.5)	(13.2–)14.8/**21.5**/62.0(–26.9)
LeafWidt	leaf width [mm]	(0.2–)0.4/**0.5**/0.8(–0.6)	(0.1–)0.2/**0.5**/0.8(–0.7)
LengSeed	seed length [μm] (Fig. 7)	(323–)377/**412**/507(–467)	(336–)357/**397**/473(–448)
PapHei	papilla height [μm] (Fig. 7)	(12–)15/**18**/23(–21)	(16–)17/**19**/24(–21)
PapNum	number of papillae on one quarter of the seed circumference (papillae density)	(5–)8/**12**/19(–15)	(10–)13/**15**/20(–18)
PapRat	ratio of the papilla upper part ("head") width and papilla lower part ("neck") width (papilla shape)	(1.01–)1.10/**1.24**/1.55(–1.42)	(1.04–)1.08/**1.21**/1.50(–1.35)
Ped-Cap*	pedicel/capsule length ratio	(0.57–)0.73/**1.27**/2.28(–2.10)	(1.21–)1.39/**1.80**/3.25(–2.34)
PlHeight*	height of the longest stem [cm]	(4–)5/**8**/16(–12)	(5–)6/**8**/12(–10)
SeedRat	seed length/width ratio	(1.09–)1.22/**1.29**/1.51(–1.38)	(1.23–)1.26/**1.34**/1.48(–1.42)
StemWidth*	stem width [mm]	(0.4–)0.4/**0.6**/1.3(–0.7)	(0.2–)0.3/**0.5**/0.8(–0.6)
StpLt	stipule length [mm]	(0.9–)1.1/**1.3**/1.8(–1.6)	(0.9–)1.1/**1.2**/1.5(–1.4)
StpRT	stipule length/width ratio	(0.48–)0.60/**0.72**/1.01(–0.86)	(0.58–)0.63/**0.74**/0.90(–0.83)
StpWd	stipule width [mm]	(1.3–)1.6/**1.9**/2.5(–2.2)	(1.3–)1.5/**1.7**/2.0(–1.8)
WidtSeed	seed width [μm] (Fig. 7)	(242–)289/**321**/383(–358)	(252–)265/**297**/341(–338)

14 quantitative and 7 derived ratio characters were measured (Table 2). Seed color was used as an ordinary variable. All the individuals could be unambiguously classified into two groups, one possessing black and the other brown seeds (Fig. 1). Special caution was given to evaluating well-developed seeds only.

The data were processed by multivariate statistical analyses. Quantitative characters that deviated most from a normal distribution in each of the pre-defined groups were log-transformed to improve normality (Table 2). Principal component analysis (PCA) was used to visualize the overall pattern of morphological variation in the data (CANOCO 5; Šmilauer & Lepš 2014). To find out which characters significantly separated the seed color groups, canonical discriminant analysis (CDA) was applied. The significance of individual characters was tested using both marginal effects (i.e., when a character is alone in the model) and unique contributions of the characters (i.e., the addition of each character into the model with all other characters) (Koutecký 2015). Forward selection of characters was employed to detect the combination of characters most contributing to the separation of the groups. The threshold significance level was set to $\alpha = 0.05$ and a Monte-Carlo permutation test (1000 permutations) used. The predictive ability of the selected characters was tested by classificatory discriminant analysis based on the posterior group membership probabilities and cross-validation using whole populations as leave-out units. The percentage of misclassified samples in each group served as a measure of the predictive ability. The discriminant analyses were computed using the MorphoTools scripts (Koutecký 2015) in R 3.2.3 (R Development Core Team 2015).

We also reanalyzed the data by classification trees that create a hierarchical classification based on univariate splits that can then be visualized as an easily interpretable tree diagram (Breiman *et al.* 1984). The function rpart (package rpart) in R 3.2.3 (R Development Core Team 2015) was used. The minimum split parameter (minsplit) was set to 1 and

the initial complexity parameter (cp) to 0.001. A cross-validation using the populations as the leave-out subsamples was used to assess the optimal tree complexity, instead of random subsamples as implemented in the original method (Venables & Ripley 2002). The resulting tree was selected on the basis of the 1-SE rule (Venables & Ripley 2002).

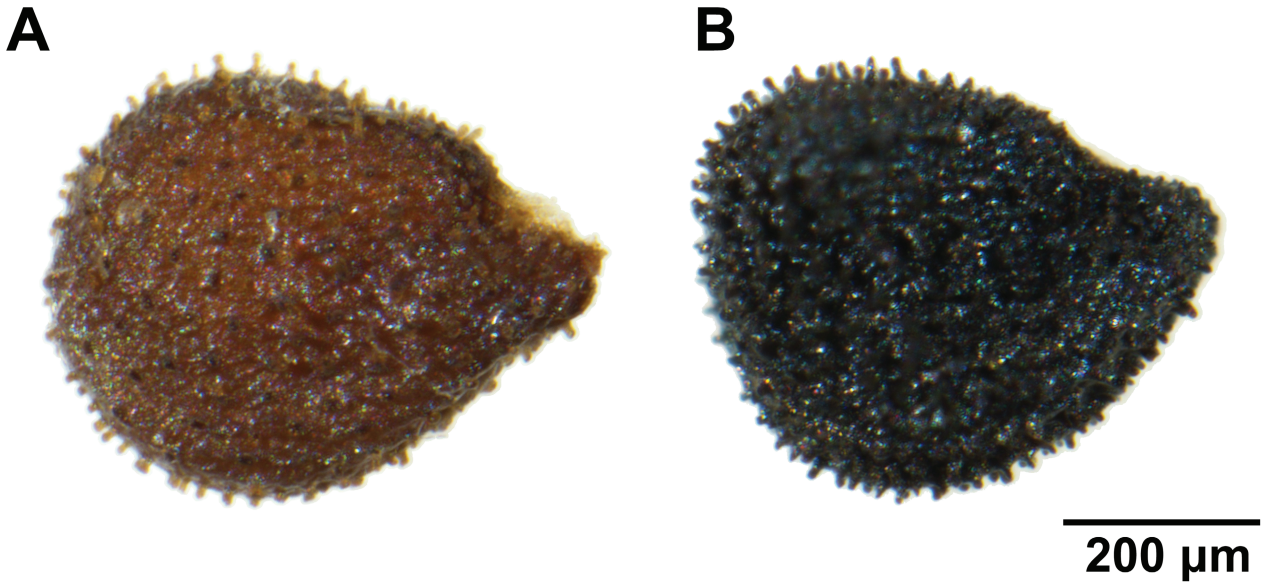

FIGURE 1. A typical seed of the brown-seeded (A) and black-seeded (B) morphotypes of *Spergularia echinosperma*.

Results

By using all 21 morphological characters, PCA did not produce any clear and distinct clusters of plants. However, the black-seeded and brown-seeded plants separated slightly along the second ordination axis (Fig. 2).

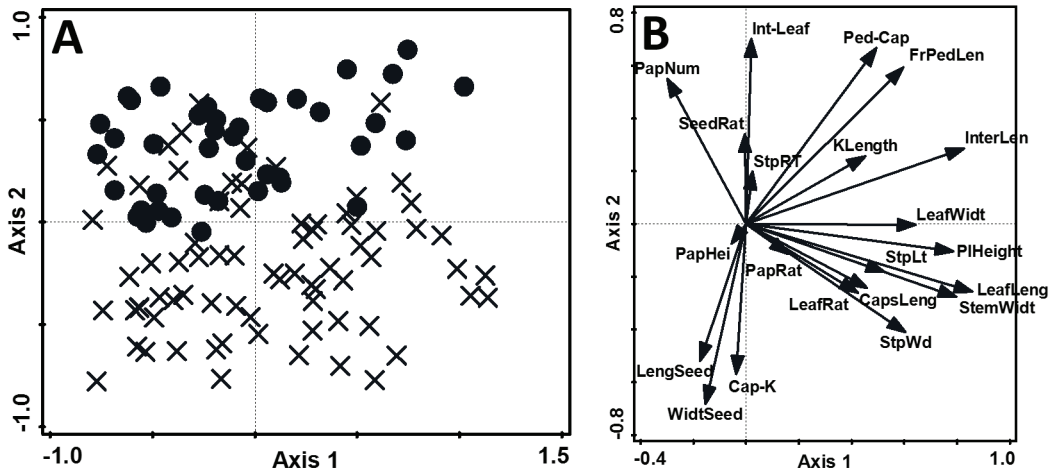

FIGURE 2. PCA analysis: (A) distribution of individuals in the ordination space (circles – black-seeded plants, X-marks – brown-seeded plants), (B) fit of the 21 morphological characters to the ordination axes. The first and the second ordination axes explain 24.3% and 16.4% of the total variation, respectively.

On the contrary, CDA highlighted some characters which significantly separate the groups (Table 3). The 7 best predictors were mainly seed characters (papillae density, papilla head/neck ratio, papilla height, seed width) plus other characters referred to the vegetative parts (i.e. pedicel/capsule length ratio, stipule width, and internode/leaf length ratio). The predictive ability of the selected characters was 84% of correctly classified samples (Fig. 3, Table 4).

TABLE 3. Morphological characters tested in the forward selection with their conditional and marginal effects, unique contributions, and their contributions to the canonical axis (biplot scores).

Character	Conditional effects		Marginal effects		Unique contributions		Biplot scores
	F	p	F	p	F	p	
PapNum	60.8	0.005	60.8	0.005	17.1	0.001	0.426
Ped-Cap	30.8	0.005	36.9	0.005	7.4	0.005	0.332
StpWd	19.7	0.005	23.3	0.005	2.3	0.146	-0.263
PapRat	13.7	0.005	2.0	0.200	12.6	0.001	-0.077
WidtSeed	8.8	0.005	18.8	0.005	3.4	0.064	-0.237
PapHei	6.3	0.015	7.8	0.005	22.3	0.001	0.152
Int-Leaf	4.9	0.020	30.2	0.005	0.3	0.639	0.300
FrPedLen	n. s.		25.9	0.005	7.9	0.003	0.278
StemWidth	n. s.		12.9	0.005	3.2	0.071	-0.196
SeedRat	n. s.		11.3	0.005	2.2	0.141	0.183
LeafLeng	n. s.		10.6	0.005	0.0	0.972	-0.178
StpLt	n. s.		8.0	0.015	0.4	0.539	-0.154
Cap-K	n. s.		7.5	0.010	0.0	0.920	-0.149
CapsLeng	n. s.		6.5	0.020	3.4	0.065	-0.139
LeafWidt	n. s.		5.4	0.030	0.0	0.925	-0.127
LengSeed	n. s.		4.7	0.035	2.2	0.140	-0.118
StpRT	n. s.		1.2	0.275	0.8	0.384	0.060
LeafRat	n. s.		1.0	0.305	0.4	0.568	-0.056
InterLen	n. s.		0.5	0.505	0.1	0.785	0.038
PlHeight	n. s.		0.2	0.690	6.3	0.011	-0.023
KLength	n. s.		0.0	0.940	0.0	1.000	-0.002

TABLE 4. Summary of the classification matrices of the black-seeded and brown-seeded morphotypes of *Spergularia echinosperma* resulting from the classificatory discriminant analysis and classification trees.

Classificatory discriminant analysis				Classification trees			
observed	black	brown	Total	observed	black	brown	Total
predicted				predicted			
black	37 (90.2%)	14 (19.2%)		black	24 (58.5%)	23 (31.5%)	
brown	4 (9.8%)	59 (80.8%)		brown	17 (41.5%)	50 (68.5%)	
Percent correct	90.0%	80.8%	84.2%	Percent correct	58.5%	68.5%	64.9%

The final classification tree selected had 3 terminal nodes (complexity parameter cp = 0.1). It confirmed the high discrimination power of pedicel/capsule length ratio. The second character selected was leaf length, contrary to the CDA forward selection (Fig. 4). The overall predictive power of this model was lower with 65% of correctly classified samples (Table 4).

The revision of herbarium specimens showed that the black-seeded morphotype is, with two exceptions, restricted to the southwestern part of the Czech Republic (especially the South Bohemian fishpond basins). The exceptions were one locality in Germany (Rathenow) and one locality in the eastern part of the Czech Republic (Kadolecký fishpond near Křižanov). The brown-seeded morphotype, on the other hand, occurs in the eastern part of the Czech Republic (especially in the Bohemian-Moravian Highlands) and Germany (along the Elbe) only (Fig. 5).

Four distinct types of habitats were found: fishponds, river reservoirs, alluvial pools, and river banks (Table 5). Nearly all localities from the Czech Republic came from drained fishponds. An exception was one population from an exposed margin of a river reservoir. In Germany, *S. echinosperma* occurred nearly exclusively in alluvial pools or river banks along the Elbe River. An exception was the only German locality of black-seeded *S. echinosperma* which was located in the vicinity of the river Havel, ca. 20 km off the Elbe. The type of habitat was unfortunately not specified on the herbarium label.

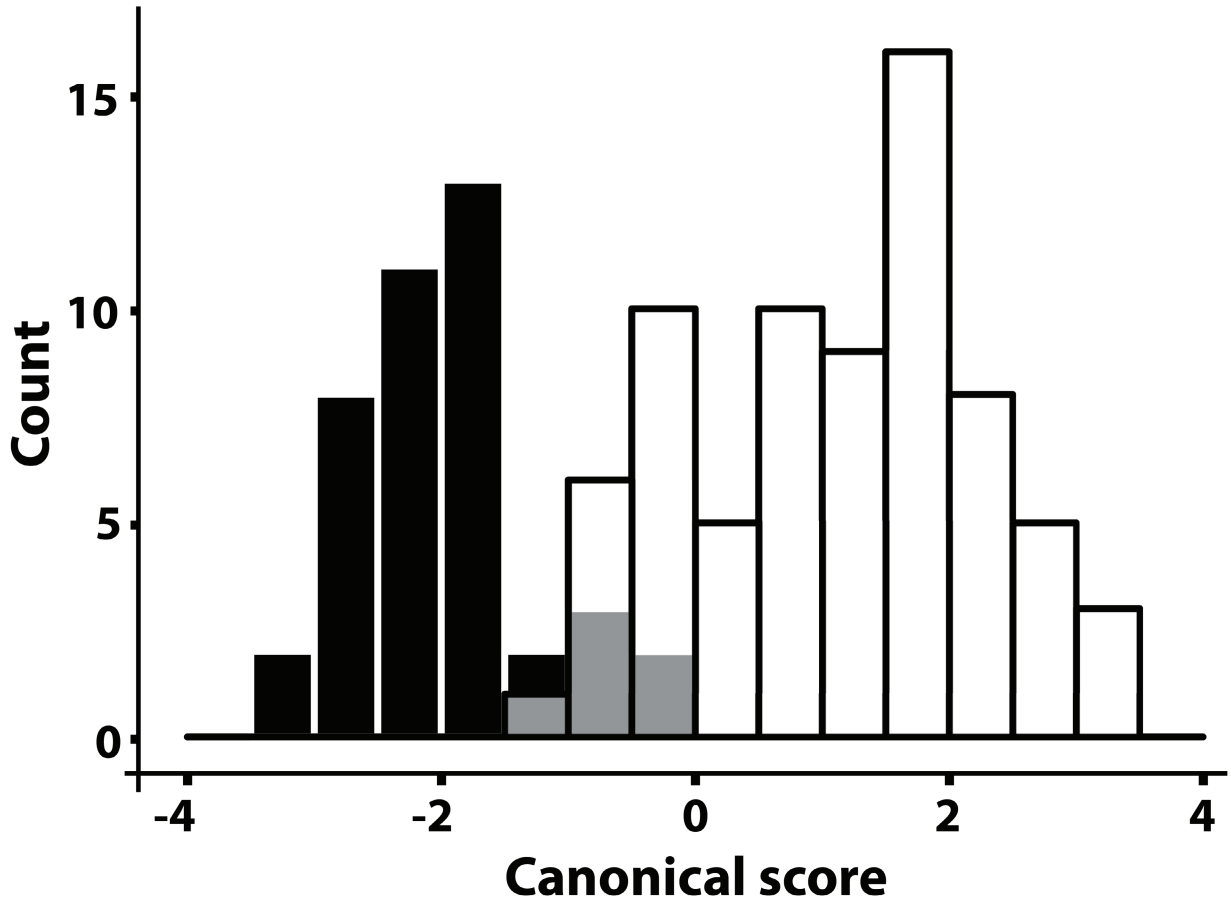

FIGURE 3. Distribution of the canonical scores from CDA for the black-seeded (black) and brown-seeded (white) morphotypes of *Spergularia echinosperma*.

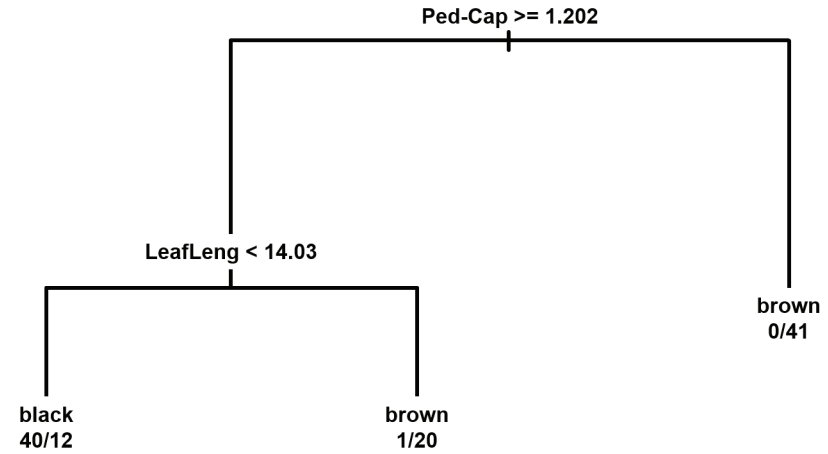

FIGURE 4. Classification tree of the individuals of the black-seeded and brown-seeded morphotype of *Spergularia echinosperma*. If a character value matches the classification rule, the determination continues to the left branch, otherwise to the right branch. Lengths of the branches correspond to the relative discriminatory powers of the respective rules. The group names at the terminal nodes indicate the predicted classification of a particular node, whereas the numbers separated by slashes indicate actual membership of samples classified to a particular node (black/brown).

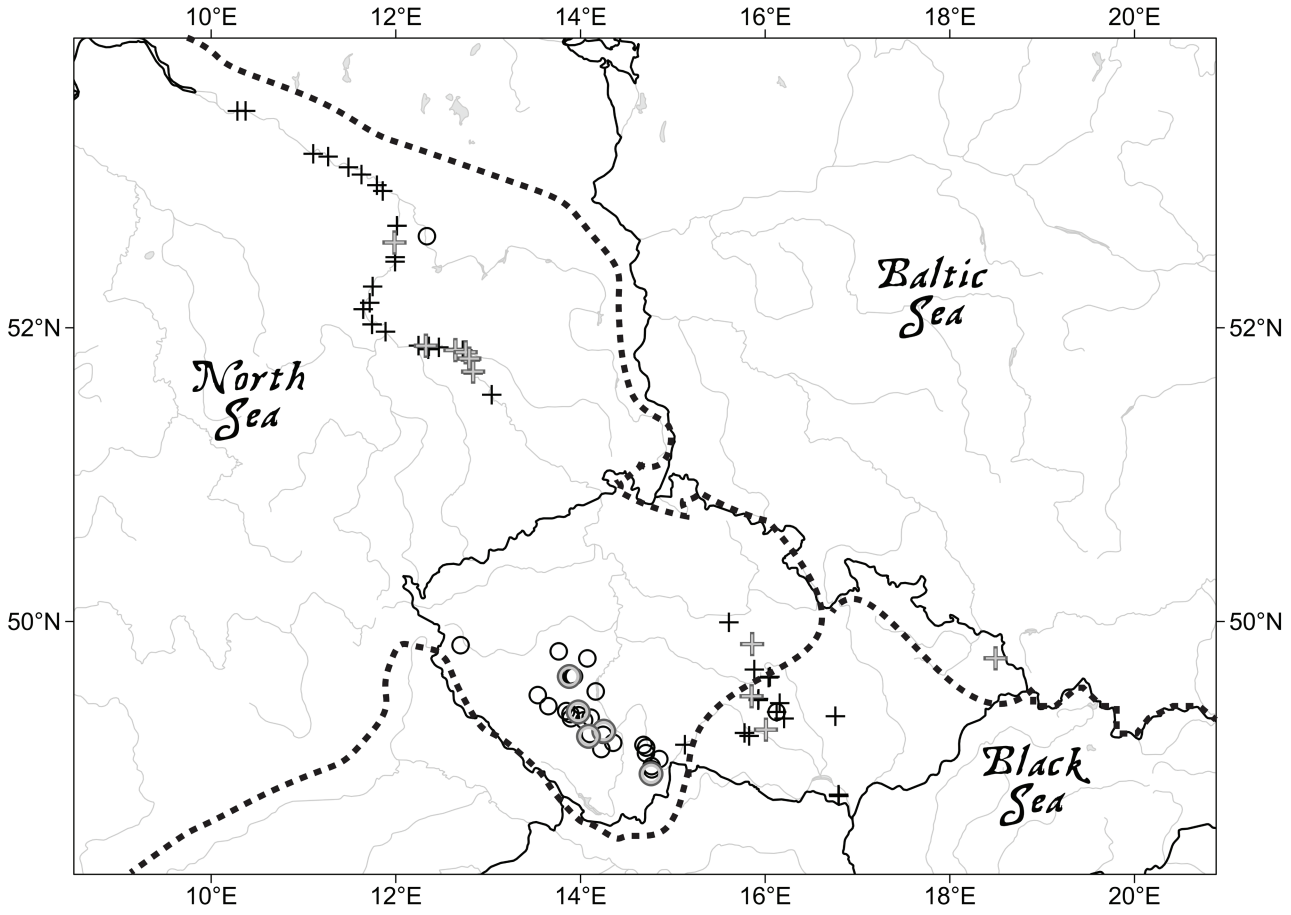

FIGURE 5. Distribution map of the black-seeded (circles) and brown-seeded (crosses) morphotypes of *Spergularia echinosperma*. Populations used for morphometric analyses are highlighted in gray. The dashed lines denote the main European drainage divides.

TABLE 5. Absolute frequencies of habitat types in which *Spergularia echinosperma* was recorded, summarized across morphotypes and countries.

Country	**Czech Republic**		**Germany**	
Seed color	black	brown	black	brown
fishpond	47	63	.	.
reservoir	.	1	.	.
alluvial pool	.	.	.	31
river bank	.	.	.	34
unknown	.	.	1	.

Discussion

We confirmed that the observed differences in seed color in *Spergularia echinosperma* are related with the differences in some other morphological characters. Most of them are related to seed morphology, but there are also some well-discriminating vegetative characters. Even if the determination of the morphotypes without the information about the seed color is not fully reliable, their morphological separation seems to be well supported.

The morphotypes display also a clearly vicariant distribution. The occurrence of brown-seeded *S. echinosperma* in Germany is in accordance with the descriptions of this species by German authors (Jage 1974, Friedrich 1979). From the Czech Republic, however, only the black-seeded morphotype has been reported so far (Dostál 1989, Dvořák 1990, Kúr 2012).

We can now only speculate about the origin and migration history of the different lineages of *S. echinosperma*. The black-seeded morphotype may have its origin in natural lakes which existed in South and South-West Bohemia

and were frequently transformed into fishponds during the Middle Ages and the Early Modern Age (Chvojka *et al.* 2010, Pokorný 2015). The brown-seeded morphotype, on the other hand, may be indigenous to periodically exposed substrates in river alluvia. This is easily conceivable in the case of the Elbe where the species still grows in this type of habitats. The same may hold true for the populations of brown-seeded *S. echinosperma* in the eastern part of the Czech Republic. In the latter region, natural lakes were not common (Chlupáč *et al.* 2002, Břízová 2009), and alluvial pools seems to be a more probable primary habitat for *S. echinosperma*. Periodically exposed alluvial pools still occur in this region (especially along the Morava River), but they were vastly destroyed in the 20th century. Unfortunately, there are no historical records of *S. echinosperma* from these habitats in this region to corroborate its indigenous status here.

Even if the two morphotypes of *S. echinosperma* have probably evolved in different regions, they nearly complete vicariance is surprising. As the Bohemian populations of black-seeded *S. echinosperma* lie within the Elbe river catchment, one would expect their occurrence at the lower reaches of the Elbe too. This could be explained by different ecological adaptations of the morphotypes. The black-seeded morphotype may be adapted to the management of the South Bohemian fishponds where there is only a relatively short and unpredictable period of substrate exposure during the spring, which makes a strong selection pressure on shortening life cycle and the presence of primary seed dormancy (Šumberová *et al.* 2005). In contrast, alluvial pools in river floodplains are usually exposed for a longer period in late summer and fall, and their water regime is more predictable probably relaxing existing selection pressures (Šumberová 2011). Differences in seed dormancy may also be the direct cause of different seed morphology. Seeds of the black-seeded morphotype probably have thicker testa than those of the brown-seeded morphotype, which is a trait that is related to increased seed dormancy (Bewley *et al.* 2012).

The absence of the black-seeded morphotype in the eastern part of the Czech Republic as well as the absence of the brown-seeded morphotype in south-western part of the Czech Republic are harder to explain as it is the same type of habitats, i.e. fishponds. This may be the result of dispersal limitation. As the majority of the populations of the black-seeded morphotype lie within a different drainage basin than do the populations of the brown-seeded morphotype, a limited diaspore exchange between the two regions seems logical. In addition, there may also exist some sort of environmental filtering. As far as we know, fishponds in the Bohemian-Moravian Highlands, where most of the Czech localities of the brown-seeded morphotype lie, are usually dried for a longer period than the South-Bohemian fishponds (K. Šumberová, pers. comm.). This may create ecological conditions more similar to those of exposed substrates in river alluvia.

Clearly, further studies, including macrofossil analyses and the study of dormancy and germination biology, are needed to elucidate the evolution history of *S. echinosperma*. Partial insight should be provided by an ongoing genetic study (based on published microsatellite markers; Kúr *et al.* 2014).

Based on the obvious geographic and ecological differentiation of the two morphotypes, as well as the morphological differences, their taxonomic treatment as separate subspecies seems justified. Since the type of *S. echinosperma* belongs to the black-seeded morphotype (deposited in the herbarium PR, No. 374981; Kúr *et al.* 2012), we decided to describe the brown-seeded morphotype as the new subspecies.

Taxonomic treatment

Spergularia echinosperma (Čelak.) Ascherson & Graebner (1893: 517) subsp. ***echinosperma*** ≡ *Spergularia rubra* subsp. *echinosperma* Čelakovský (1881: 867)

Lectotype (designated by Kúr *et al.* 2012: 921):—"*Spergularia echinosperma* n. sp. forma *pallens*, u Švarcenberského rybníka u Protivína se *Scirpus Michelianus*, Aug 1876 leg. Čelak.", PR 374981, left bottom individual.

Specimina Visa:—Czech Republic: U Švarcenberského rybníka u Protivína se Scirpus Michelianus, 49.20507, 14.2445 (±100 m), August 1876, *Čelakovský* (PR); Bei dem St. Stephans Teiche unweit von Zbirow, 49.79192, 13.76418 (±500 m), September 1902, *Domin* (B, JE, PRC); Zbirožské Brdy, m demolatis ad piscinam Sv. Štěpánský rybník prope Mýto divulgata., 49.79192, 13.76418 (±500 m), August 1902, *Domin* (PRC, PR); Jižní Povltaví: dno bývalého rybníka U Bulana u Vorlíka v množství, 49.51097, 14.16476 (±1000 m), August 1902, *Domin* (PRC); Vorlík, 49.51097, 14.16476 (±1000 m), August 1902, *Domin* (PRC); Suché dno rybníka Sv. Štěpánského u Zbiroha, 49.79192, 13.76418 (±500 m), September 1903, *Domin* (PRC); Bohemia merid., distr. Písek, nudofundo piscinae emmissae Dolní Luskovec prope Albrechtice apud Protivín, 425 m, 49.21083, 14.1031 (±100 m), 03 October 1943, *Hejný* (PRC); Třeboňsko, obnaž. dno ryb. Opatovického u obory u Třeboně, 48.98308, 14.77017 (±500 m), 10 July 1956, *Kurka*

(CB); Bohemia meridionalis - distr. Strakonice: ad ripam piscinae Horní Mlýnecký inter pagos Drahonice et Skočice, haud procul ab opp. Vodňany, 49.19467, 14.08694 (±100 m), 26 July 1967, *Pučelíková* (LIM); Horažďovicko, Jámský rybník u Kvášňovic, 49.40762, 13.652 (±100 m), 14 August 1968, *Vaněček* (CB); jižní Čechy, pánev Třeboňská, SZ okraj ryb. Ponědražského S od Lomnice n. Luž., 49.11521, 14.71102 (±500 m), 04 July 1972, *Kurka* (CB); Třeboňsko, Ponědrážka, obnažený S břeh Ponědrážkovského rybníka Z od obce, 49.11521, 14.71102 (±500 m), 09 July 1972, *Slaba* (CB); Jižní Čechy, Třeboňská pánev, Veselí nad Lužnicí, na obnaženém dně rybníka u obce Ponědráž, na písku, 49.11521, 14.71102 (±1500 m), 09 July 1972, *Deyl* (OLM); Česká republika: 39. Třeboňská pánev, Jindřichův Hradec, Ponědrážka. Třeboň: dno Veselského (Ponědrážkovského) rybníka Z obce Ponědrážka, 49.11521, 14.71102 (±500 m), *Čejka* 9 July 1972 (PL); Bohemia australis, distr. Lomnice nad Lužnicí, ad ripam piscinae Ponědrážkovský rybník prope pagum Ponědraž., 49.11521, 14.71102 (±500 m), 09 July 1972, *Deylová* (PR); Flora bohemica. Třeboňská pánev: jihovýchodní okraj Ponědrážkovského rybníka jižně od Veselí nad Lužnicí. S. m. ca 410 m, 49.11521, 14.71102 (±500 m), 20 September 1979, *Kurka* (BRNU); Flora bohemica. Třeboňská pánev: jihovýchodní okraj Ponědrážkovského rybníka jižně od Veselí nad Lužnicí. S. m. ca 410 m, 49.11521, 14.71102 (±500 m), 20 September 1979, *Kurka* (BRNU); Flora bohemica. Třeboňská pánev: jihovýchodní okraj Ponědrážkovského rybníka jižně od Veselí nad Lužnicí. S. m. ca 410 m, 49.11521, 14.71102 (±500 m), 20 September 1979, *Kurka* (BRNU); Flora bohemica. Třeboňská pánev: jihovýchodní okraj Ponědrážkovského rybníka jižně od Veselí nad Lužnicí. S. m. ca 410 m, 49.11521, 14.71102 (±500 m), 22 August 1979, *Dvořák* (BRNU); Pičín u Příbrami. Rybník Pilka. Dno zaplavovaného rybníka , 49.74266, 14.07421 (±200 m), 27 June 1981 (LIT); Veselí nad Lužnicí, Ponědrážka, obnažený rybník Hliníř, 49.1336, 14.68207 (±200 m), 04 July 1984, *Kučera* (CB); Příbram - Vranovice. Strýčkovy; dno rybníka, 49.61687, 13.92982 (±1000 m), 07 July 1985, *Zelenka* (OLM); Distr. Příbram: Nesvačily. Podhlubocký rybník, obnažené dno, 0.5 km SSZ obce, 49.61313, 13.91118 (±200 m), 07 July 1985, *Grulich* (PR); Distr. Jindřichův Hradec: Ponědrážka, vypuštěný rybník Hliníř 1.2 km Z obce, 49.1336, 14.68207 (±200 m), 09 July 1988, *Grulich* (PR); Bohemia, distr. Strakonice. Černěves: jihovýchodní část Černěveského rybníka, 1,2 km JV od středu obce; písčitý okraj letněného rybníka; 420 m s. m., 49.10472, 14.22555 (±200 m), 24 July 2000, *Šumberová* (BRNU); Dist. Strakonice, Lažany: bare bottom of the Cky pond, 49.35192, 13.89136 (±4 m), 25 June 2008, *Kúr* (CBFS); Dist. České Budějovice, Dříteň: bare pond bottom of the Kočínský rybník pond, 49.14892, 14.35417 (±4 m), 25 June 2008, *Kúr* (CBFS); Dist. Písek, Písek: bare bottom of the Jenšovský rybník pond, 49.32661, 14.10972 (±4 m), 25 June 2008, *Kúr* (CBFS); Dist. Strakonice, Sedlice: bare bottom of the Malobor pond, 49.36678, 13.97556 (±4 m), 25 June 2008, *Kúr* (CBFS); Bohemia, distr. Písek, Písecko. Dobešice: rybník Jenšovský jižně obce, za silnicí E49. S. m. 380 m, 49.32661, 14.10972 (±200 m), 17 June 2008, *Šumberová* (BRNU); Ditr. Písek, In fundo piscinae emmisae Jenšovský rybník inter vicos Dobešice et Oldřichov, 49.32661, 14.10972 (±200 m), 12 August 2009, *Paulič et Nedvědová* (CB); Dist. Strakonice, Záboří: bare bottom of the Hůrka pond, 49.37306, 13.84564 (±4 m), 21 June 2009, *Kúr* (CBFS); Dist. Strakonice, Velká Turná: bare bottom of the Babák fishpond, 49.34353, 13.9785 (±4 m), 26 June 2010, *Kúr* (CBFS); Dist. Příbram, Hoděmyšl: bare bottom of the Velký hoděmyšlský fishpond, 49.616, 13.87864 (±4 m), 21 June 2011, *Kúr* (CBFS); Dist. Jindřichův Hradec, Hrachoviště: bare bottom of the Hrachovišťský fishpond, 48.92864, 14.76408 (±4 m), 26 June 2011, *Kúr* (CBFS); Dist. Strakonice, Radomyšl: bare bottom of the Chválovec fishpond, 49.32208, 13.89681 (±4 m), 22 June 2011, *Kúr* (CBFS); Dist. Strakonice, Drahonice: bare bottom of the Mlýnský horní fishpond, 49.19467, 14.08694 (±4 m), 25 June 2011, *Kúr* (CBFS); Dist. Příbram, Vranovice: bare bottom of the Podhůrecký fishpond, 49.62389, 13.89206 (±4 m), 21 June 2011, *Kúr* (CBFS); Dist. Písek, Nová Ves u Protivína: bare bottom of the Skopec fishpond, 49.23108, 14.25231 (±4 m), 25 June 2011, *Kúr* (CBFS); Distr. Písek. Dobev - obnažené břehy polovypuštěného rybníka Stašov severně od obce, písčité dno, hojně., 49.30818, 14.03891 (±100 m), 26 June 2012, *Paulič* (R. Paulič); Dist. Jindřichův Hradec, Lomnice nad Lužnicí: exposed bottom of the Služebný fishpond, 49.07507, 14.70887 (±4 m), 16 June 2012, *Kúr* (CBFS); Dist. Písek, Stará Dobev: exposed bottom of the Stašov fishpond, 49.30818, 14.03891 (±4 m), 23 June 2012, *Kúr* (CBFS); Dist. Jindřichův Hradec, Stříbřec: exposed bottom of the Stolec fishpond, 49.03375, 14.85335 (±4 m), 08 June 2012, *Kúr* (CBFS); Dist. Jindřichův Hradec, Branná: exposed bottom of the Tobolky fishpond, 48.96055, 14.7718 (±4 m), 16 June 2012, *Kúr* (CBFS); Kadolecký rybník u Křižanova, 49.36663, 16.12426 (±4 m), 20 June 2012, *Čech* (L. Čech); Distr. Tachov, Nahý Újezdec: bare margin of the Sítina fishpond, at the end of the manipulation road, 49.83501, 12.70116 (±4 m), 08 July 2016, *Kúr* (P. Kúr); Nepomucko. Rybník Nový vypuštěný. Velmi hojná na poněkud oschlém bahně dna., 49.48527, 13.53709 (±200 m), s.d., *Mencl* (PL); **Germany:** Flora von Brandenburg, Rathenow, 52.60601, 12.33664 (±2000 m), 15 September 1899, *Kirschstein* (B).

Spergularia echinosperma subsp. ***albensis*** Kúr, Amarell, Jage & Štech, *subsp. nova*

Type:—GERMANY. Distr. Wittenberg, Priesitz: oxbow lake 1 km East by North of the church in the village; lat.: +51.7047, long.: +12.8393; 12. 10. 1971; leg. H. Jage (holotype GLM-0168069!, (Fig. 6), isotypes PR-878041!, BRNM-792025!, LI-802800!, B-100673696!).

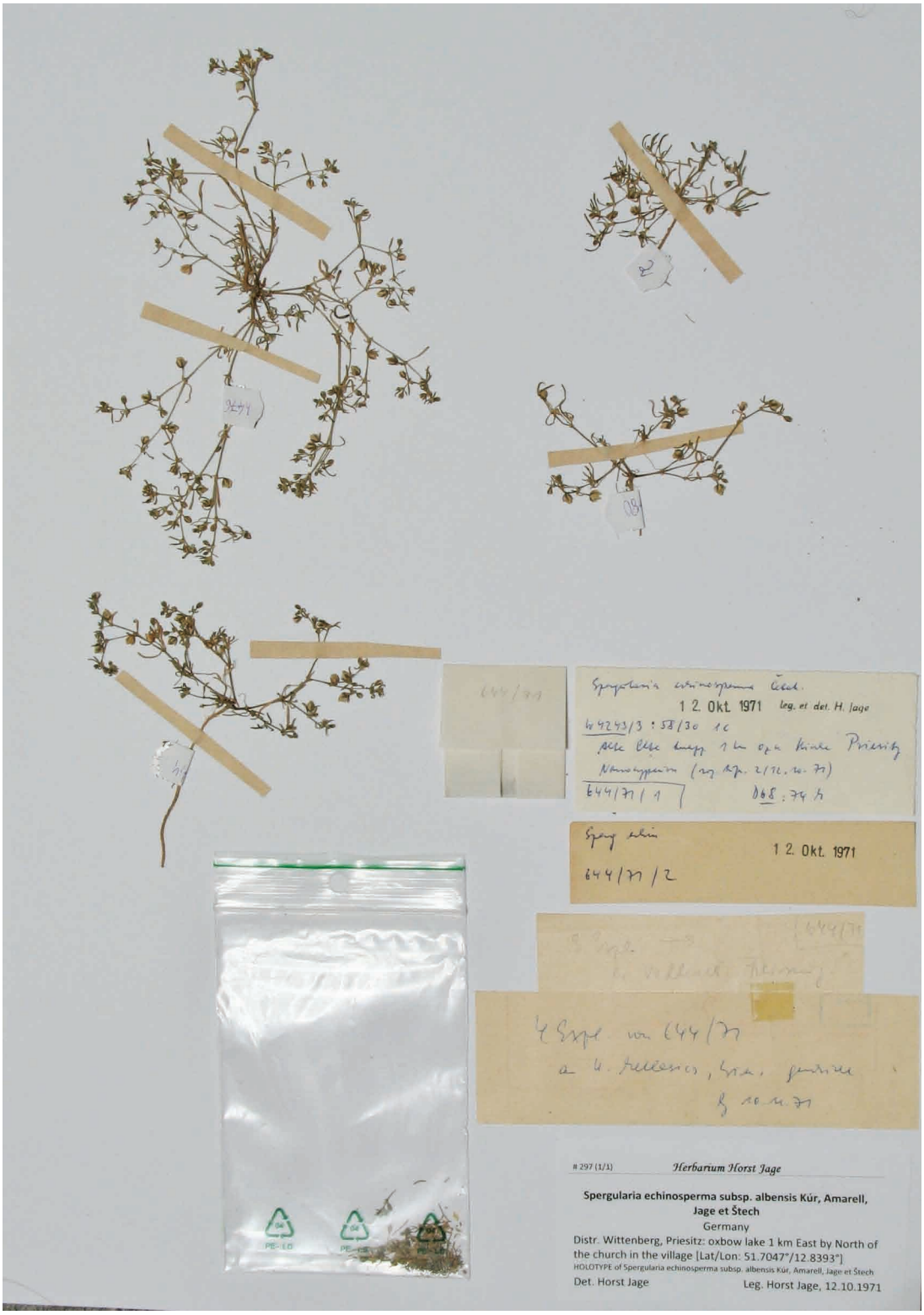

FIGURE 6. Holotype of *Spergularia echinosperma* subsp. *albensis* (GLM-0168069).

Diagnosis:—*Spergularia echinosperma* subsp. *albensis* differs from *S. echinosperma* subsp. *echinosperma* in having seeds with brown to dark-brown testa. In addition, the subsp. *albensis* has, on average, shorter fruit pedicels, lower pedicel length / fruit length ratio and longer leaves than *S. echinosperma* subsp. *echinosperma*.

Etymology:—This subspecies is named after the river Elbe where it has its main center of distribution.

Distribution and habitat:—*Spergularia echinosperma* subsp. *albensis* is currently known only from Germany and the Czech Republic. In Germany, it grows on exposed margins of alluvial pools and river banks of the Elbe, while in the Czech Republic it occurs on drained bottoms of fishponds and river reservoirs. We expect it to occur also in the neighboring countries (Austria, Slovakia, Poland).

Phenology:—Flowering time May–October, fruiting time June–November.

Conservation status:—*Spergularia echinosperma* subsp. *albensis* currently occurs in 176 known localities, in 39 of which it has been reported after 2000. Its estimated extent of occurrence (EOO) is about 20000 km^2, and its area of occupancy (AOO) is about 200 km^2. Following the recommendations of IUCN (2016), we propose treating the taxon as endangered [EN, B2b(iv)c(iii)].

Taxonomic notes:—On the basis of recent phylogenetic analyses (Smissen *et al.* 2002, Fior 2006) and the alleged lack of reliable discriminatory characters between the genera *Spergularia* and *Spergula* (López González 2010), some authors accept to merge them in a single genus (see e.g., López González 2010, Raab-Straube & Raus 2013). In our opinion, however, reliable arguments justifying the union of these two genera are still lacking as there has been no study focusing on the phylogeny and biosystematics of the *Spergula*/*Spergularia* clade yet. In addition, the genus *Spergularia* is accepted in most of the recent floras and checklists (e.g. Danihelka *et al.* 2012, Goliašová 2012, Peruzzi *et al.* 2015, Kaplan *et al.* 2016, Lorite 2016, Villaseñor 2016). We therefore use the name *Spergularia* in the present study.

Specimina Visa:—Czech Republic: Flora Moravica. Jedovnice., 49.33658, 16.76052 (±1000 m), July 1918, *Vitek* (BRNM); U Steklého rybníka u Třebíče, 49.21847, 15.77636 (±400 m), 17 September 1924, *Krajina* (PRC); Flora moravica. Třebíč: ad ripam piscinae Zteklý rybník pr. Starč, 49.19746, 15.82644 (±1000 m), September 1924, *Krajina* (BRNU); Flora moravica. Třebíč: ad ripam piscinae Zteklý rybník pr. Starč, 49.19746, 15.82644 (±1000 m), September 1924, *Krajina* (BRNU); Flora moravica. Třebíč: ad ripam piscinae Zteklý rybník pr. Starč, 49.19746, 15.82644 (±1000 m), September 1924, *Krajina* (BRNU); Flora moravica, na břehu Steklého rybníka u Hvězdoňovic (okres Třebíč), 49.21847, 15.77636 (±400 m), 16 August 1925, *Jičínský* (ZMT); Flora moravica: Osová Bítýška: Vlkovský rybník, v písku. S. m. 500 m, 49.32056, 16.20528 (±500 m), 07 September 1928, *Vybíralová* (BRNU); Flora moravica, Lednice, obnažené dno Středního rybníka. S. m. 165 m, 48.78028, 16.79556 (±300 m), 25 June 1931, *Zapletálek* (BRNU); Flora moravica, Lednice, obnažené dno Středního rybníka. S. m. 170 m, 48.78028, 16.79556 (±300 m), 25 June 1931, *Zapletálek* (BRNU); Jedousov, okr. Pardubice, obnažené dno rybníka v čistém porostu, 49.99432, 15.60735 (±200 m), 01 July 1934, *Hadač* (PR); Brno: Jedovnice, ad finstune niccum pisc. Olšovec; 500 m, 49.33658, 16.76052 (±500 m), 06 October 1943, *Podpěra* (PR); Flora moravica, Vojnův Městec: Malé Dářko., 49.66472, 15.88222 (±200 m), 26 June 1948, *Podpěra* (BRNU); Morava: Nové Město, dno letněného rybníka Medlova u Tří Studní, 49.61395, 16.05096 (±300 m), 14 July 1951, *Smejkal* (BRNM); Flora moravica. Nové Město na Moravě: ad fundum (sicaum) piscinae Medlov dictae prope pagum Tři Studně. S. m. 700 m, 49.61395, 16.05096 (±300 m), 30 July 1951, *Smejkal* (BRNU); Moravia occid., Nové Město na Moravě: ad fundum piscinae dict. Medlov apud pag. Tři Studně. S. m. cca 710 m, 49.61395, 16.05096 (±300 m), 14 July 1951, *Smejkal* (BRNU); Flora moravica, Nové Město na Moravě: na dně letněného rybníka Medlova u Tří Studní v masách. S. m. 715 m, 49.61395, 16.05096 (±300 m), 14 July 1951, *Smejkal* (BRNU); Flora moravica, Nové Město na Moravě: v masách na dně letněného rybníka Medlova u Tří Studní. S. m. 700 m, 49.61395, 16.05096 (±300 m), 14 July 1951, *Smejkal* (BRNU); Moravia occid., Nové Město na Moravě: ad fundum piscinae Medlov dict. ap. pag. Tři Studně. S. m. 710 m, 49.61395, 16.05096 (±300 m), 14 July 1951, *Smejkal* (BRNU); Flora moravica, Nové Město na Moravě: na dně letněného rybníka Medlova u Tří Studní /v masách/. S. m. 710 m, 49.61395, 16.05096 (±300 m), 14 July 1951, *Smejkal* (BRNU); Žďár nad Sázavou, Sykovec ad pagum Tři Studně, 720 m, 49.60871, 16.03817 (±200 m), 14 July 1961, *Smejkal* (BRNM, HAL, JE, MJ, OP, PRC); Moravia occidentalis, distr. Žďár nad Sázavou: in fundo paludoso piscinae aestate vacuefactae Sykovec dictae ad pagum Tři Studně. S. m. cca 720 m, 49.60871, 16.03817 (±200 m), 14 July 1961, *Smejkal* (BRNU); Flora Moravica: Nové Město na Moravě: ad fundum piscinae vacuefactae Sykovec dictae prope pagum Tři Studně. S. m. cca 720 m, 49.60871, 16.03817 (±200 m), 14 July 1961, *Smejkal* (BRNU); Flora Moravica: Nové Město na Moravě: ad fundum piscinae vacuefactae Sykovec dictae prope pagum Tři Studně. S. m. cca 720 m, 49.60871, 16.03817 (±200 m), 14 July 1961, *Smejkal* (BRNU); Distr. Lednice: Allahovy rybníčky /prostřední/ mezi Valticemi a Lednicí., 48.76986, 16.79811 (±100 m), 23 July 1962, *Hejný* (PRA); Flora moravica. Vlkov u obce Velká Bíteš, okraj Vlkovského rybníka. S. m. ca 490 m, 49.32056, 16.20528 (±500 m), 05 August 1969, *Dvořák* (BRNU); Žďárské vrchy - Tři studně, dno vypuštěného Medlov. rybníka, uprostřed, 49.61395, 16.05096 (±300 m), 10 September 1970, *Pospíšil* (BRNM); Flora moravica: Nové město na Moravě: na dně vypuštěného (letněného) rybníka Medlov u Tří Studní, masově., 49.61395, 16.05096 (±300 m), 22 July 1970, *Smejkal* (MJ); Flora moravica. Moravia occident., distr. Žďár nad Sázavou: in fundo piscinae aestatae vacuefactae Medlov dictae prope pag. Tři Studně. S. m. cca 720 m, 49.61395, 16.05096 (±300 m), 22 July 1970, *Smejkal* (BRNU); Distr. Jižní Morava: Lednické rybníky (rybn. Aloch IV), 48.7775, 16.79456 (±100 m), 13 June 1971, *Husák* (PR); Flora moravica, dist. Břeclav, Lednice, obnažená rybničná půda rybníčka Alah 4, řídce. S. m. ca 160 m, 48.7775, 16.79456 (±100 m), 18 September 1973, *Vicherek* (BRNU); Flora moravica, dist. Břeclav, Lednice, obnažená rybničná půda rybníčka Alah 4, řídce. S. m. 160 m, 48.7775, 16.79456 (±100 m), 18 September 1973,

Vicherek (BRNU); Distr. Žďár nad Sázavou: na jižním obnaženém písčitém okraji rybníka Sykovec u obce Tři Studně. cca 720 m, 49.60871, 16.03817 (±200 m), 22 July 1975, *Smejkal* (MJ); Flora moravica. Moravia occident., Nové Město na Moravě: locis paludosis arenosis que ad marginem oriental. piscinae Sykovec dictae prope pagum Tři Studně. S. m. cca 720 m, 49.60871, 16.03817 (±200 m), 22 July 1975, *Smejkal* (BRNU); Flora moravica. Moravia occident., Nové Město na Moravě: locis paludosis arenosis que ad marginem oriental. piscinae Sykovec dictae prope pagum Tři Studně. S. m. cca 720 m, 49.60871, 16.03817 (±200 m), 22 July 1975, *Smejkal* (BRNU); Flora moravica. Moravia occident., Nové Město na Moravě: locis paludosis arenosis que ad marginem oriental. piscinae Sykovec dictae prope pagum Tři Studně. S. m. cca 720 m, 49.60871, 16.03817 (±200 m), 22 July 1975, *Smejkal* (BRNU); Flora moravica. Moravia occident., Nové Město na Moravě: locis paludosis arenosis que ad marginem oriental. piscinae Sykovec dictae prope pagum Tři Studně. S. m. cca 720 m, 49.60871, 16.03817 (±200 m), 22 July 1975, *Smejkal* (BRNU); Flora moravica. Moravia occident., Nové Město na Moravě: locis paludosis arenosis que ad marginem oriental. piscinae Sykovec dictae prope pagum Tři Studně. S. m. 720 m, 49.60871, 16.03817 (±200 m), 22 July 1975, *Smejkal* (BRNU); Flora moravica. Moravia occident., Nové Město na Moravě: locis paludosis arenosis que ad marginem oriental. piscinae Sykovec dictae prope pagum Tři Studně. S. m. 720 m, 49.60871, 16.03817 (±200 m), 22 July 1975, *Smejkal* (BRNU); Flora moravica. Moravia occident., Nové Město na Moravě: ad marginem oriental. piscinae Sykovec dictae prope pagum Tři Studně, locis paludosis arenosisque. S. m. 720 m, 49.60871, 16.03817 (±200 m), 22 July 1975, *Smejkal* (BRNU); Flora moravica. Moravia occident., distr. Žďár nad Sázavou: in fundo piscinae aestatae vacuefactae Medlov dictae prope pag. Tři Studně, copiose! S. m. 720 m, 49.61395, 16.05096 (±300 m), 22 July 1975, *Smejkal* (BRNU); Flora Moravica. Lednice: obnažené dno Valachovského ryb. II [Alah 2]. S. m. ca 170 m, 48.76986, 16.79811 (±100 m), 03 July 1980, *Zapletálek* (BRNU); Flora moravica. Hills Žďárské vrchy, the pond Sykovec near the village Tři Studně, 5 km NNW from the Nové Město na Moravě. The locality no. 14 (Dvořák 1979: 115). S. m. ca 720 m, 49.60871, 16.03817 (±200 m), 26 October 1982, *Dvořák* (BRNU); Flora moravica. Distr. Žďár nad Sázavou, obec Tři Studně: obnažené dno vypuštěného rybníka Sykovec severně od Nového Města na Mor. S. m. ca 720 m, 49.60871, 16.03817 (±200 m), 26 October 1982, *Dvořák* (BRNU); Flora moravica. Distr. Žďár nad Sázavou, obec Tři Studně: obnažené dno vypuštěného rybníka Sykovec. S. m. ca 720 m, 49.60871, 16.03817 (±200 m), 26 October 1982, *Dvořák* (BRNU); Flora moravica. Distr. Žďár nad Sázavou, obec Tři Studně: obnažené dno vypuštěného rybníka Sykovec severně od Nového Města na Mor. S. m. ca 720 m, 49.60871, 16.03817 (±200 m), 26 October 1982, *Dvořák* (BRNU); Flora moravica. Distr. Žďár nad Sázavou, obec Tři Studně: obnažené dno vypuštěného rybníka Sykovec severně od Nového Města na Mor. S. m. ca 720 m, 49.60871, 16.03817 (±200 m), 26 October 1982, *Dvořák* (BRNU); Flora moravica. Distr. Žďár nad Sázavou, obec Tři Studně: obnažené dno vypuštěného rybníka Sykovec severně od Nového Města na Mor. S. m. 720 m, 49.60871, 16.03817 (±200 m), 26 October 1982, *Dvořák* (BRNU); Flora moravica. Distr. Žďár nad Sázavou, obec Tři Studně: obnažené dno vypuštěného rybníka Sykovec. S. m. ca 720 m, 49.60871, 16.03817 (±200 m), 26 October 1982, *Dvořák* (BRNU); Flora moravica. Distr. Žďár nad Sázavou, obec Tři Studně: obnažené dno vypuštěného rybníka Sykovec. S. m. ca 720 m, 49.60871, 16.03817 (±200 m), 26 October 1982, *Dvořák* (BRNU); Flora moravica. Hills Žďárské vrchy, the pond Sykovec near the village Tři Studně, 5 km NNW from the Nové Město na Moravě. The locality no. 14 (Dvořák 1979: 115). S. m. ca 720 m, 49.60871, 16.03817 (±200 m), 26 October 1982, *Dvořák* (BRNU); Flora moravica. Distr. Žďár nad Sázavou, obec Tři Studně: obnažené dno vypuštěného rybníka Sykovec. S. m. ca 720 m, 49.60871, 16.03817 (±200 m), 26 October 1982, *Dvořák* (BRNU); Distr. Nové Město na Moravě, rybník Sykovec, obnažené břehy rybníka. S. m. ca 650 m, 49.60871, 16.03817 (±200 m), 18 September 1985, *Smejkal* (BRNU); Pavlov, 12 km J od Žďáru nad Sázavou: obnažené dno při S břehu rybníka Podvesník V od obce., 49.45019, 15.92491 (±200 m), 12 July 2001, *Čech* (L. Čech); Okr. Žďár nad Sázavou: Rudolec: obnažené a právě znovu zaplavované dno rybníka Pařezný 1 km V od obce, J od silnice do Bohdalova., 49.47744, 15.85317 (±200 m), 04 July 2005, *Kaplan* (Z. Kaplan); Dist. Třebíč, Kojatín: bare bottom of the Kojatínský fishpond, 49.24172, 16.00847 (±4 m), 06 June 2011, *Kúr* (CBFS); Dist. Žďár Nad Sázavou, Bohdalov: bare bottom of the Pařezný fishpond, 49.47744, 15.85317 (±4 m), 04 June 2011, *Kúr* (CBFS); Dist. Chrudim, Švihov: bare bottom of the Švihov fishpond, 49.84264, 15.85931 (±4 m), 05 June 2011, *Kúr* (CBFS); Dist. Jindřichův Hradec, Malý Ratmířov: bare bottom of the Vosecký fishpond, 49.13522, 15.13089 (±4 m), 07 June 2011, *Kúr* (CBFS); Dist. Žďár Nad Sázavou, Kadolec: bare bottom of the Kadolecký fishpond, 49.36663, 16.12426 (±4 m), 18 June 2012, *Kúr* (CBFS); Dist. Žďár nad Sázavou, Radkov: bare bottom of the Nohavice fishpond, 49.42881, 16.15753 (±4 m), 19 June 2012, *Kúr* (CBFS); Dist. Karviná, Těrlicko: exposed margin of the Těrlicko water reservoir, 49.74336, 18.49515 (±4 m), 25 October 2012, *Kúr* (CBFS); Distr. Žďár nad Sázavou, Znětínek: bare bottom of the Znětínecký fishpond., 49.4592, 15.92774 (±4 m), 21 August 2014, *Kúr* (P. Kúr); Moravia, Pavlov (dist. Žďár nad Sázavou): hojně na obnaženém dně rybníku Podvesník, přibližně 1017 m východně kostela svatého Filipa a Jakuba v centru Pavlova., 49.45019, 15.92491 (±200 m), 11 September 2015, *Zámečník et Ducháček* (J. Zámečník); **Germany:** Magdeburg, 52.12388, 11.64658 (±20000 m), 17 October 1898

(POZ); Magdeburg: An der Elbe nahe Pratau. 2n, mit Corrigiola., 52.12388, 11.64658 (±20000 m), October 1898 (POZ); Flora von Anhalt, Elbstrand beim Sieglitzer und weiter an der Elbe beim niedrigen Wasserstrand, 51.85718, 12.35127 (±500 m), 01 August 1908, *Zobel* (MNVD); Flora der Altmark, Sandiges Elbufer bei Arneburg im Kreis Stendal (Provinz Sachsen), 52.67469, 12.01255 (±1000 m), September 1909, *Schuster* (GAT, DR); Wittenberg, Elbufer, 51.85968, 12.6326 (±2000 m), 24 August 1911, *Matthies* (GAT); Elbestrand r[echtes]. Ufer oberhalb Schönebeck., 52.0229, 11.74255 (±2000 m), 17 September 1911 (GAT); Flora der Altmark, Schlickbuhne an der Elbe bei Arneburg im Kreise Stendal., 52.67469, 12.01255 (±1000 m), August 1911, *Schuster* (GAT, DR); Sachsen. Elbstrand bei Schönebeck, 52.0229, 11.74255 (±2000 m), 10 August 1911, *Meißner* (MNVD); Anhalt. Elbstrand Sieglitzer – Vockerode, 51.85718, 12.35127 (±500 m), 19 August 1911, *Zobel* (MNVD); Anhalt. Elbstrand beim Sieglitzer, 51.85718, 12.35127 (±500 m), 09 August 1911, *Zobel* (MNVD); Alte Elbe bei Magdeburg (Strand), 52.16631, 11.71703 (±500 m), 10 August 1911, *Meißner* (MNVD); Elbstrand zw. Roßlau und Brambach, 51.88124, 12.24526 (±2000 m), 12 August 1911, *Zobel* (MNVD); Elbstrand bei Schönbeck, auch an der alten Elbe bei Magdeburg, 52.0229, 11.74255 (±2000 m), 10 August 1911, *Meißner* (MNVD); Strand der Elbe von Schönbeck bis Magdeburg, 52.12388, 11.64658 (±20000 m), 10 August 1911, *Meißner* (MNVD); Strand der Elbe bei Barby, 51.97425, 11.88841 (±1000 m), 03 August 1911, *Meißner* (MNVD); Strand der Elbe bei (Barby) Roßlau, 51.88124, 12.24526 (±2000 m), August 1911, *Meißner* (MNVD); Strand der Elbe oberhalb Schönbeck, rechtes Ufer., 52.0229, 11.74255 (±2000 m), 17 August 1911, *Meißner* (MNVD); Strand der Elbe oberhalb Schönbeck, rechtes Ufer., 52.0229, 11.74255 (±2000 m), 17 August 1911, *Meißner* (MNVD); Elbstrand bei Barby, 51.97425, 11.88841 (±1000 m), *Meißner* 03 September 1911, *Meißner* (MNVD); Sachsen, Wittenberg, Elbe-Ufer, 51.85968, 12.6326 (±2000 m), 24 August 1911, *Mayer* (STU); Elbe, südl. Hamburg, Winsen, bei Elbstorf., 53.42157, 10.28427 (±500 m), 24 August 1916, *Junge* (STU); Elbe, südl. Hamburg, Winsen, bei Marschhacht., 53.42215, 10.37284 (±500 m), 10 September 1916, *Junge* (STU); Elbufer bei Rosslau (Anhalt), 51.88124, 12.24526 (±2000 m), 06 September 1928, *Linstow* (B); Flora Germanica, Eldeufer bei Kl[ein] Schmölen, 53.12642, 11.26686 (±1000 m), 16 July 1960, *Bisse* (JE); Kreis Roßlau: Rechtes Elbufer im Luch bei Coswig, auf Elbschlick, selten, 51.86963, 12.46612 (±1000 m), 08 October 1962, *Jage* (H. Jage); Rechtes Elbufer südlich an Wittenberg, stark sandiger Schlick, selten, 51.85968, 12.6326 (±2000 m), 10 October 1962, *Jage* (H. Jage); Kreis Stendal: rechtes Elbufer gegenüber Tangermünde, wenig unterhalb der Straßenbrücke, 52.56491, 11.98564 (±500 m), 14 October 1963, *Jage* (B, H. Jage); Kreis Wittenberg: Altwasser nördlich Pratau (östlich der Fernverkehrsstraße Nr. 2), sandiger Teichschlamm, 51.85115, 12.64539 (±500 m), 07 October 1963, *Jage* (HAL, H. Jage); Kreis Wittenberg: Dorfteich Bleddin (Altwasserrest), Teichschlamm, 51.79411, 12.79542 (±500 m), 30 July 1963, *Jage* (H. Jage); Kreis Wittenberg: Nordende des Bleddiner Risses (Elbaltwasser) östlich Wartenburg, Sandbank, massenhaft!, 51.79411, 12.79542 (±500 m), 07 September 1963, *Jage* (H. Jage,); Kreis Wittenberg: Mühlanger: Südlich am Ortsteil Hohndorf, Mündungsgebiet des Mühlgrabens in die Alte Elbe, fetter Teichschlamm, 51.85029, 12.72451 (±500 m), 13 September 1963, *Jage* (H. Jage); Kr. Wittenberg: N Pratau, Altwasser östl. F2 (nahe Bude 100), 51.85115, 12.64539 (±500 m), 07 October 1963, *Jage* (H. Jage); Kreis Wittenberg: oso Wartenburg am Bleddiner Riß (Elbaltwasser), fetter Teichschlamm, 51.79411, 12.79542 (±500 m), 05 November 1963, *Jage* (H. Jage); Altkreis Roßlau: wenig oberhalb Autobahnbrücke bei Vockerode, 3. Buhne am rechten Elbufer, 51.85718, 12.35127 (±500 m), 06 September 1964, *Jage* (H. Jage); Fundort: Elbtal, Kurzer Wurf am Matzwerder, 51.88031, 12.32558 (±500 m), 05 September 1965, *Jage* (MNVD); Mittelelbegebiet zwischen Wittenberg u. Dessau: trockengefaller Uferschlamm eines Elbarmes westl. Vorwerk Werder sw Klieken. Nanocyperion; Schlamm - Sand, 51.87596, 12.3582 (±500 m), 10 September 1967, *Hilbig* (HAL); Kreis Wittenberg: Kolk oberhalb Fähre Gallin, rechts der Elbe, 51.8368, 12.75619 (±200 m), 11 September 1967, *Jage* (H. Jage); Verlandungssaum des Dorfteiches von Bleddin, 51.79411, 12.79542 (±500 m), 22 September 1968, *Zenker* (MNVD); Altkreis Roßlau: Kurzer Wurf WSW Klieken, 51.88031, 12.32558 (±500 m), 09 September 1968, *Jage* (H. Jage); Mittleres Elbtal 14 km südöstlich von Wittenberg. Bleddin: Schluft (alter Elbarm), 51.79411, 12.79542 (±500 m), 25 September 1969, *Werner et Günther* (HAL); Im Teich Die Schluft, Bleddin b. Trebitz (Elbe), 51.79411, 12.79542 (±500 m), 13 September 1970 (HAL); Flora von Anhalt, Wittenberg: Bleddin, Schluff (= Bleddiner Dorfteich), 51.79411, 12.79542 (±500 m), 13 September 1970, *Manitz* (JE); Kurzer Wurf (Elbaltwasser) reichl. 3 km WSW Klieken, 51.88031, 12.32558 (±500 m), 08 November 1971, *Jage* (H. Jage); Altkreis Torgau. Alte Elbe wenig OSO Werdau, 51.55185, 13.03868 (±300 m), 06 November 1971, *Jage* (H. Jage); Deutschland, mittleres Elbtal: Kreis Wittenberg, Alte Elbe knapp 1 km ozn Priesitz; sandig-schlammiges Ufer eines Elbatwassers, häufig im Cypero-Limoselletum (Nanocyperion), 51.70727, 12.83715 (±200 m), 12 October 1971, *Jage* (H. Jage); NNO Sachau: Kolk zwischen Elb-Damm u. Alte Elbe, 51.69527, 12.8375 (±300 m), 09 September 1972, *Jage* (H. Jage); Deutschland (DDR): Elbtal bei Wittenberg, Bleddiner Riß, 1 km nördl. Bleddin., 51.79411, 12.79542 (±500 m), 16 September 1973, *Diholz* (B); Mittleres Elbtal: Elbe-Altwasser zwischen Wittenberg u. Pratau, 51.85115, 12.64539 (±500 m), 10 June 1973, *Rauschert* (HAL); Altwasser bei Bude 100 nördl. Pratau, 51.85115, 12.64539 (±500 m), 22 October 1973, *Jage*

(H. Jage); Kreis Wittenberg: ca 1.5 km NNO Sachau, Elbaltwasser, 51.69527, 12.8375 (±300 m), 13 September 1973, *Jage* (H. Jage); Lüchow-Dannenberg: bei Gartow, Elbholz, sand. Buhnenschotter., 53.057, 11.48683 (±500 m), 30 August 1982, *Ketelhut/Meyer* (B); Kr. Wittenberg: Bleddiner Riß, Uferschlamm, 51.79411, 12.79542 (±500 m), 26 June 1982, *Jage* (H. Jage); Flora von Sachsen-Anhalt, Wittenberg, 2 km N Bleddin, Sandufer eines Elbaltarmes, 51.79411, 12.79542 (±500 m), 31 October 1990, *Korsch* (JE); Mecklenburg, Dömitz, auf Sandbänken der Elbaue bei Strachau in Menge mit Sp. rubra vergesellschaftet, 53.14466, 11.10518 (±500 m), 02 October 1990, *Henker* (LI); Sachsen-Anhalt, Kreis Stendal. Shore of r. Elbe E Grieben., 52.4387, 11.9919 (±2000 m), 18 October 1992, *Müller* (JE); Sachsen-Anhalt, Kreis Wittenberg. Bleddiner Riß NE Bleddin., 51.79411, 12.79542 (±500 m), 30 August 1992, *Müller* (JE); Sachsen-Anhalt, Kreis Wolmirstedt. Elbe meadows S Heinrichsberg., 52.2741, 11.74874 (±1000 m), 18 October 1992, *Müller* (JE); Sachsen-Anhalt, Kreis Stendal. Shore of r. Elbe SSE Schelldorf, 52.46844, 11.99283 (±1000 m), 10 October 1993, *Müller* (JE); Krs. Wittenberg, OSO Wartenburg, Bleddiner Riß nahe N-Ende, Sandbank, 51.79411, 12.79542 (±500 m), 25 September 1998, *Jage* (H. Jage); Kreis Wittenberg: östl. Priesitz, Altwasser Schluft bei Kote 77,8, auf getrockenen Sand, 51.70727, 12.83715 (±200 m), 24 August 2003, *Jage* (H. Jage); Altkreis Jessen: W Schützberg, Klödener Riß, rechtes Ufer, Sand, 51.78215, 12.8079 (±300 m), 26 August 2003, *Jage* (H. Jage); Kreis Wittenberg: OSO Wartenburg, Bleddiner Riß N-Teil (Angelgewässer Falkenweiden), ausgedehnte Sandbank, 51.79411, 12.79542 (±500 m), 07 August 2003, *Jage* (H. Jage); Elbufer / Sand NO Klein Wanzer, 53.00946, 11.62762 (±1000 m), 20 September 2003, *Frank* (H. Jage); Brandenburg, Deich: exposed shore of the oxbow lake Haken, 52.90258, 11.85815 (±4 m), 16 July 2012, *Kúr* (CBFS); Saxony-Anhalt, Beuster: exposed shore of an oxbow lake 600 m E of the village, 52.94019, 11.79487 (±4 m), 16 July 2012, *Kúr* (CBFS).

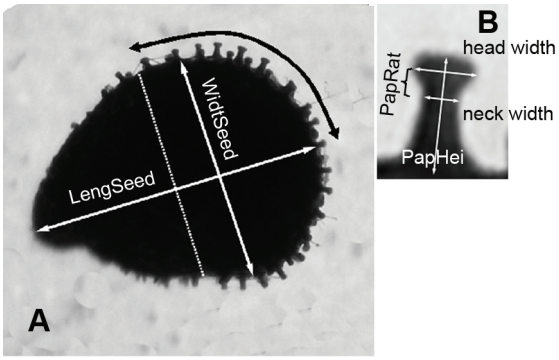

FIGURE 7. Characters measured on the seeds (A) and surface papillae (B). The black curved line specifies the part of the seed circumference where the density of papillae was determined. The longitudinal border of this part is a plane halving the vector of maximal seed length and perpendicular to it (indicated by a dotted line). The character PapRat was computed by dividing the width of the papilla head by the width of the neck.

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Appendix 3: Diversity of Rhinanthoid *Orobanchaceae*

Paper 8: Chlumský J., Koutecký P., Plačková I., & Štech M. (2016): Is genetic diversity congruent with morphological diversity across the distributional range of the Melampyrum subalpinum group (Orobanchaceae)? – Flora - Morphology, Distribution, Functional Ecology of Plants 220: 74–83.

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Is genetic diversity congruent with morphological diversity across the distributional range of the *Melampyrum subalpinum* group (Orobanchaceae)?

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ABSTRACT

The *Melampyrum subalpinum* group consists of morphologically diverse populations traditionally treated as closely related taxa with patchy distribution limited to Central Europe. The centre of the morphological variability and geographical distribution of the group lies on the north-eastern edge of the Alps in the Vienna Forest, while marginal, morphologically uniform populations occur in the Czech Republic and Slovakia. Genetic variation and population structure within the distribution range of the group remains unknown; we hypothesise that the marginal population are genetically depauperate. Allozymes were used to assess the genetic structure of 27 populations present throughout the distribution area; four *Melampyrum nemorosum* populations from the Vienna Forest were also analysed because of the presumed hybridization. An artificial pollination experiment was carried out to examine the possibility of autogamy. Four enzyme systems were clearly resolved and scored for one polymorphic locus each with a total of 20 alleles.

Seven out of 49 flowers with preserved stamens developed seeds after self-pollination. Genetic variation was generally congruent with the known pattern of the morphological variation of the group. The allelic richness was higher in the Austrian populations than in marginal Czech and Slovak populations. Some wide-leaved populations from the Vienna Forest had a rather high number of alleles which may be caused by allelic enrichment due to former hybridization with *M. nemorosum*. Czech and Slovak populations are genetically derived from Austrian populations. The high differentiation among populations suggests that the current gene flow between populations is limited. The high inbreeding coefficient in some populations indicates that there is a certain level of selfing within the populations. The pollination experiment does not contradict the possibility of autogamy. In general, our data are congruent with the central-marginal model with more variable Austrian populations and less variable isolated and probably partly inbreeding Czech and Slovak populations.

1. Introduction

Genetic variation of a species can reveal historical events connected with postglacial colonization (Wróblewska, 2008; Chung et al., 2013), historical and present population size changes (Leimu et al., 2006), the impact of environmental factors and anthropogenic disturbances, as well as the species breeding system (Aparicio et al., 2002; Leimu and Mutikainen, 2005), hybridization (Phillipp and Siegismund, 2003), and polyploidization (Rosenbaumová et al., 2004). High levels of variability are seen as healthy, conferring the ability to respond to threats such as disease, parasites and predators, and environmental changes (Amos and Hardwood, 1998). A number of studies has stated that small and scattered peripheral populations tend to be less variable than populations from the centre of distribution of the particular species. The peripheral populations can face the negative effects of inbreeding, genetic drift and the lowered genetic variation caused by bottleneck and founder effects (Lynch et al., 1995; Young et al., 1996; Tomimatsu and Ohara, 2003; Leimu et al., 2006; Chung et al., 2013). However, in some cases small limited populations do not seem to be influenced by these negative effects (Gitzendanner and Soltis, 2000; Mandák et al., 2005; Wróblewska, 2008).

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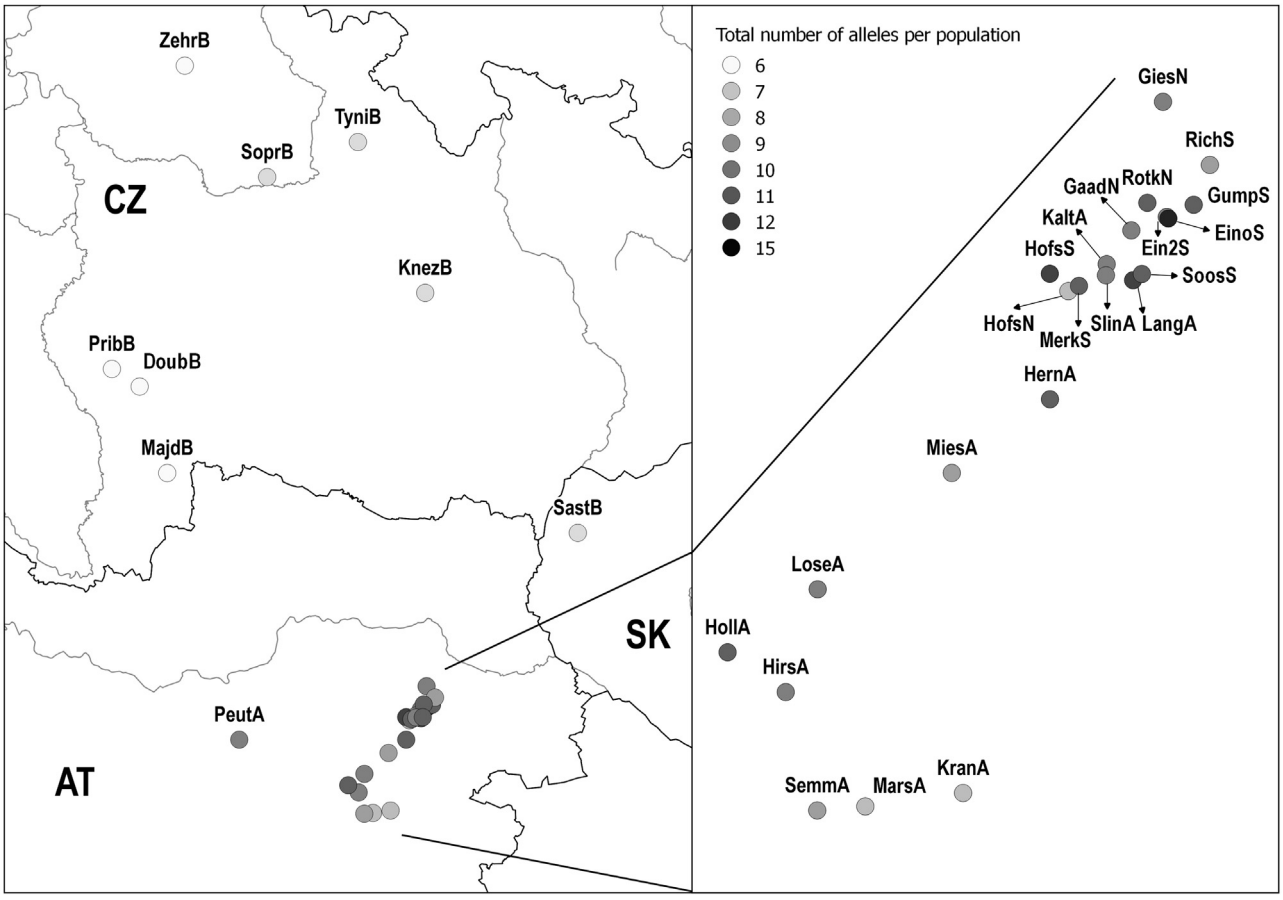

Fig. 1. Sampled populations of the *M. subalpinum* group with the total number of alleles per population represented by different shades of grey.

The *Melampyrum subalpinum* group consists of morphologically diverse populations of annual hemiparasitic plants traditionally treated as closely related taxa with a distribution area reaching from the Eastern Alpine foothills to the north of the Czech Republic and the south-western part of Slovakia (cf. Fig. 1). Following the conventional taxonomic concept, at least four taxa are recognized. The centre of morphological diversity lies in the Vienna Forest (Wienerwald), Austria (Štech, 2006). Plants from this region, traditionally designated as *M. subalpinum* (Jur.) A. Kern. s. str. (e.g., Beck, 1882, 1893), have rather wide leaves and bracts, intensively blue-coloured bracts, and relatively dense indumentum on calyx and bracts. These morphotypes are labelled as *M. subalpinum* var. *thermale* in the current Austrian literature (Fischer et al., 2008; Staudinger, 2009). Less variable populations with narrow leaves and bracts and sparse indumentum occur in an area reaching from the Vienna Forest towards the Lower Austria/Styria Alps. Traditionally they are recognized as *M. angustissimum* Beck but in recent Austrian literature the name *M. subalpinum* var. *subalpinum* is used (Fischer et al., 2008; Staudinger, 2009). Morphologically very similar and very uniform marginal populations known from the Czech Republic and Slovakia are traditionally labelled as *M. bohemicum* A. Kern. (Hadač, 1966). However, based on a comprehensive investigation of morphological variation, Štech (2006) considers these populations conspecific with *M. angustissimum.* The early flowering morphotypes of *M. angustissimum* restricted to the higher altitudes of the Alps are described as *M. grandiflorum* A. Kern. Seasonality is a common phenomenon in the genus *Melampyrum* as well as in related hemiparasitic species and genera (Soó, 1926 Soó, 1926-1927; Zopfi, 1993a,b, 1997) and it does not seem to be systematically important because the early flowering plants are quite rare and it is not possible to analyse them separately. Therefore, in this study one early flowering *M. grandiflorum* population (MarsA) is included in the narrow-leaved group together with *M. angustissimum*.

Genetic variation and population structure within the whole distribution range of the group remains unknown. According to the group's morphological variability and geographical distribution, Štech (2006) presumes that the centre of genetic diversity of the group lies on the north-eastern edge of the Austrian Alps in the Vienna Forest. The marginal morphologically uniform populations from the Czech Republic and Slovakia are expected to be genetically less variable. Some of the marginal populations are at present declining; whether this is due to a loss of genetic diversity in these small isolated populations remains unknown.

Allozymes are reliable and often used markers for the study of population genetic structure (e.g., Hamrick et al., 1981; Phillipp and Siegismund, 2003; Chrtek and Plačková, 2005; Chung et al., 2013). They are easily detected codominant markers which allow (compared to dominant markers, such as AFLP) to calculate for example allelic frequencies or standard population genetic parameters based on heterozygosity (F-statistics, deviations from the Hardy-Weinberg equilibrium). No previous genetic knowledge of the species is required, large numbers of individuals can be analysed at one time for multiple enzymes, and methods of data interpretation and analysis are well developed (Lowe et al., 2004).

1.1. In the study we addressed the following questions

(1) What is the extent and pattern of allozyme variation in the *M. subalpinum* group? (2) Is the level of genetic variation of the

M. subalpinum group correlated with its morphological variation across the whole distributional range? (3) Are marginal isolated populations in the Czech Republic threatened by a loss of genetic variation? (4) Is it possible to appraise any colonization history events of the *M. subalpinum* group in Central Europe?

2. Methods

2.1. Sampling

Sampled populations of the *M. subalpinum* group were chosen to cover the whole distributional range and all morphotypes. Sampling was thorough especially in the Vienna Forest (esp. broader surroundings of Baden) with morphologically diverse populations (Fig. 1, Table 1). Outside of the Alps and Vienna Forest, all regions in which *M. subalpinum* occurred are represented by at least one population. Because of the assumed former hybridization of *M. subalpinum* with *M. nemorosum* in the area of Vienna Forest, 4 local populations of *M. nemorosum* were also included. From each population 10 randomly selected plants were collected and in total 31 populations were sampled. A sample from each plant contained approximately 8 fresh leaves or bracts. Upon collection leaves were wrapped in wet tissues and stored in plastic bags on ice until extraction. Voucher specimens from all sampled populations are deposited in CBFS herbarium in České Budějovice (Czech Republic).

2.2. Extraction

Extraction was carried out within 24 h of collection. Approximately 80 mg of leaf tissue was ground in ice-cold Tris–HCl extraction buffer with the addition of a small amount of DOWEX 1 × 8–100 (Cl) and quartz sand. The extraction buffer contained 0.1 M TRIS–HCl pH 8.3, 1% (w/v) L-glutathione reduced, 10 mM $MgCl_2 \cdot 6H_2O$, 5% (w/v) sucrose and 0.1% (v/v) 2-mercaptoethanol. Crude homogenates were centrifuged for 10 min at 15,000 rpm. Clear supernatant was stored in deep freeze at −75 °C.

2.3. Electrophoresis

The extracts (30 μl per sample) were subjected to electrophoresis in vertical polyacrylamide gel slabs (Hoefer SE 600 vertical unit) using separating gel (8.16%) with 1.82 M Tris–HCl buffer, pH 8.9, and stacking gel (4%) with 0.069 M Tris–H_3PO_4 buffer, pH 6.9. Electrode buffer was 0.02 M Tris, 0.24 M glycine, pH 8.3. Ice-refrigerated electrophoresis was carried out by applying a pulsed current at 80 mA for ca. one hour (until the front of samples left the stacking gel) and subsequently at 100 mA for 3 h and 15 min. The following 18 enzymes were tested with a focus on well stained and clearly interpretable zymograms exhibiting some variability: SKDH (1.1.1.25), ADH (1.1.1.1), SOD (1.15.1.1), AAT (2.6.1.1), ENP (3.4.23.6), PRX (1.11.1.7), MDH (1.1.1.37), IDH (1.1.1.42), GDH (1.4.1.2), ACP (3.1.3.2), HEX (2.7.1.1), 6-PGDH (1.1.1.44), PGM (2.7.5.1), PGI (5.3.1.9), DIA (1.6.-.-), LAP (3.4.11.-), EST (3.1.1.-), G-6-PDH (1.1.1.49). From these enzymes 4 were chosen for further analysis (shikimic acid dehydrogenase SKDH, alcohol dehydrogenase ADH, superoxide dismutase SOD and endopeptidase ENP). The other tested enzymes were either invariable or did not provide zymograms of the required quality.

2.4. Staining

The staining procedures followed Vallejos (1983) to visualize ADH and Wendel and Weeden (1989) for ENP, SOD and SKDH with the following modifications. The SOD staining ingredients were 50 ml of 0.05 M Tris–HCl (pH 8.2), 5 mg of NBT, 4.5 mg of EDTA and 1.5 mg of riboflavin. The gel was incubated in the dark at 37 °C for 20 min and then placed under lamplight until bands appeared on the dark background. The SKDH ingredients were 30 ml of 0.1 M Tris–HCl (pH 8.4), 30 mg of shikimic acid, 5 mg of NADP, 6 mg of MTT and 1 mg of PMS. The gel was incubated in the dark at 32 °C until bands appeared. Ingredients for ENP for solution A were 50 ml 0.2 M Tris–maleic acid (pH 5.5) and 50 ml for rinsing of the gel, 20 mg of Fast Black K salt and 50 mg of $MgCl_2 \cdot 6H_2O$. Ingredients for solution B were 2 ml *N*,*N*-dimethylformamide and 25 mg BANA. Solution A was poured into solution B in the dark. The gel was rinsed in chilled Tris–maleic acid and then incubated in a mixture of solutions A and B at 37 °C until bands appeared. ADH staining ingredients for solution A were 40 ml 0.1 M Tris–HCl (pH 7.5), 15 mg NAD, 10 mg MTT and 1 mg PMS. Solution B was 10 ml of chilled ethanol. Solution A was poured over the gel and left to incubate at 32 °C. After 3 min solution B was added. If the gel was not sufficiently stained, more ethanol was added after 1 h of incubation. All gels were rinsed in distilled water and wrapped in two cellophane sheets and dried.

2.5. Data analysis

Zymograms were scored according to Soltis and Soltis (1989). One variable and easily interpretable locus was chosen in each enzyme system. Within this locus in monomeric SKDH and ENP zymograms alleles were numbered with increasing migration distance from the origin. In dimeric SOD and ADH zymograms heterozygotes possess 3 bands. From these only outer bands were scored as alleles whereas the middle (heterodimeric) band was not considered as an allele. Occasionally occurring secondary bands with notably lower intensity were not scored as alleles. Allele frequencies, percentage of polymorphic loci (P), mean number of alleles (A), mean effective number of alleles (A_e), observed (H_o) and expected (H_e) heterozygosity and fixation index (F_{st}) were calculated using POPGENE version 1.31 (Yeh et al., 1999). Coefficients of inbreeding (F_{is}) were calculated using FSTAT version 2.9.3.2 (Goudet, 1995); their significance (assuming the null hypothesis of $F_{is} = 0$) was tested by a permutation test with 1000 replicates. A dendrogram based on Nei's genetic distances between populations (Nei, 1972) was generated using POPGENE. Neiís genetic distances were used as a metric in a Principal Coordinate Analysis, which was calculated using Canoco version 5.01 (ter Braak and Šmilauer, 2012),

To determine if genetic distances were correlated with geographic distances, a Mantel test (Mantel, 1967) was performed for different subsets of the studied populations (all populations of *M. subalpinum* agg.; Czech, Slovak, and Austrian narrow-leaved; all Austrian; Austrian narrow-leaved; Austrian wide-leaved; only Czech and Slovak populations). Correlation coefficients between matrices of Neiís genetic distances of populations and their geographical distances were calculated using zt software (Bonnet and van de Peer, 2002); significance of the correlation coefficients was tested by a permutation test with 10,000 replicates.

2.6. Reproduction experiment

A pollination test was carried out on the Czech DoubB population. Very young unopened flowers on several plants were used for the test. Flowers were alternately either castrated and pollinated a few days later with pollen from another plant (allogamy), not castrated and left to self-pollinate (autogamy) or only castrated (control). After the treatment, the experimental plants were covered with monofilament sacks to prevent access by pollinators. The flowers were visually checked for the formation of capsules and seeds (well formed × aborted/defective × absent).

Table 1
Sampled populations with description of locality, altitude, geographical coordinates, date of sampling and approximate number of plants per population.

Population code	Locality	Altitude (m a. s. l.)	Coordinates (WGS84)	Date of collection	Size of the population (order)
M. subalpinum					
Czech and Slovak populations					
DoubB	Doubí u Tábora: forest near the road 230 m SSW of the railway station	410	49°19′8.8″N 14°43′1.7″E	26.8.2008	10^2–10^3
KnezB	Kněževes: forest along the path ca 0.7 km NW of the village	550	49°35′30.4″N 16°24′59.8″E	7.8.2006	10^3
MajdB	Majdaléna: forest edge along the path 2.5 km NNW of the village	445	48°59′4.8″N 14°50′39.7″E	30.8.2005	10^2
PribB	Příběnice: forest around of the castle ruin of Příběnice	420	49°23′32.8″N 14°33′44.8″E	5.9.2006	10–10^2
SastB	Šaštín: pine forest near the road to Borský Mikuláš 1.4 km ESE of the village	185	48°37′52.3″N 17°10′12.8″E	29.8.2006	10^2–10^3
SoprB	Sopreč: forest near the crossroad 1.8 km SSE of the village	230	50°4′42.9″N 15°32′46.3″E	5.9.2006	10^3
TyniB	Křivice: forest 1.2 km WNW of the village	280	50°11′1.7″N 16°5′48.4″E	5.9.2006	10^3
ZehrB	Žehrov: forest near the road 0.9 km SE of the village centre	280	50°31′22.8″N 15°6′23.5″E	5.9.2006	10^2
Austrian narrow-leaved populations					
HernA	Hernstein: forest margin along the road to Neusiedel 1.6 km NNW of the village	390	47°54′26.6″N 16°5′41.2″E	24.6.2008	10^3
HirsA	Hirschwang an der Rax: forest above the Höllental road 1.9 km NNW of the village	525	47°43′20.9″N 15°48′15.9″E	21.8.2008	10^2
HollA	Höllental: forest margin and shrubs near the mouth of the G. Kesselgraben valley	580	47°45′9.6″N 15°44′55.5″E	23.6.2008	10^3
KaltA	Baden, Kaltenberger Forst: pine forest along the path on the northeast slope of the Soosser Lindkogel 5 km W of the town	590	47°59′46.2″N 16°9′48.0″E	21.8.2008	10^2
KranA	Kranichberg: hazel shrubs SE of the castle of Kranichberg	660	47°38′38.6″N 15°58′31.4″E	23.6.2008	10^3
LangA	Sooß: forest along the path on the ridge southward of the Langer Graben valley	430	47°59′1.5″N 16°11′19.7″E	21.8.2008	10^2
LoseA	Losenheim: forest along the path 200 m N of the castle ruin of Losenheim	760	47°47′26.8″N 15°50′40.6″E	30.8.2005	10^3
MarsA	Maria Schutz: spruce forest margin along the road 0.5 km ESE of the Mariaschutz church	730	47°38′25.0″N 15°52′32.9″E	23.6.2008	10^3
MiesA	Miesenbach: forest margin along the road 2.5 km N of the village	440	47°51′45.7″N 15°59′22.0″E	21.8.2008	10^2–10^3
PeutA	Peutenburg: pine forest above the path 0.3 km WNW of the Peutenburg railway station	430	47°57′20.5″N 15°9′15.1″E	29.8.2006	10^2
SemmA	Semmering: forest along the road 250 m N of the Hotel Panhans	1030	47°38′24.4″N 15°49′37.3″E	29.8.2006	10^4
SlinA	Sooß: forest along the path 0.3 km S of the Soosser Lindkogel hilltop	640	47°59′19.2″N 16°9′42.0″E	21.8.2008	10^2
Austrian wide-leaved populations					
Ein2S	Einöde: forest and shrubs along the road to Gaaden 750 m NW of the village centre	320	48°01′29.7″N 16°13′41.8″E	21.8.2008	10^3
EinoS	Einöde: forest and shrubs along the road to Gaaden 600 m NW of the village centre	320	48°01′25.6″N 16°13′47.0″E	30.8.2005	10^3
GumpS	Gumpoldskirchen: open forest on the rock outcrops ca 500 m WNW of the village	420	48°2′45.4″N 16°15′40.9″E	29.8.2006	10^2
HofsS	Rohrbach, Hofstätten: shrubs (Corylus, Carpinus) along the road to the hermitage Hofstätten	420	47°59′34.4″N 16°6′17.5″E	30.8.2005	10^3

Table 1 (*Continued*)

Population code	Locality	Altitude (m a. s. l.)	Coordinates (WGS84)	Date of collection	Size of the population (order)
MerkS	Merkenstein: forest above the castle ruin of Merkenstein	450	47°58′58.6″N 16°8′0.3″E	29.8.2006	10^2–10^3
RichS	Gumpoldskirchen, Richardhof: shrubs and forest edge along the road 300 m NNE of the Hotel Richardhof	360	48°3′28.2″N 16°16′34.9″E	21.8.2008	10^3
SoosS	Sooß: shrubs along the road 1.4 km W of the centre of the village centre	310	47°59′15.0″N 16°11′53.7″E	21.8.2008	10^3
M. nemorosum populations					
GaadN	Rosental: shrubs along the road ca 1.7 km SSE of the village	260	48°1′4.0″N 16°11′28.2″E	24.6.2008	10^3
GiesN	Gießhübl, Tirolerhof–Siedlung: forest on the western edge of the settlement	420	48°6′12.6″N 16°14′2.6″E	30.8.2005	10^4
HofsN	Bad Vöslau, Großau: forest along the road ca 4.3 km WNW of the village Großau	380	47°58′48.0″N 16°7′20.0″E	30.8.2005	10^2
RotkN	Rotes Kreuz: forest margin ca 2 km SSE of the village Gaaden near Rotes Kreuz crossroad	400	48°2′8.3″N 16°12′34.8″E	29.8.2006	10^3

3. Results

3.1. Allozymes

For SOD two loci were detected, but only the faster locus provided clear and variable pattern (dimeric, 2 alleles). For ADH two loci were detected as well and only the faster locus was variable and clear enough for scoring (dimeric, 7 alleles). SKDH yielded one monomeric locus with 7 alleles and ENP had one monomeric locus with 4 alleles.

The total number of alleles per population ranged from 6 to 7 in Czech and Slovak populations, 8 to 12 in narrow-leaved Austrian populations and 9 to 15 in wide-leaved populations from the Vienna Forest (Fig. 1). The highest number of alleles (15) was detected in the EinoS population which is morphologically close to *M. nemorosum*. The number of alleles in *M. nemorosum* populations ranged from 8 to 11.

The rarest alleles were ADH 7 with a frequency of 0.002 (LoseA population), SKDH 7 with a frequency of 0.005 (HirsA population), ADH 1 with a frequency of 0.006 (EinoS and HollA populations) and ADH 4 with a frequency of 0.032 (EinoS and SastB populations). The most frequent alleles were ENP 2 with an overall frequency of 0.640 and SOD 2 and ADH 6 with a frequency of 0.613. These alleles were present in most or all of the populations including some *M. nemorosum* populations. Apart from rare alleles that occurred only in one or two populations, we found a unique allele (ADH 5) that occurred only in *M. nemorosum* populations and one wide-leaved *M. subalpinum* population (EinoS) that is morphologically close to *M. nemorosum*. The SKDH 6 allele was found only in 3 wide-leaved *M. subalpinum* populations morphologically close to *M. nemorosum*, but not in any *M. nemorosum* population. Allele ADH 2 occurred only in Austrian populations, both wide- and narrow-leaved, from the Vienna Forest region and in one *M. nemorosum* population, and was absent in narrow-leaved populations from the Alps and Czech and Slovak populations. The ADH 3 allele was very common in both Austrian *M. subalpinum* morphotypes, however it also occurred in one *M. nemorosum* population and in one isolated Czech *M. subalpinum* ZehrB population. The ENP 4 and SKDH 5 alleles occurred in wide- and narrow-leaved populations, both Austrian and Czech, but did not occur in *M. nemorosum*. The SKDH 2 and 3 alleles did not occur in the Czech populations, but were present in *M. nemorosum* and wide- and narrow-leaved Austrian populations. The SKDH 4 allele occurred in all *M. nemorosum* populations and 1 wide-leaved population (MerkS) and 1 narrow-leaved Austrian population (LoseA); hence, although it is not unique for any group, it is clearly much more common in *M. nemorosum* populations than in the *M. subalpinum* agg. The allelic frequencies for each locus and each population are given in Table 2.

The effective number of alleles (A_e) per population ranged from 1.112 to 2.940 (Table 3). Among different groups (Table 4), the wide-leaved *M. subalpinum* group had the highest value (A_e = 2.160) and the Czech narrow-leaved group had the lowest value (A_e = 1.340).

Observed heterozygosity (H_o) ranged from 0.050 to 0.593 (mean 0.340), and mean expected heterozygosity (H_e) ranged from 0.092 to 0.572 (mean 0.373). The Czech and Slovak populations had the lowest H_e suggesting the lowest genetic variation. The inbreeding coefficient (F_{is}) of Czech and Slovak populations ranged from high (0.481) to very low (−0.416), which was also the lowest from all studied populations. The highest value of F_{is} was 0.570 for the Austrian LoseA population. Population characteristics are given in Table 3.

The total fixation index (F_{st}) for all populations was 0.378 which indicates a very high genetic differentiation between populations. In the case of groups of populations, the highest F_{st} was found for the Czech-Slovak group (0.514) and the lowest, but still a considerably high F_{st} was found for the *M. subalpinum* group (0.224). A summary of population characteristics for the groups is given in Table 4.

The UPGMA dendrogram (Fig. 2) and the PCoA based on Nei's genetic distances showed similar population relationships. The most isolated population in UPGMA is the Austrian narrow-leaved PeutA population. The remainder of the populations clustered into two major groups. The first group contains all *M. nemorosum* populations and three *M. subalpinum* populations (EinoS, Ein2S, HofsS), which are morphologically closest to *M. nemorosum*. The structure of the second major group is less clear. The remaining Austrian wide-leaved populations are dispersed between narrow-leaved populations. Most of the Czech populations are grouped together, yet the KnezB population, the Slovak SastB population, and especially ZehrB are distant from the rest of the Czech populations. These populations have a rather isolated position as seen in the PCoA analysis (outlying *M. nemorosum* populations were excluded for better resolution). *M. subalpinum* populations morphologically close to *M. nemorosum* (EinoS, Ein2S, HofsS) lie remote from the main patch of the Austrian populations with HofsS being the closest (Fig. 3).

Table 2
Allelic frequencies at four polymorphic loci for each population. Alleles are numbered for each enzyme system/locus.

Population	Locus/allele																			
	SOD		SKDH							ENP				ADH						
	1	2	1	2	3	4	5	6	7	1	2	3	4	1	2	3	4	5	6	7
HofsN	0.850	0.150	0.000	0.050	0.400	0.550	0.000	0.000	0.000	0.000	1.000	0.000	0.000	0.000	0.000	0.000	0.000	0.444	0.556	0.000
GiesN	1.000	0.000	0.050	0.050	0.450	0.450	0.000	0.000	0.000	0.500	0.300	0.200	0.000	0.000	0.000	0.000	0.000	0.350	0.650	0.000
GaadN	0.950	0.050	0.150	0.000	0.200	0.650	0.000	0.000	0.000	0.150	0.650	0.200	0.000	0.000	0.000	0.000	0.000	0.750	0.250	0.000
RotkN	0.950	0.050	0.100	0.000	0.100	0.800	0.000	0.000	0.000	0.000	0.950	0.050	0.000	0.000	0.050	0.200	0.000	0.250	0.500	0.000
EinoS	0.900	0.100	0.150	0.150	0.000	0.300	0.050	0.350	0.000	0.000	0.650	0.000	0.350	0.050	0.550	0.150	0.100	0.100	0.050	0.000
Ein2S	0.850	0.150	0.300	0.000	0.000	0.250	0.000	0.450	0.000	0.000	0.500	0.200	0.300	0.000	0.389	0.000	0.000	0.000	0.611	0.000
HofsS	0.550	0.450	0.150	0.150	0.000	0.000	0.250	0.450	0.000	0.000	0.900	0.050	0.050	0.000	0.389	0.167	0.000	0.000	0.444	0.000
GumpS	0.250	0.750	0.800	0.000	0.100	0.000	0.100	0.000	0.000	0.056	0.611	0.333	0.000	0.000	0.050	0.600	0.000	0.000	0.350	0.000
MerkS	0.650	0.350	0.150	0.000	0.350	0.100	0.400	0.000	0.000	0.000	0.750	0.050	0.200	0.000	0.000	0.550	0.000	0.000	0.450	0.000
RichS	0.200	0.800	0.000	0.000	0.650	0.000	0.350	0.000	0.000	0.000	0.800	0.000	0.200	0.000	0.050	0.400	0.000	0.000	0.550	0.000
SoosS	0.250	0.750	0.250	0.100	0.200	0.000	0.450	0.000	0.000	0.000	0.550	0.250	0.200	0.000	0.111	0.000	0.000	0.000	0.889	0.000
LoseA	0.050	0.950	0.850	0.000	0.000	0.150	0.000	0.000	0.000	0.000	0.450	0.400	0.150	0.000	0.000	0.400	0.000	0.000	0.550	0.050
PeutA	0.050	0.950	0.050	0.950	0.000	0.000	0.000	0.000	0.000	0.050	0.050	0.400	0.500	0.000	0.000	0.750	0.000	0.000	0.250	0.000
SemmA	0.000	1.000	0.600	0.100	0.300	0.000	0.000	0.000	0.000	0.000	0.550	0.300	0.150	0.000	0.000	0.250	0.000	0.000	0.750	0.000
HernA	0.400	0.600	0.450	0.100	0.000	0.000	0.450	0.000	0.000	0.000	0.600	0.350	0.050	0.000	0.050	0.250	0.000	0.000	0.700	0.000
HollA	0.050	0.950	0.750	0.000	0.050	0.000	0.200	0.000	0.000	0.000	0.700	0.200	0.100	0.150	0.000	0.150	0.000	0.000	0.700	0.000
HirsA	0.200	0.800	0.600	0.250	0.000	0.000	0.000	0.000	0.150	0.000	0.600	0.150	0.250	0.000	0.000	0.350	0.000	0.000	0.650	0.000
KaltA	0.200	0.800	0.000	0.000	0.800	0.000	0.200	0.000	0.000	0.000	0.550	0.350	0.100	0.000	0.050	0.300	0.000	0.000	0.650	0.000
KranA	0.200	0.800	0.900	0.100	0.000	0.000	0.000	0.000	0.000	0.000	0.600	0.000	0.400	0.000	0.000	0.100	0.000	0.000	0.900	0.000
LangA	0.300	0.700	0.400	0.250	0.300	0.000	0.050	0.000	0.000	0.000	0.650	0.300	0.050	0.000	0.286	0.143	0.000	0.000	0.571	0.000
MarsA	0.400	0.600	0.250	0.750	0.000	0.000	0.000	0.000	0.000	0.000	0.500	0.000	0.500	0.000	0.000	0.111	0.000	0.000	0.889	0.000
MiesA	0.150	0.850	0.950	0.050	0.000	0.000	0.000	0.000	0.000	0.000	0.800	0.150	0.050	0.000	0.000	0.450	0.000	0.000	0.550	0.000
SlinA	0.400	0.600	0.450	0.000	0.250	0.000	0.300	0.000	0.000	0.000	0.450	0.550	0.000	0.000	0.150	0.600	0.000	0.000	0.250	0.000
MajdB	0.500	0.500	1.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.700	0.300	0.000	0.000	0.000	0.000	0.000	0.000	1.000	0.000
SastB	0.300	0.700	1.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.950	0.000	0.050	0.000	0.000	0.000	0.000	0.900	0.000	0.100	0.000
PribB	0.150	0.850	0.950	0.000	0.000	0.000	0.050	0.000	0.000	0.000	1.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	1.000	0.000
SoprB	0.300	0.700	0.900	0.000	0.000	0.000	0.100	0.000	0.000	0.000	0.950	0.050	0.000	0.000	0.000	0.000	0.000	0.000	0.000	1.000	0.000
ZehrB	0.000	1.000	0.000	0.000	0.000	0.000	1.000	0.000	0.000	0.000	0.700	0.000	0.300	0.000	0.000	0.800	0.000	0.000	0.200	0.000
TyniB	0.200	0.800	1.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.667	0.111	0.222	0.000	0.000	0.000	0.000	0.000	1.000	0.000
KnezB	0.750	0.250	1.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.150	0.300	0.550	0.000	0.000	0.000	0.000	0.000	1.000	0.000
DoubB	0.000	1.000	1.000	0.000	0.000	0.000	0.000	0.000	0.000	0.400	0.550	0.000	0.050	0.000	0.000	0.000	0.000	0.000	1.000	0.000
Mean	0.387	0.613	0.490	0.098	0.134	0.105	0.127	0.040	0.005	0.037	0.640	0.169	0.154	0.006	0.069	0.217	0.032	0.061	0.613	0.002

Table 3
Summary of genetic population characteristics for all studied populations based on allozymes. P (%) = percent of polymorphic locus; A = average number of alleles per locus; A_e = effective allele number; H_o = observed heterozygosity; H_e = expected heterozygosity; F_{is} = inbreeding coefficient (values above 0.25 in bold; values marked with * are significant at 5% level).

Population	P (%)	A	A_e	H_o	H_e	F_{is}	No. of alleles
HofsN	75	2.000	1.710	0.342	0.339	−0.010	8
GiesN	75	2.500	1.640	0.525	0.438	−0.212	10
GaadN	100	2.500	1.720	0.350	0.395	0.119	10
RotkN	100	2.750	1.550	0.250	0.309	0.200	11
EinoS	100	3.750	2.940	0.533	0.506	−0.034	15
Ein2S	100	2.500	2.174	0.569	0.526	−0.089	10
HofsS	100	3.000	2.220	0.547	0.526	−0.044	12
GumpS	100	2.750	1.690	0.431	0.459	0.067	11
MerkS	100	2.750	2.161	0.400	0.534	**0.262***	11
RichS	100	2.250	1.160	0.350	0.429	0.192	9
SoosS	100	2.750	2.123	0.350	0.488	**0.294***	11
LoseA	100	2.500	1.880	0.175	0.395	**0.570***	10
PeutA	100	2.500	1.490	0.275	0.303	0.096	10
SemmA	75	2.250	1.590	0.350	0.395	0.119	9
HernA	100	2.750	2.049	0.375	0.533	**0.308***	11
HollA	100	2.750	1.970	0.325	0.372	0.133	11
HirsA	100	2.500	1.950	0.425	0.496	0.150	10
KaltA	100	2.500	1.540	0.475	0.445	−0.072	10
KranA	100	2.000	1.820	0.350	0.305	−0.156	8
LangA	100	3.000	2.350	0.593	0.572	−0.038	12
MarsA	100	2.000	1.230	0.364	0.411	0.120	8
MiesA	100	2.250	1.280	0.23	0.311	**0.286**	9
SlinA	100	2.500	2.180	0.500	0.572	0.133	10
MajdB	50	1.500	1.431	0.300	0.242	−0.256	6
SastB	75	1.750	1.220	0.125	0.183	**0.328**	7
PribB	50	1.500	1.112	0.050	0.092	**0.471**	6
SoprB	75	1.750	1.220	0.175	0.183	0.045	7
ZehrB	50	1.500	1.870	0.200	0.195	−0.029	6
TyniB	50	1.750	1.150	0.183	0.215	0.155	7
KnezB	50	1.750	1.240	0.350	0.253	−0.416	7
DoubB	25	1.500	1.760	0.075	0.141	**0.481**	6

Table 4
Summary of genetic characteristics for groups of populations. P (%) = percent of polymorphic locus; A = average number of alleles per locus; A_e = effective allele number; H_o = observed heterozygosity; H_e = expected heterozygosity; F_{st} = fixation index.

Group	P (%)	A	A_e	H_o	H_e	F_{st}
CZ + SK	100	2.750	1.340	0.182	0.369	0.514
AU narrow	100	4.250	2.120	0.368	0.523	0.226
AU wide	100	4.500	2.160	0.457	0.615	0.224
M. nemorosum	100	3.250	1.740	0.368	0.419	0.146

Table 5
Results of the Mantel test. r = correlation coefficient, p = significance by probability test with 10,000 replicates (value below 0.01 in bold).

Group	r	p
All populations of *M. subalpinum* agg.	0.090	0.224
CZ + SK + AU narrow	0.158	0.113
AU narrow + AU wide	0.396	0.015
AU narrow	**0.698**	**<0.001**
AU wide	−0.350	0.027
CZ + SK	0.213	0.205

The results of the Mantel test show a significant positive correlation ($r = 0.698$, $p < 0.001$) between Neiís genetic distances and the geographic distances of the Austrian narrow-leaved populations. Correlations in the most remaining subsets of populations (all populations of *M. subalpinum* agg., Czech, Slovak, and Austrian narrow-leaved, all of the Austrian populations, and Czech and Slovak populations) are positive as well, but markedly lower and they are not, or are only weakly, significant. Weakly significant but negative correlation was revealed in Austrian wide-leaved population (Table 5).

3.2. Reproduction experiment

From 35 flowers without stamens pollinated by pollen from different plants (allogamy), 31 flowers developed into a ripe capsule, 1 developed into an aborted capsule and 3 flowers did not produce a capsule. From 49 non-pollinated flowers with preserved stamens (autogamy), 42 did not develop into a capsule and 7 flowers self-pollinated and developed capsules. From 58 non-pollinated control flowers with removed stamens 56 did not develop, 1 developed into the aborted capsule and 1 developed into a ripe capsule.

4. Discussion

The central-marginal concept claims that within-population genetic diversity declines and among-population differentiation increases from the centre of the species' geographical range to the periphery (Eckert et al., 2008). Although there are some studies questioning the central-marginal concept in the genetic variation of populations (e.g., Mandák et al., 2005; Wróblewska, 2008), our results are consistent with this theory.

The highest genetic variation of *M. subalpinum* agg. among Austrian wide-leaved populations ($H_e = 0.615$) and the highest effective number of alleles per locus ($A_e = 2.160$) is in accordance with the assumptions based on morphology about the diversity centre of the group in the Vienna Forest (Štech, 2006). The adjacent narrow-leaved Austrian populations occurring from the Vienna Forest towards the Lower Austria/Styria Alps display a lower level of variation ($H_e = 0.523$, $A_e = 2.120$) and the marginal Czech and Slovak narrow-leaved populations have the lowest variation ($H_e = 0.369$,

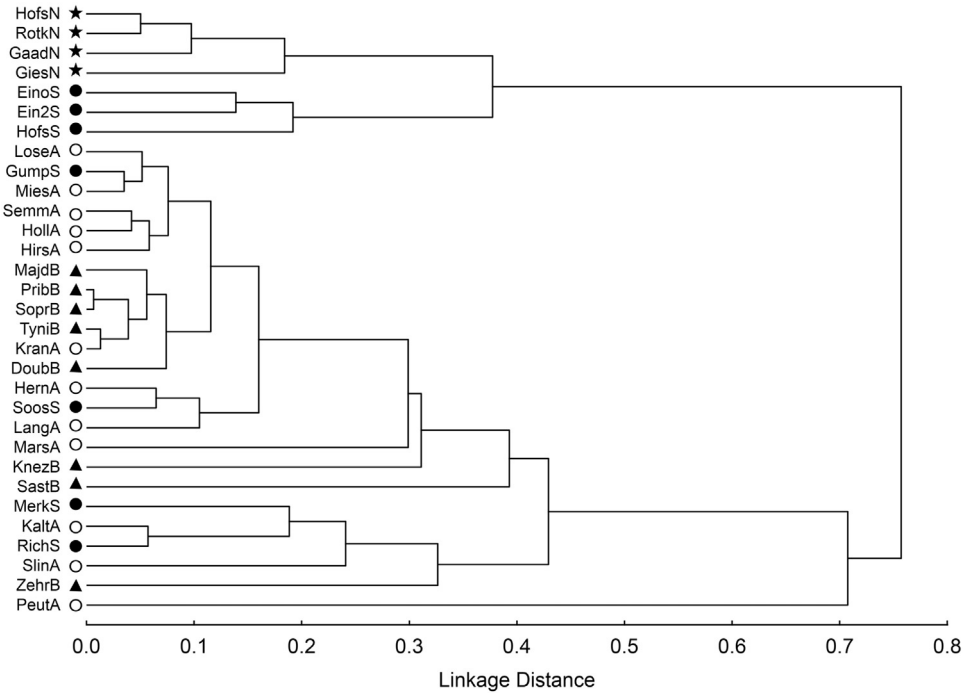

Fig. 2. The UPGMA dendrogram based on Nei's genetic distances (Nei, 1972). Symbols explanation: * *M. nemorosum*; ● wide-leaved *M. subalpinum*; ○ Austrian narrow-leaved *M. subalpinum*; ▲ Czech or Slovak *M. subalpinum*.

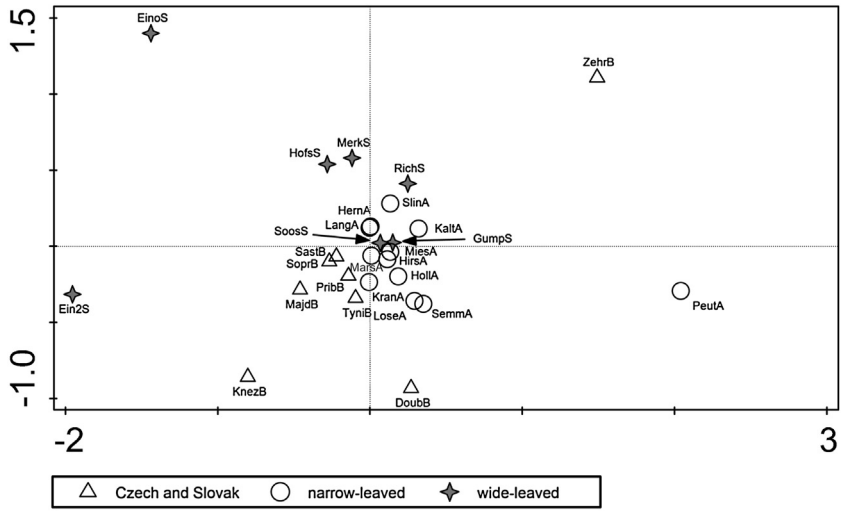

Fig. 3. Principal coordinates analysis for all studied populations of *M. subalpinum*. For better resolution *M. nemorosum* populations were removed.

A_e = 1.340) and lowest number of alleles per population as well as a lower percentage of polymorphic loci. The highest genetic variation of the wide-leaved populations may also be partly influenced by an assumed hybridization with *M. nemorosum* populations (Štech, 2006).

This assumption is partly supported by the Mantel test results which showed the highest significant correlation of genetic and geographic distance among Austrian narrow-leaved populations. For wide-leaved populations the correlation was weaker (yet still significant) and negative. The lower correlation is probably caused by the low number of wide-leaved populations which are also restricted to a relatively small area. Another reason might be the possible hybridogenous origin of wide-leaved populations. Hybridogenous populations were observed to possess no significant geographic component in genetic variation, whereas obvious correlations between genetic and geographic distances were detected in non-hybrid populations of the genus *Lotus* (Kramina, 2013).

The highest average fixation index (F_{st} = 0.514) for the Czech and Slovak populations supports the marginal status of this group and clearly shows that the disjunction of these populations is not a recent event. It is possible that the ancestral taxon was spread in the early Holocene across large areas which included today's localities and started to withdraw when the tree canopy became more dense, and later due to substantial changes to suitable habitats made by man. Recent localities are often found in sparse woods on well-illuminated sites such as slopes and terrace edges of river floodplains, forest margins, or the surroundings of forest roads and clearings (Chlumský and Štech, 2011). Another possibility is that *M. subalpinum* was never widespread in today's marginal areas, but was restricted to small regions with appropriate environmen-

tal conditions, and recent localities are the remains of scattered historical distribution.

Nevertheless, genetic drift (Amos and Hardwood, 1998) as a result of either persistent isolation, bottleneck, or founder effect has had enough time to substantially enhance the genetic differentiation between populations.

The average inbreeding coefficient (F_{is}) was close to 0 in all groups suggesting that there is not a high risk of inbreeding depression. However, a closer look at single populations shows a more complicated pattern. There were 8 populations that had a rather high value for the inbreeding coefficient ($F_{is} > 0.25$, Table 3; note that in four of them it is not significantly different from 0 in a permutation test, which is, however, caused by their low overall variation or even invariability of some loci, which are then omitted from the computation). One of the reasons could be the isolation of populations and therefore higher inbreeding due to crossing with close relatives. The pollination test also showed the partial ability to self-fertilise, which may as well add to the amount of inbreeding. Adaptations for self-fertility are known to increase the probability of establishment following dispersal (Larson and Barrett, 1998) and self-fertility may also be selected in peripheral populations (Lipow and Wyatt, 2000). However, allogamy is obviously still the preferred reproduction mode in *M. subalpinum* agg.

A common feature of populations with high inbreeding coefficient is that they occupy secondary stands which were presumably colonised by rapid radiation from a small number of plants originating from vanished primary stands. The density and regular distribution of these populations is often very high. It has been observed that land use changes may lead to a decreased genetic diversity within populations shortly after the colonization of secondary stands (Vellend, 2004; Jacquemyn et al., 2004, 2009).

On the contrary, the populations with the lowest inbreeding coefficients are often spatially structured and comprised of a system of patches. Although their sites are usually secondary, they often occur close to their putative primary habitats such as the edges of terraces above watercourses. Disturbance dynamics realized by the river ensured proper light conditions for the survival of the species during the Holocene. The continual occurrence of appropriate conditions can reduce a bottleneck effect and such populations may act as allelic refugia and present higher genetic variability (Comps et al., 2001). In case of sufficient genetic diversity in the population and gradual expansion of population size there is still sufficient opportunity for non-relative allogamy and F_{is} thus may reach even moderate negative values.

In concordance with Honnay and Jacquemyn (2007) there was no significant relationship between population size and the inbreeding coefficient.

Despite the low number of studied loci (but high total number of alleles), the dendrogram (Fig. 2) and principal coordinates analysis (Fig. 3) based on Nei's genetic distances were easily interpretable. The dendrogram separated a cluster containing *M. nemorosum* populations together with some wide-leaved Austrian populations that are morphologically closest to *M. nemorosum*. This pattern supports a hypothesis supposing old hybridization between *M. subalpinum* and *M. nemorosum* assumed on the basis of morphological characteristics (Štech, 2006).

The EinoS population had also the allele ADH 5 which was otherwise specific for *M. nemorosum* populations. Another interesting allele, SKDH 4, common for *M. nemorosum populations*, was present in 3 wide-leaved populations. However, it is also present in the population LoseA, which morphologically belongs to the narrow-leaved populations and occurs in a region without the presence of any *M. nemorosum* population. It is hard to say if SKDH 4 is an ancestral allele or evidence for an old hybridization event with an inconspicuous morphological manifestation.

As expected, *M. grandiflorum* MarsA population did not differ in any way from the rest of the narrow-leaved Austrian populations.

The allelic pool of Czech and Slovak populations is obviously pauperized compared to the Austrian populations and there are no unique alleles present for this area. An interesting fact is that the ADH 4 allele is shared by the Slovak SastB population and Austrian wide-leaved EinoS population, which are morphologically different, but geographically relatively close.

The ZehrB population was, due to its geographic isolation and rather late year of discovery, considered to be introduced (Holub, 1996). However, within the Czech populations unique ADH 3 allele discovered in the ZehrB population (common for Austrian *M. subalpinum* agg. populations and one Austrian *M. nemorosum* population) suggests that the ZehrB population might be considered relic.

5. Conclusions

Genetic variation estimated by isozyme analyses is congruent with the known pattern of morphological variation of *M. subalpinum* agg.

The allelic richness is higher in the Austrian populations than in the marginal Czech and Slovak populations.

Some wide-leaved populations from the Vienna Forest have a rather high number of alleles. This allelic abundance may be caused by allelic enrichment due to an old hybridization with *M. nemorosum*. The Czech and Slovak populations traditionally designated as *M. bohemicum* are, according to their allozymes, genetically derived from Austrian narrow-leaved populations.

The high differentiation among populations suggests that the current gene flow between populations is not common and populations do not interbreed often. The highest between-population differentiation (F_{st}) in the group of Czech and Slovak populations suggests that they have been isolated long enough for the genetic drift to divide these populations.

The high inbreeding coefficient (F_{is}) in some populations together with the pollination experiment indicates that there might be selfing within the populations.

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Roles of species-preferential seed dispersal by ants and endozoochory in *Melampyrum* (Orobanchaceae)

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Abstract

Aims

Melampyrum pratense and *M. subalpinum* are two myrmecochorous species, which possess similar habitat requirements and frequently occur together. Despite this, their population sizes differ markedly. *Melampyrum pratense* populations are usually very large, whereas *M. subalpinum* has rather small and isolated populations. We suggest that such an imbalance might be partially influenced by the difference in ant-mediated seed-removal rates.

Genus *Melampyrum* is considered to be exclusively myrmecochorous, though to achieve the recent distribution of some *Melampyrum* species during the Holocene myrmecochory would be highly insufficient. We suggest that endozoochory takes place in the long-distance migration, whereas myrmecochory is important for the removal of seeds on a local scale.

Methods

For seed-preference analysis, *M. pratense* and *M. subalpinum* mixed seed samples were placed around *Formica polyctena* anthills. After a period of time, the remaining seeds of both species were counted for each sample. The results were analysed by analysis of variance and generalized linear mixed-effect model. To test myrmecochorous removal distances, *M. pratense* seeds were covered with fluorescent dactyloscopic powder and placed in the vicinity of a large ant trail. The area around the starting plot was searched in the dark using UV LED torchlight 7 h after the beginning. The distance from the starting plot was measured for each seed found. Birds, rodents, leporine and a ruminant were fed with *M. pratense* seeds and fresh plants to test the possibility of endozoochorous dispersal of the species. Animal droppings were searched for intact seeds.

Important Findings

Our field studies show that from mixed seed samples, containing both species, ants significantly preferred the seeds of *M. pratense*. This may be one of factors that has positive influence on *M. pratense* success in seed dispersal on mixed stands and consequently in the colonization of favourable sites. Experiments focusing on ant-mediated dispersal distance revealed that *F. polyctena* ants are able to move seeds over a distance of 36 m in only 7 h. This distance is among the furthest known myrmecochorous removals of forest plant seeds. A new *Melampyrum* seed disperser *Oligolophus tridens* (Opiliones) was observed repeatedly. Our pilot study documented that *Melampyrum* seeds are able to pass through the digestive tract of a cow intact. This suggests that large ruminants such as deer, bison or forest-grazing livestock may function as important long-distance dispersers of *Melampyrum* species.

Keywords: endozoochory • *Melampyrum* • myrmecochory • Orobanchaceae • seed dispersal

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INTRODUCTION

Myrmecochory (seed dispersal by ants) is a relatively common means of seed distribution among plants and is known in >3000 species (Beattie and Hughes 2002). Many plants are considered to be solely myrmecochorous, yet little is known about the real contribution of myrmecochory to population sizes, total distribution of species and interactions between myrmecochorous species. The benefits of this symbiotic relationship for plants may, apart from dispersal, also include seed-predator avoidance (Culver and Beattie 1978; Gibson 1993b), escape from seedling competition and the competition

of seedlings and parental plants (Bronstein *et al.* 2006; Stamp and Lucas 1990) and transport to favourable sites for germination and growth (Gibson 1993a).

Myrmecochory has a crucial role in the dispersal at a local scale. The longest published myrmecochorous removal distance is 180 m (Whitney 2002). However, this value refers to *Acacia ligulata* seeds in the arid zones of Australia, which are known to be extreme in terms of ant-mediated dispersal distances. In relation to temperate forest herbs, the maximum reported distances of dispersal by ants are 35 m for *Asarum canadense* (Cain *et al.* 1998) and up to 70 m for *Melica nutans* and *Viola hirta* (Sernander 1906). Nevertheless, an average distance for forest herbs is just a few meters per year (Cain *et al.* 1998; Gómez and Espadaler 1998). The long-distance dispersal of myrmecochorous plants is obviously often achieved in other ways than that of myrmecochory; however, it still remains poorly documented (Cain *et al.* 1998; Heinken 2004; Vellend *et al.* 2003).

For our study, we have selected two species of the genus *Melampyrum* (Orobanchaceae), *M. pratense* L. and *M. subalpinum* (Jur.) A. Kern. The genus includes myrmecochorous annual hemiparasitic herbs mainly from the temperate zone of Eurasia (one species is present also in North America). The habitat requirements of the studied species widely overlap (both occur in light oak, pine, or spruce forests) and can often be found growing together. Despite this, their population sizes differ markedly. *Melampyrum pratense* populations are usually very large, whereas *M. subalpinum* has a patchy distribution with small populations (Chlumský and Štech 2011). The Czech populations of *M. subalpinum* used in this study are traditionally labelled as *M. bohemicum* A. Kern. However, both morphological and molecular data support conspecifity of populations from the former Czechoslovakia and populations from the main distribution area in the Eastern Alpine foothills described as *M. subalpinum* (Štech 2006).

We have found prosperous *M. pratense* populations at the most Czech and Slovak localities of *M. subalpinum* (Chlumský and Štech 2011). At localities hosting both species *M. subalpinum* usually occurs as a small compact population and does not expand to favourable sites in the vicinity, whereas *M. pratense* grows abundantly at all suitable sites in the area. We hypothesize that one of the reasons for such a contrast might be the unequal dispersal of the two species, parallel to differing preference of seeds by the ants.

There are two studies focussed on dispersal distance in *Melampyrum*. Heinken (2004) estimated the average distance for *M. pratense* 0.91 m per year with a maximum of 6.48 m and Gibson (1993a) reported the value of 1.08 m per year for *M. lineare* with a maximum of 4.5 m. However, these values are too low considering the migration from Holocene refugia, especially in regard to the huge distribution area of *M. pratense* in glaciated areas. Seeds would have to be transported at least several hundred meters per year to achieve present distribution. *Melampyrum* seeds are too heavy for anemochory and their oval shape and smooth surface exclude epizoochory. The most probable means of long-distance dispersal would, therefore, be endozoochory, which, however, has not been reported in the genus *Melampyrum* so far.

Questions:

(1) Are seeds of one of the species (*M. pratense* or *M. subalpinum*) dispersed by ants preferentially?
(2) What are myrmecochorous dispersal distances in the area with high ant density?
(3) Is endozoochorous dispersal in the genus *Melampyrum* possible?

MATERIALS AND METHODS

Study site

Seed removal experiments were carried out in a spruce forest near the town of Tábor, Czech Republic (49°27′52″N, 14°49′55″E, WGS84). The locality is suitable for myrmecochorous experiments due to a high density of *Formica polyctena* ant colonies and sparse ground vegetation.

Seed preference

Seeds were collected from three populations of *M. pratense* and two populations of *M. subalpinum* (Table 1) 1 day prior to the experiment and were stored on ice over night.

The average weight of seeds is ~0.02 g. Both species have similar oblong ellipsoid seeds 3.5–7 mm long with smooth surface and elaiosome attached to one side of the seed (Štech 2000). In the experiment, we needed to distinguish between seeds of the two species. Therefore, seeds of one species were slightly cut opposite to the elaiosome (each species marked at half of the squares).

Twenty seeds of each species were placed on a cardboard square (10 × 10 cm) and 10 squares were placed at a distance of 10 m from the anthill in a half-circle formation with a distance

Table 1: seed-source localities for experiments

Species	Locality	Coordinates (WGS84)	Repeat of the experiment
Melampyrum pratense	Doubí u Tábora: 0.6 km NNW from train stop	49°19′38″N14°42′55″E	First
	Zahájí: 1.65 km SE from the church	49°04′43″N14°23′07″E	Second
	Včelná: 1.25 km SSW from the train station	48°54′50″N14°26′35″E	Third
Melampyrum subalpinum	Doubí u Tábora: 0.6 km NNW from train stop	49°19′38″N14°42′55″E	First
	Ústrašice: 1.5 km SW from the cemetery	49°20′01″N14°40′39″E	Second and third

of 5 m between the neighbouring squares. The squares were placed around three anthills of *F. polyctena*. After 15, 40 and 90 min, the remaining seeds of both species were counted for each square. The experiment was repeated on three different days. For each day and anthill, one optimal check-time data set (seed counts after 15, 40 or 90 min depending on ant activity was chosen for the analysis to avoid uninformative too short (no seeds removed from most of the squares) or too long times (all seeds removed). However, within the selected check-time data set all squares were analysed, including those with zero values of remaining/removed seeds. The number of removed seeds was analysed using two approaches. Firstly, we computed analysis of variance (ANOVA) with arcsin transformation of the proportion of removed seeds. The model included two factors with fixed effects (species and which species was marked at the particular plot) and three hierarchically nested factors with random effects (experimental day, anthill and plot number). Statistica 9 (StatSoft 2010) was used for the analysis. Since arcsin transformation is not optimal for the data with such a complicated structure, we have also tested fixed effects factors using the generalized linear mixed-effect model (GLMM) with binomial distribution using the *glmer* function as implemented in the *lme4* package (Bates and Maechler 2010) in R 2.11 software (R Development Core Team 2010). The fixed-effect factors were tested by comparing the models including/not including the tested factor using a likelihood ratio test. The structure of random factors was the same as in ANOVA; however, since this branch of statistics is still under development tests of random factors itself are not available yet.

Dispersal distance

Totally, 1200 seeds of *M. pratense* were covered with fluorescent dactyloscopic powder (Sirchie, Youngsville, NC, USA) and placed in the vicinity of a large ant trail ~50 m from the nearest anthill. The area around the starting plot was searched in the dark using UV LED torchlight 7 h after the beginning to a distance of 60 m. The distance from the starting plot was measured for each seed found.

Endozoochory

Four groups of vertebrates were tested for the possibility of endozoochorous transport of *M. pratense* seeds: Birds (1 great tit—*Parus major* and 1 house sparrow—*Passer domesticus*), rodents (2 common black rats—*Rattus norvegicus*, 1 djungarian hamster—*Phodopus sungorus* and 1 common vole—*Microtus arvalis*), leporine (10 domestic rabbits—*Oryctolagus cuniculus* f. *domesticus*) and ruminants (1 cattle—*Bos primigenus* f. *taurus*). Birds and rodents were fed with 200 fresh seeds, rabbits with ~2 kg of fresh plants carrying ripe capsules and the cattle with ~20 kg of fresh plants. The cattle droppings were collected into plastic bags for 3 days and checked for intact seeds. The droppings of the remaining animals were collected for 24 h and checked afterwards. The germination capability of retrieved seeds was tested by sowing them on two experimental sites with favourable vegetation and host plants (hazelnut shrubs and pine forest).

RESULTS

Seed preference

Ants preferred the seeds of *M. pratense* to *M. subalpinum* (Fig. 1). The results of both statistical methods (ANOVA and GLMM) are highly congruent. The difference between species is highly significant ($P << 0.001$) in both ANOVA (Table 2) and GLMM. On average, 53.7% of *M. pratense* and 34.2% of *M. subalpinum* seeds were removed after 90 min. The effect of marking

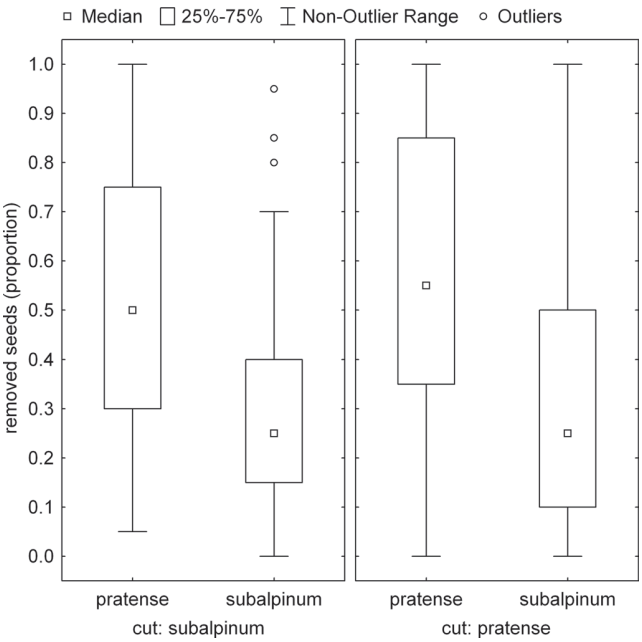

Figure 1: average rate of removed seeds. Left-hand chart represents squares with *Melampyrum subalpinum* seeds cut, right-hand chart represents squares with *Melampyrum pratense* seeds cut. Central points indicate medians, boxes are 25–75% quantiles, whiskers non-outlier range, outliers are depicted as separate points.

Table 2: ANOVA of proportion of removed seeds

Factor	Effect	Sum of Squares	df	*F*	*P*
Species	Fixed	2.710	1	113.027	1×10^{-15}
Cut	Fixed	0.056	1	2.340	0.130
Day	Random	2.649	2	15.993	0.004
Anthill (day)	Random	0.497	6	0.358	0.903
Square (anthill (day))	Random	18.736	81	9.647	1×10^{-15}
Error		2.110	88		

Fixed factors were identity of species ('species') and which species was marked by cutting ('cut'). Hierarchically nested random factors were sampling day, anthill and square. Raw data were arcsin transformed prior to the analysis.

seeds by cutting was insignificant (P = 0.130 in ANOVA and P = 0.122 in GLMM). The slight cutting of seeds thus did not influence seed recognition or preference in the ants.

Dispersal distance

We discovered 179 seeds outside the starting plot 7 h after the beginning of the experiment (Fig. 2). The average distance of all removed seeds was 9.2 m, 90% of seeds were found <15 m away. The maximum distance was 36.5 m. Seeds were intact or had partly bitten off elaiosomes; however, none were completely without the elaiosome.

Apart from ants, the dispersal of *Melampyrum* seeds by harvestmen, *Oligolophus tridens* (Opilionida), was recorded repeatedly (16 observations). The longest distance a harvestman was found carrying a seed was 18.6 m from the starting plot. However, it is not clear whether the harvestman carried the seed the whole distance or just found a seed dropped by ants.

Endozoochory

All experimental animals consumed the provided *M. pratense* seeds or plants willingly. No seeds were found in the droppings of the tested animals except for that of the cattle, where we found several hundred seeds. It is obvious that when seeds were not damaged mechanically by chewing, subsequent digestive processes did not damage them either. The only parts that were digested from all the seeds were the elaiosomes. About two-thirds of the seeds in the cattle droppings were blackened, which is a clear sign of degradation in *Melampyrum*, and seeds would thus probably be unable to germinate. Blackening of seeds was most probably caused by 3-day storage in plastic bags during collecting of droppings, which caused overheating of seeds. However, approximately one-third of the seeds seemed to be intact with no changes in colour.

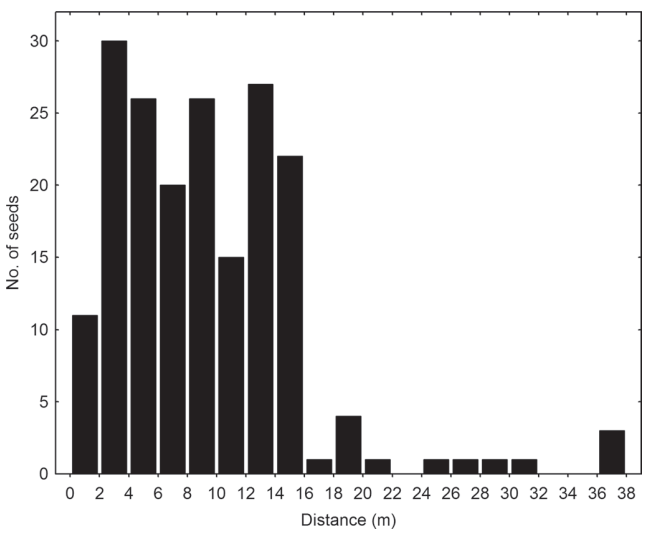

Figure 2: histogram of distances *Melampyrum* seeds were dispersed to in 7 h.

Approximately 60 seeds with no colour changes were sown on experimental sites and their germination was checked during March and May 2011. Unfortunately, none of the sown seeds germinated.

DISCUSSION

Seed preference

The studied species, *M. pratense* and *M. subalpinum*, markedly differ in their population sizes. The discovered preference of *M. pratense* seeds in ants suggests that myrmecochory may be one of the factors that play a role in the diversification of population sizes between the studied species.

It is known that during foraging ants choose from various food sources the most nutritionally effective or simply the most attractive one (Peters *et al.* 2003). Ants adapt their foraging strategy according to elaiosome size (Manzaneda *et al.* 2009; Peters *et al.* 2003), elaiosome size to seed size ratio (Edwards *et al.* 2006), the nutritional composition of the elaiosome (Boulay *et al.* 2006; Fischer *et al.* 2008) and chemical attractants or pheromones contained in the elaiosome or seed (Pfeiffer *et al.* 2009). Gómez *et al.* (2005) suggest that elaiosomes are important also as handles during removal of seeds by ants. However, in the case of *M. pratense* and *M. subalpinum*, average elaiosome size including seed size and shape widely overlap (Štech 2000), which excludes possible handling difficulties in one of the species. Hence, ants probably choose according to nutritional composition or chemical attractants.

A less frequent opinion suggests that the attractiveness of seeds may also depend on ontogenetic or phylogenetic historical constraints (Johnson 1991; Roces 1994). The experience of handling seeds of certain species by ants is in some cases known to increase seed removal rates (Gorb and Gorb 1999; Johnson 1991). On our experimental site, *M. pratense* plants can be found ~100 m from the studied *F. polyctena* colonies and, therefore, ants probably have some historical or recent experience with the seeds of this species, and thus may prefer them to the rare, and in the study area absent, *M. subalpinum* (the nearest locality is ~20 km from the study site). To the contrary, Peters *et al.* (2003) suggest that historical constraints in seed preferences may play a role in exceptional cases, but more importantly seed attractiveness depends on the diaspore characteristics that ants evaluate to increase foraging efficiency. This theory is also supported by Boulay *et al.* (2006) who noted that free fatty acid content and composition in *Helleborus foetidus* seeds from two 750-km-distant populations differ markedly and that ants from both localities, contrary to the historical constraint theory, prefer seeds from only one constant locality. In our removal experiments, seeds of both species in the mixed seed samples were from close or overlapping populations just as from very distant populations. In all cases, *M. pratense* seeds were prefered.

Habitat requirements of both studied species are highly similar, though there is a range of minor factors that can together lead to different population sizes in studied species.

Based on our field experience, there may be a small difference in the light requirements of the two species (*M. pratense* being slightly more shade tolerant). Nevertheless, this minor difference itself cannot fully explain such heterogeneity in population sizes between the two species.

From the acquired results, we conclude that the difference in population sizes of *M. pratense* and *M. subalpinum*, particularly on common sites, is apart from other factors influenced also by differences in seed preference during their dispersal by ants. The common occurrence of both species possibly has influence on *M. subalpinum* dispersal. In the frequent event of the ants having enough seeds of the preferred *M. pratense*, there is much smaller interest in the less attractive *M. subalpinum* seeds, which will suffer from lower removal rate and possibly remain mostly under parental plants.

Together with population sizes also distribution areas of both studied species differ markedly (Fig. 3). *Melampyrum pratense* is widespread almost all over Europe (apart from the most southern parts), at its easternmost extreme reaching the Altai Mountains (Meusel *et al.* 1978; Štech 2000). In contrast, *M. subalpinum* has a patchy distribution limited only to Central Europe (Hadač 1966; Hartl 1974). While we assume only a marginal role of observed preference of *M. pratense* in the formation of total distribution area, differences in seed preference may be one of the important factors shaping internal structure of the distribution area (e.g. number of localities, their population size and connectivity, etc.).

Dispersal distance

Dispersal distances (both maximum and average) found in the present study rank among the longest known myrmecochorous removals of temperate forest plant seeds. Cain *et al.* (1998) observed the long-distance carries of *Asarum canadense* seeds for up to 35 m and refers to these observations as the largest distance ants are known to move seeds of any woodland herb. Our observation exceeds this distance by another 1.5 m and compared to the *Melampyrum pratense* maximum distance (Heinken 2004), our observation was almost 30 m longer. Contrary to Cain *et al.* (1998), our starting plot was placed next to a large ant trail, which certainly facilitated the long-distance dispersal of seeds.

Sernander (1906) mentions a myrmecochorous dispersal of up to 70 m (acquired by direct observation of *Formica rufa* workers carrying the seed) for *Melica nutans* and *Viola hirta*, which would rank among the extreme even for arid zones (Gómez and Espadaler 1998). Even though Heinken (2004) questions Sernander's (1906) observations, our results suggest that such dispersal distances may be possible given enough time and optimal conditions (e.g. large system of ant colonies, flat terrain with sparse vegetation, long foraging ant trails, large ant species).

Important factor in myrmecochorous dispersal is also ant size, which is strongly correlated with dispersal distance (Ness *et al.* 2004). Our study included one of the largest European ant species *F. polyctena* and, therefore, dispersal distances in our experiment were expected to reach rather high values. (We did not observe any other ant species in the locality.) The large *Formica* species (*Formica* s. str.) are common in localities of both studied *Melampyrum* species; hence, similar distances may be frequent. Sernander (1906) observed the 70-m dispersal by *F. rufa*, which is closely related to *F. polyctena* and has similar body size. Unfortunately, Cain *et al.* (1998) did not mention which ant species was the disperser in their study; however, larger ants such as *Formica* s. str. are highly probable.

Although most studies state mean dispersal distance by myrmecochory from ~1 m up to 5 m (Andersen 1988; Cain *et al.* 1998; Gibson 1993a; Gómez and Espadaler 1998; Heinken 2004; Heinken and Winkler 2009; Ohkawara *et al.* 1997), in our experiment, all seeds outside the starting plot ($n = 179$) were dispersed in ~7 h to an average distance of 9.2 m (Fig. 2). About 10% of all seeds were found >15 m away. These values are probably influenced by the very dense system of *F. polyctena* colonies in the area. Some studies are also based on a different method in which the dispersal distance of seeds is estimated indirectly by counting newly appeared plants next year (e.g. Heinken 2004; Heinken and Winkler 2009; Ohkawara *et al.* 1997). With increasing distance, the number of dispersed seeds decreases significantly. Together with the generally low germination success and seedling viability of *Melampyrum* (Smith 1963), the later method indicates rather 'typical' dispersal distances in which there is reasonable probability of finding an established plant. On the contrary, the direct tracking method using fluorescence-marked seeds is more suitable in documenting occasional extreme values. However, our results are still exceptional even among studies using direct observations (Andersen 1988; Cain *et al.* 1998; Gómez and Espadaler 1998). Despite the fact that seeds were placed in the vicinity of an ant trail leading to a large anthill ants dispersed seeds in all directions. Seeds were often dispersed in the opposite direction from the anthill (up to 27.3 m from the starting plot) into the area where no other *F. polyctena* colony was present.

Even though ants are the main dispersers of *Melampyrum* seeds on a local scale, they are not exclusive. Seeds can also be carried by carabid ground beetles (Carabidae), which feed on elaiosomes (e.g. Gibson 1993b). We have found a new arthropod disperser of *Melampyrum* seeds—the harvestman *Oligolophus tridens* (Opiliones). Harvestmen are preferably predators of small invertebrates; however, they are also known to scavenge dead animals and plant matter (Halaj and Cady 2000). The reason for their interaction with *Melampyrum* seeds is therefore probably the nutritious elaiosome. The fact that harvestmen moved seeds quite long distances is apparently caused by the presence of large numbers of aggressive ants, which they tried to avoid.

Endozoochory

The genus *Melampyrum* is usually considered as solely myrmecochorous (Hartl 1974) and no other means of seed transport has been described so far. However, the recent

wide distribution of some *Melampyrum* species, such as *M. pratense* (Fig. 3), is in conflict with the slow dispersal speed achieved by myrmecochory (Gibson 1993a; Heinken 2004). Dispersal distances needed to achieve the total distribution of *M. pratense* during Holocene clearly exclude myrmecochory. Anemochory and epizoochory are impossible due to rather large oval seeds with smooth surface. Therefore, the endozoochorous long-distance transport of seeds is self-evident. Contrary to Heinken's (2004) opinion, our pilot study shows that *M. pratense* seeds are able to pass through the digestive system of a cattle physically intact and only a minority of them are damaged by chewing. The rest of the tested animals are probably highly specialized for seed digestion (birds) or they grind food too finely (small rodents and rabbits) and are therefore inadequate in endozoochorous transport of *Melampyrum* seeds.

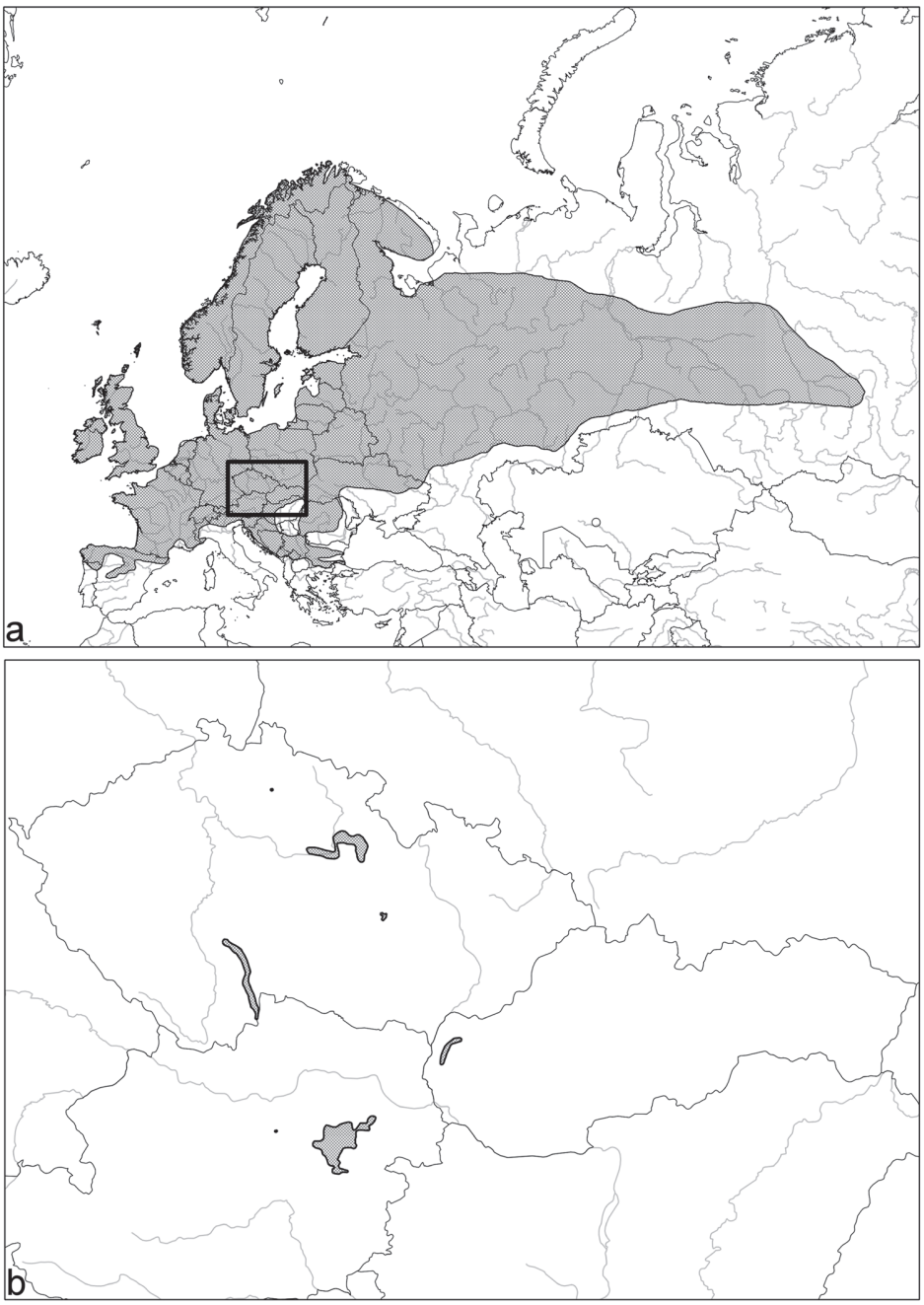

Figure 3: distribution area of *Melampyrum pratense* (A) and *Melampyrum subalpinum* (B). The rectangle in the map (A) delimits the region displayed in the map (B). Maps were processed in Quantum GIS software (QGIS Development Team 2011).

The fact that none of the seeds acquired from the cow droppings germinated may be the consequence of a number of factors and does not necessarily mean that seeds were damaged by the digestive processes. The artificial germination and cultivation of *Melampyrum* plants is a rather sensitive process and it is not always successful (Smith 1963). It is known that *Melampyrum* seeds exhibit much higher germination success if they are ant-mediated planting (Gibson 1993a). In the case of slightly inconvenient conditions (e.g. higher temperature, non-optimal moisture), seeds tend to blacken, which is a sign that they are no longer capable of germination. The blackening of a large part of the seeds in our experiment was probably a result of their 3-day-long storage in plastic bags, in which they probably became overheated. The fact that the seeds were physically intact and the colour of some of them was unchanged is, in our opinion, an indication that *Melampyrum* seeds are at least partly able to pass intact through the digestive tract of ruminants. Furthermore, *M. pratense* was also previously recorded as germinating in the dung of cattle (Stender *et al.* 1997), which strongly supports the possibility of endozoochorous dispersal. Based on our observations, *Melampyrum* plants are also very often grazed by deer. This suggests that large ruminants such as deer, moufflon, bison, or forest-grazing livestock can be important long-distance dispersers of some *Melampyrum* species, whereas ants are important for the removal of seeds on a local scale. Relatively high number of seeds obtained from droppings of a single cow suggests that endozoochory can be rather common event in *Melampyrum*; it is promising hypothesis for further studies. Endozoochory thus can offer an elegant explanation for the long-distance migration of some myrmecochorous plants during the Holocene period or in the event of the loss or formation of new favourable habitats (Cain *et al.* 1998; Heinken 2004; Heinken and Winkler 2009).

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Paper 10: Těšitel J. & Štech M. (2007): Morphological variation in Melampyrum sylvaticum group within the transitional zone between Melampyrum sylvaticum L. s. str. and Melampyrum herbichii Woł. – Preslia 79: 83–99.

Morphological variation in the *Melampyrum sylvaticum* group within the transitional zone between *M. sylvaticum* s. str. and *M. herbichii*

Morfologická variabilita *Melampyrum sylvaticum* agg. v přechodné zóně mezi *Melampyrum sylvaticum* s. str. a *Melampyrum herbichii*

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The *Melampyrum sylvaticum* group is a complex of three closely related species. This group is most variable in the Carpathian region. Interactions among different levels (within-population to interspecific) of genetic variation and influence of the environment are considered to be the main sources of the complicated morphological variation in this region. Morphological variation in the *M. sylvaticum* group was studied in mountain ranges of the Hercynian Massif and in the Western and Ukrainian Carpathians. Several populations were sampled at different altitudes within each mountain range. Hierarchical partitioning of morphological variation at different levels (within populations, among populations within a mountain range and among mountain ranges) was calculated. Correlations among groups of morphological characters and altitude were calculated. The largest proportion of variation on a large geographic scale (i.e. among mountain ranges) was detected in anther length and several corolla characters (length of the lower corolla lip, height of upper corolla lip), whereas these traits were homogeneous at a local scale (within populations and among populations in one mountain range). An opposite pattern (i. e. high proportion of variation at the low levels, which blurred possible large scale differences) was found in bract traits and several calyx characters. Moreover, a strong correlation between bract length and altitude was observed. The observed changes in the proportions of morphological variation and response to altitude suggest a close connection between bract characters and environmental factors (or lower levels of genetic variation). On the other hand, some of the flower characters seem to be genetically determined and thus might reflect evolutionary processes (early diversification, potential hybridization, introgression) on which the taxonomic treatment of the group should be based. The most distinct differences were detected between samples from the Ukraine and south-western part of Bohemia. Populations from the the Sudeten Mts and the Western Carpathians were variable and morphologically intermediate, forming a continuum between the two extremes.

Keywords: *Melampyrum herbichii*, *Melampyrum sylvaticum*, morphometrics, Sudeten Mts, taxonomy, variance components, Western Carpathians

Introduction

The genus *Melampyrum* L. belongs to the family *Orobanchaceae* (Olmstead et al. 2001), which consists of species mostly having a (hemi)parasitic life cycle. Diversification of individual microspecies within relatively distinct species complexes in most of the genera is supposed to have taken place in the late Pleistocene. Quaternary climatic cycles (glacial/interglacial periods) and consequent migration are thought to have played an important role in the diversification and distribution pattern of individual microspecies (Wesselingh & van Groenendael 2005). Their recent origin by various evolutionary pro-

cesses (hybridization, introgression) may complicate the identification and delimitation of particular taxa (Wesselingh & van Groenendael 2005).

Three probably closely related species are distinguished in the *M. sylvaticum* group. These species have usually been delimited from each other on the basis of flower traits, especially length of the anthers, colour and size of the corolla (Baumgarten 1816, Wołoszczak 1888, Jasiewicz 1958). Typical specimens can be classified appropriately using these characters. *Melampyrum sylvaticum* L. is characterized by a yellow and relatively small corolla, (5–) 7–9 (–11) mm long and the shortest anthers, (1.4–) 1.6–2.1 (–2.3) mm long, within the group (Jasiewicz 1958). This species is the most widespread of the three microspecies. Its geographical range corresponds to the range of the whole group, i.e. it covers the European boreal zone and mountains in the European temperate zone from the Pyrenees to the Urals (Meusel et al. 1978).

Melampyrum herbichii Woł. is similar to the previous species, particularly in corolla colour. The length of the anthers and corolla, which reach (2.0–) 2.4–3.6 (–4.2) mm and (7.0–) 9.0–12.5 (–14.0) mm respectively, are supposed to be the main diagnostic characters differentiating this species from *M. sylvaticum* s. str. (Jasiewicz 1958) although significant overlaps are obvious in both characters. This species was first described from three localities in the Ukrainian Carpathians by Wołozsczak (1888). The description of the species is based on samples from three localities including the Hoverla massif in the Chernogora region. Its geographical range was believed to be restricted to the Eastern and Southern Carpathian region (Soó & Webb 1972). However, populations classified as *M. herbichii* are reported from the regions of the Western Carpathians and the Sudeten Mts (e.g. Jasiewicz 1958, Šípošová 1997, Štech 2000) together with specimens featuring diagnostic characters with values transitional between *M. herbichii* and *M. sylvaticum* (Štech 1998, Štech & Drábková 2005). In addition, significant variation in bract shape has been detected in samples from this region (Štech 1998). Bract morphology was investigated in the most recent studies (Štech 1998, Štech & Drábková 2005) suggesting that bract proportions can be used to discriminate between species in the transitional (potential hybrid) zone. Beside the morphological differentiation, a significant difference in habitat preferences between *M. herbichii* and *M. sylvaticum* is described by Šípošová (1997). According to this taxonomical treatment, the habitat preferences of *M. sylvaticum* are supposed to be relatively wide, as it grows in mountain spruce forests up to the dwarf-pine communities at the tree-line. By contrast, *M. herbichii* appears to be restricted to montane meadows around the tree-line (Šípošová 1997). However, Jasiewicz (1958) also reports *M. herbichii* from forests at lower altitudes.

The third species, *M. saxosum* Baumg., is characterized by a white corolla. Quantitative morphological characters seem to overlap completely those of *M. herbichii*; thus the corolla colour is the only diagnostic trait separating these two species (Jasiewicz 1958). *Melampyrum saxosum* occurs in the Eastern (and probably Southern) Carpathians (Soó & Webb 1972), any overlap with the other species reported elsewhere can be considered as a misidentification (Štech 2000).

Although several authors have attempted to elucidate and interpret morphological variation in the *M. sylvaticum* group (the exact delimitation between *M. herbichii* and *M. sylvaticum*, and relationships among transitional populations are usually considered to be the most important questions), their effort have never been completely successful, because a few factors confuse the large-scale morphological variation gradient. A high

phenotypic plasticity is expected to have a great impact on the overall morphology of plants; however its impact is unlikely to be the same for all morphological traits. So-called seasonal variation, a specific type of low-scale genetic variation affecting plant architecture, characteristic of many hemiparasitic *Orobanchaceae* (Wettstein 1895, Sóo 1926–1927, Zopfi 1993a, 1993b, 1995, 1997, 1998a, 1998b), is another phenomenon with considerable influence on morphology. Seasonal variation produces polytopic locally adapted ecotypes, which differ primarily in stem internode number (Zopfi 1993b); however, some flower and bract traits might be directly correlated with this character. Phenotypic plasticity together with seasonal variation and their interactions produce local gradients in morphological variation, which can interfere with large geographical gradients in variation resulting in the geographical distribution of individual morphotypes showing a very complex pattern (Štech & Drábková 2005, Těšitel 2005). Thus, neglecting the low-scale variability of phenotypic factors (e.g. building a taxonomical treatment of the group on the basis of sampling a single population within individual mountain ranges) may lead to biased conclusions.

There exist many environmental gradients that may affect the morphological features of specimens of the *M. sylvaticum* group (e.g. light conditions, climate, host plant species and competition with other species). But the major factors are directly connected to altitude and seasonal variation (Štech 1998, Těšitel 2005). The altitudinal gradient is suitable for direct analyses and easy to interpret, which is why this variable was chosen as a reference for gradients in low-scale morphological variation in this study.

The aim of the current study is to elucidate patterns in morphological variability in the *M. sylvaticum* group in the Western Carpathian and Sudeten regions, where transitional morphological types between *M. sylvaticum* and *M. herbichii* prevail (typical specimens of individual species were also included for reference). Detailed analysis of variation in morphological traits is a crucial part of this assessment. It should help to exclude the characters, which were considered discriminatory by previous authors but display significant low-scale variation. Consequent analysis of geographical distribution of morphotypes can be used as a basis for formulating phylogeographic hypotheses and a taxonomic treatment of the group. Population sampling differed slightly and was more complex than in other recent studies (Štech 1998, Štech & Drábková 2005). The objective of this was to obtain a more robust data set suitable for such a rigorous assessment.

Material and methods

Material

Morphometrical data were collected from 24 populations (658 plants) of the *M. sylvaticum* group within the transitional zone between *M. sylvaticum* and *M. herbichii* (Fig. 1 and Appendix 1). The localities were selected to cover the whole transitional zone including typical populations of the microspecies. Several populations within a homogeneous geographically-defined mountain range were sampled in order to evaluate the distribution of variation among these geographical units (further referred to also as regions) and among populations within them. Populations from the Šumava Mts and the Brdy massif were combined into one region named "South and Central Bohemia" due to the low number of localities studied (two and one, respectively) and overall morphological similarity among the

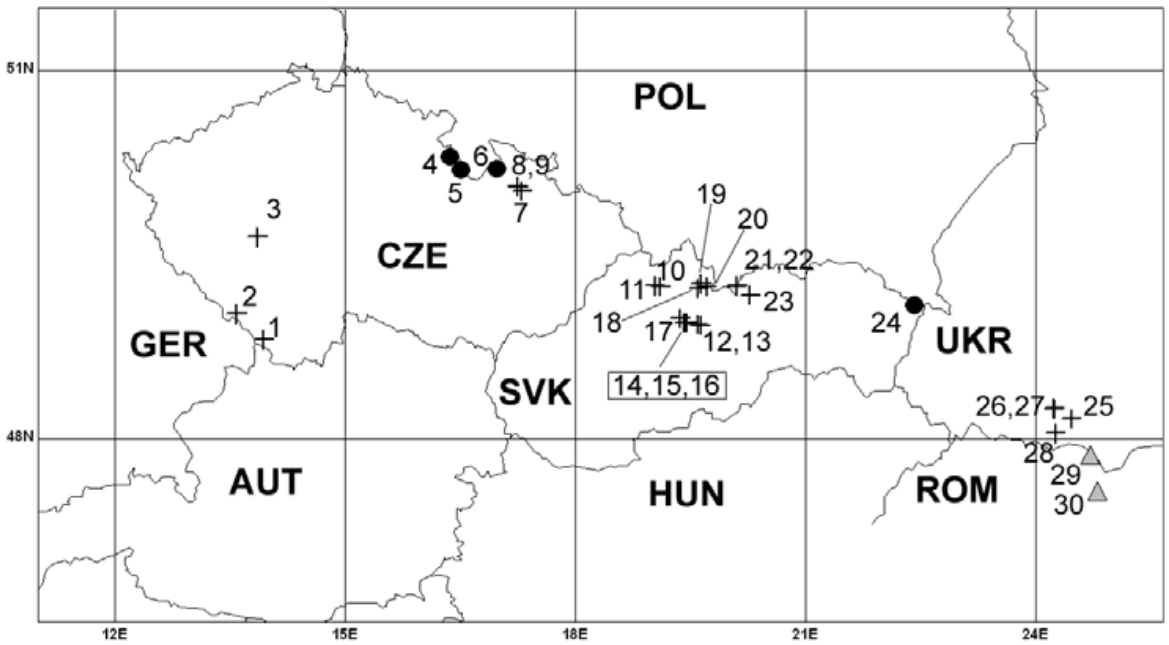

Fig. 1. – Map of the localities of the *Melampyrum sylvaticum* group included in this study. Borders of Central European countries are also displayed. CZE – Czech Republic, SVK – Slovakia, UKR – The Ukraine, ROM – Romania, HUN – Hungary, AUT – Austria, GER – Germany, POL – Poland. + populations included in morphometric analyses, ● additional populations referred to in Discussion ▲ populations of *M. saxosum*.

plants growing in these districts, which were always classified as typical *M. sylvaticum* (Štech 2000). Considering the other end of the geographical gradient, all the population samples collected in the Ukrainian Carpathians are a priori classified as *M. herbichii*, as this species was described from this region (Wołozsczak 1888). Beside the specimens included in the analyses, several additional populations were included in a survey of the geographical distribution of variation. These are the specimens collected in the Orlické hory Mts and the Rychlebské hory Mts, for which data on bract shape are lacking, and populations of *M. saxosum*, which were used as a reference. The sampling was conducted over a short period of time at the start of the flowering season in order to minimize the influence of phenological divergence on the morphology of the flowers (1st to 3rd, exceptionally 4th lowermost flower of all the plants were taken and analyzed). The only exception was the sample of plants from the Bukovské vrchy Mts, which were collected at a later onthogenetical stage and 5th or 6th lowermost flower had to be taken.

A population sample of 21–30 (– 38) plants were collected at every locality (see Appendix 1 for the exact number at each locality). The calyx and corolla of one flower of each plant were put into an Eppendorf-tube filled with ethanol and stored until measured. The first and the fifth bracts were stuck on a sheet of paper using a transparent tape. The other parts of the whole plants were processed as standard herbarium specimens and are kept in the herbarium of the Faculty of Biological Sciences, University of South Bohemia (CBFS).

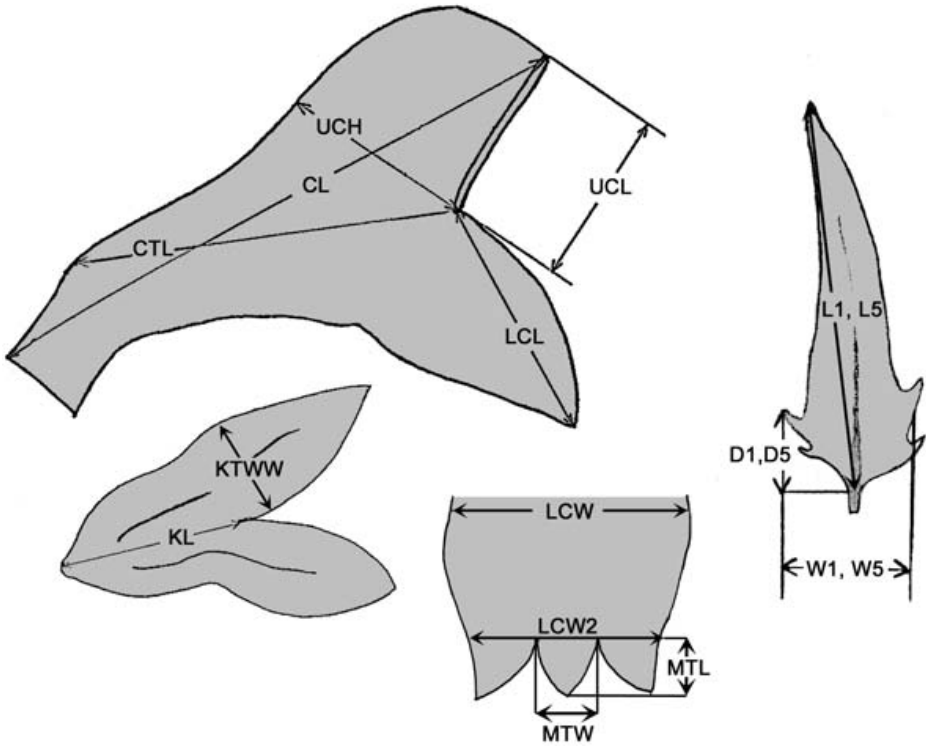

Fig. 2. – Morphological characters of the corolla, calyx, lower lip of corolla and bracts. See the "Morphological characters" paragraph in the methods section for definitions.

Morphological characters

Morphological characters were obtained by a series of measurements conducted on each plant. Twelve traits were measured on flowers (anther length – AL, corolla length – CL, length of the corolla tube – CTL, length of the lower corolla lip – LCL, length of the upper corolla lip – UCL, height of the upper corolla lip – UCH, width of the lower corolla lip – LCW, width of the lower corolla lip at the base of corolla teeth – LCW2, width of the middle tooth of lower corolla lip – MTW, length of the middle tooth of lower corolla lip – MTL, calyx tube length – KL, width of the upper calyx tooth at its widest point – KTWW) and three traits (length – L, width – W, distance of the widest part from its base – D) on the 1st and 5th bract (see Fig. 2). Some of the traits were not available in a few cases due to damaged material. This happened rather frequently for the fifth bract which was often not developed, particularly when the plants were sampled at an early ontogenetic stage (i.e. immediately after flowering).

Statistical analyses

Multivariate statistical techniques were employed to investigate the morphological variation in all characters together. An unconstrained ordination method, principal component analysis (PCA, Lepš & Šmilauer 2003), based on the matrix of correlations among the trait values (and thus centered and standardized data), was used to display general patterns in

the variation. The patterns were correlated with the altitude of the sites, the most obvious environmental gradient in the data, using a constrained ordination method, redundancy analysis (RDA, Lepš & Šmilauer 2003). Furthermore, Pearson correlation coefficients were calculated to quantify the relationships detected.

Proportional variation was estimated for single traits at different geographical levels by extracting the variance components from a linear mixed effect model (Quinn & Keough 2002). The regions and populations were random-effect terms in this model. Expected mean squares estimation (EMS) and restricted maximum likelihood estimation (REML; Quinn & Keough 2002, Pinheiro et al. 2005) were employed for processing multivariate and univariate data, respectively.

Multivariate statistical analyses were performed using Canoco for Windows, version 4.52 (terBraak & Šmilauer 2002). Variance components were computed with the R package, version 2.2 (R Core Development Team 2005), package nlme version 3.1-65 (Pinheiro et al. 2005). Statistica for Windows, version 6 (StatSoft 2001) was used for correlation analyses and other basic statistics.

Results

Overall variation in morphological characters

Within-population morphological variation of all characters accounted for 48.6% of the total variation. Regarding particular characters, considerable differences can be identified in their patterns of variation (Fig. 3, Table 1). A highly significant proportion of the variation among populations was detected in all traits (see fourth and fifth column in Table 1). However, the structure of this variation differed noticeably among individual traits. The length of anthers (AL) had the largest proportion of variation connected to the among-region level but the lowest proportion of within-regional variability and within-population variation. Some corolla traits (LCL, UCH, LCW, CL, and CTL) appeared to have a similar pattern of variation, but the proportion of within-population and within-regional variation was substantially higher. In contrast, there was no bract trait with a significant variance component based on the differences among regions. Variation in these characters was concentrated at the within-regional and within-population levels.

Two independent groups of traits can be distinguished in PCA plots based either on the variation among individual plants, or on the means of character values within particular populations (Figs 4 and 5, respectively). These show a rather strong correlation with the first or the second principal axes. The group correlated with the first axis is formed entirely by flower traits whereas the second group consists of bract characters. Populations from different regions appear not to be clearly separable from each other using these morphological traits, but there is only a very small overlap in the morphological features of the populations from the regions at the opposite sides of the gradient of the first PCA axis (Figs 5, 6).

Morphological variation in relation to altitude

Morphological variability induced by differences in environmental conditions was investigated by evaluating the relationships between morphometric trait values and altitude. Results of the RDA (Fig. 7) indicate that variation in most flower traits (except for LCW,

Table 1. – Likelihood-ratio test results for the variance components of individual morphological traits (see Fig. 3). Significant results (P < 0.05) are displayed in bold.

Morphological character	Variation among regions		Variation among populations	
	Likelihood-ratio	P	Likelihood-ratio	P
Anther length (AL)	37.13	**< 0.0001**	796.73	**< 0.0001**
Length of the lower corolla lip (LCL)	19.43	**< 0.0001**	583.01	**< 0.0001**
Height of the upper corolla lip (UCH)	13.96	**0.0002**	415.90	**< 0.0001**
Width of the lower corolla lip (LCW)	11.31	**0.0008**	448.99	**< 0.0001**
Corolla length (CL)	12.57	**0.0004**	490.30	**< 0.0001**
Width of the lower corolla lip at the base of corolla teeth (LCW2)	11.77	**0.0006**	410.41	**< 0.0001**
Length of the corolla tube (CTL)	10.44	**0.0012**	356.58	**< 0.0001**
Length of the upper corolla lip (UCL)	6.37	**0.0116**	558.78	**< 0.0001**
Width of the middle tooth of lower corolla lip (MTW)	5.28	**0.0216**	210.69	**< 0.0001**
Calyx tube length (KL)	5.76	**0.0164**	248.54	**< 0.0001**
Distance of the widest part of the 5th bract from its base (D5)	1.64	0.2007	250.44	**< 0.0001**
Length of the middle tooth of lower corolla lip (MTL)	2.37	0.1239	203.96	**< 0.0001**
Width of the upper calyx tooth at its widest point (KTWW)	0.65	0.4213	340.91	**< 0.0001**
Length of the 1st bract (L1)	0.45	0.5040	351.84	**< 0.0001**
Distance of the widest part of the 1st bract from its base (D1)	0.13	0.7139	294.17	**< 0.0001**
Length of the 5th bract (L5)	0.00	0.9440	322.26	**< 0.0001**
Width of the 5th bract (W5)	0.00	0.9995	283.73	**< 0.0001**
Width of the 1st bract (W1)	0.00	0.9995	300.59	**< 0.0001**

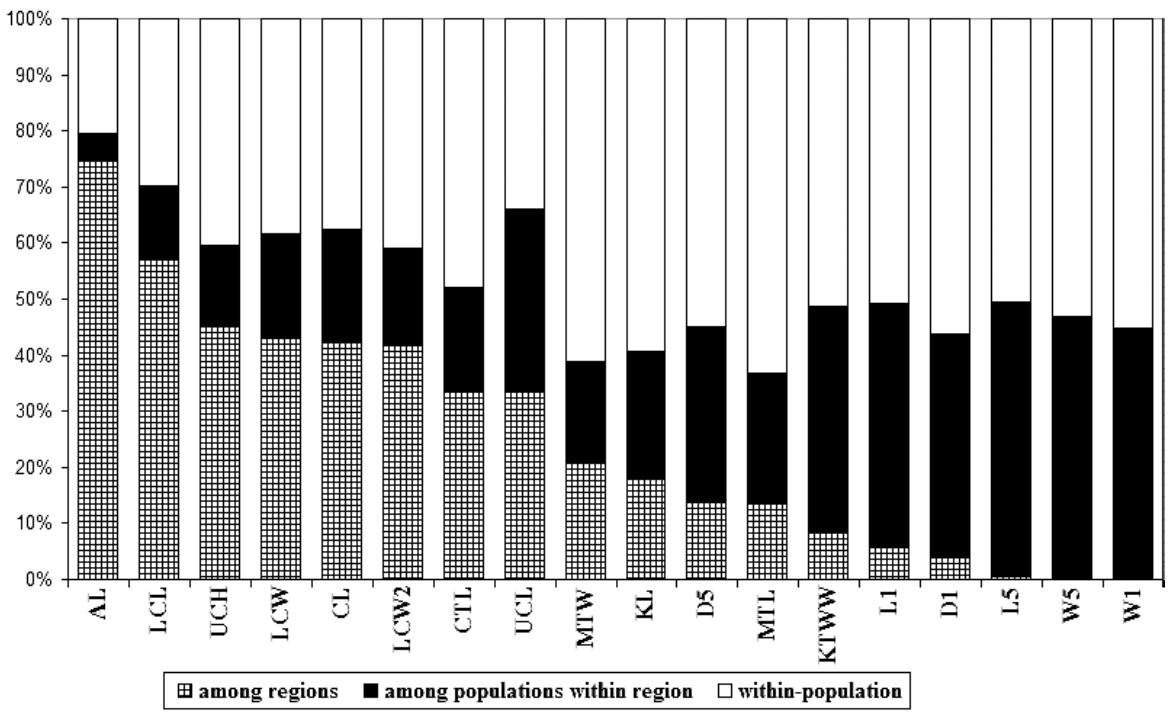

Fig. 3. – Variance components of individual characters corresponding to the hierarchical geographical levels. Variance component estimates are based on a random effect extraction from a linear mixed effect model using restricted maximum likelihood estimation (REML). See Table 1 for significance tests of the variance components. See Fig. 2 and the "Morphological characters" paragraph in the methods section for definitions of the traits.

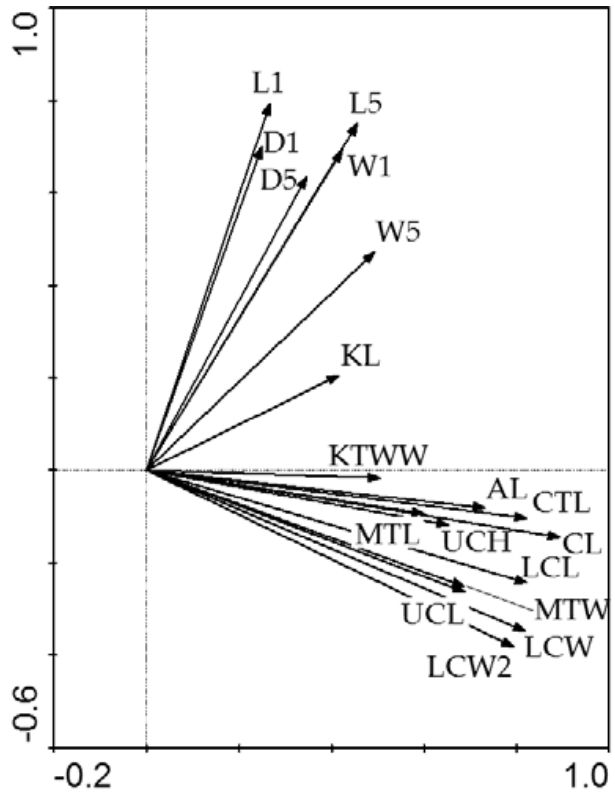

Fig. 4. – PCA plot based on individual plants. Directions of changes in morphological characters are displayed in relation to the first two principal component axes. The first and second ordination axes explain 39% and 18.6% of the variation, respectively.

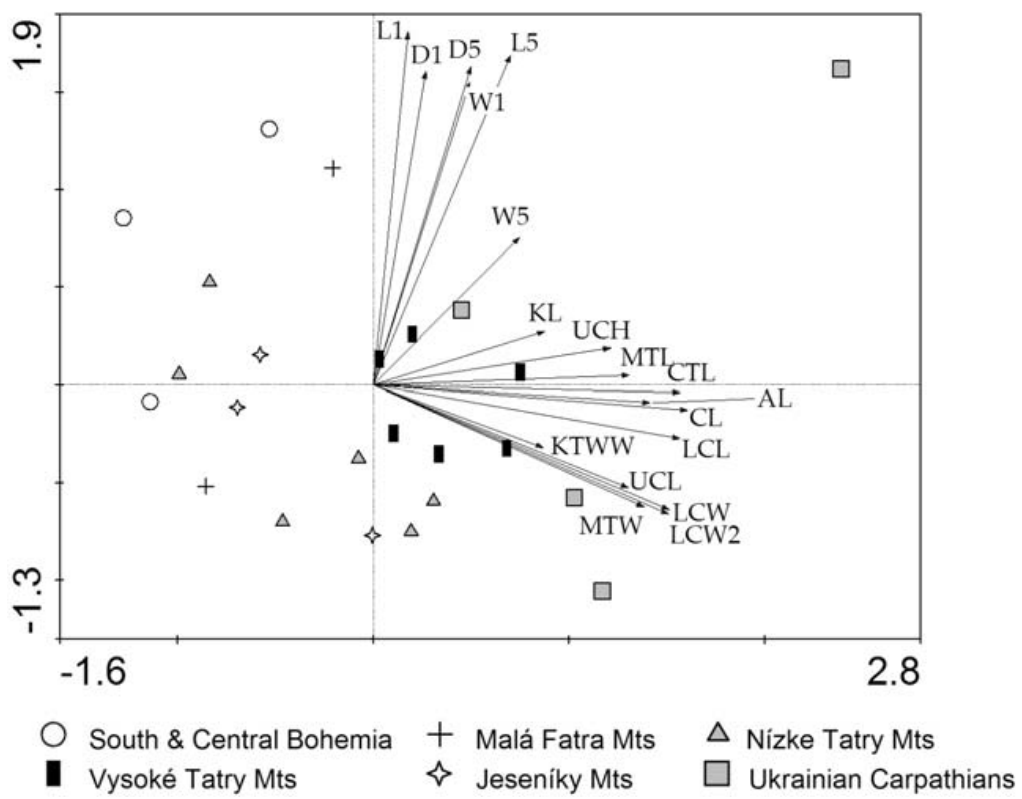

Fig. 5. – PCA plot based on means of character values within populations. Directions of changes in the morphological characters are demonstrated. Regional geographical distribution of the populations is depicted using different symbols for the ordination scores of individual populations. First two ordination axes are displayed. First axis explains 46.7% and the second 21.0% of the total variation.

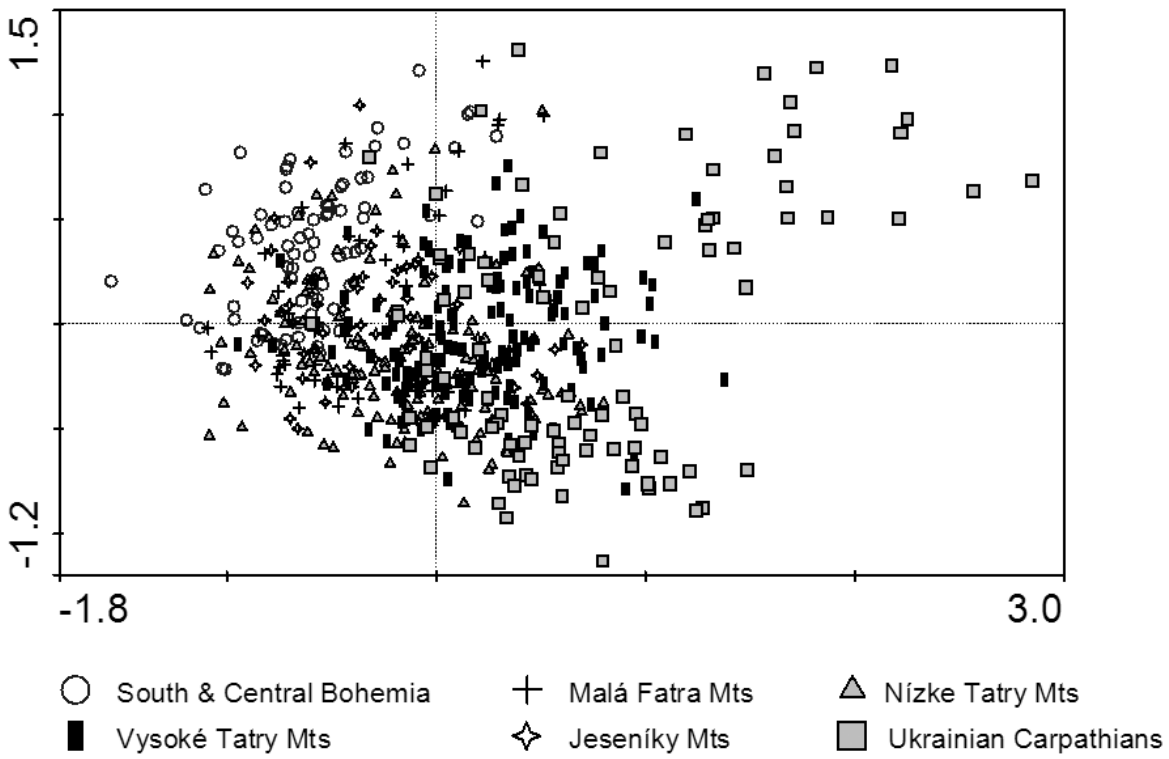

Fig. 6. – PCA plot of individual plants. Ordination scores of the plants are displayed. Regional geographical distribution of the specimens is displayed using different symbols for the ordination scores of individual plants. The first and second ordination axes explain 39% and 18.6% of the variation, respectively.

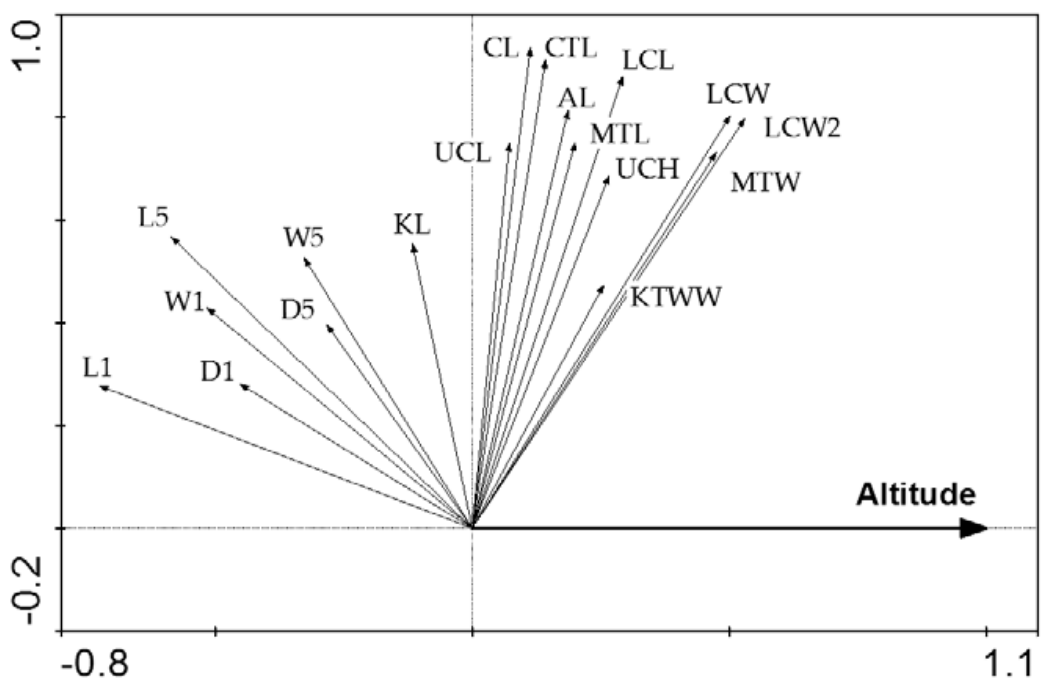

Fig. 7. – RDA plot describing directions of changes in morphological characters in relation to altitude. RDA is based on mean trait values for individual populations thus only among-population variation is considered. The constrained (horizontal) axis explains 14.6% of the variability. Monte-Carlo permutation test of significance of the canonical axis: F = 3.76, P = 0.012 (with 999 permutations).

LCW2 and MTW, for which there is a weak correlation) are independent of altitude, whereas bract traits (especially L1 and L5) seem to be correlated with this environmental variable. A more detailed survey of these relationships is provided by a correlation analysis (Table 2) confirming the pattern visible on the RDA plot (Fig. 7). A significant correlation was detected for most of the bract traits (except for W5 and D5), whereas LCW, LCW2 and MTW were the only flower characters for which there were significant correlations.

Table 2. – Pearson correlation coefficients (r) and significance of the correlation (p) between the means of morphological trait values within the populations of the *Melampyrum sylvaticum* group and altitude. Significant ($P < 0.05$) relationships are displayed in bold.

Morphological character	Correlation with altitude	
	r	P
Corolla length (CL)	0.11	0.5975
Length of the corolla tube (CTL)	0.14	0.5066
Length of the upper corolla lip (UCL)	0.07	0.7378
Height of the upper corolla lip (UCH)	0.27	0.2081
Length of the lower corolla lip (LCL)	0.29	0.1647
Width of the lower corolla lip (LCW)	**0.50**	0.0125
Width of the lower corolla lip at the base of corolla teeth (LCW2)	**0.53**	0.0077
Width of the middle tooth of lower corolla lip (MTW)	**0.47**	0.0191
Length of the middle tooth of lower corolla lip (MTL)	0.20	0.3480
Anther length (AL)	0.19	0.3821
Calyx tube length (KL)	–0.12	0.5887
Width of the upper calyx tooth at its widest point (KTWW)	0.26	0.2274
Width of the 1st bract (W1)	**–0.52**	0.0098
Distance of the widest part of the 1st bract from its base (D1)	**–0.45**	0.0265
Length of the 1st bract (L1)	**–0.73**	0.0001
Width of the 5th bract (W5)	–0.33	0.1182
Distance of the widest part of the 5th bract from its base (D5)	–0.28	0.1795
Length of the 5th bract (L5)	**–0.59**	0.0026

Discussion

Evaluation of morphological characters

The distribution of variation in particular morphological characters (Fig. 3, Table 1) provides an important baseline for further considerations. Assuming that a great genetic divergence manifests itself at the higher geographical levels, the traits that vary most at the among-mountain-range level are the only potentially appropriate diagnostic characters for delimiting *M. sylvaticum* and *M. herbichii*. Variation in the length of anthers (AL) and some of the corolla traits (LCL, UCH, LCW, CL and LCW2) appear to show such a pattern, whereas an opposite constellation was detected in most bract traits. The PCA plots (Figs 4, 5, 6) imply that the first principal axes on these diagrams correspond to higher-scale variation, whereas the second axes can be interpreted as lower-scale morphological variation gradients (resulting either from low-scale genetic variability or phenotypic plasticity).

In accordance with the analyses of overall variation, the correlation analyses comparing values of the morphological traits with altitude (Table 2, Fig. 7) suggest that the proportions of bracts (especially the length) and the width proportions of the lower corolla lip (LCW, LCW2, MTW) are mostly related to either phenotype plasticity or small-scale genetic variation. In contrast, the stability of anther length (AL) and lower corolla lip length (LCL) in relation to altitude confirms the results of previous analyses and suggests that these traits reflect high-scale genetic divergence more closely than any of the other characters.

This pattern of variation supports the delimitation of *M. sylvaticum* and *M. herbichii* on the basis of anther length, as found in previous studies (Jasiewicz 1958, Soó & Webb 1972). However, corolla length, another trait frequently used for determination (e.g. Jasiewicz 1958, Šípošová 1997), is obviously less robust in differentiating among populations from different regions than some other corolla characters. This might be caused by differences in the curvature of the corolla base (see Fig. 2) which adds some error variance in its values. Using another trait, the length of the lower corolla lip (LCL), instead of simple corolla length for classification seems to be more appropriate as this character appears to reflect geographical distribution pattern more precisely than any other corolla trait.

These conclusions do not agree with those of Štech (1998) and Štech & Drábková (2005). In these studies, within-population variation seemed to blur substantially the differences between individual species (even considering the traits considered diagnostic such as AL or CL), which led to a search for other traits, which can be used to delimit *M. sylvaticum* and *M. herbichii*. This disagreement can be explained by a difference in the sampling technique. As stated in the methods section, plant specimens for the current study were collected over a short period of time at the beginning of the reproductive season. Thus, the 1st – 3rd (exceptionally 4th) lowermost flower from the main inflorescence was collected for measurement. In the previous studies (Štech 1998, Štech & Drábková 2005), the sampling continued over the whole flowering season. Flowers for measurement were chosen randomly within the plant (i. e. flowers from higher nodes on the main inflorescence and from branch inflorescences were also processed). Hence, this flower trait values may have included additional variance associated with differences in the morphological features of flowers from different positions on a plant. This explanation is furthermore supported by the genetic variation detected by RAPDs (Štech & Drábková 2005). The proportion of within-population variation detected in the whole set of morphological characters in the current study coincides closely with that obtained by a RAPD marker analysis (48.6% versus 47.6% for RAPD data) but is considerably larger (68.5%) for the set of morphological characters analyzed by Štech & Drábková (2005).

Variation in the proportions of bracts needs detailed evaluation. The length of bracts (L1 and L5) does not seem to differ between the two species. However, this trait was found to have a great discriminatory power between the two species in the most recent study dealing with variation in the *M. sylvaticum* group (Štech & Drábková 2005). Such a discrepancy might be caused by slight differences in the environmental conditions experienced by the populations analyzed by Štech & Drábková (2005) and those analyzed in the current study. The populations chosen as training data-sets for classification by discriminant analysis by Štech & Drábková (2005) differed not only in their geographical distribution but also in the environmental conditions at the sites from which the samples were collected. The samples of *M. herbichii* from the Ukraine and the Bukovské vrchy

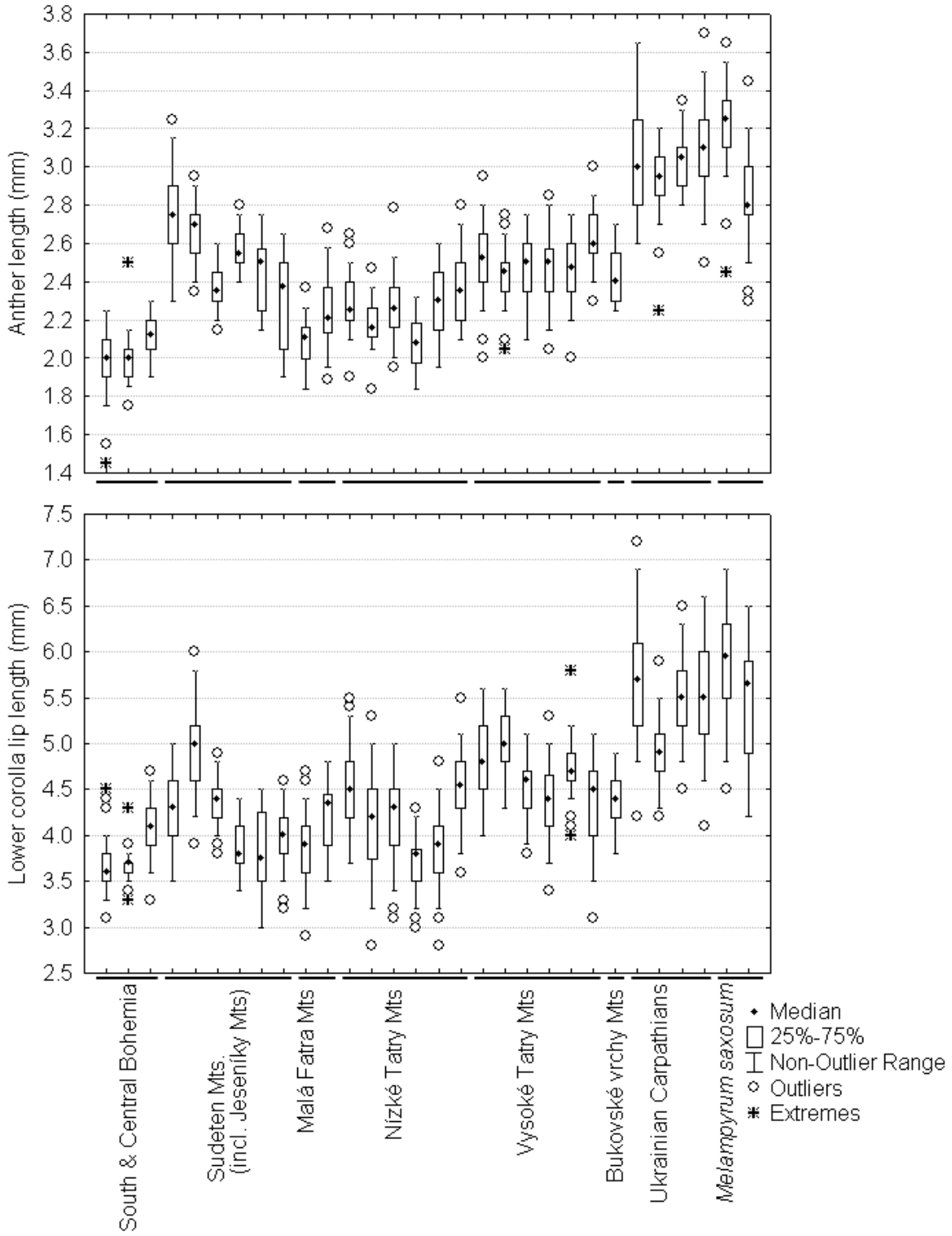

Fig. 8. – Box-and whisker plots displaying the values of anther length (AL) and lower corolla lip length (LCL) at individual localities in different regions. The populations are displayed along the horizontal axes in the order (from left to right) of the descriptions of localities in Appendix 1.

Mts included in that study were collected from alpine meadows at altitudes, mainly between 1150 and 1580 m a.s.l., whereas specimens of *M. sylvaticum* from the western group of localities (the Alps and Bohemian Forest) originated from both meadows and forests at altitudes between 500 and 1100 m a.s.l. (see Appendix in Štech & Drábková 2005). Two populations from the Alps, at 1600 and 1900 m a.s.l., were the only exceptions, but these included a very small number of plants (4 and 6, respectively) and had little influence on the coefficients of the discriminatory function. This difference in environmental conditions between the training data-sets must have biased the discriminatory function so that their consequent classification was not only based on the morphological divergence between the typical samples of the two species but also on the basis of morphological differences induced by the environment (altitude and habitat type). The a priori classification of plants from the Bukovské vrchy Mts as *M. herbichii* poses another problem because there is no evidence that these populations are closely genetically related to the Ukrainian populations of *M. herbichii*, they even seem to be morphologically more proximate to plants from the Vysoké Tatry Mts than to plants from the Ukraine (Fig. 8). It is obvious that other bract characters (W1, W5, D1, D5) are also strongly affected by various environmental conditions (e.g.the bract width is influenced by the presence of bract teeth, which was observed to be more frequent at sites exposed to direct sunlight; Štech 2000); however, it is still likely that they also reflect some large-scale genetic differences. Specimens of *M. sylvaticum* tend to have narrower bracts and the distance of the widest point of the bract from its base is usually greater than in *M. herbichii*, moreover significant differences are expected regarding frequency and shape of bract teeth (Štech 1998, 2000).

Obviously, conclusions on the pattern of variation in particular traits cannot be made based only on the data presented in this study. In particular, it is not possible to decide whether the low-scale variation detected is due to phenotypic plasticity or low-level genetic divergence (local adaptations), because these factors are correlated with each other. However, future transplantation experiments are expected to differentiate between phenotypic plasticity and genetically determined morphological variation and thus provide useful information.

Geographical distribution of variation

Regarding variation in the morphological characters (Figs 4, 5, 6), it is clear, that differences among populations form a continuum within which no obvious split can be identified. The Ukrainian populations appear the most diverse compared to the other regions. The population from Rakhiv (no. 28) is the most distinct not only from the other Ukrainian populations but from the complete set of populations studied. This could suggest some kind of differentiation from the other populations of the same region; however the morphological differences are based mainly on bract traits and some flower characters which are strongly intercorrelated, which increases the weight of a single independent measure. This biased the PCAs and made the differentiation more apparent than it is. Considering the traits reflecting large-scale variation (AL, LCL), the differentiation between the Rakhiv population and other Ukrainian populations is negligible (Fig. 8).

The largest differences in anther and lower corolla lip length can be found between populations from the Ukraine and the south-western part of Bohemia (Fig. 8). The Ukrainian plants, described as *M. herbichii*, had significantly higher values for both of these characters.

The overlap between the two morphological extremes was negligible. The other populations display intermediate values and form a continuum between them. Differentiation among these regions was substantially smaller, although same slight differences can be detected and interpreted. Plants from localities in the Vysoké Tatry Mts, Bukovské vrchy Mts, Orlické hory Mts, Rychlebské hory Mts and Jeseníky Mts appear to have longer anthers and lower corolla lips. In some of these populations, values of these traits reach those of Ukrainian plants. Thus, populations in these regions are slightly less distinct from *M. herbichii* than other populations. This supports the conclusions of previous studies (Jasiewicz 1958, Šípošová 1997, Štech 1998, 2000, Štech & Drábková 2005), which report plants similar to *M. herbichii* in the Western Carpathians and the Sudeten Mts (Jasiewicz 1958, Šípošová 1997). Lower values for both anther length and lower corolla lip length were detected in the population from the Bukovské hory Mts than reported by Štech & Drábková 2005. This can be explained by the sampling of plants at later onthogenetic stages (see methods section) and consequent analyzing of flowers from higher nodes (which are sometimes reported to differ slightly from the basal ones; Těšitel 2005).

The background of this pattern in morphological variation remains to be resolved. There are two hypotheses. The morphologically transitional populations may have arisen through hybridization between different groups of plants migrating from their glacial refuges in the early Holocene. The alternative explanation is based on the evolution of different morphotypes within isolated mountain ranges after the immigration of a common variable ancestor. Application of molecular tools is the only way to decide which of these hypotheses is correct because only a phylogeographic, genetically-based analysis can reconstruct the migratory pathways of particular groups of populations in the Holocene and explain their origin.

The stability of diagnostic traits within different mountain ranges and the lack of a correlation between their values and altitude suggest that there are hardly any differences in habitat preferences between *M. sylvaticum* and *M. herbichii*. Both species were found to grow in both montane forests and meadows at the tree-line, even though typical samples of *M. herbichii* growing in the Transcarpathian Region in the Ukraine seem to be rare at low altitudes. Considering transitional morphotypes from the Western Carpathians and Sudeten Mts, no ecological differentiation can be found between populations proximate to *M. herbichii* and populations similar to *M. sylvaticum*. Thus, the ecological differences between the two species reported in the past (Šípošová 1997) should be reconsidered. In contrast, the pattern of morphological variation and its connection to their ecology described by Jasiewicz (1958) appears to correspond well with the results of our study.

It is not possible to evaluate the morphological variation in *M. saxosum* on the basis of two population samples. The overlap in quantitative morphological characters with *M. herbichii* reported by Jasiewicz (1958) was recorded in these specimens. Difference in anther length between the two populations may suggest the possibility of a significant pattern of variation in *M. saxosum*.

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Souhrn

Melampyrum sylvaticum agg. patří mezi velmi variabilní a taxonomicky problematické skupiny flóry Střední Evropy. Komplikovaná morfologická variabilita je způsobena interakcemi mezi různými úrovněmi genetické variability a vlivy prostředí. Cílem této studie bylo oddělit jednotlivé úrovně variability a nalézt tak morfologické znaky, které vykazují malou variabilitu v lokálních měřítkách (na úrovni vnitropopulační či mezi populacemi v rámci jednoho pohoří), minimální závislost na podmínkách prostředí v různých biotopech a zároveň dostatečnou variabilitu na vyšší geografické škále. Geografická škála tak byla použita jako reference vyjadřující přibližně i genetickou rozdílnost mezi vzorky. Za účelem zjištění, jaké rozdíly v morfologii odpovídají jednotlivým geografickým úrovním (mezi homogenními, geograficky definovanými pohořími, mezi populacemi v rámci pohoří, uvnitř populací), byl proveden hierarchický rozklad variability jednotlivých znaků. Závislost morfologie rostlin na vlastnostech biotopu v němž rostou byla testována pomocí korelací mezi hodnotami znaků a gradientem nadmořské výšky, s nímž jsou těsně korelovány i další potenciální rozdíly mezi podmínkami prostředí v jednotlivých biotopech.

Délka prašníku a spodního korunního pysku se ukázaly jako znaky nejlépe odrážející variabilitu ve velkých měřítkách, což částečně odpovídá výsledkům některých starších studií (jako diagnostický znak se však tradičně používá celková délka koruny, její hodnota však vykazovala poměrně velkou vnitropopulační variabilitu). Hodnoty těchto znaků byly navíc dostatečně odlišné v různých regionech, což umožnilo provést zhodnocení geografického rozložení variability v populacích a vytvořit základ pro taxonomické hodnocení skupiny. Naopak se ukázalo, že převážná část variability ve tvaru listenů je zapříčiněna buď fenotypovou plasticitou anebo velmi lokálními genetickými rozdíly. Zároveň byla zjištěna i silná korelace znaků na listenech s gradientem nadmořské výšky.

Značné rozdíly v délkách prašníků a spodních korunních pysků byly zjištěny mezi populacemi *M. herbichii* z Ukrajiny a *M. sylvaticum* s. str. ze Šumavy a Brd. Populace ze Západních Karpat a Sudet dosahují v těchto znacích intermediárních hodnot, které v konkrétních případech poněkud blíží buď ukrajinským populacím (rostliny z Vysokých Tater a Sudet) anebo šumavským a brdským populacím (rostliny z Malé Fatry a Nízkých Tater). Mezi těmito skupinami však neexistuje žádná ostrá hranice, která by přerušila kontinuum v morfologické variabilitě. Otázka druhové klasifikace těchto rostlin tak zůstává otevřená, přestože v některých starších studiích jsou tyto rostliny přiřazovány k druhu *M. herbichii*.

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Appendix 1. – List of localities of the *Melampyrum sylvaticum* group studied. Those marked with asterisk are only discussed, not included in the analysis.

C z e c h R e p u b l i c . **Southern & Central Bohemia** (Šumava Mts and Brdy Massif): 1 – Šumava Mts, Ovesná: spruce forest next to the railway station; 48°48'26"N, 13°56'21"E, 740 m a.s.l.; 10. 7. 2004; 30 specimens analyzed. 2 – Šumava Mts, Kvilda: group of spruce trees on a knoll in valley of Kvildský potok stream 0.5 km E of the village; 49°01'04"N, 13°35'02"E, 1045 m a.s.l.; 11. 7. 2004; 29 specimens analyzed. 3 – Brdy Massif, Zalány: spruce forest at the N border of the village; 49°38'35"N, 13°51'25"E, 645 m a.s.l.; 12. 7. 2005; 26 specimens analyzed. **Orlické hory Mts** (Sudeten Mts): *4 – Deštné v Orlických horách: forest border between ski slopes ca 0.5 km S of the village; 50°17'49"N, 16°21'59"E, 730 m a.s.l.; 7. 6. 2003; 37 specimens analyzed. *5 – Rokytnice v Orlických horách: forest border ca 100 m N of the Hanička settlement ca 5 km NE of the town; 50°11'22"N, 16°30'38"E, 750 m a.s.l.; 7. 6. 2003; 38 specimens analyzed. **Rychlebské hory Mts** (Sudeten Mts): *6 – Velké Vrbno: forest edge ca 0.5 km W of the village; 50°11'56"N, 16°59'09"E, 840 m a.s.l.; 6. 6. 2003; 37 specimens analyzed. **Jeseníky Mts** (Sudeten Mts): 7 – Karlov: small forest on the left bank of the Moravice river in S part of the village; 50°01'12"N, 17°18'12"E, 670 m a.s.l.; 7. 7. 2004; 21 specimens analyzed. 8 – Karlov, Velká kotlina Valley: montane spruce forest ca 1.5 km SE of peak of Mt Vysoká Hole; 50°03'10"N, 17°14'52"E, 1110 m a.s.l.; 7. 7. 2004; 28 specimens analyzed. 9 – Karlov, Mt Vysoká hole: montane meadows on the E slope of the mountain; 50°03'31"N, 17°14'30"E, 1290 m a.s.l.; 7. 7. 2004; 30 specimens analyzed.

S l o v a k i a . **Malá Fatra Mts**: 10 – Mt Veľký Rozsutec: spruce forest on the N slope of the mountain; 49°14'20"N, 19°06'20"E, 1385 m a.s.l.; 22. 6. 2004; 29 specimens analyzed. 11 – Terchová: NW slope of the

Sokolie massif; 49°14'42"N, 19°02'11"E, 695 m a.s.l.; 23. 6. 2004; 28 specimens analyzed. **Nízke Tatry Mts**: 12 – Trangoška: margin of pathway in spruce forest between Trangoška settlement and Štefánikova chata chalet; 48°55'34"N, 19°37'58"E, 1375 m a.s.l.; 25. 6. 2004; 30 specimens analyzed. 13 – Trangoška: montane meadows on the S slope of Mt Chopok, ca 100 m N of the Kosodrevina Hotel; 48°55'57"N, 19°35'28"E, 1525 m a.s.l.; 26. 6. 2004; 28 specimens analyzed. 14 – Magurka: montane meadows near the summit of Mt Mestská hora E of the village; 48°56'51"N, 19°27'06"E; 1505 m a.s.l.; 28. 6. 2004; 29 specimens analyzed. 15 – Magurka: clearing in spruce forest on the W slope of Mt Mestská hora on E of the village; 48°56'46"N, 19°26'23"E, 1175 m a.s.l.; 28. 6. 2004; 24 specimens analyzed. 16 – Magurka: margins of a road next to Kapustisko settlement 1 km E of the village; 48°56'50"N, 19°25'09"E, 960 m a.s.l. ; 29. 6. 2004; 25 specimens analyzed. 17 – Lužná: montane shrubs on the summit of Mt Salatín above the village; 48°58'53"N, 19°21'47"E, 1615 m a.s.l.; 30. 6. 2004; 30 specimens analyzed. **Vysoké Tatry Mts**: 18 – Huty: montane forest near a starting point of a pathway leading to Mt Biela skala, ca 2 km E of the village; 49°13'24"N, 19°35'59"E, 930 m a.s.l.; 1. 7. 2004; 30 specimens analyzed. 19 – Zuberec: montane forest near Zverovka chalet ca 4,5 km E of the village; 49°14'33"N, 19°42'36"E, 985 m a.s.l.; 3. 7. 2004; 25 specimens analyzed. 20 – Zuberec: montane forest around road leading to Zverovka chalet, ca 1 km E of the village; 49°15'37"N, 19°38'10"E, 820 m a.s.l.; 3. 7. 2004; 29 specimens analyzed. 21 – Lysá Poľana: montane forest at a tourist shelter ca 3 km S of the village; 49°14'53"N, 20°06'07"E, 990 m a.s.l.; 4. 7. 2004; 25 specimens analyzed. 22 – Lysá Poľana: meadow at the road leading from the village to a gamekeeper's lodge, ca 3.5 km S of the village; 49°14'27"N, 20°06'05"E, 1005 a s. l.; 6. 7. 2004; 29 specimens analyzed. 23 – Tatranská Lomnica: montane spruce forest on the N border of the town; 49°10'11"N, 20°16'31"E, 910 m a.s.l.; 6. 7. 2004; 26 specimens analyzed. **Bukovské Vrchy Mts**: *24 – Runina: alpine pastures at Sedlo pod Ďurkovcom Saddle, 3.2 km NNE of the village; 49°05'08"N, 22°25'24"E, 1128 m a.s.l.; 8. 7. 2005; 29 specimens analyzed.

U k r a i n e . **Ukrainian Carpathian Mts**: 25 – Chernogora Mts, Lazeshchina: alpine pastures between Mt Hoverla and Mt Pietrosh ca 2.75 km W of the Hoverla summit, ca 12 km S of the village; 48°09'37"N, 24°27'50"E, 1570 m a.s.l.; 11. 7. 2003; 21 specimens analyzed. 26 – Svydovets Mts, Yasinya: alpine pastures at NE slopes of the Mt Blyznitsa ca 1.75 km N of the Blyznitsa summit, 48°14'25"N, 24°14'24"E, 1410 m a.s.l.; 12. 7. 2005; 30 specimens analyzed. 27 – Svydovets Mts, Yasinya: forest edge by ski slopes at tourist base ca 8 km W of the town; 48°14'50"N, 24°14'11"E, 1375 m a.s.l.; 12. 7. 2003; 29 specimens analyzed. 28 – Rakhiv: montane forest on slope ca 2 km ESE of the town; 48°02'36"N, 24°15'13"E; 950 m a.s.l.; 30. 6. 2005; 27 specimens analyzed.

Localities of *Melampyrum saxosum*:

*29 – Ukrainian Carpathians, Chivchin Mts, Burkut: alpine meadows at Mt Chivchin, ca 0.5 km N of the summit, ca 8.5 km S of the village; 47°52'09"N, 24°42'38"E, 1640 m a.s.l.; 9. 7. 2003; 26 specimens analyzed. *30 – Romania, Munţii Rodnei Mts: Staţiunea Borşa: N slope below mountain edge ca 6 km S of the village; 47°34'15"N, 24°48'00"E, 1848 m a.s.l.; 12. 8. 2004; 26 specimens analyzed.

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Variation in the *Melampyrum sylvaticum* group in the Carpathian and Hercynian region: two lineages with different evolutionary histories

Variabilita *Melampyrum sylvaticum* agg. v karpatské a hercynské oblasti: dvě odlišné evoluční linie

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Těšitel J., Malinová T., Štech M. & Herbstová M. (2009): Variation in the *Melampyrum sylvaticum* group in the Carpathian and Hercynian region: two lineages with different evolutionary histories. – Preslia 81: 1–22.

We investigated variation in the *Melampyrum sylvaticum* group in the Carpathian and Hercynian regions using morphological and molecular tools. The aim of our study was to examine differences in the pattern of variation between the Eastern Carpathians and region of the Western Carpathians and the Hercynian Massif. We also tested correlations between putatively taxonomically important variation in corolla colour present in the *Melampyrum sylvaticum* group in the Eastern Carpathian region and other morphological and molecular traits. Samples were collected from populations of the *M. sylvaticum* group in the Hercynian Massif and the Eastern and Western Carpathians. Morphometric analyses of the size and shape of the corolla (based on thin plate spline with sliding semilandmarks), length of the anthers and especially molecular analyses based on sequencing the nuclear ITS and *trn*L-*trn*T regions of chloroplast DNA, confirmed that the populations occurring on the opposite sides of the Eastern-Western Carpathian biogeographic boundary are very different. It is likely that the eastern and western lineages have been isolated for a long time and the extant pattern of variation with character disagreement within the border zone, originated from hybridization and introgression. The differences in corolla colour did not coincide with the variation in morphological traits or molecular markers within the North-Eastern Carpathian region. In addition, the geographical distribution of the populations with contrasting corolla colours lacked any pattern and there are populations with both corolla colours as well as plants with transitional pale-yellow flowers. Therefore, it is suggested that *M. saxosum* and *M. herbichii*, microspecies delimited on the basis of corolla colour, are conspecific. The high level of molecular variation and its pattern indicate that the *M. sylvaticum* group may have survived in or near the Eastern Carpathians during the Weichselian Ice Age. This hypothesis is supported by several recent phytogeographical and palaeoecological studies, which indicate the existence of a glacial refuge in the Eastern Carpathian region. Molecular uniformity of the Western Carpathian and Hercynian populations might in contrast indicate recent (Holocene) migration from assumed perialpine refuges.

K e y w o r d s : Carpathians, geometric morphometrics, haplotype lineage, *Melampyrum*, molecular variation, phylogeography, refuge

Introduction

The hemiparasitic genus *Melampyrum* (*Orobanchaceae*) is an important part of the European flora and is most diverse in the Balkan Peninsula followed by Caucasus and temperate Europe (Meusel et al. 1978). *Melampyrum* originated probably in the Mid Tertiary (Wolfe et al. 2005), evolved a number of species a few of which migrated outside Europe and the Caucasus and constituted taxa that do not occur in Europe (e.g., *Melampyrum roseum* and a few related species in E Asia, *M. lineare* in E North America; Soó

1926–1927, Štech 1998). Having survived the Quaternary climatic cycles, temperate species of *Melampyrum* maintained a high diversity, unlike most of the European Tertiary flora (Ložek 1973, Lang 1994). Their diversity might have even increased as a result of isolated evolution in glacial refuges.

The genus *Melampyrum* is still actively speciating, which has resulted in the evolution of several complexes of closely related microspecies that are hardly distinguishable from each other, of which the *M. nemorosum* and *M. sylvaticum* groups are good examples. The origin and distribution pattern of individual microspecies are supposed to have been predominantly affected by the migration of populations, their isolation and subsequent coming into contact in the late Pleistocene and Holocene (Wesselingh & van Groenendael 2005).

The *M. sylvaticum* group is a widespread element of the European montane and subalpine flora. Its geographical range covers mountain ranges from W Europe (the Pyrenees, Scottish Highlands; Dalrymple 2007) to the Urals and lowlands in the boreal zone (Meusel et al. 1978). Forming large populations, it is relatively common in most of its range. Nonetheless, it is sometimes considered rare or even endangered in countries near its geographical boundary, e.g. in Britain (Dalrymple 2007).

Three taxa are usually distinguished at the species level in the *M. sylvaticum* group, based on anther length, corolla size and colour. *Melampyrum sylvaticum* L. s. str. defined by short anthers and a small yellow corolla (see Soó & Webb 1972 or Těšitel & Štech 2007 for the exact range of values) is the most widespread type, believed to grow across the entire range of the group (Meusel et al. 1978). Although certain levels of variability in *M. sylvaticum* s. str. are reported from the Alps (e.g., Ronniger 1911, Soó 1926–1927) there are currently no other species level taxa in this group (Soó & Webb 1972). Long anthers and long (large) corolla characterize *M. herbichii* Woł. and *M. saxosum* Baumg., which differ from each other only in their corolla colour, being yellow and white, respectively. Beside being similar in terms of morphology, the center of the geographical distribution of the latter two species is in the Eastern Carpathians (Soó 1926–1927, Jasiewicz 1958, Paucă & Nyárady 1960, Soó & Webb 1972) and they are also often reported from the Southern Carpathians (Paucă & Nyárady 1960, Soó & Webb 1972). The taxonomic concept of the group is complicated by populations exhibiting diagnostic traits that are intermediate between those of *M. sylvaticum* s. str. and the Eastern Carpathian species. These are frequent in the Western Carpathians and eastern part of the Hercynian Massif, where there is a large zone of morphologically transitional types (Jasiewicz 1958, Štech & Drábková 2005, Těšitel & Štech 2007). Nonetheless, it is not clear whether this morphological similarity reflects genetic similarity with the Eastern Carpathian populations, which has resulted from gene flow across the boundary between the Eastern and Western Carpathians. An alternative hypothesis is that the similarity in morphological features resulted from convergent evolution under similar ecological conditions in both mountain ranges.

The main objective of this study was to evaluate the pattern of variation in the *M. sylvaticum* group across the Hercynian Massif and the Eastern and Western Carpathians using molecular markers and modern morphological methods. We asked the following questions: (i)What is the relationship between populations occurring on the opposite sides of the biogeographical boundary between the Eastern and Western Carpathians? Are they closely related or do they present two distinct lineages within the complex? (ii) Can we detect gene flow between the two Carpathian massifs? (iii) Do the Eastern Carpathian populations display any morphological or genetic variation (in non-coding loci) associated with the two corolla colour forms?

Material and methods

Material

The present study is based on plant material from 31 populations (596 individual specimens) of the *M. sylvaticum* group collected from the Eastern and Western Carpathian region and Hercynian Massif (Table 1, Fig. 1). Up to 31 plants were sampled from each population and used in the morphometric analysis of all the specimens collected (596 plants). The corolla of one flower per plant was put into an Eppendorf-tube filled with concentrated (96%) ethanol (denatured) and stored for digitalization. Several leaves or bracts from up to three plants per population were desiccated using silica-gel and kept at –20°C for DNA extraction (in total, 72 specimens were analyzed using molecular tools). The other parts of each plant were processed as a standard herbarium voucher and are in the herbarium of the Faculty of Science, University of South Bohemia (CBFS).

Some of the samples used in the present investigation were originally collected for a previous study of variation in the *M. sylvaticum* group (Těšitel & Štech 2007). Unfortunately, this material was destroyed during processing for the previous analysis and, therefore, not available for the current morphometric analyses. Nonetheless, those samples are a valuable source of material for the molecular part of this study (Table 1). These populations were chosen so as to represent all the mountain ranges from where the samples for our previous study originated (Těšitel & Štech 2007).

Digitalization and morphometric analysis

Thin plate spline method with sliding semilandmarks (Bookstein 1997, Zelditsch et al. 2004) was employed to analyze corolla shape. This is an efficient way of describing the outline of an object, in particular the edges (presence of which causes difficulties when outline-based methods are used) and has been used in a number of studies on the variation in shape of biological objects (e.g., Neustupa & Hodač 2005, Macholán 2006). Preliminary trials clearly showed that this method is superior to the traditional distance-based morphometrics previously used (Štech & Drábková 2005, Těšitel & Štech 2007). The semilandmarks managed to capture e.g., variation in the corolla curvature, an important diagnostic character completely overlooked by the conventional approach based on a series of linear measurements of corolla shape (see Těšitel & Štech 2007).

The corollas kept in ethanol were flattened and scanned at 1200 dpi using CanoScan 4200 (Canon Inc., Tokyo). The images were saved as RGB colour images in JPG format (low compression). Twenty-seven landmarks were digitized on the outline of each corolla (Fig. 2a), using version 2.05 of tpsDig software (Rohlf 2006). The images were ordered randomly before performing the landmark digitization, which should minimize subjective bias caused by potential similar misplacement of some landmarks in successive images. Twenty-five landmarks were defined as semilandmarks allowing them to slide along the abscissa between their neighbours during the superimposition. Although landmarks 11, 12 and 21 seem to be well defined in two dimensional space (Fig. 2a), we decided to use them as semilandmarks. True landmarks have a slightly higher influence on the analysis than semilandmarks (Zelditsch et al. 2004), which is undesirable in the case of these points. Their position is strongly affected by bending of the lower corolla lip, which occurs when the three dimensional corolla is flattened, and the curvature of the corolla base,

Table 1. – List of the details of the samples of the *Melampyrum sylvaticum* group used in this study. Localization, type of the nuclear DNA (ITS) and chloroplast DNA (*trn*L-*trn*T) haplotypes are indicated. Samples marked by an asterisk are from populations included in our previous study (Těšitel & Štech 2007). Numbers of specimens analyzed by morphometric and molecular methods are indicated before and after the slash, respectively.

No.	Country	Locality	Latitude	Longitude	Altitude (m)	Date of sampling	ITS haplotypes	cpDNA haplotypes	Corolla color	Number of specimens
1*	Ukraine	Rakhiv: spruce forest on slope ca 2 km ESE of the town	48°02'36"N	24°15'13"E	950	30.6.2005	B	a	yellow	27/3
2*	Ukraine	Yasinya: forest edge abbuting the ski slopes at the tourist resort ca 8 km W of the town	48°14'25"N	24°14'24"E	1410	12.7.2005	B, B1	a4	yellow	30/3
3	Romania	Staţiunea Durău resort: side of path between Fîntînele and Dochia chalets on N slope of Mt Ceahlau, ca 3 km ESE of the mountain resort.	46°59'03"N	25°57'25"E	1610	2.7.2006	A6, A7	a6	white	30/3
4	Romania	Vatra Dornei: rocky massif Piatrele Doamnei ca 1 km SW of Mt Rărau, ca 20 km NE of the town	47°26'51"N	25°33'53"E	1590	3.7.2006	A, A5	a	yellow	30/3
5	Romania	Vatra Dornei: side of path in a spruce forest on the ridge between Mt Rărau and Mt Giumalau, ca 13 km NE of the town	47°27'01"N	25°29'60"E	1416	4.7.2006	A	a2	yellow	20/2
6	Romania	Vatra Dornei, Mt Giumalau: *Pinus mugo* vegetation on the E slope of the mountain ca 250 m E of the summit, ca 11 km NE of the town	47°26'13"N	25°29'04"E	1788	4.7.2006	–	–	yellow	27/–
7	Romania	Vatra Dornei: mountain meadows of Poiană Obcina Mică ca 5 km NE of the town	47°22'46"N	25°22'39"E	1250	5.7.2006	A, A2	a3	mixed	29/3
8	Romania	Vatra Dornei: side of path in meadows on the NE boundary of the town	47°21'38"N	25°22'14"E	946	5.7.2006	A	a3	yellow	22/3
9	Romania	Gura Haitii: *Pinus mugo* vegetation along a path ca 1 km S of the rocky massif Stîncile doispresece apostolii	47°13'04"N	25°13'28"E	1589	5.7.2006	A	a	white	30/3
10	Romania	Iacobeni: edge of spruce forest on the S slope of Mt Tarniţa ca 5 km W of the village	47°24'49"N	25°14'13"E	1421	6.7.2006	A	a	yellow	27/4
11	Romania	Rotunda settlement: *Pinus mugo* vegetation around a path ca 1.5 km SE of the summit of Mt Omu, ca 8 km SE of the settlement	47°29'18"N	25°06'23"E	1737	7.7.2006	A	a	white	30/3
12	Romania	Rotunda settlement: side of the road between the Rotunda settlement and the Pasul Rotunda saddle ca 2 km SW of the settlement	47°33'27"N	25°01'14"E	1128	8.7.2006	A	a	mixed	29/3
13	Romania	Rotunda settlement: side of the road between the Rotunda settlement and the Pasul Rotunda saddle ca 1.5 km SW of the settlement	47°33'33"N	25°01'52"E	1080	8.7.2006	A	a3	white	27/2
14	Romania	Daneşti (Izvoru Oltului): spruce forest and spring area on N slope of a hill ca 1 km NW of the village	46°34'46"N	25°46'47"E	908	1.7.2006	A4, A5, A7	a1	yellow	29/3

15	Romania	Baile Tuşnad: edge of spruce forest at the W boundary of the town ca 1 km SW of the railway station	46°08'42"N	25°51'09"E	683	30.6.2006	A, A1	a, a5	yellow	31/2
16	Romania	Timişu de Jos: path margin in the valley of the Şipaia creek ca 2 km SE of the railway station	45°34'41"N	25°38'13"E	838	28.8.2006	A, A2, A3	a	yellow	24/3
17*	Slovakia	Runina: alpine pastures at Sedlo pod Ďurkovcom Saddle, 3.2km NNE of the village	49°05'08"N	22°25'24"E	1128	8.7.2005	B	a	yellow	29/3
18*	Czech Republic	Zalány: *Picea abies* forest on the N border of the village	49°38'35"N	13°51'25"E	645	12.7.2005	B	b1	yellow	26/3
19*	Czech Republic	Ovesná: *Picea abies* forest next to the railway station	48°48'26"N	13°56'21"E	740	15.6.2006	B	b	yellow	30/2
20	Czech Republic	Volary: edge of forest ca 1 km W of Mt Doupná hora, ca 3.5 km ESE of the town	48°53'45"N	13°55'28"E	790	21.6.2006	–	–	yellow	30/–
21	Czech Republic	Javorník: edge of meadow ca 700 m S of the village	49°07'58"N	13°39'37"E	900	21.6.2006	–	–	yellow	15/–
22	Czech Republic	Pec pod Sněžkou: edge of forest near the lower station of the cableway to Mt Sněžka.	50°42'28"N	15°44'02"E	870	17.6.2006	B	b	yellow	24/2
23*	Czech Republic	Kvilda: group of spruce trees on a knoll in valley of Kvildský potok stream 0.5 km E of the village	49°01'04"N	13°35'02"E	1045	11.7.2004	B	b1	yellow	–/2
24*	Czech Republic	Karlov, Velká kotlina Valley: montane spruce forest ca 1.5 km SE of peak of Mt Vysoká Hole	50°03'10"N	17°14'52"E	1110	7.7.2004	B	b	yellow	–/2
25*	Slovakia	Mt Veľký Rozsutec: spruce forest on the N slope of the mountain	49°14'20"N	19°06'20"E	1385	22.6.2004	B	b	yellow	–/2
26*	Slovakia	Trangoška: montane meadows on the S slope of Mt Chopok, ca 100 meters N of Kosodrevina Hotel	48°55'57"N	19°35'28"E	1525	26.6.2004	B	b	yellow	–/2
27*	Slovakia	Huty: montane forest near the start of the pathway leading to Mt Biela skala, ca 2 km E of the village	49°13'24"N	19°35'59"E	930	1.7.2004	B	b	yellow	–/2
7967 28*	Slovakia	Lysá Poľana: meadow beside the road between the village and a gamekeeper's lodge, ca 3 km S of the village	49°14'27"N	20°06'05"E	1005	4.7.2004	B	b	yellow	–/2
29	Slovakia	Oravská Polhora: alpine meadows with *Pinus mugo* shrubs on Mt Babia hora, ca 0.5 km SSW of the summit, ca 8 km NE of the village	49°34'09"N	19°31'34"E	1580	6.7.2008	B	b	mixed	–/3
30*	Ukraine	Burkut: alpine meadows on Mt Chivchin, ca 0.5 km N of the summit, ca 8.5 km S of the village	47°52'09"N	24°42'38"E	1640	9.7.2003	A	b	white	–/2
31*	Ukraine	Lazeshchina: alpine pastures between Mt Hoverla and Mt Pietrosh ca 2.75 km W of the summit of Hoverla, ca 12 km S of the village	48°09'37"N	24°27'50"E	1570	11.7.2003	A	c	yellow	–/2

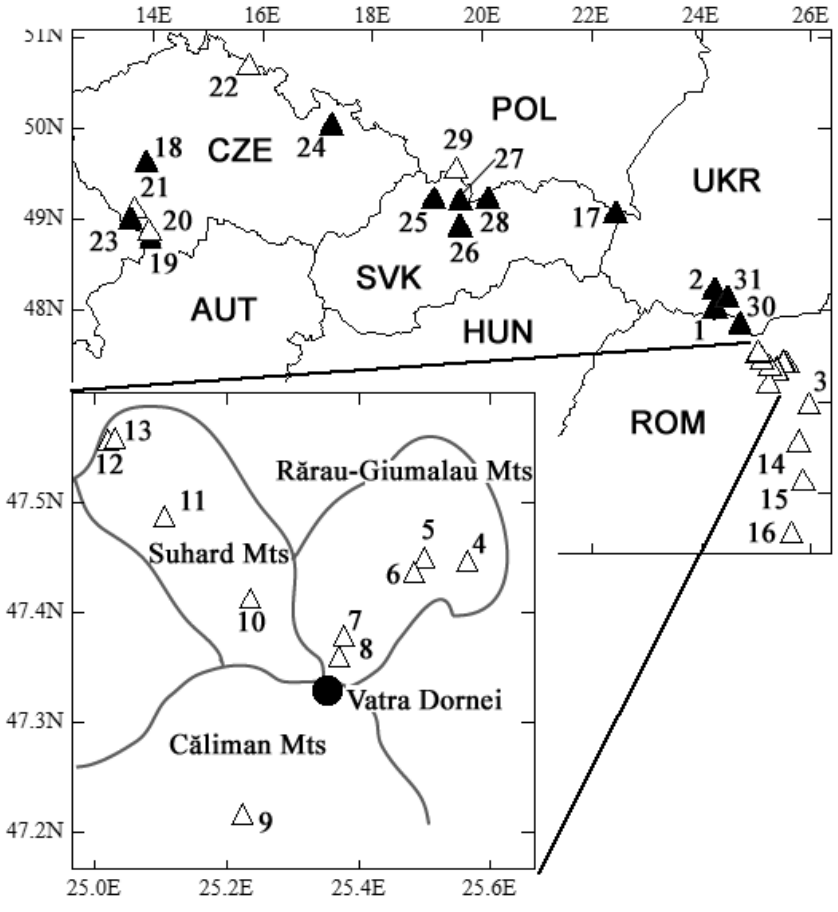

Fig. 1. – Map of the *Melampyrum sylvaticum* group localities included in this study. A magnified view of the surroundings of the town of Vatra Dornei is provided as many samples were collected in this area. Populations displayed by ▲ were sampled in our previous study (Těšitel & Štech 2007) and those depicted by △ were sampled in this study. Borders of the following Central and Eastern European countries are shown: CZE – Czech Republic, AUT – Austria, SVK – Slovakia, POL – Poland, UKR – The Ukraine, ROM – Romania, HUN – Hungary.

which is more or less stochastic and potentially connected to the phenological stage of individual flowers. Nonetheless, there was no apparent difference in the results when we performed a reference analysis in which these points were true landmarks.

Individual landmark constellations were aligned using the Procrustes superimposition (Zelditsch et al. 2004) in tpsRelw, version 1.42 (Rohlf 2005). A maximum of 10 iterations was allowed in the superimposition procedure aiming to minimize the bending energy among the shapes. Resulting scatter of superimposed landmarks can be seen in Fig. 2b. Relative warp analysis (RWA, Rohlf 1993) was subsequently performed with the parameter α set to 0 (resulting in shape principal component analysis) using tpsRelw, version 1.42 software (Rohlf 2005). Centroid size (i.e., sum of distances between individual landmarks and the central point defined as the hypothetical center of gravity) was extracted during the superimposition procedure and employed in subsequent analyses as a measure of size independent of shape.

Anther length was measured in individual flowers in addition to the acquisition of corolla shape and size data. The measurements were done under a dissection microscope. The metering accuracy was 0.05 mm.

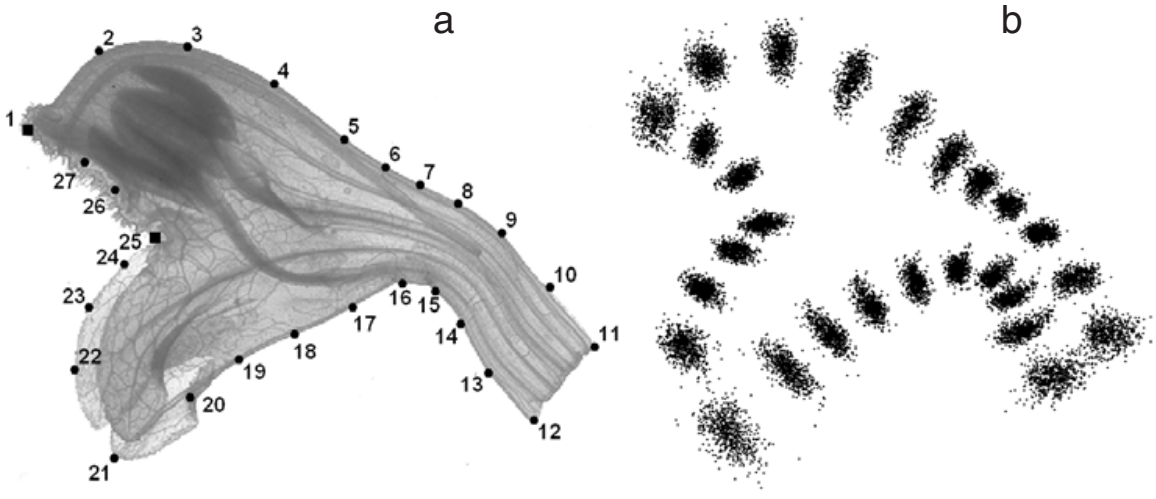

Fig. 2. – (a) Position of landmarks on flattened corolla. Landmarks are marked by boxes, semilandmarks by circles. (b) Scatter plot of superimposed specimens.

DNA sequencing

DNA was extracted from dried leaf tissue using a commercial Invitek Plant Extraction Kit (Invitrogen) and following the standard protocol provided by the manufacturer. Polymerase chain reaction (PCR) performed on a Biometra T3000 thermal cycler was employed to amplify the *trn*L-*trn*T region of chloroplast DNA and the ITS1, 5.8S and ITS2 region of ribosomal DNA under the following conditions. PCR was performed in a total volume of 25 µl consisting of 1X PCR Buffer, 200 µM each of dNTPs, 1.25U Taq DNA polymerase (TopBio), 1 µl DNA template solution and 7.5 pmol of each of the primers *trn*L (5'-GAGATTTTGAGTCTCGCGTGTC-3'; primer d in Taberlet et al. 1991), *trn*T2F (5'-CAAATGCGATGCTCTAACCT-3'; Cronn et al. 2002) for cpDNA amplification, or plant-specific ITS1P (5'-CTTTATCATTTAGAGGAAGGAAG-3'; Selosse et al. 2002) and ITS4 (5'-TCCTCCGCTTATTGATATGC-3'; White et al. 1990) for ITS. The amplification profile for cpDNA consisted of denaturation at 95°C (300 s), 30 cycles of denaturation at 95°C (60 s), annealing at 62°C (90 s), extension at 72°C (90 s) and final extension at 72°C (600 s). The amplification profile for ITS consisted of denaturation at 95°C (300 s), 32 cycles of denaturation at 95°C (60 s), annealing at 52°C (90 s), extension at 72°C (90 s) and final extension at 72°C (600 s). The PCR products were subsequently purified using JetQuick PCR Purification Kit (Genomed). Sequencing reaction was performed using BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) in the Sequencing Centre of the University of South Bohemia.

The sequencing procedure resulted in unambiguous data for both loci. The nucleotide 583 in the ITS alignment was the only exception, oscillating erratically between C and T. It was treated as an ambiguous base Y in all specimens, which set its influence in any analysis to zero. The ITS data otherwise displayed complete concerted evolution (see e.g., Álvarez & Wendel 2003 for explanation) and were directly used in sequence grouping for haplotype definition.

Data analysis

We employed standard statistical techniques for detecting variability in morphological characters. The axes constructed by the relative warp analysis (RWA) are suitable for direct visualization by ordination plots as this method is identical with a principal component analysis (PCA), if an appropriate parameter setting is applied. Proportions of within-population variation were calculated using an expected mean square procedure (EMS; Quinn & Keough 2002). Indicators of populations were regarded as random-effect predictors in the calculation. Differences in the quantitative morphometric traits among populations displaying different corolla colours were analyzed only within the North-Eastern Carpathian region due to lack of morphological data for the Western Carpathian populations and the uniformity of the South-Eastern Carpathian populations. An analysis of variance (ANOVA) and a redundancy analysis (RDA) based on the relative warp scores were used to test the relationship between corolla colour and univariate morphometric characters, and corolla shape. Based on the population means or mean relative warp scores of populations (corolla shape), these tests treated populations as independent observations. Corolla colour entered this analysis as a predictor defined as a binary-coded two variable matrix (describing presence of yellow/white colour in the population); hence populations of mixed or transitional colours received 1 for both predictor variables.

We used Statistica for Windows, version 6.0 (StatSoft 2001) for basic statistical procedures, graphical visualization of data and calculation of EMS for univariate variables. Package R, version 2.3.1 (R Development Core Team 2006) was employed for ANOVA calculations. Canoco for Windows, version 4.53 (ter Braak & Šmilauer 2002) was used for the multivariate statistics and for an extraction of sum of squares from the relative warps, which served as a basis for subsequent manual calculation of EMS using a formula in Quinn & Keough (2002). A PCA based on consensual landmark configurations was computed in PAST package, version 1.67 (Hammer et al. 2001) using a singular value decomposition algorithm (which improved the PCA stability when more variables than samples were present in the analysis).

Sequences of each of the analyzed loci were aligned using Clustal W (Thomson et al. 1994) and the alignment was subsequently improved manually. Identical sequences were grouped to define haplotypes. Phylogenetic network of nuclear and chloroplast haplotypes was constructed by means of statistical parsimony (Posada & Crandall 2001) using software package TCS, version 1.21 (Clement et al. 2000). Indels were treated as independent binary characters (coded as A for absence and C for presence as TCS does not support 0/1 coding). Individual gap positions were treated as missing data.

Results

Continuous morphometric characters

Within-population variation accounted for 50.3% of the variation in the shape of the corolla, 32.9% of that of the corolla centroid size and 23.8% of anther length (inferred from EMS analyses). Variation in all analyzed traits displayed continual patterns, which were more or less congruent with each other.

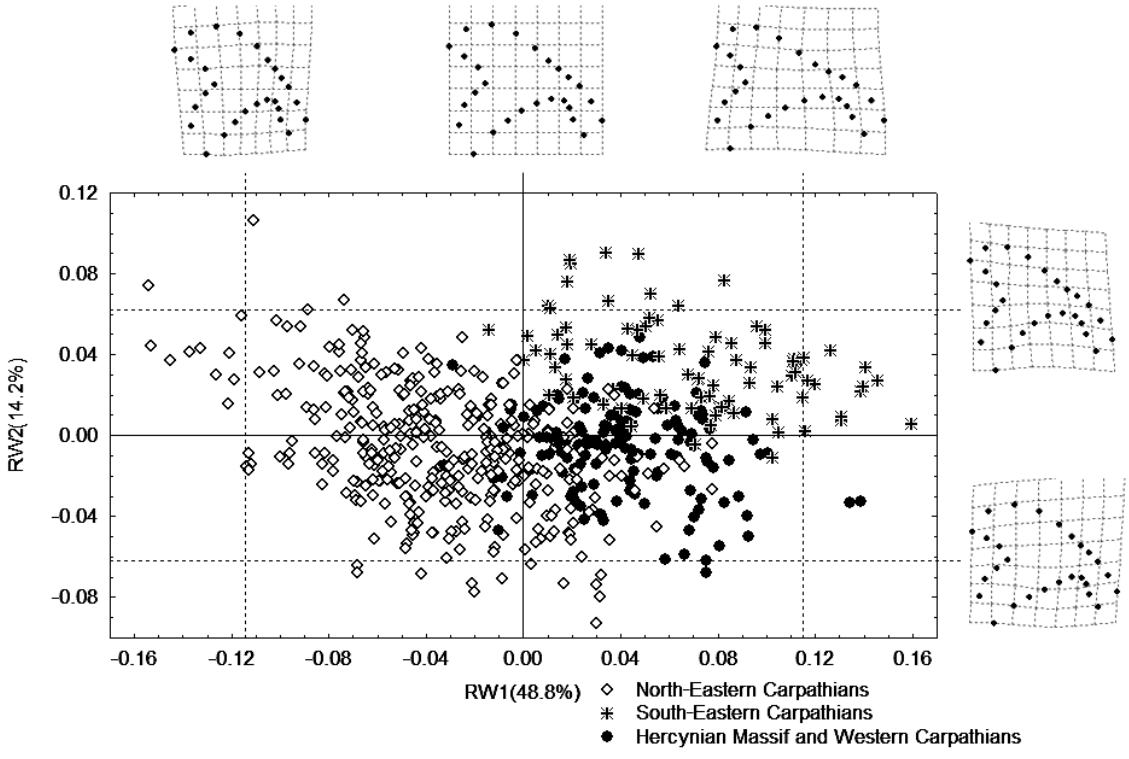

Fig. 3. – RWA ordination plot based on variation in corolla shape of individual plants. Points representing the specimens are classified according to the geographical distribution of the populations. Mean corolla shape and the shape changes associated with the first two principal warps are depicted (shapes corresponding to ± 2 SD positions are displayed on each axis).

Classification of individual specimens (Fig. 3) or populations (Fig. 4) in the relative warp ordination plots revealed that plants growing in different geographical regions tend to concentrate in certain parts of the ordination space. Most of the specimens (and all when the consensual corolla shapes within populations are considered) from the North-Eastern Carpathians (north of the southern slopes of the Câliman Mts and Ceahlau Massif) occupy the left side of the first ordination axis, and tend to have a concave shaped shorter corolla with a slightly more prominent lower lip. Differing mainly in the convex shape of their corolla, the Western Carpathian and Hercynian plants (populations) are generally located on the opposite side of this gradient. Three populations in the South-Eastern Carpathians (south of the northern limit of the Harghita Mts) differ from both of these groups, especially those in the geographically proximate North-Eastern Carpathians. Featuring very long and strongly convexly curved corollas, these plants appear similar to some extreme specimens from the Western Carpathian – Hercynian region.

The plot of variation in univariate morphometric characters showed similar opposite tendencies in samples from North-Eastern Carpathians versus Hercynian and Western Carpathian populations (Fig. 5). The former group has longer anthers and larger corollas than the latter group. The three populations in the southern part of the Eastern Carpathians differ in that their corollas are very large but anther length is variable, as one population has long and the other two rather short anthers, similar to the Hercynian specimens.

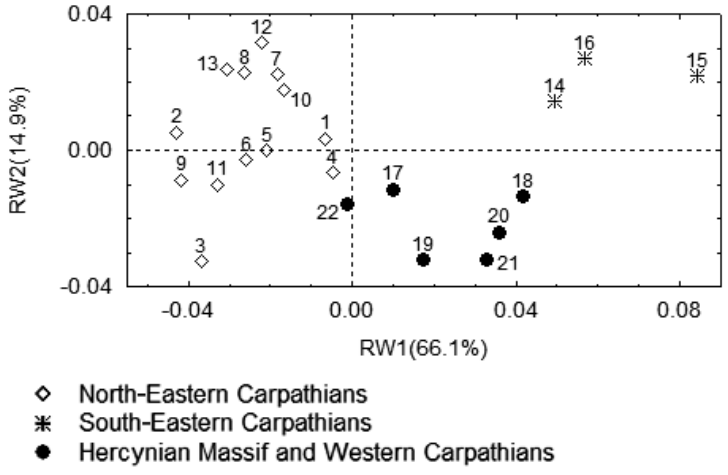

Fig. 4. – RWA ordination plot based on consensual corolla shapes in each population. Percentages of variance explained by the axes correspond only to the variation among populations. The populations are labelled with the numbers of the localities (Table 1).

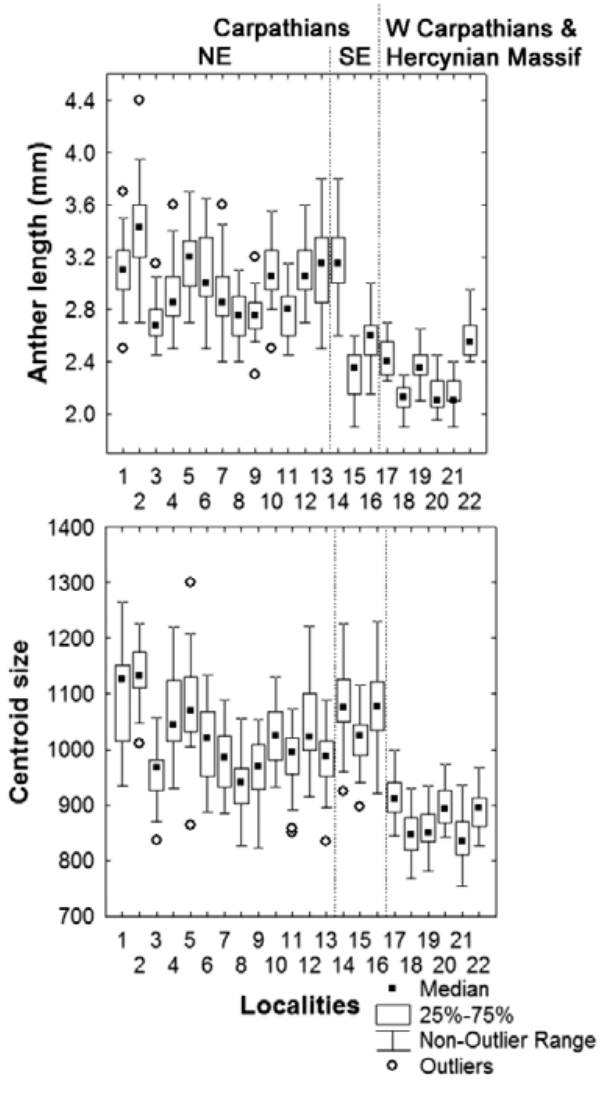

Fig. 5. – Box-and-whisker plots displaying the values of anther length (AL) and centroid size at individual localities. Population numbers correspond to those in Table 1. Their geographical location is indicated by dotted lines separating populations in the North-Eastern Carpathians (1–13), South-Eastern Carpathians (14–16) and Western Carpathians and Hercynian Massif (17–22).

Table 2. – Chloroplast DNA haplotypes defined by variable positions in the *trn*L-*trn*T cpDNA region. Substitutions within indels are indicated by white font on a black background. Number of plants and populations (sites) in which individual haplotypes were found are indicated.

Haplotype	GenBank accession number	Number of plants (sites)	Variable positions (bp)										
			131	306	307–315	347–355	484	559	615–622	637	678–700	701–723	798–799
a	EU653274	27 (10)	A	C	–	–	C	G	–	A	TTATATTTCTAGAGACACTATAT	–	–
a1	EU653275	3 (1)	A	C	–	–	C	G	–	A	TTATATTTCTAGAGA**T**ACTATAT	–	–
a2	EU653276	2 (1)	A	C	–	–	C	A	–	A	TTATATTTCTAGAGACACTATAT	–	–
a3	EU653277	8 (3)	A	C	–	–	C	G	–	A	TTATATTTCTAGAGACACTATAT	TTATATTTCTAGAGACACTATAT	–
a4	EU653278	3 (1)	A	C	TATAA	–	C	G	–	C	TTATATTTCTAGAGACACTATAT	–	–
a5	EU653279	1 (1)	A	C	–	–	C	G	–	A	TTATATTTCTAGAGACACTATAT	–	GC
a6	EU653280	3 (1)	A	G	–	–	T	G	–	A	TTATATTT**A**TAGAGACACTATAT	–	–
b	EU653282	17 (8)	A	C	–	–	C	G	AATATAGA	A	–	–	–
b1	EU653283	5 (2)	C	C	–	–	C	G	AATATAGA	A	–	–	–
c	EU653281	2 (1)	A	C	–	AGTAATTAA	C	G	–	A	–	–	–

Table 3. – Nuclear DNA haplotypes defined by variable positions in the internal transcribed spacer (ITS1, 5.8S, ITS2) sequences. Number of plants and populations (sites) in which individual haplotypes were found are indicated.

Haplotype	GenBank accession number	Number of plants (sites)	Variable position (bp)														
			50	59	86-87	91	187	406	408–409	414	442	509	558–560	567	571–572	577–579	597
A	EU624125	27 (13)	C	G	TC	A	C	T	GT	C	A	A	–	C	CG	–	C
A1	EU624126	1 (1)	C	G	TC	C	C	T	GT	C	A	A	–	C	CG	–	C
A2	EU624127	2 (2)	C	G	TC	A	C	T	GT	C	G	A	–	C	CG	–	C
A3	EU624128	1 (1)	C	G	CC	C	C	T	GT	C	A	A	–	C	CG	–	C
A4	EU624129	1 (1)	C	G	TC	C	C	T	GT	C	G	A	–	C	CG	–	C
A5	EU624130	3 (2)	C	A	TC	C	C	T	GT	C	A	A	–	C	CG	–	C
A6	EU624131	1 (1)	C	G	CT	C	C	T	GT	C	G	A	–	C	CG	–	C
A7	EU624132	3 (2)	T	G	CT	C	C	T	GT	C	G	A	–	C	CG	–	C
B	EU624133	28 (13)	C	A	TC	A	A	C	GT	T	A	C	TTG	T	TC	GTA	A
B1	EU624134	2 (1)	C	A	TC	A	A	C	AC	T	A	C	TTG	T	TC	GTA	A

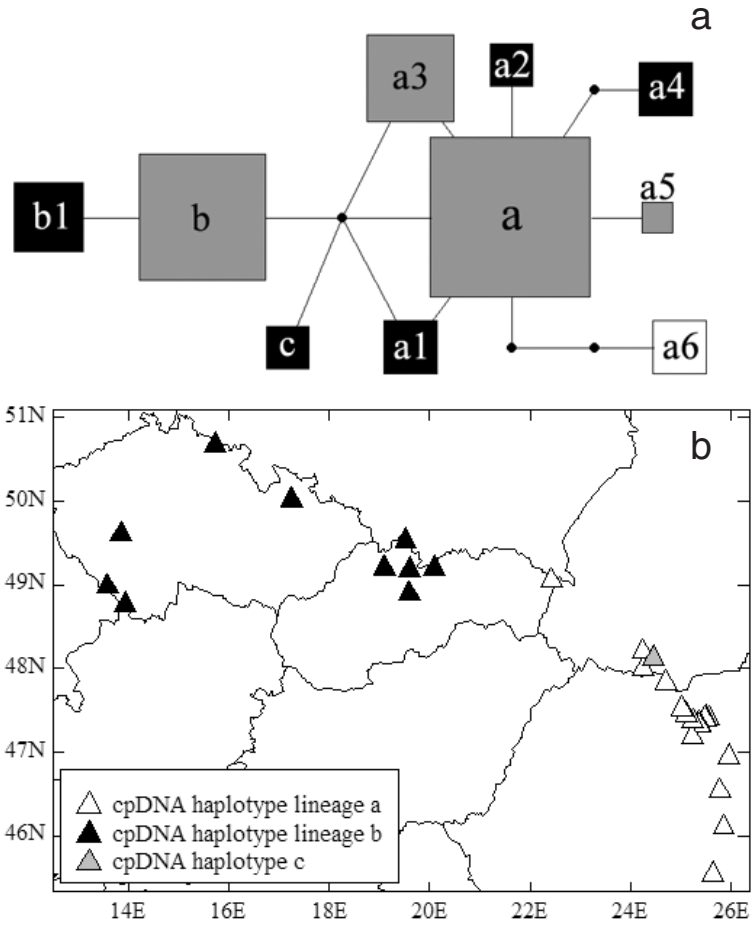

Fig. 6. – (a) The most parsimonous phylogenetic network of the cpDNA haplotypes of the *Melampyrum sylvaticum* group detected in the populations studied. Size of boxes is proportional to the number of plant specimens in which individual haplotypes were detected. Small circles symbolize missing haplotypes. Corolla colour trait is mapped onto the network: haplotypes of populations with only yellow-coloured flowers ■, haplotypes of populations with only white-coloured flowers □, haplotypes of populations with flowers of both colour types or mixed corolla colour ■. (b) Map displaying the distribution of the cpDNA haplotype lineages of the *Melampyrum sylvaticum* group in the populations studied.

Variation revealed by molecular markers

Both loci analyzed were variable enough to provide valuable information on the relationships among the populations. Ten haplotypes were detected in the *trn*L-*trn*T region of cpDNA (Table 2). Most of them in two haplotype lineages (**a, b**), which differed in two relatively large indel mutations (Table 2, Fig. 6a). Within each of these lineages there is a basic and widespread haplotype (haplotypes a and b) from which other generally much less frequent haplotypes were derived (these are marked by numbers) by both indel and point substitutions. Haplotype c could not be assigned to either of the large lineages and formed an independent group characterized by a unique indel combination, which positioned it between haplotype groups **a** and **b**. Lineage **a** was found in the whole Eastern Carpathian region including the Bukovské vrchy Mts (Fig. 6b), with its basic haplotype present in most of the specimens and populations (Table 1, Fig 6a). The derived haplotypes were either characteristic of small populations or only found in one population, resulting in com-

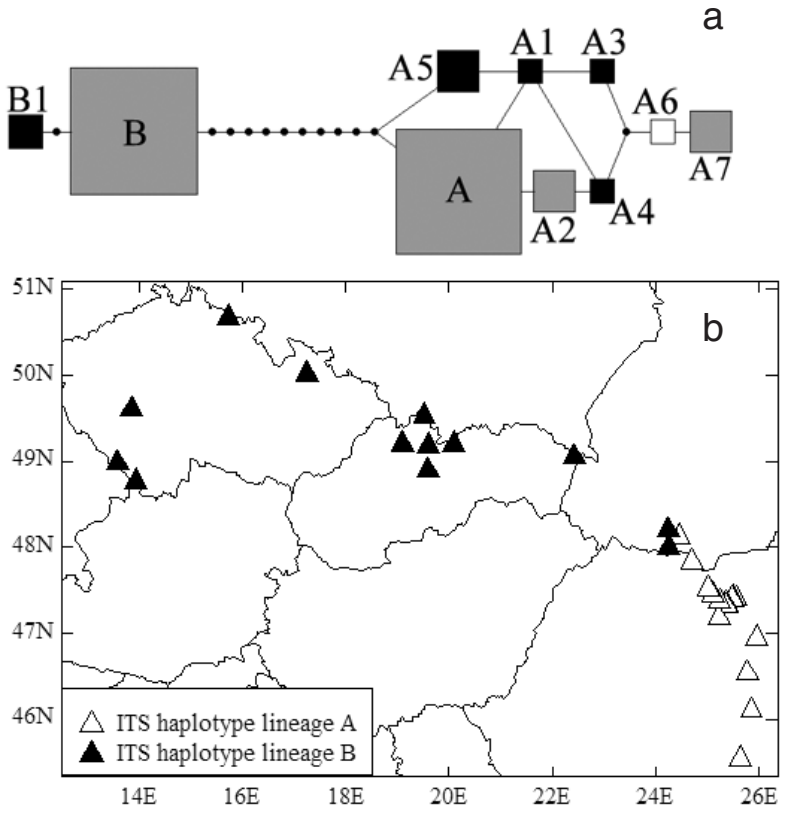

Fig. 7. – (a) The most parsimonous phylogenetic network of the ITS haplotypes of the *Melampyrum sylvaticum* group detected in the populations studied. Size of boxes is proportional to the number of plant specimens in which individual haplotypes were detected. Small circles symbolize missing haplotypes. Corolla colour trait is mapped onto the network: haplotypes of populations with only yellow-coloured flowers ■, haplotypes of populations with only white-coloured flowers □, haplotypes of populations with flowers of both colour types or mixed corolla colour ■. (b) Map displaying distribution of the ITS haplotype lineages of the *Melampyrum sylvaticum* group in the populations studied.

paratively high genetic differentiation among populations. In contrast, within-population variation was rather low as multiple (two) haplotypes were found only in one population (Table 1). The Hercynian and Western Carpathian populations are similar in only containing haplotype b and its variant b1, distinguished by a point substitution present in two populations in the southern half of Bohemia (Table 1). Haplotype c was only found in a single population on Mt Hoverla (Table 1, Fig. 6b).

The ITS haplotypes could be assigned to the two major lineages **A** and **B**, which differ in the number of single base substitutions and two three-base indels (Table 3). The most parsimonous network describing relationships among individual haplotypes revealed a very pronounced genetic difference (corresponding to a high number of missing haplotypes) between these haplotypic groups (Fig. 7a). Occurring at more than one site, the derived haplotypes were in general not characteristic of individual populations. There was more than one haplotype in many Eastern-Carpathian populations despite the small number of specimens analyzed per population (Tables 1, 3). This was particularly pronounced in the three populations in the southern part of the mountain range where almost no two plants share the same haplotype. Therefore, the genetic pattern is characterized by low differentiation between populations and high within-population variation, at least in the Eastern Carpathians where the genetic variability is high enough for such an estima-

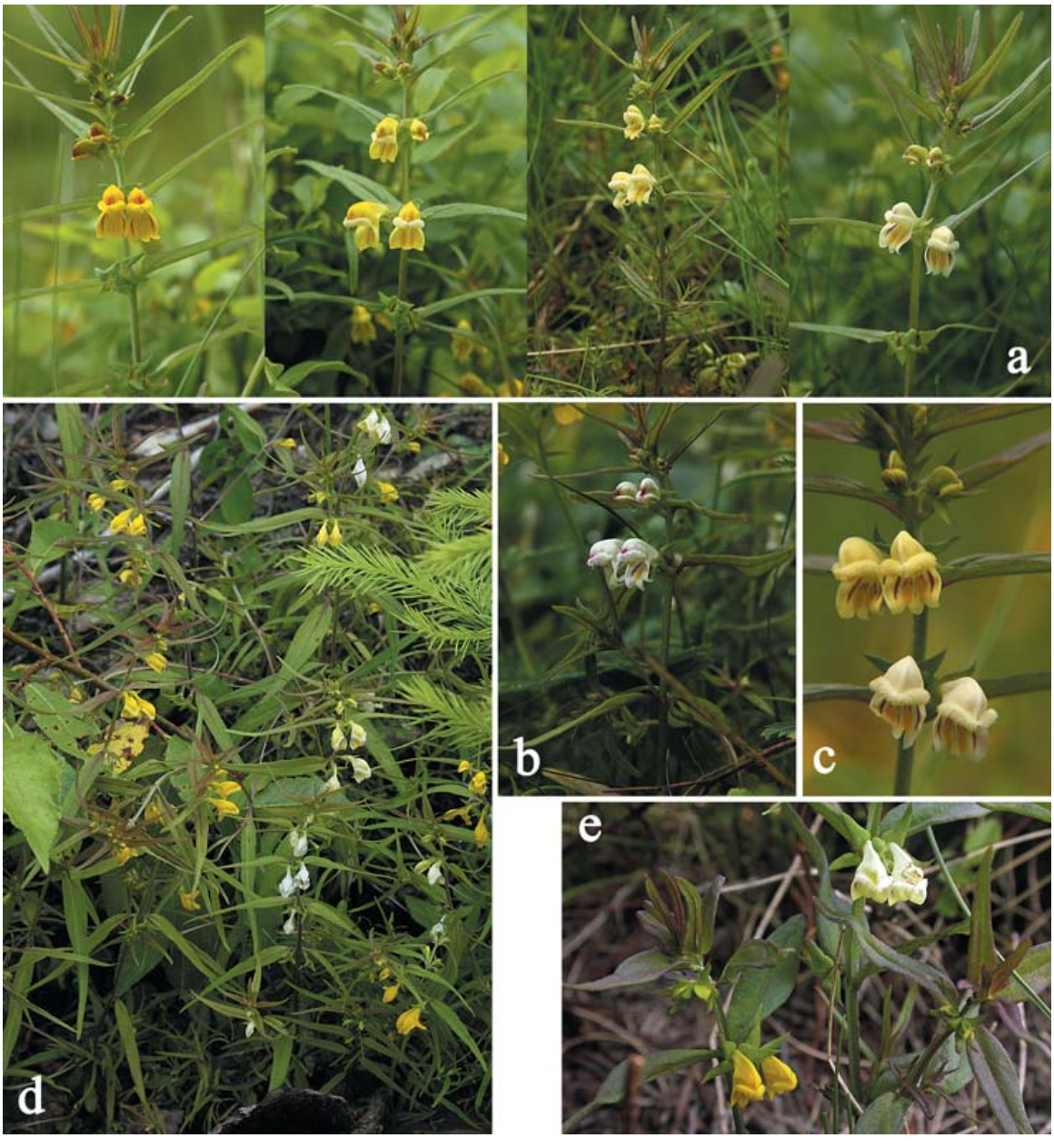

Fig. 8. – Variation in the corolla colour of populations of the *Melampyrum sylvaticum* group. (a) Variation in corolla colour of the population growing in the Poiană Obcina Mică meadows near Vatra Dornei, Romania. The plants are ordered from the yellow on the left to the lightest (almost white) specimen on the right. 5 July 2006. (b) A typical example of a white-flowered plant, Ceahlau Massif, 2 July 2006. (c) An unusual plant with flowers of different colours at different nodal positions, Poiană Obcina Mică meadows near Vatra Dornei, 5 July 2006. (d) A mixture of yellow-, white- and pale-yellow-flowered plants growing along the side of the road near Pasul Rotunda saddle, 8 July 2006. (e) Yellow- and whitish-flowered specimens from Mt Babia hora, Slovakia, 6 July 2008.

tion. The geographical range of lineage **A** includes most of the Eastern Carpathian sites except for those occurring near the Eastern-Western Carpathian boundary (Fig. 7b). In contrast to the cpDNA lineages, lineage **B** is not restricted to the Hercynian and Western Carpathian populations but also occurs in the Eastern Carpathians. The valley of the Tisa River, which crosses the Eastern Carpathians in the Ukraine, appears to be its eastern limit (Fig. 7b).

Variation in both molecular markers appears substantially higher in the Eastern Carpathians than in the western populations, which were found to be almost uniform. In spite of clear differentiation between the Eastern Carpathian and the western populations revealed by both molecular markers, the geographical borders of the distributions of the haplotype lineages do not coincide, resulting in discordance of the phylogeographical patterns. That is, there is a transitional zone on the boundary between the Eastern and Western Carpathians.

Variation in corolla colour

There were both yellow- and white-flowered plants in the North-Eastern and Western Carpathian populations of the *Melampyrum sylvaticum* group (Figs 8a, 8b) but only yellow-flowered plants in the populations sampled in the South-Eastern Carpathians and Hercynian Massif. White-flowered specimens were frequent in the North-Eastern Carpathian region, where they formed entire populations, but very rare in the Western Carpathians where only one population was found with yellow, intermediate pale yellow and almost white-flowered specimens on Mt Babia hora (population no. 29, Fig. 8e). The same within-population pattern in corolla colour was recorded at one North-Eastern Carpathian site near Vatra Dornei (population no. 7). In the population below the Rotunda saddle in the Suhard Mts (no. 12) there were plants with intermediate pale-yellow flowers and both extreme corolla colours (Fig. 8d). There was a continuum in corolla colour from white (or almost white in the first two cases) to yellow (Fig. 8a) in all these populations. Slight differences in colour were rarely observed even among flowers on an individual plant (Fig. 8c).

There were no significant relationships between the variation in corolla colour and anther length (logarithmic transformation; ANOVA, $F_{2,10} = 2.59$, $P = 0.226$), centroid size (square-rooted; ANOVA, $F_{2,10} = 1.73$, $P = 0.124$) or corolla shape (RDA, Monte-Carlo permutation test with 999 permutations: $F = 2.47$, $P = 0.146$). There was also no apparent agreement between corolla colour and genetic variation in either of the analyzed loci (Fig. 6a, 7a). Moreover, there were no conspicuous patterns in the geographical distributions of populations featuring different corolla colours (Fig. 9).

Discussion

Differentiation and gene flow on the border between the Eastern and Western Carpathians

Our analyses revealed solid and in general concordant phylogeographical patterns in variability in all continuous morphometric characters and both molecular markers. The differentiation between the Eastern Carpathian and western populations suggested by previous studies (e.g., Jasiewicz 1958, Těšitel & Štech 2007) is clearly supported by two distinct lineages within the *Melampyrum sylvaticum* group in our data. The differences delimiting the Eastern and Western Carpathian populations were especially pronounced in the ITS sequences and in congruent patterns in variation of several morphological characters (despite continual nature of their variation and overlaps). The marked differences in the western (**B**) and eastern (**A**) ITS haplotype lineages is good evidence that the Eastern Carpathian and western types were isolated from one another for a long time in their evolutionary history.

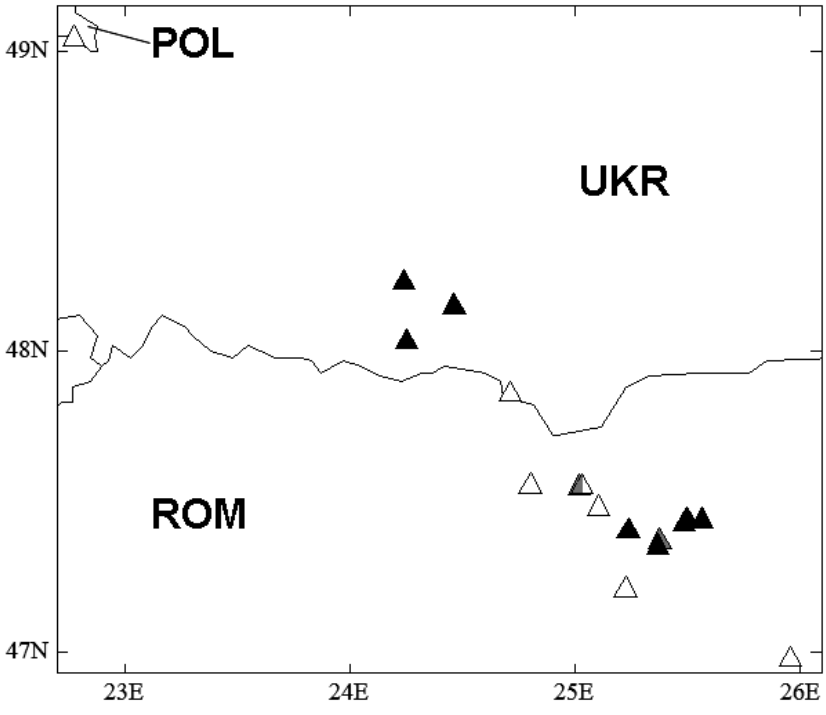

Fig. 9. – Map of the localities for the *Melampyrum sylvaticum* group in the northern part of the Eastern Carpathians for which reliable data are available. Position of an additional Polish white-flowered population is estimated on the basis of data in Zając & Zając (2001) and on the web site of the Bieszczady National Park (Anonymus 2008). The populations are classified according to corolla colour (▲ yellow corolla, △ white corolla, ▲ populations with mixed- and pale-yellow-flowered plants). Borders of countries are shown: UKR – The Ukraine, ROM – Romania, POL – Poland.

The origin of the transitional zone observed in the border region between the Eastern and Western Carpathians can be attributed to the meeting and subsequent hybridization of the two lineages. Although there are few samples from this area, it is likely that this zone extends from the Tisa valley (and neighbouring Pass of Yablunjitsa) to the Bukovské vrchy Mts (probably its western margin). Reaching only around 850 and 700 m a.s.l., respectively, these sites are very low and narrow parts of the Carpathian ridge, and are likely to impede the migration of alpine and upper-montane floristic elements. The pattern in variation apparently reflects these gene-flow barriers, although the *M. sylvaticum* group is generally regarded as a montane to subalpine taxon (Soó & Webb 1972, Šípošová 1997, Štech 2000) growing in mountain spruce (less frequently beech) forests and ascending to the tree-line. This description of its ecology is however based predominantly on observations in the Alps or Western Carpathians, where many populations indeed grow under spruce or beech forest canopy albeit not in heavily shaded areas (e.g., populations 19, 23 and 27 in the present dataset; Table 1). By contrast, all the populations in the North-Eastern Carpathians apparently prefer open habitats, either natural subalpine grasslands or dwarf-pine vegetation near the tree-line or man-made meadows, clearings and road sides at lower altitudes. This may account for the limited gene flow from the Eastern Carpathians westwards but not in the reverse direction. The low altitude of the Eastern Slovakian part of the main Carpathian ridge combined with the comparatively ineffective myrmecochorous seed dispersal strategy of *Melampyrum* (Winkler & Heinken 2007) may have prevented a mass migration of the central Western Carpathian populations in an east-

erly direction. The region between the Vysoké Tatry and Bukovské vrchy Mts comprises ca 100 km wide zone within which the altitude only fluctuates between 500 and 800 m a.s.l. The natural vegetation of this region is a continuous closed-canopy beech forest unfavourable for *M. sylvaticum*. On the other hand, *M. sylvaticum* might have migrated at certain periods in the Holocene when *Picea abies* forests formed a more substantial part of the local vegetation, probably between ca 8000 and 4000 BP (Latałova & van der Knaap 2006). The presence of western-type ITS haplotype in the Eastern Carpathian populations west of the Tisa valley may, therefore, be attributed to gene flow from the central part of the Western Carpathians in the past and subsequent introgression.

The pronounced genetic and morphological differences in the *M. sylvaticum* group on the East-West Carpathian boundary are similar to the recently reported patterns in genetic variation in *Hypochaeris uniflora* (Mráz et al. 2007) and *Campanula alpina* (Ronikier et al. 2008). The relatively wide transitional zone is in agreement with the continuous nature of the biogeographical boundary characterized by a gradual decrease in the diversity of Eastern Carpathian alpine floristic elements (such as *Rhododendron kotschyi*, *Alnus viridis*, *Laserpitium krapfii* subsp. *krapfii* and several diploid species of *Hieracium*; Polívka et al. 1928, Mráz & Szeląg 2004) in a westerly direction (Zemanek 1991). Many lower-montane species (i.e., those occurring mainly in beech forests), however, crossed this border and reached the Western Carpathians (e.g., *Veronica urticifolia*, *Aconitum moldavicum*, *Aposeris foetida*) and even the Hercynian Massif (e.g., *Anthriscus nitida* and *Doronicum austriacum*; Slavík 1997, Štech 2004). The latter case was recently well documented for *Rosa pendulina* using a phylogeographical study based on chloroplast DNA sequence variation (Fér et al. 2007).

Evolution of the extant variation pattern and its palaeoecological background

The high level of molecular variation in both chloroplast and nuclear DNA sequences in populations in the Eastern Carpathians indicates that large populations sufficient to maintain such variability have been present there for a long time. These loci are almost uniform in the Hercynian and Western Carpathian populations, which indicates markedly different evolutionary histories. Populations in the North-Eastern Carpathians probably survived the last glacial period (Weichsel, Würm) in one, or more likely, several refuges located probably either in the Eastern Carpathians or their vicinity. Locations and size of these favourable sites might have been relatively dynamic and dependent on climatic oscillations. Evolution in refuges that were isolated but connected periodically can result in the observed pattern in the genetic variation. Molecular uniformity of the populations in the Western Carpathian and Hercynian Massif indicate a recent (Holocene) migration from refuges located probably in perialpine areas.

Several recent studies have demonstrated that it is highly likely that a glacial refuge existed in the Eastern Carpathians, which supports our hypothesis of the long-term persistence of the *M. sylvaticum* group in this region. Robust evidence comes from a review of palaeobotanical finds of charcoal in Central Europe (Willis & van Andel 2004), which indicates the presence of *Picea* (one of the main *M. sylvaticum* group host species; e.g., Štech 2000) and *Alnus* in the eastern surroundings of the North-Eastern Carpathians between 35 000 and 20 000 years BP (calibrated ^{14}C chronology), i.e. during a significant part of the Last Glacial Maximum (LGM). Genetic and pollen data indicate that *P. abies*

survived in the North-Eastern Carpathian region (Tollefsrud et al. 2008). In addition, the North-Eastern Carpathian populations of *Pinus mugo,* another important species associated with the *M. sylvaticum* group, differ morphologically from other Central European and Balkan populations, which indicates their genetic isolation and that they have probably been present in the region for a long time (Boratyńska, Muchewicz & Drojma 2004). This is also supported by the results of several palynological sequences that indicate the presence of *Pinus* (probably *P. mugo*) in the late ice age (Farcas et al. 1999, Feurdean 2004), and that a refuge or several isolated refuges suitable for *Melampyrum* might have existed at favourable sites at the base of the mountains in the North-Eastern Carpathian region.

It is suggested that Siberian taiga-type boreal forest existed in the Western Carpathians during the LGM (Jankovská & Pokorný 2008) This does not accord with our hypothesis that the Hercynian Massif and Western Carpathians were recolonized by *M. sylvaticum* during the Holocene, as it suggests the species might have survived in the area during the full-glacial period. On the other hand, Tollefsrud et al. (2008) have demonstrated not only the survival of *Picea abies*, a characteristic and often dominant tree species in European boreo-montane forests, in the Western Carpathians during the Weichselian Ice Age but also very low genetic diversity in populations of this species in this region. These authors suggest a bottleneck resulting from a substantial decrease in population size during either the LGM or Younger Dryas (Tollefsrud et al. 2008), associated with a decrease in the area covered by vegetation favourable for *M. sylvaticum* and its putative local extinction. Moreover, the present distribution of *M. sylvaticum* is limited by the Uralian mountain range and does not extend substantially into Siberia (Meusel et al. 1978), where the vegetation is nowadays analogous with that reconstructed by Jankovská & Pokorný (2008). It is possible that the distribution of *Melampyrum* is limited by permafrost as it germinates in autumn and has an active overwintering stage with roots. This would account for its present distribution limit and extinction in the Western Carpathians if the reconstructed LGM forest grew on permafrost (Jankovská & Pokorný 2008). The conditions in the Eastern Carpathians were certainly more favourable as at least the southern part of the mountain range was in the permafrost free zone even during the LGM (Taberlet et al. 1998) and permafrost free sites could have occurred at more northerly situated sites (e.g., on southern slopes).

The substantial divergence between *Picea abies* genetic lineages in the northern and southern parts of the Eastern Carpathians (Tollefsrud et al. 2008) suggests a possible explanation for the morphological divergence and differences in the pattern of genetic variation found between the *M. sylvaticum* group populations occurring in these regions. Both species have similar ecological preferences and, therefore, might share the same evolutionary history characterized by the isolation in the past of the populations inhabiting the South- and North-Eastern Carpathians. The little data on *Melampyrum*, however, make this hypothesis very speculative. Nonetheless, it is an interesting idea worthy of further study.

Taxonomic conclusions

Our results demonstrate that the current taxonomic concept of the *Melampyrum sylvaticum* group (Jasiewicz 1958, Soó & Webb 1972) needs to be reviewed. The insignifi-

cant relationships between corolla colour and other traits, lack of a pattern in the geographical distributions of populations with different corolla colours and the presence of whitish-flowered specimens in the Western Carpathians decrease the taxonomic value of this character. Therefore, we propose that *M. saxosum* and *M. herbichii* are conspecific as their delimitation is based entirely on corolla colour. Under the terms of the priority rule, the correct name for most Eastern Carpathian plants is *Melampyrum saxosum* Baumg., as this name was published earlier (Baumgarten 1816) than *Melampyrum herbichii* Woł. (Wołoszczak 1887). Nonetheless, this nomenclatorical solution must be regarded as preliminary. The final designation of plant names must be based on type herbarium vouchers, which have not yet been studied.

We suggest that the Central European populations of the *M. sylvaticum* group be classified into two species *M. sylvaticum* s. str. and *M. saxosum* differing in the shape and size of the corolla and anther length. These species have different evolutionary histories and geographic distributions, with the approximate border zone between them on the Eastern-Western Carpathian boundary. Nevertheless, this morphological delimitation between these species applies only to the populations in the northern part of the Carpathian mountain range. The presence of morphologically specific populations in the South-Eastern Carpathians that are genetically closer to the North-Eastern Carpathian samples prevents the generalization of this delimitation between *M. sylvaticum* s. str. and *M. saxosum*, which requires further study (especially the collection of more samples from the Southern Carpathian region).

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Souhrn

Studovali jsme variabilitu *Melampyrum sylvaticum* agg. v Hercynském Masivu a ve Východních a Západních Karpatech pomocí morfometrických a molekulárních metod. Našim cílem bylo především zjistit, jak se ve variabilitě studované skupiny odráží výrazná biogeografická hranice, oddělující Východní a Západní Karpaty a jaké jsou rozdíly ve struktuře variability na opačných stranách této hranice. Zároveň jsme hledali molekulární a další morfologické znaky, které by byly korelované s variabilitou v barvě koruny ve východokarpatských populacích. Ty se na základě barevných rozdílů obvykle oddělují do mikrospecií *M. saxosum* (s bílou barvou koruny) a *M. herbichii* (se žlutou barvou koruny).

Analýzy tvaru koruny, její velikosti a délky prašníku a zejména molekulární analýzy založené na sekvencích chloroplastové (region *trn*L-*trn*T) a jaderné ribozomální DNA (region ITS) potvrdily výrazné odlišnosti typů rostoucích na opačných stranách hranice mezi Východními a Západními Karpaty. Zjištěné rozdíly na molekulární úrovni se ukázaly natolik výrazné, že se velmi pravděpodobně jedná o dvě poměrně značně vzdálené linie v rámci komplexu. Pozorované nesoulady ve struktuře genetické variability mezi molekulárními markery přímo na hranici Východních a Západních Karpat připisujeme hybridizačnímu procesu mezi těmito liniemi a následné introgresi. Nenalezli jsme žádný znak oddělující od sebe různě barevně kvetoucí východokarpatské populace a zároveň jsme objevili bíle kvetoucí jedince i v populaci na lokalitě Babia hora v Západních Karpatech, která však byla molekulárně identická se žlutě kvetoucími rostlinami z okolních pohoří. Obě barvy květů se nevyznačují ani žádným

charakteristickým geografickým rozšířením v rámci severní části Východních Karpat, kde se společně vyskytují nejhojněji. Proto jsme navrhli považovat obě východokarpatské mikrospecie za jediný druh.

Vysoká molekulární variabilita východokarpatských populací svědčí o tom, že mohly přečkat poslední glaciál v refugiích v blízkosti svého současného areálu, což podporují i biogeografické studie druhů preferujících stejný typ vegetace (především subalpínské klečové formace). Možnou existenci glaciálního refugia v prostoru Východních Karpat podporují též paleobotanická data. Populace *M. sylvaticum* s. str. ze Západních Karpat a Hercynského masivu jsou naopak značně molekulárně uniformní, což svědčí o holocenní expanzi tohoto typu z pravděpodobně perialpských refugií.

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REGULAR PAPER

Microsatellite analysis of four similar *Euphrasia* (Orobanchaceae) species changes the traditional view of this group

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Background and aims – The genus *Euphrasia* comprises a taxonomically intricate group. In Central Europe, *E. nemorosa* and *E. stricta* are widely accepted species. However, the occurrence of putative intermediate morphotypes considered to be the result of regular hybridization makes identification of populations often difficult. Besides these mostly late-flowering species, two mostly early-flowering species, *E. coerulea* and *E. slovaca*, are distinguished in the Sudeten and in the Carpathians, respectively. Because of the doubtful nature of intermediate forms and difficult distinction of early-flowering morphotypes, the aims of this study were to find genetically supported groups and test morphological differences among them.
Methods and key results – We conducted a survey of the genetical and morphological diversity in 42 populations, which were assigned to four species based on morphology. Using microsatellite analysis, we discovered three genetic groups within our data set. Whereas *E. stricta* and *E. nemorosa* comprised separate clusters, most of the early-flowering populations identified as *E. coerulea* and *E. slovaca* formed one common cluster. Traditional characters such as corolla length, branching and the presence of a long awn on the bracts were identified in multivariate analyses as the most reliable morphological differences between genetically defined *E. stricta* and *E. nemorosa*. Early-flowering populations differed generally by their low number of nodes. In spite of their genetic similarity, they differed morphologically between the two geographical areas. In spite of the assumption of different selfing rates correlated with corolla size, differences in genetic diversity among populations with different corolla sizes were not found.
Conclusions – There are three well supported groups in the studied dataset of *Euphrasia* species. Delimitation of *E. stricta* and *E. nemorosa* is in concert with traditional views, but delimitation of the third group changes the traditional distinction of two mostly early-flowering species in the study area.

Key words – *Euphrasia*, microsatellites, genetic diversity, morphometrics.

INTRODUCTION

The genus *Euphrasia* L. (eyebright) comprises annual hemiparasitic plants (rarely perennials or semishrubs) distributed in temperate areas and in mountainous areas of tropical zones (e.g. von Wettstein 1896, Hartl 1974, Barker 1982, Smejkal & Dvořáková 2000, Gussarova et al. 2008). Because of their diverse breeding systems, interspecific hybridization, rapid and relatively recent radiation (Gusarova et al. 2008), rapid adaptations to specific conditions (Karlsson 1976, Vitek 2011) resulting in high intraspecific variation (Kolseth & Lönn 2005), seasonal dimorphism and phenotypic plasticity, the genus *Euphrasia* represents one of the most taxonomically intricate and challenging genera in the European flora.

In the European flora there are two ploidy levels in *Euphrasia*: diploid (2n = 2x = 22) and tetraploid (2n = 4x = 44) (Yeo 1954, Vitek & Kiehn 1990). The tetraploids are the most widespread and most critical taxonomically.

The breeding system is supposed to depend on flower size and shape. Several types of flowers and prevailing breeding modes can be found in this genus. Each of them differs in the degree of autogamy, which is very frequent in small-flowered species (von Wettstein 1896, Gómez 2002, Vitek 1998). Species with large flowers reproduce mostly by outcrossing, but if no pollinator visits the flower, they can self-pollinate as well. Species with mid-sized flowers reproduce mostly by allogamy, but autogamy occurs more often compared to large-flowered species (Hartl 1974, Vitek 1998).

Seasonal dimorphism is another important source of intraspecific variability in the genus *Euphrasia*, as well as in other genera of hemiparasitic Orobanchaceae (von Wettstein 1895, von Sterneck 1901, Ronniger 1911, Zopfi 1997, 1998a,

1998b, Štech 2000) and in some Gentianaceae (Zopfi 1991). Two (or more) types of phenologically different forms can be found: early flowering (aestival) and late flowering (autumnal). These two types differ in growth habit, which is used as one of the species determination criteria. Plants of the early-flowering type have no branches (or very few), a small number of internodes and their leaves often persist during flowering. The stems of late-flowering plants are usually branched, have many short internodes and there are usually no stem leaves during flowering (von Wettstein 1895, Smejkal & Dvořáková 2000).

In general, hybridization is common in perennial plants (Ellstrand et al. 1996), while in annuals it is quite rare (Solbrig 1970). However, in *Euphrasia*, as well as in the related genus *Rhinanthus* (von Sterneck 1901, Kwak 1978, Ducarme & Wesselingh 2005), hybridization is assumed to be relatively frequent and is considered to be one of the main causes of the current variability in this genus (Smejkal 1960, Karlsson 1976, Yeo 1978). Hybridization in the genus *Euphrasia* is assumed to occur between all species that come into contact with one another (Vitek 1998). While hybridization between diploid species is not reported very often (Vitek 1982, Smejkal & Dvořáková 2000), hybridization between tetraploids is considered to be very common (Yeo 1954) and many hybrids have been described (e.g. von Wettstein 1893–1895).

Euphrasia is a monophyletic genus (Bennet & Mathews 2006, Gussarova et al. 2008, Těšitel et al. 2010) with 100–300 species depending on the authors (Hartl 1974, Smejkal & Dvořáková 2000, Vitek 2002). The original narrow species concept resulted in the description of hundreds of species and intraspecific taxa, which are connected with many names of different taxonomic categories (von Wettstein 1893–1895, Sennen 1930, Rothmaler 1935, Vitek 1985a, 1985b, 1986). Many early taxonomic treatments (Smejkal 1963, Hartl 1974, Yeo 1978) were based on the narrow species concept used by von Wettstein (1893–1895, 1896). Increasing knowledge on different aspects of variability in this genus may reduce the number of recognized taxa. Recent authors prefer a relatively wide species concept (Vitek 1998, 2002, 2011, Krok et al. 2013). On the contrary, a rather narrow species concept has been accepted in the Czech Republic (Smejkal 1963, Smejkal & Dvořáková 2000, Dvořáková 2002), Slovakia (Králik 1997) and in the Ukraine (Peregrym 2010).

Similar to other parts of Europe, the tetraploid taxa are the principal source of taxonomic uncertainties and identification difficulties in the Czech Republic and there has been a strong need to revise their taxonomy in the country. In addition to morphologically distinct *E. micrantha* Rchb., *E. frigida* Pugsley and the extinct *E. corcontica* (Smejkal) Smejkal & Dvořáková, six other taxa are recorded from the Czech Republic at this ploidy level (Smejkal & Dvořáková 2000, Dvořáková 2002). *Euphrasia stricta* J.P.Wolff ex J.F.Lehm. and *E. nemorosa* (Pers.) Wallr. are widely accepted in the recent European literature (Marhold 2011). On the other hand, *E. tatarica* auct. and *E. curta* subsp. *glabrescens* (Wettst.) Smejkal differ from *E. stricta* or *E. nemorosa* by the presence of short eglandular hairs, which are today usually included in this widely accepted species in most studies (Yeo 1971, Vitek 2005, 2011, Marhold 2011). *Euphrasia coerulea* Tausch and *E. slovaca* (Yeo) Holub are taxonomically the most uncertain species. *Euphrasia coerulea* is described from the Jizera Mts in the Sudeten (Tausch 1834, Szeląg 2014). It is usually considered as an early-flowering ecotype of *E. nemorosa*, and this concept is accepted at the species (Yeo 1978, Králik 1997, Smejkal & Dvořáková 2000) or subspecies level (Danihelka et al. 2012). However, this taxon is often considered as a part of morphological variation of *E. nemorosa* to include in this species by some authors (Vitek 2011). *Euphrasia slovaca* was described originally as a subspecies of *E. arctica* Lange ex Rostr. (Yeo 1971), which is morphologically similar to the *E. stricta* group, and some authors do not accept their separation (Hartl 1974, Krok et al. 2013). The *E. arctica* group differs from *E. stricta* by leaf and bract shape, bract teeth shape and capsule width and separation of the two groups was supported especially by Yeo (1971). Until then, the presence of glandular hairs was considered as the most important characteristic and *E. stricta* was regarded as a species without short glandular hairs, in contrast to the short-glandular pubescent *E. brevipila* Burnat & Gremli ex Wettst. (von Wettstein 1896) and *E. slovaca* (Smejkal 1963, Králik 1997).

Thus, in the recent Czech literature, the four main tetraploid species have been distinguished based on flower size, bract characteristics and indumentum, phenology and occurrence in different geographic regions of Central Europe (Smejkal & Dvořáková 2000, Dvořáková 2002). *Euphrasia stricta* and *E. slovaca* belong to species with medium-sized flowers, and *E. nemorosa* and *E. coerulea* belong to small-flowered species. *Euphrasia stricta* and *E. slovaca* have awns at the end of the bract teeth which lack in the other two species. *Euphrasia nemorosa* is more branched than *E. stricta* and its branches are thicker, while *E. slovaca* and *E. coerulea* are early-flowering species with their first flowers on lower nodes (2^{nd}–6^{th}) as well as branches that are short and thin. All of the species may occur without branches, depending on the surrounding vegetation, their vigour and growth stage.

Euphrasia stricta is distributed in most parts of Europe and is a relatively common species throughout the Czech Republic, while *E. nemorosa* can be found in mountains and hills of Atlantic and subatlantic Europe and occurs only in the suboceanic climate regions of the Czech Republic (Smejkal 1963, 1964, Smejkal & Dvořáková 2000). Due to phenotypic plasticity and a broad range of variation, there are many morphologically intermediate populations which make identification of these species problematic. Additionally, a putative hybrid between these two species, *E.* × *haussknechtii* Wettst., was described (von Wettstein 1893–1895, Yeo 1978), and the frequent occurrence of hybrid populations is reported in the Czech Republic (Smejkal 1960). *Euphrasia slovaca* is considered to be endemic to the western and Ukrainian Carpathians and is recorded in the Czech Republic only from the Moravian Carpathians (Yeo 1971, Smejkal & Dvořáková 2000). *Euphrasia coerulea* is a Sudeten-Carpathian endemic and is recorded from mountains and hills of northern Bohemia (incl. Krkonoše Mts) and from Carpathian regions (Smejkal 1964, Smejkal & Dvořáková 2000).

This study is focused on genetic and morphological variation in these four *Euphrasia* species. Molecular data were used (1) to test hypotheses of differentiation among traditionally distinguished morphotypes, (2) to test morphological

differences among genetically defined groups and (3) to test whether the presumed pollination syndromes are reflected in patterns of genetic diversity.

MATERIAL AND METHODS

Plant material

Euphrasia populations were collected in the Czech Republic, Slovakia, Poland, Austria and Germany (table 1 & fig. 1). In total, 42 populations and 398 plants (vouchers are deposited in CBFS herbarium) were sampled. After preliminary identification in the field, based on flowering time, branching, flower size and colour, presence and amount of glandular hairs (table 2) and the region of sampling, *E. stricta*, *E. nemorosa*, *E. coerulea* and *E. slovaca* were equally covered. Populations showing intermediate morphological characters were classified into one of the species based on prevailing similarity. From most of the populations, ten individuals were collected. In four very small populations (VLH2, HAVR, JAV, VKAR), only five plants were collected. From each individual, several leaves and bracts were silica-dried and taken for genetic analyses. One flower and one bract from the middle of the main inflorescence were attached to paper with transparent tape and scanned at a resolution of 600 dpi. Two plants from the BBAR population were too tiny to obtain material for both genetic and morphometric analyses. Thus, material was taken only for microsatellite analysis.

Microsatellite analysis

DNA extraction was performed using Invisorb Spin Plant Mini Kit (Invitek, Berlin, Germany) or the NaOH method (Werner et al. 2002). Eight microsatellite loci were used for this study. Five microsatellite loci (Ene1, Ene2, Ene3, Ene4 and Ene5) were amplified in 5-µL reactions using primers and PCR conditions as described by French et al. (2003), except that 2.5 µL of 2x Plain PP Master Mix (Top-Bio, Prague, Czech Republic) was used. To obtain higher resolution in the data, microsatellite loci developed by Wang et al. (2009) were tested on European *Euphrasia* species. Three loci (En-B, En-G and En-I) showed variability and were selected for this study. These loci were amplified using M13-tailed primers (Schuelke 2000). PCR contained 2.5 µL 2x Plain PP Master Mix, 0.3 µM of fluorescently labelled M13 primer and reverse primer, 0.075 µM of M13-tailed forward

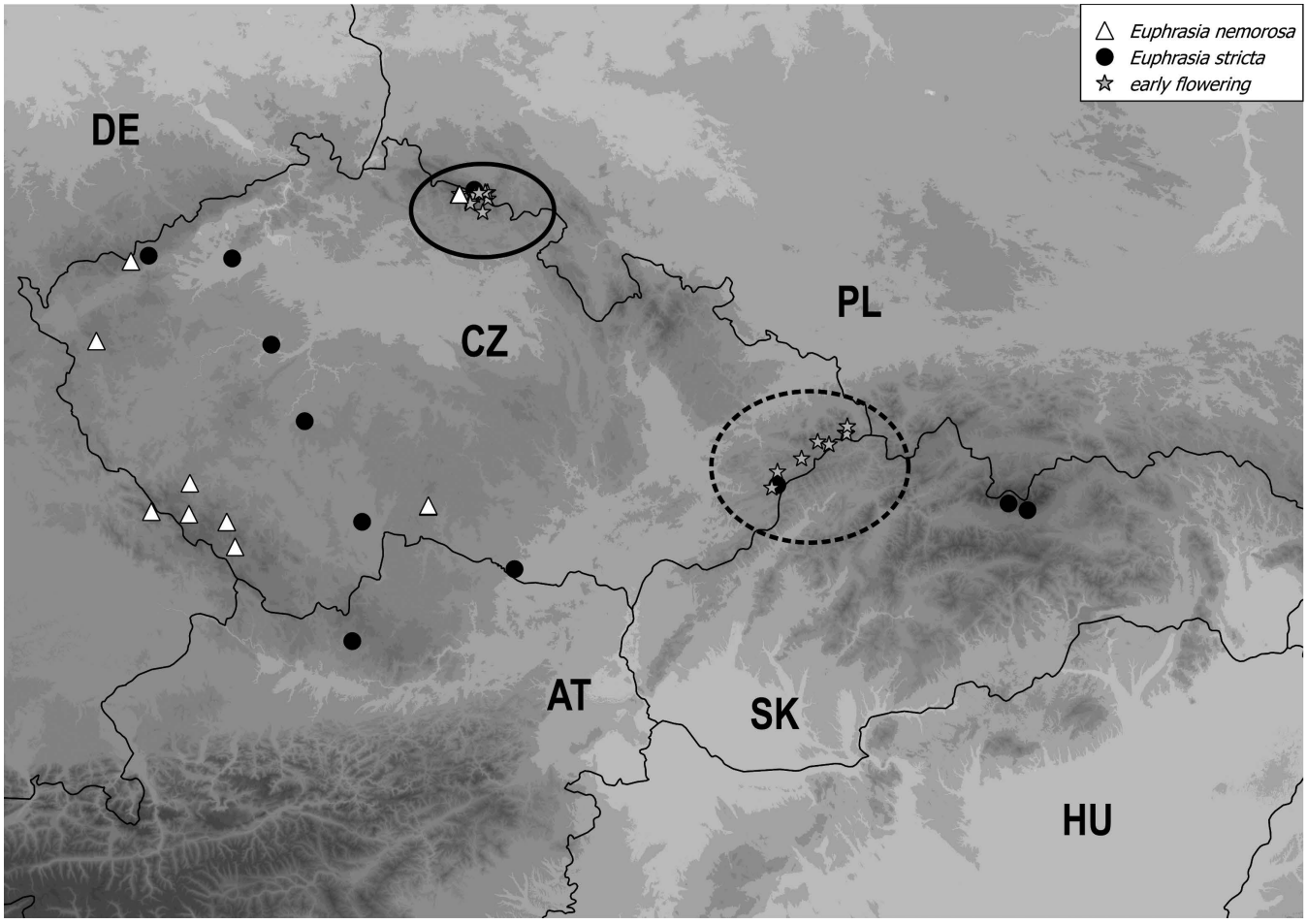

Figure 1 – Localities where the samples used in the present study were collected. *Euphrasia* populations are marked according to the results of genetic analyses. Solid ellipse indicates the Krkonoše Mts (Sudeten Mts), dotted ellipse indicates a part of the Czech Carpathians. Borders of the following central-European countries are displayed: CZ – Czech Republic, AT – Austria, SK – Slovakia, PL – Poland, HU – Hungary, DE – Germany.

Table 1 – List of *Euphrasia* populations used in this study.
Spec. prelim. = species according to preliminary identification, Genet. group = genetic group according to STRUCTURE results, N = number of individuals in population, Ng = number of multilocus haplotypes in population, D_G = diversity index, Na = number of microsatellite alleles in population.

Spec. prelim.	Genet. group	Pop ID	Country	Population	Latitude	Longitude	Altitude (m)	N	Ng	D_G	Na
E. stricta	*E. stricta*	MED	Czech Republic	Měděnec	50°25′28.5″N	13°06′40.8″E	905	10	10	1.000	23
E. stricta	*E. stricta*	BER	Czech Republic	Beroun	49°58′25.1″N	14°04′59.8″E	280	10	10	1.000	20
E. stricta	*E. stricta*	STUD	Czech Republic	Studánky	48°35′24.9″N	14°18′51,0″E	750	10	10	1.000	22
E. stricta	*E. stricta*	POP	Slovakia	Popradské pleso	49°07′24.6″N	20°04′28.6″E	1240	10	7	0.911	19
E. stricta	*E. stricta*	PODB	Slovakia	Podbanské	49°09′28.5″N	19°55′28.3″E	990	10	9	0.978	18
E. stricta	*E. stricta*	SKO	Czech Republic	Skoupý	49°34′56.2″N	14°20′51.6″E	545	10	10	1.000	22
E. stricta	*E. stricta*	RANA	Czech Republic	Raná	50°24′38.7″N	13°46′23.3″E	340	10	10	1.000	26
E. stricta	*E. stricta*	WBW	Austria	Weinsberger Wald	48°26′24.6″N	14°43′29.3″E	660	10	7	0.911	18
E. stricta	*E. stricta*	HAVR	Czech Republic	Havraníky	48°49′01.8″N	16°00′36.3″E	315	5	5	1.000	14
E. nemorosa	*E. stricta*	VO	Czech Republic	Lužnice	49°03′46.8″N	14°48′09.3″E	445	10	8	0.933	23
E. nemorosa	*E. nemorosa*	VLH2	Czech Republic	Velká Lhota 2	49°08′35.3″N	15°19′34.3″E	470	5	2	0.400	13
E. nemorosa	*E. nemorosa*	KLI	Czech Republic	Klínovec	50°23′47.0″N	12°58′15.7″E	1210	10	10	1.000	25
E. nemorosa	*E. nemorosa*	MIS	Czech Republic	Horní Mísečky	50°44′0.8″N	15°34′09.8″E	1000	10	8	0.933	16
E. nemorosa	*E. nemorosa*	HMCP	Czech Republic	Medvědín - late flow.	50°44′0.8″N	15°34′28.9″E	1035	10	3	0.644	12
E. nemorosa	*E. nemorosa*	VIMP	Czech Republic	Vimperk	49°03′44.9″N	13°43′49.2″E	900	10	9	0.978	22
E. nemorosa	*E. nemorosa*	VBOR	Czech Republic	Velký Bor	49°06′09.5″N	13°25′49.3″E	890	11	3	0.378	16
E. nemorosa	*E. nemorosa*	VLH	Czech Republic	Velká Lhota	49°08′50″N	15°19′38.7″E	600	9	2	0.222	13
E. nemorosa	*E. nemorosa*	ML	Czech Republic	Mariánské lázně	49°59′32.5″N	12°41′49.9″E	520	10	5	0.861	13
E. nemorosa	*E. nemorosa*	LEN	Czech Republic	Lenora	48°55′58.7″N	13°47′46.2″E	765	10	10	1.000	23
E. nemorosa	*E. nemorosa*	HMB	Czech Republic	Hory Matky Boží	49°15′46.3″N	13°26′16.9″E	685	10	9	0.978	20
E. nemorosa	*E. nemorosa*	JAV	Germany	Javor	49°06′57″N	13°07′59.1″E	1320	5	1	0.000	13
E. coerulea	early flow.	SML	Czech Republic	Nad Spáleným mlýnem	50°42′36.2″N	15°47′58.4″E	775	10	10	1.000	21
E. coerulea	early flow.	PJE	Czech Republic	Pod Jelenkou	50°44′29.8″N	15°47′45.0″E	1035	10	10	1.000	25
E. coerulea	early flow.	JLN	Czech Republic	Jelenka	50°44′31.5″N	15°46′41.8″E	1255	10	10	1.000	22
E. coerulea	early flow.	CHO	Czech Republic	Černá hora	50°38′33.6″N	15°45′27.8″E	1070	10	10	1.000	25
E. coerulea	early flow.	MOS	Czech Republic	Modré sedlo	50°43′39.7″N	15°41′37.4″E	1410	10	1	0.000	13
E. coerulea	early flow.	JLN2	Czech Republic	Jelenka 2	50°44′31.5″N	15°46′39.3″E	1260	10	8	0.956	20
E. coerulea	early flow.	SB	Czech Republic	Slezská Bouda 1	50°44′21.7″N	15°43′41.5″E	1390	10	1	0.000	12

Table 1 (continued) – List of *Euphrasia* populations used in this study.

Spec. prelim.	Genet. group	Pop ID	Country	Population	Latitude	Longitude	Altitude (m)	N	Ng	D_G	Na
E. coerulea	early flow.	SB2	Czech Republic	Slezská Bouda 2	50°44'20.3"N	15°43'43.1"E	1385	10	3	0.378	17
E. coerulea	early flow.	PRB	Czech Republic	Přední Renerovy boudy	50°41'42.8"N	15°39'22.6"E	1225	10	8	0.933	23
E. coerulea	early flow.	HB	Czech Republic	Husí Boudy	50°41'08.4"N	15°39'32.8"E	1040	10	9	0.978	18
E. coerulea	early flow.	HMCC	Czech Republic	Medvědín - early flow.	50°44'0.8"N	15°34'28.9"E	1036	10	3	0.378	16
E. coerulea	*E. stricta*	VST	Poland	Velký Stav	50°45'20.2"N	15°41'28.4"E	1405	10	3	0.378	15
E. slovaca	*E. stricta*	ZDE	Czech Republic	Zděchov	49°15'10.3"N	18°05'34.0"E	660	10	1	0.000	12
E. slovaca	early flow.	SIV	Czech Republic	U Sivků	49°19'17.5"N	18°05'37.3"E	640	10	3	0.378	14
E. slovaca	early flow.	HLU	Czech Republic	Horní Lomná - Úpalony	49°31'18.3"N	18°37'56.2"E	605	10	8	0.822	17
E. slovaca	early flow.	PREL	Czech Republic	Přelač	49°31'0.7"N	18°38'31.3"E	780	10	4	0.711	15
E. slovaca	early flow.	BBAR	Czech Republic	Baraní	49°27'42.0"N	18°30'5.8"E	575	8	8	1.000	27
E. slovaca	early flow.	KAM	Czech Republic	Pod Kamenitým	49°33'16.9"N	18°38'51.3"E	685	10	9	0.978	19
E. slovaca	early flow.	HUT	Czech Republic	Staré Hamry	49°28'32.2"N	18°24'44.3"E	535	10	10	1.000	24
E. slovaca	early flow.	VKAR	Czech Republic	Velké Karlovice	49°23'20.9"N	18°17'03.5"E	715	5	3	0.700	18
E. slovaca	early flow.	LUZ	Czech Republic	Lužná	49°14'18.5"N	18°2'55.9"E	660	10	5	0.667	16

primer, 0.4 µL of DNA template, and sterile water to a final volume of 5 µL. PCR conditions for all three loci were as follows: 3 min of initial denaturation at 94°C, followed by 30 cycles of 30 s at 94°C, 30 s at 50°C and 30 s at 72°C, then followed by 8 cycles of 30 s at 94°C, 30 s at 46°C and 30 s at 72°C, and then a final extension step for 10 min at 72°C. PCR products were pooled and subjected to fragment analysis (SEQme s.r.o., Dobříš, Czech Republic).

The lengths of microsatellite alleles were read using GeneMarker ver. 1.80 (SoftGenetics, LLC, USA) and scored as dominant data (0/1) because all studied species are tetraploids and allelic scoring was not possible. The genetic structure was inferred by a Bayesian clustering approach using STRUCTURE 2.3.3 (Pritchard et al. 2000) at MetaCentrum VO (https://metavo.metacentrum.cz/). Both admixture with no prior information and correlated allele frequencies and no admixture models with no prior information and uncorrelated allele frequencies were used. The burn-in period was 200,000 and 1,500,000 iterations were run afterwards. Analyses were performed for K from 1 to 10 with 20 replicate runs for each K. The optimal number of clusters K in the dataset was selected using the methods described by Evanno et al. (2005) using Structure Harvester (Earl & vonHoldt 2012). Moreover, the analysis of population subsets of only the populations morphologically classified as *E. slovaca* and *E. coerulea* was performed.

Jaccard's coefficient was used to calculate the distance matrix for principal coordinate analysis (PCoA), which was computed in Canoco for Windows ver. 5 (ter Braak & Šmilauer 2012). STRUCTURE clustering was independently displayed in PCoA. The number of microsatellite alleles and the number of multilocus genotypes per population and shared genotypes were calculated using Arlequin 3.5.1 (Excoffier & Lischer 2010). Genotype diversity was estimated as a modification of the Simpson's index (Pielou 1969, Berg & Hamrick 1994): $D_G = 1 - \Sigma\, n_i(n_i - 1)/N(N - 1)$, where ni is the number of individuals of genotype i and N is the total number of individuals in population. Differences among groups delimited by STRUCTURE in the genotype diversity index and the number of alleles per population were tested by non-parametric Kruskal-Wallis one-way analysis of variance using Statistica 12 (StatSoft 2001), as well as the correlation between corolla length and the genotype diversity index.

Morphological analysis

In total, twenty characters were measured on each plant (table 3 & fig. 2). Software tpsDig ver. 2.10 (Rohlf 2006) was used for measurements of the scanned material. The normality of traits was checked visually on histograms. The character "number of branching nodes", which distribution markedly deviated from normal, was log-transformed. The correlation between traits was examined with the use of Pearson's correlation coefficients. The main components of variation were evaluated using principal component analysis (PCA). To find out which characters significantly separated groups defined by the microsatellite analysis, canonical discriminant analysis (CDA) with forward selection of traits was applied. The threshold significance level was set to $\alpha = 0.05$ with Bonferroni correction and a Monte-Carlo permutation

Table 2 – Characters used to identify species in the field.

Species	Seasonal type	Branches	Flower size	Flower colour	Glandular hairs
E. stricta	late	erect or divergent	6–10 mm	lilac to white	none
E. nemorosa	late	frequent, thick, ascending	5–7 mm	white to lilac	none
E. slovaca	early	short or none	6–8.5 mm	deep lilac to white	many
E. coerulea	early	short or none	5–7 mm	purple to white with lilac upper lip	infrequent

Table 3 – List of characters studied in the morphometric analysis.
The accuracy of the measurements was on one decimal place.

ID	Description of character	Unit
V2	height of plant to the first flower	cm
nodes	number of nodes up to first flower	count
nodes_br	number of branching nodes	count
CL	corolla length	mm
CTL	corolla tube length	mm
CTW	corolla tube width	mm
CH	corolla height	mm
UCL	length of upper corolla lip	mm
CLU	length of side of lower corolla lip	mm
CLL	length of lower corolla lip	mm
CLW2	1/2 of width of lower corolla lip	mm
LCD	diagonal of lower corolla lip	mm
BL	bract length	mm
BW	bract width	mm
BD	distance from the widest point of the bract to its base	mm
BT3W	width of third tooth on bract	mm
BT3L	length of third tooth on bract	mm
BT3O	length of awn of third tooth on bract	mm
BTLW	width of terminal tooth on bract	mm
BTLL	length of terminal tooth on bract	mm
BTLO	length of last of terminal tooth on bract	mm

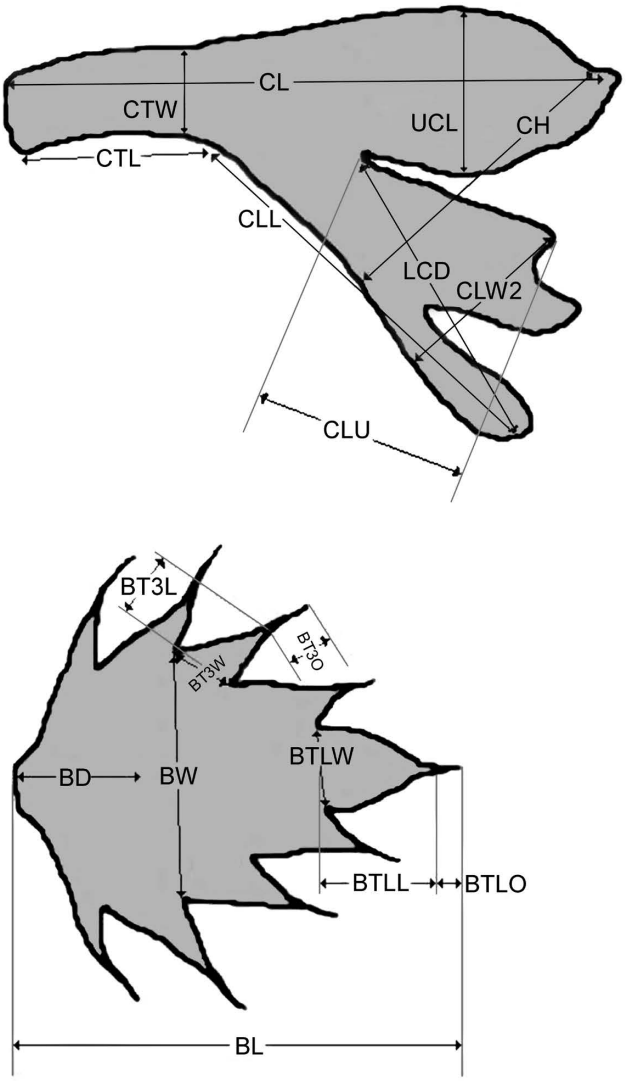

Figure 2 – Characters measured on flowers and bracts. Abbreviations are explained in table 3.

test (999 permutations) was used. Classificatory discriminant analysis was performed with cross-validation using population as the leave-out unit. All analyses were performed using MorfoTools scripts in R 3.1.2 (R Core Team 2014, Koutecký 2015) except for PCA and CDA, which were computed using Canoco for Windows ver. 5 (ter Braak & Šmilauer 2012).

RESULTS

Microsatellite analysis

A total of 63 alleles were detected across the eight analysed loci (twelve from Ene1, eleven from Ene2, twelve from Ene3, four from Ene4, four from Ene5, thirteen from En-B, two from En-G and five from En-I). All microsatellite loci were polymorphic across the whole data set. In one group of populations (*E. stricta*, see the group definition below), all loci were polymorphic. In the other two groups (*E. nemorosa* and early flowering group), the En-G locus was monomorphic with a single exception (LEN).

STRUCTURE clustering (both the no-admixture and admixed models) revealed an optimal separation of the dataset into three groups (fig. 3, data shown for no-admixture model), instead of the expected four (DeltaK for four clusters was considerably lower, electronic appendix 1). Most of the populations that were preliminarily identified as late flowering *E. stricta* and *E. nemorosa* formed separate clusters

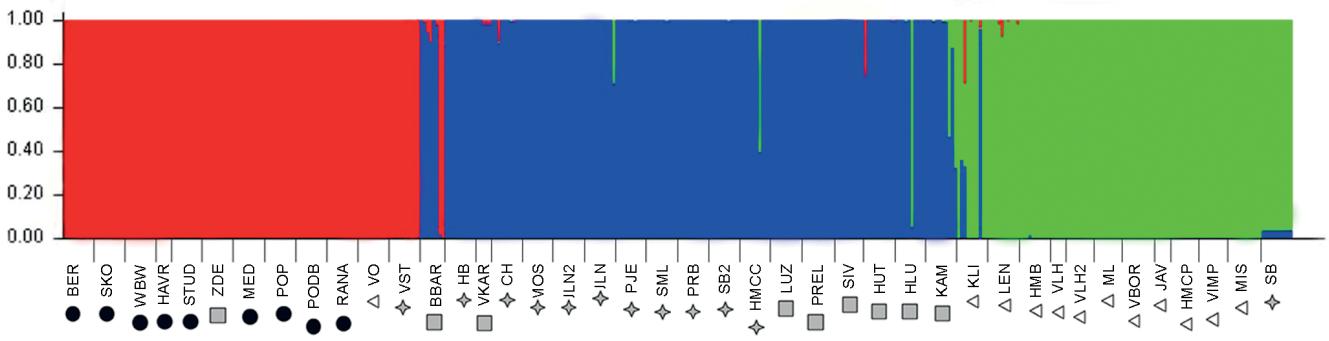

Figure 3 – Bar plot of the three genetic groups (*E. stricta*: red, *E. nemorosa*: green, early flowering populations: blue) detected by STRUCTURE. Original identification of populations is depicted with symbols (circle = *E. stricta*, square = *E. slovaca*, star = *E. coerulea*, triangle = *E. nemorosa*).

while early flowering populations corresponding to *E. coerulea* and *E. slovaca* formed one common cluster despite their provenance from two geographically distant regions. In the analyses resulting in four genetic groups, the early-flowering populations were divided into two subgroups not corresponding to morphology, ecology or geographic distribution. The same result was obtained from the analysis of the subset of early-flowering populations only (results not shown). In *E. stricta*, populations were uniform and all individuals were clearly assigned to *E. stricta* in all STRUCTURE runs. Only few populations of the rest of the *Euphrasia* taxa included in the study comprised individuals from different molecular clusters. In *E. nemorosa*, the population VO was competely assigned to *E. stricta* based on microsatelite analysis, the population KLI was admixed with both the early-flowering group and *E. stricta* group, and three other populations showed minor admixture. In the early-flowering group, more populations were slightly admixed either with *E. stricta* or *E. nemorosa* (fig. 3).

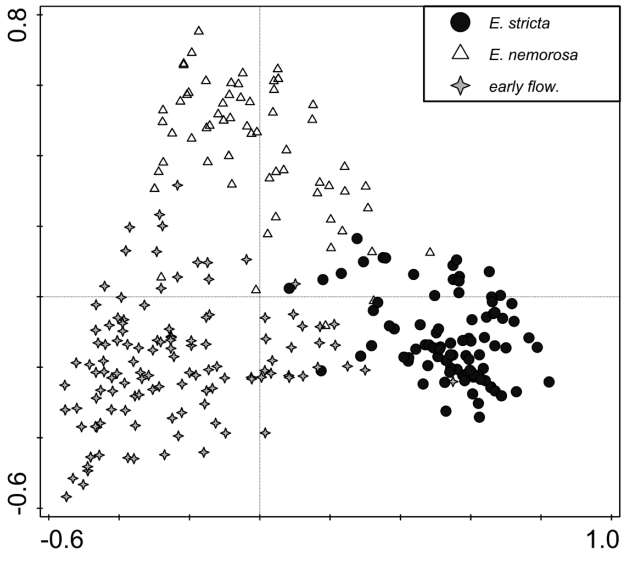

Figure 4 – Principal coordinate analysis (PCoA) of variability in microsatellites of 398 *Euphrasia* individuals based on the Jaccard coefficient. The first, second and third axis explained 15.78%, 11.3% and 6.27%, respectively, of the total observed variability. First and second ordination axes are shown.

The three genetic groups were also distinct in the PCoA plot (fig. 4). The first three axes explained 33.35% of variability in the microsatellite data. *Euphrasia stricta* was placed on the right side of the ordination plot, while *E. nemorosa* and early-flowering populations were on the left. The second axis further separated *E. nemorosa* from the early-flowering populations. The separation of all three clusters was not complete and implied shared alleles in several populations from different genetic groups. Part of this overlap was caused by the admixed populations identified by STRUCTURE (fig. 3). In most cases, individuals from particular populations formed rather compact clusters. However, some populations showed higher intrapopulation variation and individuals were scattered over a larger part of the ordination diagram.

Genetic diversity parameters of populations are shown in table 1. There was apparent variation among populations in a number of genotypes per population as well as genetic diversity.

Kruskal-Wallis one-way ANOVA revealed no significant differences among the three genetic groups in the number of alleles per population and the diversity index ($p > 0.5$). No significant correlation was revealed between corolla length and diversity index (Spearman $r = 0.2$, $p > 0.05$). Most populations of *E. stricta* had a high diversity index (> 0.9), while only two had a low diversity index (0 and 0.67 respectively). Most of the early-flowering populations showed a high diversity index. In contrast, the proportion of populations with a high diversity index and a low diversity index was rather balanced in the *E. nemorosa* group.

Except for a few exceptions, multilocus genotypes were private for all populations except two geographically very close populations of *E. nemorosa* (MIS, HMCP), which shared one multilocus genotype. Another two geographically close "early-flowering" *E. coerulea* populations (PRB and HB) shared two multilocus genotypes. One of these populations from the Krkonoše Mts (PRB) shared one multilocus genotype with a remote *E. slovaca* population from the Carpathians (HUT).

Table 4 – Results of canonical discriminant analysis of the three genetic groups of *Euphrasia* individuals with forward selection of the characters.
Characters with significant conditional effect (i.e. the effect of the variable in addition to other variables already included in the model) are listed. Explained% = part of the total variation explained by the individual variable, P = Bonferonni-corrected significance level, CorE scores = correlations with axes of the canonical discriminant analysis. Marg. – characters with significant marginal effect (i.e. when the variable is alone in the model) but insignificant conditional effect. marg.: nodes_br, CTL, UCL, CLW2, BL, BD, BT3W, BT3L, BTLO.

Character	Explained%	P	CorE scores	
			Axis 1	Axis2
CL	21.0	0.001	0.5923	-0.2625
nodes	20.1	0.001	0.2710	0.5669
BTLW	4.6	0.001	-0.2502	-0.4336
BT3O	3.6	0.001	0.5189	-0.2164
LCD	2.9	0.001	0.4107	-0.4072
BTLL	1.7	0.001	0.0910	-0.2852
CTW	1.7	0.001	0.5563	-0.0797
V2	1.1	0.001	0.1905	0.0819
BW	1.0	0.002	-0.0662	-0.2195

Morphological analysis

Based on the high correlation (Pearson coefficient > 0.85) between characters, three of them (CH, CLU and CLL), which correlated with CL, CLW2 and LCD, respectively, were excluded from further analyses.

Principal component analysis (PCA) of both, populations and individuals, revealed a rather poor structure of morphological variation in our dataset (results not shown). The canonical discriminant analysis (CDA) of populations revealed good morphological separation of the three clusters defined by STRUCTURE when all traits were included (fig. 5). Forward selection identified four characters, corolla length (CL), diagonal of lower corolla lip (LCD), number of nodes up to the first flower (nodes) and the width of the terminal tooth on bract (BTLW), which were sufficient for separation of the groups.

Although CDA of individuals showed morphological separation of the three groups as well, there was some overlap between the groups (fig. 6). The overlap between *E. stricta* and *E. nemorosa* was larger than the overlap of the early-flowering group and *E. stricta* or *E. nemorosa*. Forward selection identified nine characters that contributed most to the separation of the three groups (table 4).

The ability of all of the nine selected traits to make correct identifications was tested by classificatory discriminant analysis. In general, more than 78% of individuals were correctly classified (table 5). The incorrect identification was not evenly spread; most of the populations had more than 90% correct classifications. When there was misclassification in the *E. stricta* or *E. nemorosa* population, they were classified mostly as the early-flowering group. In three populations, PODB, VO and ZDE, classification was very poor and only 30% or less was successful. The detailed results for each population are listed in electronic appendix 2.

Discriminant analysis was also performed for the group of early-flowering populations only, as they come from different mountain ranges. The analysis revealed good morphological separation of plants from these regions (table 6, fig. 7), but this did not correspond to the genetically delimited groups. Characters selected by forward selection as the most suitable for discriminating these two groups of populations were the number of nodes, the number of branching nodes (but in a different range than the one that differentiates between aestival and autumnal taxa), the length of the terminal tooth on bract and corolla length.

DISCUSSION

Genetic structure

Based on preliminary identification, ecology and distribution of studied populations, we expected four genetic groups. However, our results based on microsatellite data revealed a clear division of the studied populations into three groups only, predominantly late-flowering *E. stricta*, late-flowering *E. nemorosa* and the early-flowering group formed by merging *E. coerulea* and *E. slovaca*. Genetically defined groups of *E. stricta* and *E. nemorosa* populations corresponded mostly with the preliminary identification of both species. The presence of short eglandular pubescence recorded in some studied populations of *E. stricta* and *E. nemorosa* have no genetic support and this result is in concert with the recent inclusion of central European populasions named as *E. tatarica* and *E. curta* in *E. stricta* or *E. nemorosa*, respectively (Vitek 2011, Krok et al. 2013).

Table 5 – Classificatory discriminant analysis of the three *Euphrasia* groups.
Number and percentage of correctly classified individuals for each of the three groups are given. Data obtained from CDA with all characters (78.47% correct) and CDA with characters after forward selection (78.75% correct) are shown.

	CDA with all characters			CDA with characters after forward selection		
	early flow.	*E. nemorosa*	*E. stricta*	early flow.	*E. nemorosa*	*E: stricta*
early flow.	141 (91.6%)	8 (5.2%)	5 (3.2%)	138 (89.6%)	9 (5.8%)	7 (4.6%)
E. nemorosa	11 (11.2%)	69 (70.4%)	18 (18.4%)	13 (13.3%)	74 (75.5%)	11 (11.2%)
E. stricta	21 (18.3%)	16 (13.9%)	78 (67.8%)	23 (20%)	15 (13%)	77 (67%)

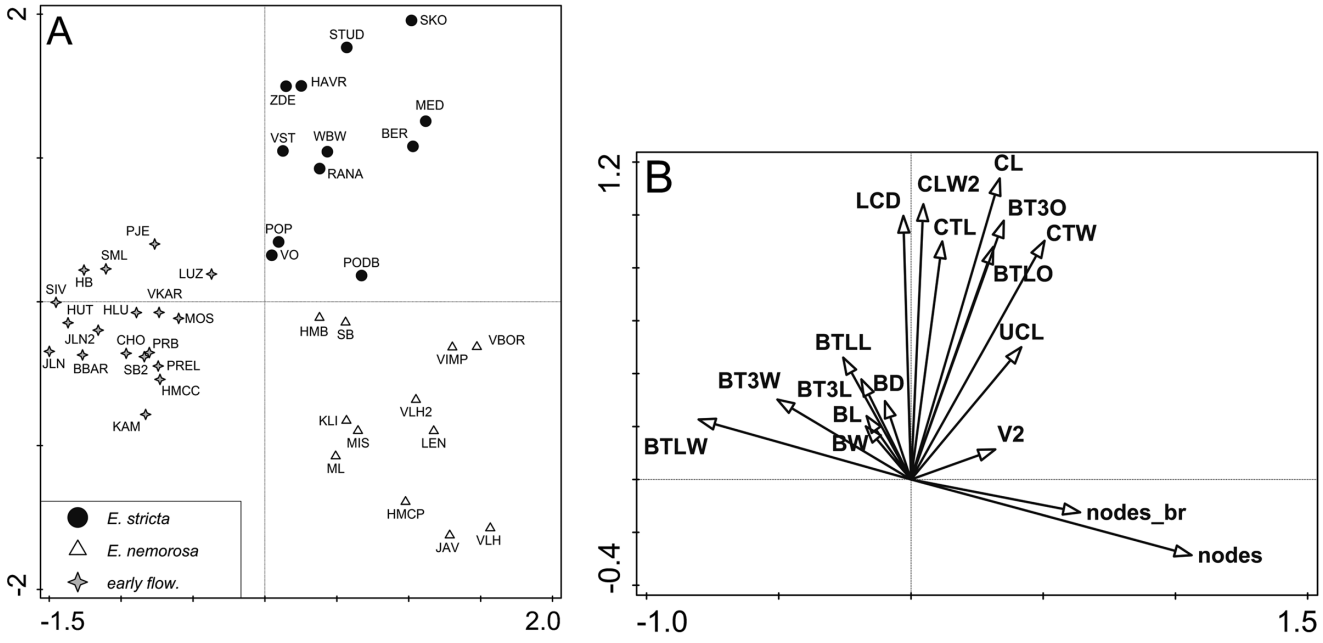

Figure 5 – Results of canonical discriminant analysis (CDA) of populations (A) and all measured characters (B) of the three genetically defined groups of studied *Euphrasia* samples. The first and second axes explained 79.50% of the total observed variability. The first and second ordination axes are shown.

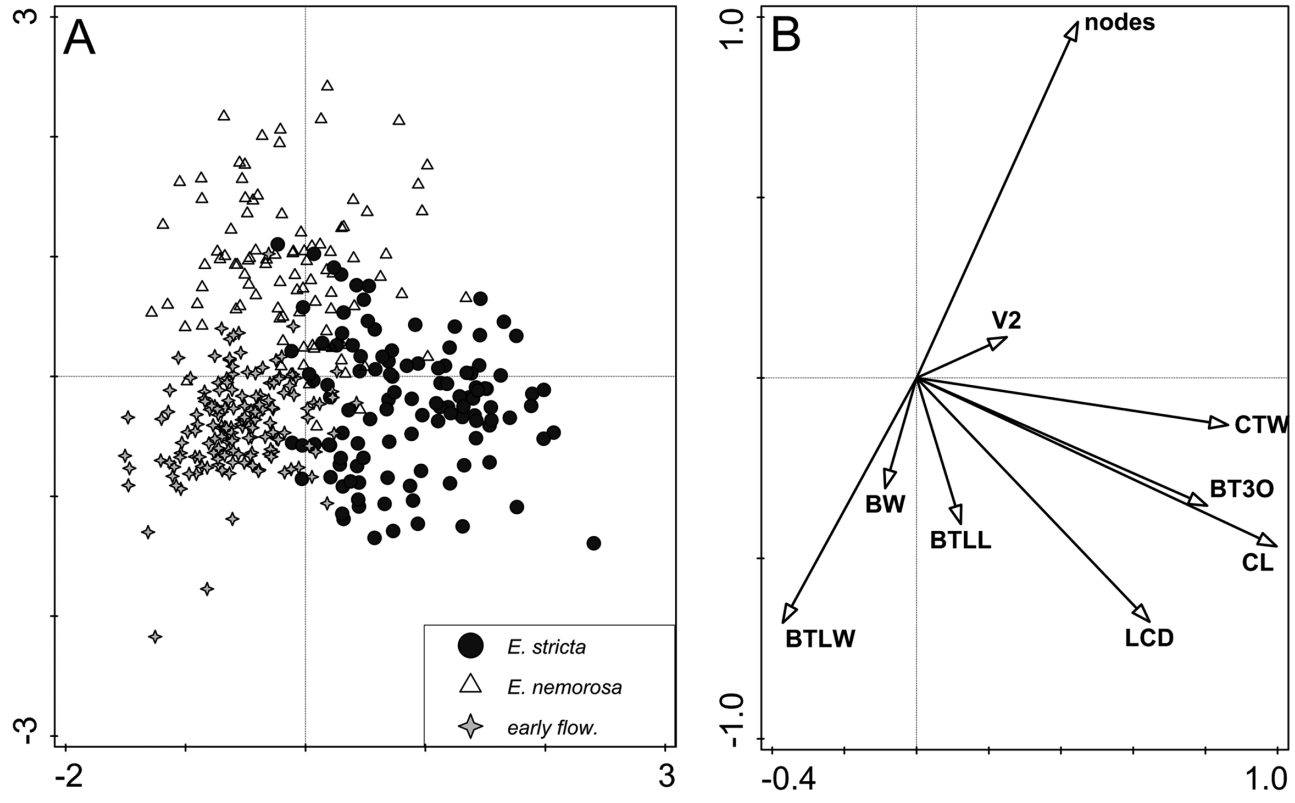

Figure 6 – Results of CDA of individuals (A) and all measured characters (B) of the three genetically defined groups of the studied *Euphrasia* samples. The first and second axes explained 57.67% of the total observed variability.

Table 6 – Classificatory discriminant analysis of the early flowering *Euphrasia* group.
Number and percentage of correctly classified individuals to the mountain range from which they originate are given. Data were obtained from CDA with characters after forward selection (93.54% correct).

	Krkonoše Mts	Carpathians
Krkonoše Mts	95 (95%)	5 (5%)
Carpathians	5 (9.1%)	50 (90.9%)

The third genetic group comprised early-flowering populations both from the Carpathians and most of the populations from the Krkonoše Mts Populations in the Carpathians occur in mowed meadows and along forest paths and are characterized by short glandular pubescence of bracts, although its density may vary considerably. The typical habitat of populations from Krkonoše Mts consists of road edges and subalpine localities. The indumentum of plants differed as well and the occurrence of glandular hairs on bracts was rare.

Differences in the presence of short glandular hairs between Sudeten populations (Krkonoše Mts) and Carpathian populations of *E. coerulea* were observed by Smejkal (1963). Moreover, the rare presence of glandular hairs was observed in many populations of *E. stricta* from different regions included in this study, which indicates variation in this character and the necessity of performing another study in regions where glandular populations classified as taxa from the *E. stricta* assemblage are common (see below). On the other hand, detection of a shared multilocus genotype between Krkonoše Mts and Carpathians suggests close genetic relationships between morphologically diverse populations from geographically separated areas.

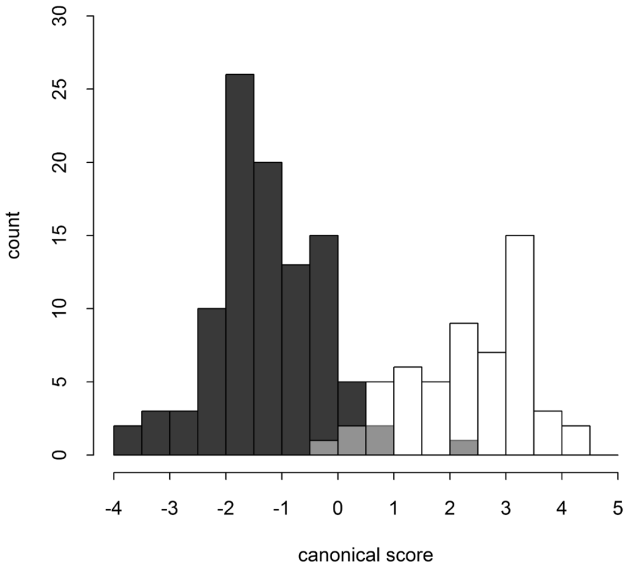

Figure 7 – Histogram of canonical scores of CDA of individuals of the early-flowering genetic group (dark grey = Krkonoše Mts, white = Carpathians; pale grey = overlap of both). Only characters chosen by forward selection were used.

The separate position of early-flowering populations is in opposition to the inclusion of early-flowering populations of *E. coerulea* in *E. nemorosa* in the recent treatment of *Euphrasia* in Germany (Vitek 2011). Smejkal (1963) considered *E. coerulea* as an early-flowering vicariant of *E. nemorosa* as well. However, unification of most of the studied early-flowering populations is an important change for a better taxonomic understanding of this group. Most of the early-flowering populations from the Carpathians have been recently identified as *E. slovaca*. *Euphrasia coerulea* is recorded as an extremely rare species from this region (Dančák 2011). The reality seems to be completely different. Most of the early-flowering populations with relatively small flowers belong to *E. coerulea*. Only one population (ZDE) with early phenology and distinctly larger flowers was classified as *E. stricta*, but was not a separate group. The case of *E. slovaca* is more complex, and our data are not sufficient to resolve this. Although this taxon was described from the Ukraine, the author recorded its occurrence from the Moravian Carpathians (Yeo 1978). Plants from the population ZDE morphologically resemble plants of the type specimen of *E. slovaca* (deposited in herbarium PRC). There is the principal question of the relation between *E. stricta* and *E. arctica* accepted by Yeo (1971) and other similar taxa with glandular hairs – e.g. *E. brevipila*, which also includes *E. slovaca* (Posz 2014). The other taxon that should be studied is *E. chitrovoi* Tzvelev, which was described from northwestern Russia, but is supposed to occur in Central Europe as well (Tzvelev 1980). All these questions must be the topic of separate studies with a large sampling area especially in Northern Europe and in the Carpathians.

Hybridization

Despite general expectations, hybridization was surprisingly rare in the studied populations. Only one population from the *E. nemorosa* group (LEN) contained several alleles typical for *E. stricta*. We should consider possible hybridization with some *E. stricta* populations that grow nearby. A quite good marker of introgression is the En-G locus, which was polymorphic in the *E. stricta* and LEN populations, but monomorphic in the rest of the *E. nemorosa* populations. A trigger of hybridization between populations could be the destruction of natural barriers caused by human activities. This might frequently be the case in mostly ruderal places and on downhill courses in ski areas connecting populations from different altitudes. We suppose this is the case in the most admixed population of the whole dataset, KLI. In spite of this, our results suggest that hybridization between *E. stricta* and *E. nemorosa* occurs much less often in the area of interest than stated in the literature (Smejkal 1963, Smejkal & Dvořáková 2000). Hybridization in the early-flowering group does not seem to be an important phenomenon, because of the relatively small number of populations with an indication of admixture of other species. The presence of markers of *E. stricta* in predominantly early-flowering BBAR population can be explained by the occurrence of non-flowering plants, which probably belonged to *E. stricta*. Actually, if there was a former hybridization event, genes of one paren-

tal species could prevail and populations could be identified rather easily as one of the parental species. Rapid population dynamics and the prevalence of markers of one parental species was detected in hybrid populations of *Rhinanthus minor* and *R. angustifolius* (Ducarme et al. 2010).

Morphological characters separating genetic clusters

The results obtained from classical morphometric analysis of populations showed separation of the three genetic groups (fig. 4). However, there were large overlaps in the morphology of all three groups caused by considerable morphological variability within populations (figs 5 & 6) and phenotypic plasticity (Karlsson 1976). Classificatory discriminant analysis gave a correct morphological identification of most of the populations, although some populations showed very low success of correct identification to genetic clusters.

The most reliable morphological characters delimiting the early-flowering group were the width of the terminal tooth on bract (BTLW) and the number of nodes under the first flower (nodes). These traits were generally used to distinguish early-flowering plants from the late-flowering plants by previous authors (Smejkal & Dvořáková 2000). Populations of *E. stricta* have the largest flowers of all of the studied populations and thus corolla length (CL) and diagonal of lower corolla lip (LCD) were the best characters to discriminate this species. However, plants from several populations of *E. stricta* with unusually small flowers (e.g. PODB, VO) were often misclassified in the classificatory discriminant analysis. The existence of small-flowering populations of *E. stricta* was mentioned by Smejkal (1963) and Karlsson (1976). This is probably the case with population VO, which was wrongly classified in the field. Similarly, misclassification of some plants from two large-flowering populations of *E. nemorosa* was detected.

Another important diagnostic characteristic, especially for *E. stricta* and *E. nemorosa*, is the presence of awns. Although most authors stated that *E. nemorosa* does not have awns (Smejkal 1963, Stace 1997), other authors (e.g. Pugsley 1930, Hartl 1974 or Yeo 1978) admitted that awns might be present. This confusion is caused by a different species concept that includes other species in *E. nemorosa*. However, in spite of following Smejkal's (Smejkal & Dvořáková 2000) species concept, some of the studied *E. nemorosa* populations had awns on bracts. We observed awns most frequently on the lowermost two or three bract teeth. However, awn length in *E. nemorosa* was less than in *E. stricta*.

There were several other populations with very low success of correct identification. The population ZDE was the most challenging. Classification in the early-flowering population is in agreement with a preliminary determination based on general habitat. However, this population belongs to *E. stricta*. Early-flowering populations of *E. stricta* are common in North Europe and some seasonal intraspecific taxa are distinguished there. These taxa are extremely rare in Central Europe, and in spite of the two subspecies (*E. stricta* subsp. *stricta* and *E. stricta* subsp. *suecica* (Murb. & Wettst.) Wettst.) being accepted for the Czech Republic (Smejkal 1963), no subspecies have been recorded in a recent Czech flora (Smejkal & Dvořáková 2000, Dvořáková 2002). However, this issue must be the other topic of a large study of *E. stricta* resemblance.

Morphological convergence in extreme climatic conditions can be another reason for misclassification in some populations. This may be the case in some populations from a mountain ridge (VST, SB), where all populations looked very similar, but they were clustered in different genetic groups than most of the populations from high altitudes of the Krkonoše Mts Gene flow between nearby growing populations can be another reason for morphological differences from typical populations. However, corolla colour seemed to be a useful character distinguishing between *E. nemorosa* and the early-flowering group in this region. On the other hand, the corolla colour is usually stated to be variable in many species (Smejkal 1963, Yeo 1978, Smejkal & Dvořáková 2000) and the situation may be different in other parts of the distribution area of this species.

The apparent morphological separation we found between genetically identical early-flowering populations from the Carpathians and Krkonoše Mts could be explained by the phenomenon of seasonal dimorphism. It is known that different seasonal types of hemiparasitic plants look morphologically distinct in spite of genetic similarity. Different seasonal types can differ not only in the number of nodes and branches but also in bract shape and flower size (von Sterneck 1901, von Soó 1926–1927). One of the important differences between populations from the Carpathians and Krkonoše Mts is the number of nodes which indicates different seasonal types. Populations from Krkonoše Mts had a higher number of nodes, which correlated with a later flowering time in this region. However, it seems that these morphotypes do not differ genetically.

Population diversity

Kolseth & Lönn (2005) observed a stronger divergence among populations than among morphologically and ecologically defined varieties of *E. stricta* in Gottland based on AFLP. The population structure of our dataset showed a similar pattern. Multilocus genotypes were virtually unique for each population. Shared multilocus genotypes were present only in three pairs of populations. In two cases, this was probably caused by seed transport along a road because these populations are in close geographical contact.

We found no significant differences in diversity index values and number of alleles per population among genetic groups of *Euphrasia*, which are likely to differ in their prevailing mode of reproduction. On the other hand, non-significant results might imply a more complex scenario that includes local factors that influence genetic variation. The highest values were found in *E. stricta* (table 1), which has the largest flowers of the species included in our study. It was supposed that this type of flowers favours outcrossing, while small-flowered species rely more often on autogamy (Vitek 1998, Gómez 2002, French et al. 2005). However, there were populations of *E. stricta* with an extremely low diversity index value (ZDE and VST). The population VST was located in the extreme climatic conditions of the mountain ridge, which may cause lack of pollinators and favour autogamy. This phenomenon could underlie the obvious reduction in

multilocus genotype numbers in populations of other species in this area. In addition to a potentially autogamous reproduction, the extremely small population size in the ZDE population caused by a bottleneck or resulting from inbreeding could be a reason for the low variation, as reduction of diversity in small populations is well-known (Young et al. 1996). In most small-flowered *E. nemorosa*, the populations usually had a reduced number of multilocus genotypes, especially in small populations (JAV, VLH2). However, the population size itself is not the only factor influencing variability in the population. There were several small-sized populations with high diversity index values, as well as large populations with low values. Another process reducing genetic diversity could be a strong directional selection pressure (Lacy 1987). This may be the case of early-flowering populations in mowed meadows in the Carpathians. Many populations there were quite homogeneous and consisted of one multilocus genotype. This situation suggests adaptation of local populations to management. If the type of management changes, these populations probably go extinct locally or rapidly change their behaviour. Several populations with large variability were found in the Carpathians as well. These populations were usually found along roads, which enables gene exchange or, as in the case of the BBAR population, were mixed with *E. stricta*.

CONCLUSION

Contrary to the traditional view, we detected only three genetically defined groups of tetraploid *Euphrasia* populations in our dataset. The species *E. stricta* and *E. nemorosa* are rather well morphologically defined and hybrid populations are not as frequent as it has been supposed. The best differences are the traditionally used corolla length and awn presence. Populations with an atypical flower size can also be found, which can complicate identification. On the other hand, there is very good support for the distinction of early-flowering populations as a separate species *Euphrasia coerulea*. In general, the number of nodes is a more useful characteristic for identification than the presence of glandular hairs, which is typical for Carpathian populations only. These populations differ from genetically similar populations from the Krkonoše Mts in the number of nodes, which was lower. However, there are large overlaps in the morphology of all three groups caused by considerable morphological variability within populations and the fact that different species react in the same way to habitat conditions. The correct identification may be very difficult due to the rare occurrence of hybrids or introgression, but especially the parallel origin of morphologically similar types as a reaction to habitat conditions. No significant differences in population genetic diversity among species differing by flower size were found.

More exhaustive research must be performed to understand the relationships and taxonomy of *E. stricta* resemblance outside of study area, where other morphotypes (especially with glandular hairs and aestival characteristics) occur.

SUPPLEMENTARY DATA

Supplementary data are available in pdf at *Plant Ecology and Evolution*, Supplementary Data Site (http://www.ingentaconnect.com/content/botbel/plecevo/supp-data), and consist of: (1) structure Harvester (Earl & vonHoldt 2012) summary of STRUCTURE results for whole microsatellite dataset of *Euphrasia* species, and (2) classificatory discriminant analysis of particular *Euphrasia* populations into the three genetic groups.

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