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# Evolutionary and conservation consequences of interspecific hybridization in rare plant species

# Evoluční a ochranářské důsledky mezidruhové hybridizace u vzácných druhů rostlin

PhD Thesis

Supervised by: Jan Suda

Prague, 2014

## Content

## Declaration

I hereby declare that I wrote this thesis independently using the mentioned references. I did not submit or present any part of this thesis for any other degree or diploma.

Průhonice, 13. 5. 2014

## Author contribution statement

I declare that I have contributed to all involved papers substantially and my contribution to particular papers are as follows:

**I.** **Vít P**., Wolfová K., Urfus T., Tájek P. and Suda J. (2014): Interspecific hybridization between rare and common plant congeners inferred from genome size data: assessing the threat to the Czech serpentine endemic *Cerastium alsinifolium* (Caryophyllaceae). – Preslia 86: 95-117.
Study design, field sampling, lab work, data analyses and manuscript preparation

**II.** **Vít P**., Lepší M. and Lepší P. (2012): There is no diploid apomict among Czech *Sorbus* species: a biosystematic revision of *S. eximia* and discovery of *S. barrandienica*. – Preslia 84: 71-96.
Field sampling, lab work, data analyses, manuscript preparation

**III.** Kabátová K., **Vít P.** and Suda J. (2014): Species boundaries and hybridization in Central-European water lilies as inferred from genome size and morphometric data. – Preslia 86: 131 - 154.
Study design, field sampling, lab work and data analyses, interpretation

**IV.** Hanušová K., Ekrt L., **Vít P**., Kolář F. and Urfus T. (2014): Continuous morphological variation correlated with genome size indicates frequent introgressive hybridization among *Diphasiastrum* species (Lycopodiaceae) in Central Europe. – Plos One (**accepted**)
Study design, field sampling, lab work, manuscript preparation

## Acknowledgment

I would hereby like to thank especially my supervisor, Jan Suda, for his advice and support over the past eight years. Without his innovative ideas, enthusiasm and assistance, I would have never completed this thesis.

Many thanks are due to my friends Tomáš Urfus, Karol Krak, Pavel Trávníček, Filip Kolář, Fred Rooks and many others for numerous helpful comments, fruitful discussions and an enjoyable time both at Charles University and the Institute of Botany. I am also grateful to my colleagues participating in hybridization research at the Department of Botany, namely Klára Kabátová, Kristýna Hanušová and Katka Wolfová. I am grateful to Martin Lepší and Petr Lepší who introduced me into the world of hybridogenous rowan trees. The biggest thanks of course have to go to my family and girlfriend Jana for their infinite support, understanding and love.

The work was supported by the Grant Agency of the Academy of Sciences (project No. KJB601110709) and Charles University Grant Agency (projects No. 250/2004, 29507/200, 90809/2009 and 116710/2010).

## Summary

Hybridization plays an important role in the evolution of vascular plants. It can have both positive and negative consequences, ranging from the origin of new species on the one hand to the extinction of taxa through introgression on the other. These effects may be pronounced in geographically restricted or rare species. The core of this thesis are three case studies addressing interspecific hybridization involving rare angiosperm species. Finally, the thesis is completed with a study considering hybridization as a source of variation and new species. The coexistence of frequent primary hybrids with their parental taxa was revealed in the system comprising the rare species *Cerastium alsinifolium* and its widespread counterpart *C. arvense*. The spatial distribution of the endemic species and its habitat preferences were elucidated. In contrast, comparatively rare hybridization events were found in the *Nymphaea alba – N. candida* complex. Although it has been assumed that water lilies hybridize freely, our karyological data do not support this hypothesis. Hybrids therefore do not present a serious risk to either of these rare species. The third study describes interspecific hybridization in the spore-bearing genus *Diphasiastrum*. Traditionally, three basic and three hybridogenous species are recognized in Central Europe. However, species boundaries are blurred through frequent introgressive hybridization. Introgression has been catalysed by human activities (disturbances), which facilitate spatial contact between originally partly allopatric species and subsequent interspecific hybridization. The origin of a new agamospermous lineage through interspecific hybridization was described in the genus *Sorbus*. Apomictic triploids most likely originated via hybridization between diploid and tetraploid taxa. Their mode of reproduction shifted from sexual to apomictic, which assured their long-term persistence.

## Souhrn

Hybridizace hraje významnou roli v evoluci cévnatých rostlin. Obecně ale může mít jak pozitivní tak negativní důsledky, sahající od vzniku nových taxonů až po možné vyhynutí druhu skrze introgresi. Tyto důsledky mohou být znásobeny obzvláště u taxonů s omezeným geografickým rozšířením nebo u vzácných taxonů. Základem předkládané dizertační práce jsou tři studie zabývající se mezidruhovou hybridizací s účastí vzácného taxonu. Práce je doplněna studií, ve které vystupuje hybridizace jako zdroj variability a nového taxonu. Koexistence početných primárních hybridů s rodičovskými taxony byla odhalena u endemického rožce Slavkovského lesa (*Cerastium alsinifolium*) a jeho široce rozšířeným protějškem (*C. arvense*). Naproti tomu, hybridizace nativních leknínů (*Nymphaea alba*, *N. candida*) je velmi vzácná. Předpokládalo se, že oba taxony mohou volně hybridizovat, avšak naše karyologická data tento předpoklad nepotvrdila. Hybridizace v obou případech není vážným ohrožením vzácných taxonů. Třetí studie osvětluje důsledky mezidruhové hybridizace v rámci rodu *Diphasiastrum*. Obecně jsou ve střední Evropě rozlišovány tři základní a tři hybridogenní taxony. Vymezení jednotlivých taxonů je však nejednoznačné a díky introgresivní hybridizaci existují přechody (jak v morfologii, tak ve velikosti genomu) mezi taxony. Tato introgrese je z velké části umožněna lidskými aktivitami (m.j. disturbance), které způsobily kontakt mezi původně prostorově izolovanými taxony a následnou hybridizaci. Původ nové agamospermické linie v rodu *Sorbus* byl popsán v poslední studii. Obdobné linie vznikají ve střední Evropě opakovaně díky mezidruhové hybridizaci. Nejčastěji vznikají agamospermičtí triploidi hybridizací diploního (*S. torminalis*) a tetraploidního taxonu (např. *S. danubialis*, *S. graeca*). Dlouhodobá existence těchto linií je umožněna přechodem k agamospermickému způsobu reprodukce.

# Introduction

## 1) Interspecific hybridization in a nutshell

Hybridization[1] plays an important role in the evolution of living organisms. It is the basic mechanism of processes such as introgression of diverse phenotypic traits between diverged taxa or hybrid speciation. Most flowering plants and ferns originated through (allo-)polyploidization (= hybridization followed by genome duplication), which is supposed to be the most powerful "engine" of plant evolution (Soltis et Soltis 2009). Hybridization plays a striking role in human "nutrition evolution". Although the first written evidence is dated back to the early 18$^{th}$ century, hybridization has been important to humans since the Neolithic era (Rieseberg et Carney 1998). When domestication and breeding of plants and animals was in its infancy, hybridization events happened mostly accidentally and inadvertently. Later, by selecting crops (or breeds) carrying required characters, hybridization of closely related species or local races became intentional and increased in intensity.

In early studies devoted to hybridization, several incorrect presumptions had been made. Hybridization had been considered a "blind alley" of evolution because of assumed hybrid sterility (Grant 1981, Rieseberg et Carney 1998, Ouyang et al. 2010). The evolutionary impact of hybridization was once enormously underestimated due to presumed rarity of this phenomenon (Knobloch 1972). Although botanists have paid considerable attention to hybridization (Rhymer et Simberloff 1996), it has been proved in the last decades that hybridization plays an inestimable role not only in plant, but also in animal evolution (Dowling et Secor 1997, Hegarty et Hiscock 2005, Wissemann 2007, Soltis et Soltis 2009).

Hybridization is of paramount importance in the conservation of many rare species. Hybridization is essential for generating new evolutionarily independent lineages (which may ultimately develop into new species); on the other hand, it can have a detrimental effect on populations of rare species. These may suffer from hybridization with common congeners, resulting in blurring of boundaries between taxa and threatening species´ genetic integrity. Low abundance of individuals, low number of populations, marginal populations and discontinuous range of distribution are the main "natural" reasons rare species are under threat. During the last century, human influence considerably increased the risk of rare species becoming extinct, eg. by landscape fragmentation, changes in traditional landscape management, habitat loss and degradation, etc. Rare species represent an important component of both local and global biodiversity and are often regarded as indicators of biodiversity richness (Heywood et Iriondo 2003). They often have narrow ecological niches, which are patchily distributed in the modern landscape. Populations of these specialists therefore often consist of a low number of individuals. Spatially-limited species include endemics of particular geographic regions (Kaplan 2012). The present thesis addresses questions concerning cases of interspecific hybridization that involve at least one rare species, with an emphasis on conservation consequences including a risk assessment.

[1] Hybridization as a term has several different meanings – from the most strict : "every fusion of two gametes", through "fusion of two gametes from two individuals from different populations of the same species", to the widely accepted "cross-fertilization between two individuals of different (and isolated) species" (eg. Rhymer et Simberloff 1996, Arnold 1997, Soltis et Soltis 2009). The latter meaning is used throughout this thesis, unless specified otherwise.

## 2) Evolutionary aspects of hybridization

Hybridization is not uniformly distributed across the plant kingdom, but unevenly across different taxonomic groups. Some taxonomic groups hybridize freely whereas many others do not (Rieseberg et Carney 1998). Some of the most "promiscuous" temperate species are members of the families Asteraceae, Rosaceae, Poaceae, Scrophulariaceae, Brassicaceae and Salicaceae (Ellstrand et al. 1996). Attempts to analyse and compare local floras may run into problems when operating with different taxonomic concepts, especially when agamospermy occurs (Stace 1975, Ellstrand et al. 1996, Danihelka et al. 2012). The so-called narrow taxonomic concept is characterized by an increased number of described taxa (microspecies; see eg. Tyler 2011, Schischkin et Bobrov 2002). The so-called broad taxonomic concept does not distinguish between hybrids and hybridogenous species, so the number of regular taxa is generally lower (Bräutigam et Greuter 2007, Aldasoro et al. 1998). Although the narrow concept reflects evolutionary patterns, numerous microspecies are impractical for non-expert botanists (Dickinson 1998, Hörandl 1998, Kirschner 1998, Stace 1998). The number of recognized taxa is frequently connected with so-called taxonomic favouritism (Bickford et al. 2007) and popularity of specific groups of taxa. There is, for example, a considerably higher proportion of hybridogenous *Sorbus* species in Germany and the British isles compared to the rest of Europe (especially Balkan Peninsula). It is open to argument whether low or high numbers of taxa reflect true biological diversity in nature and whether high numbers of recognized taxa merely indicate taxonomical bias. In spite of the mentioned difficulties, proportions of hybrid species in national floras are comparable and range from 10 to 20% (British Isles 10–20%, Czech Republic 14%, Scandinavia 10%; Stace 1975, Ellstrand et al. 1996, Danihelka et al. 2012). Hybridization is generally a widespread phenomenon in vascular plants. Approximately 25% of species are known to hybridize, but this proportion may be underestimated because hybrid origins are often difficult to prove (Wagner 1969, Mallet 2007).

### Advantages and disadvantages of hybridization

Hybridization has both evolutionarily positive and negative consequences, which all stem from the fact that two different genomes are combined. Hybridization increases genetic diversity and enables gene flow between previously isolated taxa. If backcrossing occurs, some beneficial alleles may be transferred from one species to another. Interspecific hybrids are highly variable in fertility and vigour – especially F1 hybrids (eg. between geographic races or closely related taxa) tend to exceed their parents in vegetative vigour or robustness (heterosis; Grant 1975). This phenomenon is frequently utilized in crop breeding to reach specific characters increasing yield. Heterosis may also partially explain the success of allopolyploids and many clonal hybrid lineages (eg. agamic complexes in the genera *Sorbus*, *Rubus* or *Taraxacum*). Hybrids may also possess characters, which were suppressed or inexpressive in parental generations (Rieseberg and Carney 1998), or exhibit novel or extreme characters (transgressive segregation; Rieseberg 1997, Rieseberg et al. 2003, Seehausen 2004). These characters can enable hybrids to reach novel niches and allow selection to act in favour of their establishment (Rieseberg and Carney 1998). Generally, hybrids have a broader adaptation ability (frequently combining that of their parents; Abbott 1992, Buerkle et al. 2000, Rieseberg et al. 2003). Hybridization allows genetic novelties to accumulate faster than through mutations alone (Martinsen et al. 2001). If a newly arisen hybrid is capable of independent reproduction and is reproductively isolated from its parental species, it may act as a separate species.

The main disadvantage of hybridization is the breakdown of the genetic integrity of parental taxa. Recurrent hybridization may lead to the emergence of the third group of plants (F1 hybrids) in the habitat. This model of hybridization is not harmful provided that hybridization events are rare or F1 hybrids are sterile, precluding the formation of complex hybrid swarms. If hybrids are fertile or if reproductive barriers towards (at least one) parental species is broken, backcrossing may occur, and

differences between species may get blurred, possibly leading to genetic swamping [2]of parental species (Rhymer & Simberloff 1996). Taxa with low abundance may become extinct if strong introgression occurs (Levin et al. 1996). Hybrids, even if completely sterile, present a significant burden for parental species with regard to competition (Wolf et al. 2001, Prentis et al. 2007). Plant hybrids often share the same habitat and compete with their parents for resources (nutrients, water and radiation) or may dramatically decrease the number of suitable breeding partners (Buerkle et al. 2000, Bleeker 2007). Hybrids can ultimately replace their parental species altogether (demographic swamping[3]; Wolf et al. 2001).

**Reproductive isolation mechanisms**

To avoid hybridization (and its evolutionary consequences), several breeding barriers in plants have developed. These reproductive isolation mechanisms may be classified into two major categories – prezygotic and postzygotic, referring to the ontogenetic stage in which they take effect (before or after fertilization). Prezygotic barriers include habitat, temporal (different growth period and flowering time) and behavioural isolation (pollinator fidelity, morphological adaptation avoiding pollination), gametic competition (pollen tube competition) or some kind of incompatibility (preventing pollen grains to germinate). Prezygotic mechanisms seem to represent the most efficient reproductive barriers against hybridization, although some of them may be easily overcome (eg. mentor effect in avoidance of pollen germination; Richards 1997, Krahulcová et al. 1999, Mráz 2003). Postzygotic barriers include zygote mortality, reduced hybrid vigour (hybrids fail to develop or do not reach maturity), reduced fertility (hybrids fail to produce gametes, eg. due to irregular chromosome pairing) and hybrid breakdown (descendants following the F1 generation are of various fitness, often inviable). Reproductive isolating mechanism does not work as a rigorous barrier, but more likely as permeable filters (Mallet 2007). In cases of closely related species (and species recently originated or diverged), isolation mechanisms are often weak or are not yet established. On the other hand, postzygotic barriers may decrease the fitness of parental taxa and thus are often replaced by prezygotic mechanisms of some kind.

Hybridization is frequently initiated by the breakdown of spatial reproductive isolation barriers (either due to natural drivers or human activity). Another possible trigger of hybridization is the breakdown of ecological mechanisms isolating two potentially hybridizing taxa; in this case human activities play a major role (eg. transport of goods, changes in habitats, disturbances, new types of secondary habitats; Abbott 1992, Krahulcová et Krahulec 1999, Hanfling et Kollmann 2003, Krahulec et al. 2004).

Although hybridization had been believed to be predominantly bidirectional, asymmetric patterns have often been found (Rieseberg et Carney 1998, Lepais et al. 2009, Ma et al. 2010). Unidirectional crossing is more common when hybridizing species are of different ploidy levels (ie. one ploidy level is the donor of pollen and second is the acceptor; Krahulec et al. 2004, Ludwig et al. 2013).

**Outcomes of hybridization**

When reproductive barriers between two species are broken, hybridization can follow several different evolutionary trajectories, including the formation of a hybrid zone, hybrid swarm and genesis of a new species. A hybrid zone is formed where two genetically distinct groups meet and

[2] The process when genes from a larger population dominate over the genes in a small population. Genetic diversity in the small population is thus significantly reduced.

[3] If hybrids are sterile or display reduced fitness, the population growth rate of the hybridizing taxa may decrease below that required for replacement of one or both parental species.

hybridize (Barton et Hewitt 1985). The existence of selection against hybrids stabilizes the hybrid zone and complicates later hybridizations. Hybrid zones are often formed at boundaries between different habitats – if each habitat favours one parental taxon, hybrids are selected against in both habitats (Anderson 1948, Harrison 1993), and hybridization is restricted to a relatively narrow area where the two species are in contact. More or less stable hybrid zones have been observed, for example, in *Arctium* (Repplinger et al. 2007), *Senecio* (Prentis et al. 2007) or *Cardamine* (Marhold et al. 2002, Lihová et al. 2007b); selection against hybrids has been documented in most hybrid zones studied (Arnold 1994, Allendorf et al. 2001, Seehausen 2004). An extreme example of hybrid zones are hybrid swarms, which result from introgressive hybridization. When newly arisen hybrids are (inter)fertile and backcross with their parents, the integrity of parental species is progressively blurred. After several generations of introgression, hybrids merge with their parental species, and a morphological and genetic continuum originates (plants with different proportions of parental genomes). Introgression either affects both parental taxa or may be unidirectional (backcrossing with one parental taxon only; eg. *Rhododendron* – Ma et al. 2010, *Quercus* – Lepais et al. 2009). A special and confounding product of introgression is chloroplast capture. During such an introgressive hybridization event, the cytoplasm of one species is replaced by that of another species (Rieseberg et Soltis 1991). To avoid introgression, reproductive barriers in introgressed population are often reinforced through selection for assortative mating (Arnold 1992). Introgression allows expansion into new habitats due to the production of new genotypes that may be better adapted than parental species (Arnold 1992, Rhymer et Simberloff 1996). Introgression is notoriously difficult to prove, which explains the lack of biosystematic studies dealing with it. Introgressive hybridization has been documented, for example, in the genera *Viola* (Krahulcová et al. 1996), *Populus* (Martinsen et al. 2001), *Cardamine* (Lihová et al. 2007b), *Rhododendron* (Ma et al. 2010) or *Diphasiastrum* (Hanušová et al. 2014). Rare hybridization events between two parapatric species may lead to the formation of a contact zone. Hybrids in contact zones have decreased fitness and are often sterile. As hybridization occurs at low frequency, introgression does not play a major role. A mosaic or tension contact zone may develop (Petit et al. 1999). In a mosaic zone, parental species are distributed patchily depending on ecological conditions. Where an ecological cline occurs, a tension contact zone may form. Studies of contact zones are frequently focused on the coexistence of different ploidy levels (Castro et al. 2012, Krejčíková et al. 2013), while the coexistence of parental taxa and their hybrids is often neglected.

A hybrid (or hybridogenous) taxon may originate if some hybrids become independent of their parental species and are able to reproduce themselves. Single hybridization events usually do not lead to the emergence of hybrid species; series of hybridization events are most likely needed. Different asexual or modified sexual modes of reproduction often evolve to overcome the influence of parental species (eg. autogamy, agamospermy and clonal growth). Shifts in the reproductive mode accompanied by polyploidization play a crucial role in the establishment of agamic complexes (eg. *Sorbus* – Nelson-Jones et al. 2002, *Pilosella* – Krahulcová et al. 2000, *Rubus* – Krahulcová et al. 2013, *Crataegus* – Campbell et al. 1991).

**Hybrid speciation and polyploid formation**

Hybrid speciation is more common in plants than in animals, where other speciation modes prevail (Otto et Whitton 2000). Indeterminate growth, longevity, clonality, hermaphroditism with selfing potential and limited gene flow are the main differences which favour the formation of plant hybrid species (Mallet 2007). From a speciation point of view, hybridization is a process which allows faster accumulation of genetic novelties than random evolutionary events (eg. mutation, genetic drift; Martinsen et al. 2001). The presence of transgressive characters in hybrids supports the statement that hybridization is the main source of variation upon which selection can act (Rieseberg et Ellstrand 1998). Reproductive isolation between parental and hybrid species is essential for their long-term existence, hybrid species must remain distinct even if they get in contact with their parents

secondarily (Mallet 2007). The formation of hybrid species is risky because newly arisen species always suffer (similarly to a newly formed cytotype) from processes analogous to the minority cytotype disadvantage (Levin 1975, Mallet 2007). Hybridization and hybrid speciation allow rapid evolutionary changes by generating novel gene combinations, which may lead to increased genetic variation and fitness, and to the adaptation to new environments (Ellstrand 1992). That is why hybrid speciation is common among rapidly radiating groups (Seehausen 2004, Mallet 2007, Fehrer et al. 2009). Reproductive success is crucial for newly originated hybrids. Complete or partial sterility has been detected in many homoploid hybrids (*Cirsium* – Bureš et al. 2010; *Cerastium* – Vít et al. 2014) as well as in heteroploid hybrids (*Viola* – Krahulcová et al. 1996; *Cardamine* – Lihová et al. 2007a, *Cirsium* – Bureš et al. 2010; *Sorbus* – Rich 2009; *Nymphaea* – Kabátová et al. 2014). Analogously, reduced fertility has been observed in hybrid pteridophytes (spore abortion; *Dryopteris* – Ekrt et al. 2009, *Diphasiastrum* – Hanušová et al. 2014). Reproductive success is often reduced in homoploid hybrids too, because they often face problems with chromosome pairing during meiosis (Grant 1981). After meiosis, gametes carry an unbalanced number of chromosomes because somatic cells of the hybrid contain only one chromosome set from each parent. This often results in aneuploid somatic chromosome numbers (Ramsey and Schemske 1998). However, hybrids may overcome this "blind end" through polyploidization when all chromosomes are duplicated and then undergo regular meiosis. Alternatively, hybrids can switch their reproductive system to clonal growth (which is, however, not possible for all plants, eg. annuals) or apomixis (Asker et Jerling 1992). Many plant groups are predisposed to apomixis (Asker et Jerling 1992, Catanach et al. 2006), and its occurrence generally correlates with hybridization and polyploidization (eg. in *Sorbus* – Nelson-Jones 2002, *Crataegus* – Campbell et al. 1991, *Pilosella* – Krahulcová et al. 2000, *Taraxacum* – Richards 1997).

Recent studies indicate that most angiosperms are of ancient polyploid origin (Soltis et Soltis 2009). Hybridization accompanied by chromosome doubling is thus essential for generating contemporary species diversity (Grant 1981, Soltis et Soltis 1993). Polyploidy is highly correlated with asexual modes of reproduction (apomixis, haploid parthenogenesis), selfing and longevity in plants as well as in animals (Mallet 2007, Otto et Whitton 2000). Two types of polyploidy are recognized from a genetic point of view – autopolyploids arise within a single population or between ecotypes of a single species whereas allopolyploids are derived from interspecific hybrids (Ramsey et Schemske 1998). Polyploids originate in different ways depending on the particular mechanism of chromosome doubling: 1) autopolyploidization of diploids, 2) triploid bridge (fusion of a reduced and an unreduced gamete), 3) fusion of two unreduced gametes. Unreduced gametes are rarely formed in diploids and non-hybrid taxa (mean frequency around 0,5%), but are about fifty times more frequent in hybrids (frequency around 25%; Ramsey et Schemske 1998). High numbers of aneuploid and probably also unreduced gametes seem to originate from polyploids with odd chromosome numbers (Krahulcová et al. 2000). When a polyploid successfully overcomes the phase of formation, other problems usually emerge (eg. demographic establishment of new a new cytotype facing the minority cytotype disadvantage; Ramsey et Schemske 1998). Polyploids are often reproductively independent of their diploid parents, but when they backcross, progeny with odd numbers of chromosomes occurs. Although these offspring may be viable, they frequently produce sterile gametes or gametes with aneuploid chromosome counts (Grant 1981, Ramsey et Schemske 1998). On the other hand, this triploid bridge is essential for most novel cytotype formations. The origins, distribution and spreading of many recently formed allopolyploids is well documented (eg. *Senecio cambrensis* – Abbott et Lowe 2004, *Spartina anglica* – Ainouche et al. 2004, *Tragopogon mirus* and *T. miscellus* – Soltis et al. 2004, *Cardamine schultzii* – Urbanska et al. 1997, Mandáková et al. 2013, Zozomová-Lihová et al. 2014). Moreover, the evolutionary history of allopolyploid crops selected for transgressively high yields is also well described (Anderson et Stebbins 1954, Grant 1981, Soltis et Soltis 2009).

## 3) Human-triggered hybridization involving rare species

In cases of rare species, the consequences of hybridization may be even stronger because their populations are often small or occur at the margins of the species´ distribution areas. Microevolutionary processes (eg. speciation, inbreeding depression, bottleneck effect, genetic drift) act more readily in small populations than in large populations (Rhymer et Simberloff 1996). Consequently, both beneficial and harmful consequences of hybridization (genesis of new evolutionary units vs. potential extinction of populations) are more striking (Rieseberg and Ellstrand 1993). Many rare species originated from widely distributed relatives (textbook examples from the Czech flora are *Minuartia smejkali* – Dvořáková 1988; *M. corcontica* – Dvořáková 1999; *Cerastium alsinifolium* – Novák 1960), and are therefore more prone to hybridize with their progenitors. Several other taxonomically complex groups (Ennos et al. 2006) comprise rare taxa originating (eg. via allopolyploidy) from their widely distributed counterparts.

Hybrid genotypes often vary considerably in their fitness (Rieseberg et Carney 1998). Although hybrids from early-generations are on average less vigorous than parental taxa, individuals with transgressive characters originate regularly. If reproductive isolation mechanisms are not established, further hybridization events may follow soon. Repeated rounds of hybridization, possibly leading to the establishment of hybrid swarms can dramatically jeopardize the genetic integrity of rare species. This process can ultimately result in genetic swamping of the rare species by hybrids. Parental species are more likely to get replaced by hybrids through genetic swamping than due to higher average fitness of hybrids (Rieseberg et Carney 1998). An analogous situation occurs in insular-like specialists (eg. serpentine or mountain relicts), whose distribution and gene pool is limited due to long-term isolation to specific ecological conditions, but are surrounded by many related and potentially crossable genotypes (eg. *Knautia* – Kolář et al. 2009, *Cerastium alsinifolium* – Vít et al. 2014).

One still overlooked phenomenon is so-called anthropohybridization (Wójcicki 1991), which refers to hybridization processes with the participation of cultivated or human-introduced species. Such species might have detrimental effects on related native plants. Dilution of their gene pool and gene transfer from crops/aliens are of the most important consequences of anthropohybridization (Abbott 1992, Bleeker et al. 2007, Campbell et al. 2009). Recent examples in which anthropohybridization has been recorded are *Prunus fruticosa* (Musilová 2013) or *Malus sylvestris* (Cornille et al. 2013). Anthropohybridization is largely facilitated by the absence of reproductive isolation mechanisms between crops/aliens and rare species. Crop cultivars often originated in different parts of the world. Reproductive isolation mechanisms are therefore often missing; when cultivars and native taxa come into contact, they may hybridize freely (Ellstrand et al. 1999). Contact between introduced and native taxa is facilitated by three main human activities: plant introduction, landscape fragmentation and habitat modification (Allendorf et al. 2001). Determining whether hybridization is of natural or anthropogenic origin is crucial for conservationists, whose task is to set up appropriate management plans and to take necessary actions. Human-induced changes in habitats may lead to secondary contact of previously separated species, promoting their hybridization (Rhymer et Simberloff 1996). It has been documented, for example, in *Viola lutea* subsp. *sudetica* × *V. tricolor* (Krahulcová *et al.* 1996), *Senecio hercynicus* × *S. ovatus* (Raudnitschka *et al.* 2007), *Arctium lappa / A. tomentosum* × *A. minus* (Repplinger *et al.* 2007), *Cerastium alsinifolium* × *C. arvense* (Vít et al. 2014) or *Diphasiastrum* species (Hanušová et al. 2014). A classic example of when human-induced hybridization can take place are mountain meadows in the Krkonoše Mts, where native montane and introduced lowland species meet due to long-term human activities. Hybridization between alpine and lowland *Hieracium* subg. *Pilosella* species has led to the origin of hybridogenous species and lineages restricted to these habitats (eg. *Hieracium iseranum*; Krahulec et al. 2004). The role of habitat disturbance in hybridization has been a subject of discussion since Anderson (1948). He argued that disturbances create open niches which may host a wide diversity of hybrid genotypes. Disturbances may also support the breakdown of established reproductive

isolation mechanisms. Levin et al. (1996) consider disturbances as corridors promoting movement of species and leading to sympatry (and hybridization) between allo- or parapatric species. Expansion of one species to the geographical range of another may also be prompted by habitat modification (Rhymer et Simberloff 1996). A recurrent issue in conservation biology is whether populations with hybridizing rare species should receive the same conservation effort as non-hybridizing populations (eg. Thompson et al. 2009). Efforts should be targeted at maintaining remaining pure populations rather than at trying to save population already affected by high degrees of hybridization (eg. removal of non-native species and hybrids or restoration of habitats; Allendorf et al. 2001).

Reciprocal transplant experiments are essential for obtaining environment-dependent fitness data for parental and hybrid individuals (Rieseberg and Carney 1998), but may endanger rare species populations. In a similar way, replanting experiments are often used by conservationists for strengthening population numbers or reintroducing rare species from distant (genetically different) populations. In stable populations of rare species, reproductive isolation mechanisms against closely related species may exist. Introducing "alien" individuals from distinct populations (which might be adapted to another environment) may lead to loss of local adaptation (eg. reproductive isolation mechanisms) and decrease (often substantially) the average fitness of targeted populations (Barton et Hewitt 1985, Ellstrand 1992). Moreover, the resulting outbreeding depression[4] often promotes hybridization. When hybridization occurs, well intentioned reintroduction projects can be counterproductive if hybrids become fertile (Rhymer et Simberloff 1996). Rieseberg et Carney (1998) therefore advise to avoid transplantation experiments or to manage them under special conditions (removing anthers during the flowering period and harvesting seeds before they disperse). However, such measures are time-consuming and costly.

Generally, biosystematic studies of species complexes with rare or endangered taxa threatened by hybridization with common relatives or intricate agamic complexes are highly appreciated by conservationists. Such studies allow them to better evaluate the risks of hybridization based on knowledge of reproductive modes, hybrid frequency and other important facts. The key question surrounding all conservation efforts undoubtedly is "How do we recognize hybrids?".

## 4) Current methods for hybrid identification

Hybrids have been recognized and studied at least since the times of **Linnaeus** (Rieseberg and Carney 1998), although "products of hybridization" were observed much earlier, at the dawn of agriculture (eg. descendants of cereal breeding and ancient agricultural selection of the most productive plants; Feuillet et al. 2007). As in many other areas, the sensitivity and resolution of research methods have changed rapidly over the last decades. Detection of hybridization (or ancient hybridization events) is much easier nowadays thanks to the development of highly sensitive approaches. Methods widely used for detecting hybridization and ongoing processes in populations of hybridizing taxa are discussed further below.

### Phenetic methods

Phenetic methods have been for a long time the sole technique for hybrid detection. Intermediate appearance or a combination of morphological characters of parental taxa is a classic indication of hybrid origin. However, many hybrids have eluded detection by this approach (eg. hybrid swarms; Rhymer et Simberloff 1996, Allendorf et al. 2001). Transgressive or novel characters are not an exception, but occur regularly among hybrids and become more frequent in later hybrid generations (ca 10% in F1 hybrids; Rieseberg and Ellstrand 1993). A meta-analysis of 46 hybridization studies made by Rieseberg and Ellstrand (1993) found significant deviations from presumed intermediacy.

[4] Crosses between genetically distant sources produce offspring with lowered fitness

Hybrids from the first generation were characterized by a mosaic of parental and intermediate characters, rather than possessing exclusively intermediate ones (Rieseberg 1995). Differences between particular morphological characters and their inheritance in hybrids can be explained by differences in their genetic control and interactions with the environment. Rieseberg and Ellstrand (1993) suppose, that morphological characters under multigenic control tend to attain intermediate values, while characters under simple control usually manifest as parental or intermediate appearance in the first hybrid generation. However not all morphological characters have a genetic basis (Allendorf et al. 2001) and hybrid characters constitute a mosaic of parental phenotypes. It is difficult to determine based on morphology alone whether a particular hybrid represents the first or a later generation or backcross (Allendorf et al. 2001). This knowledge is nevertheless crucial for conservationists whose job is to evaluate the risk of hybridization for rare species. Last but not least, it is necessary to take into account the quantity and potential correlation of morphometric characters when evaluating complex hybrids. Functional or developmental correlations highly reduce the informative content of each morphological character (Rieseberg and Ellstrand 1993). Only scoring of a considerable amount of characters may bring valuable results that truly indicate hybridization and backcrossing.

On the other hand individuals with intermediate morphologies are often interpreted as interspecific hybrids (eg. in the genus *Nymphaea*; Heslop-Harrison 1955, Ejankowski & Małysz 2011) even though their hybrid status is not supported by other methods (karyology or molecular markers). Many recent studies have not confirmed hypotheses about the hybrid origins of such individuals (eg. in the genus *Nymphaea*; Kabátová et al. 2014). Analogously, several putative hybrids have finally turned out to be ecomorphoses, or individuals damaged during early developmental stages or altered by suboptimal conditions (eg. occurrence of frequent hybridization in the genus *Chenopodium* was disproved using karyological techniques; Mandák et al. 2012).

## Karyology

Detection of hybrids using conventional karyology is relatively easy and straightforward when differences in chromosome number or karyotype exist. Special attention is necessary when chromosome deviations (eg. aneuploidy or chromosome rearrangements) are expected. This phenomenon is more common in hybrids (eg. higher frequency of aneuploidy; Ramsey et Schemske 1998). The need of mitotically active tissue, huge laboriousness and the need of an experienced karyologist to analyse material are the main disadvantages of this method. Advanced karyological methods based on in situ hybridization (GISH, FISH) are suitable for detection of homoploid hybrids as well as for tracking the origin of allopolyploids. Nevertheless, exact chromosome counts or ploidy determination using karyological techniques are essential for the calibration of flow-cytometric analyses (Doležel et al. 2007).

## Flow cytometry

Flow cytometry (FCM) is a fast and effective method for analysing optical characteristics of isolated particles. Estimation of genome size and detection of DNA ploidy level are routine applications of FCM in plant biology. Estimation of DNA ploidy level is much faster and easier than using conventional karyological techniques (Doležel et al. 2007). If differences in genome size or ploidy level between parental taxa exist, flow cytometry can easily be used for detecting both homoploid and heteroploid hybrids (Kron et al. 2007, Loureiro et al. 2010). Most hybridization events are not connected with changes in nuclear DNA content, and genome size of hybrids can be straightforwardly inferred from values of their putative parents (Kron et al. 2007, Loureiro et al. 2010). Several obstacles may arise, however – for example, when taxa with the same or similar genome size have different chromosome numbers (eventually holocentric chromosomes may occur; eg. Bozek et al. 2012, Pazy & Plitmann 1995, Hipp et al. 2009). The use of flow cytometry for detecting hybrids of rare species might be beneficial because it requires only small amounts of tissue.

Moreover, some benchtop flow cytometers are mobile, and samples may be analysed directly in the field. Compared to karyology, flow cytometry is not dependent on mitotically active tissues, and samples may be prepared from various types of tissue (from roots to flowers). Nevertheless, each newly detected DNA ploidy level should be confirmed by chromosome counting (Suda et al. 2006).

## Molecular methods

Data obtained from molecular markers have several advantages compared to other types of data (eg. karyological, morphological). Molecular markers are universal (they may be used to study morphologically or karyologically distant taxa and allow their direct comparison). Moreover, the number of characters obtained by molecular markers is many times higher compared to morphological ones. Molecular characters are also well defined (4 nucleotides in DNA sequences) and discrete. Nowadays, a wide spectrum of PCR-based methods is available and commonly used in studies of hybridization. As more and more sensitive methods are routinely used for hybrids detection, many new hybridization events are discovered. Ancient hybridization (eg. Grimm et Denk 2008, Fehrer et al. 2009) and cryptic hybrids (eg. Jasińska et al. 2010, Nicole et al. 2007, Paule et al. 2012) represent previously overlooked phenomena that were often for the first time unravelled using modern molecular techniques in the last decade. Hence, the selection of suitable genetic markers for studying hybridization is essential. It always depends on the required resolution and relationships of the studied taxa.

Sequences of nuclear genes are useful for detecting hybrids because of their biparental inheritance. A sequence (or restriction profile) of a targeted gene is transferred from both parents equally to hybrids. However, because of the variation in inheritance patterns, recombinations and linkages, results must be interpreted with caution. Markers from nuclear ribosomal DNA (nrDNA; ITS region) are the most widely used to detect hybrids (eg. Fuertes Aguilar et al. 1999, Lihová et al. 2007a). In cases of both ancient and recent hybridization events, potential consequences of concerted evolution (eg. homogenization of sequences) must be taken into account (Alvarez et Wendel 2003). The use of genes which are resistant to concerted evolution (eg. low copy genes) strikingly increased in the last decade (eg. Shimizu-Inatsugi et al. 2009, Krak et al. 2013, Ramadugu et al. 2013, Schneider et al. 2013). However, their application has also brought numerous difficulties, stemming mainly from population genetic processes such as incomplete lineage sorting, genetic drift or natural selection (Sang 2002, Small et al. 2004, Linder & Rieseberg 2004). Low-copy or nrDNA markers often suffer from insufficient variation, which causes problems in studies focused on the population level (eg. detection of hybridization rates, assessment of reproductive modes). More sensitive markers should therefore be adopted – traditionally, microsatellites or AFLPs are used in population based studies (Meudt et Clark 2007). For instance, SNPs[5] derived from high-throughput sequencing techniques (Rad-seq, GBS) are becoming an important source of molecular data useful for elucidating hybrid origin (Hohenlohe et al 2011, 2013, Wagner et al. 2013).

Organellar DNA (chloroplast DNA, mitochondrial DNA) can also be highly informative when detecting plant hybridization thanks to its predominantly uniparental inheritance in plants (Harris et Ingram 1991). Several aspects of microevolutionary processes may be tracked, including the direction of hybridization or introgression. Sufficient variation between the hybridizing taxa is the main prerequisite, despite the plethora of cpDNA markers (Taberlet et al. 1991, Demesure et al. 1995, Shaw et al. 2005, Shaw et al. 2007). The use of cpDNA seems to be easy, but one must bear in mind that cpDNA reflects only one genetic line (parental species), while the second remains hidden. It is therefore appropriate to use cpDNA markers in combination with nuclear markers to enable the reconstruction of relationships between both parental species and hybrids.

[5] Single nucleotide polymorphisms

## 5) Species under study

### *Cerastium alsinifolium*

*Cerastium alsinifolium* Tausch (Smejkal 1990, Kaplan 2012) is an outcrossing serpentine endemic of western Bohemia (Slavkovský les Mts). The total area occupied by this species does not exceed 15 $km^2$, with all sites situated within the Protected Landscape Area Slavkovský les (Tájek et al. 2012). *Cerastium alsinifolium* is a critically endangered plant in the Czech flora (Grulich 2012, Kaplan 2012). Currently, *C. alsinifolium* is reported from two rather contrasting types of habitat on serpentine bedrock, namely dry open grassland on rocky outcrops, and (semi)shaded springs and seeps in coniferous forests (Melichar 2005, Tájek et al. 2012). At several sites in the Slavkovský les Mts, it co-occurs with another perennial large flowered species, *C. arvense*, which is widely distributed in Europe and usually inhabits dry grasslands or semiruderal sites (Smejkal 1990). *Cerastium arvense* is relatively tolerant of soils with a high heavy metal content (Levine & Greller 2004), and in the Slavkovský les Mts it occasionally grows on outcrops of serpentine or in their immediate vicinity.

Potential hybridization between serpentine endemic *Cerastium alsinifolium* and its widespread counterpart *C. arvense* in the Slavkovský les Mts has been suspected for a long time (Smejkal 1990, Hrouda 2002, Rybka et al. 2004). It has, however, not been proved by biosystematic approaches. Possible evolutionary consequences of the hybridization also remain unknown.

### *Sorbus*

In the genus *Sorbus*, taxonomic difficulties stem from recognizing and describing new species originating through hybridization of diploid sexual [*S. torminalis* (L.) Crantz, *S. aucuparia* L. and *S. chamaemespillus* (L.) Crantz] and tetraploid [taxa from the group of *Sorbus aria* (L.) Crantz] species. Primary hybrids occur spontaneously and are of the same ploidy level as their parents (Meyer et al. 2005). Hybrid lineages and stabilized hybridogenous species (or so-called microspecies) have higher ploidy levels, indicating their formation through unreduced gametes or through hybridization of polyploids. Their evolutionary success is connected to agamospermy (apospory), which can be accompanied by residual sexuality (Proctor et Groenhof 1992, Robertson 2004). Newly originated lineages can persist *in situ* for many years and further shape the population structure of parental species (eg. as pollen donors; Ludwig et al. 2013). Microspecies may originate recurrently from the same parental combination and exhibit highly similar morphology with negligible differences due to distinct parent genotypes. Most discovered stabilized lineages are subsequently described as new species (see the number of *Sorbus* taxa in the flora of the Czech Republic; Kaplan 2012). This approach may, however, spoil the taxonomy, especially when each and every local lineage is formally described as a separate species. *Sorbus quernea*, a formerly recognized endemic rowan from Prague, is an example of an apomictic taxon that was sunk into synonymy after a thorough taxonomic revision (Lepší et al. 2013).

The study of agamic complexes in the frame of conservation efforts is a very difficult task. With respect to taxonomy, agamic complexes are a “dynamic system” of newly described and rejected taxa (see Lepší et al. 2013). This approach is, however, often difficult to digest for conservationists. Conservation of higher taxonomic units (eg. at the subgeneric level; Pellicer et al. 2012) or the conservation of evolutionary units that generate taxonomic diversity seems to be an alternative (Rhymer et Simberloff 1996). The questions remain: What should be protected (the product or the speciation trigger), and which phenomenon is more valuable – endemism or speciation? One may argue that hybridogenous agamic species are “blind alleys” of evolution that do not deserve protection due to their asexual mode of reproduction. This is not the case, however, as they may still enter further hybridizations as pollen donors (production of viable pollen grains is relatively high; Rich 2009). Hybridization is a significant evolutionary process, and hybridizing populations of parental species and hybrids alike are extremely important from several standpoints. To protect taxonomically

complex groups and processes taking place within them, it is crucial to conserve these “engines” of evolution (Stace 1998, Ennos et al. 2006).

Biosystematic evaluation of *Sorbus eximia* is presented in the second study. It was a textbook example of diploid-tetraploid taxon with agamospermous mode of reproduction (including agamospermy at the diploid level; Jankun et Kovanda 1988). This exceptional mode of reproduction was several times cited (Nelson-Jones 2002, Talent et Dickinson 2006, Dobeš et al. 2013), but never been reliably confirmed.

### *Nymphaea*

Two native *Nymphaea* species occur in Central Europe – *N*. *alba* and *N*. *candida*. Species boundaries between them are blurred by overall morphological similarities, high phenotypic plasticity and possible interspecific hybridization. The situation is further complicated by the occurrence of many garden cultivars. Morphological similarities are at least partly caused by close evolutionary relationships between *N. alba* and *N. candida* (Volkova et al. 2010). Individuals with intermediate morphologies have often been interpreted as interspecific hybrids (Heslop-Harrison 1955, Ejankowski & Małysz 2011) although their hybrid status has only rarely been evidenced. The few exceptions include crosses between *N. alba* and *N. candida* (= *N. × borealis* Camus) from several sites in Germany and Sweden, confirmed by AFLP fingerprints (Werner & Hellwig 2006, Nierbauer et al. 2014). Natural interspecific hybridization in *Nymphaea* seems to be quite extensive, as indicated by the great number of horticultural crosses (Slocum 2005). Garden cultivars have been repeatedly introduced, be it accidentally or intentionally, into natural habitats, where they can survive for long periods and potentially interact (compete or mate) with native plants. Reliable discrimination between escaped white-flowered cultivars and native species on the basis of morphological traits is difficult, if not impossible.

### *Diphasiastrum*

Six diploid *Diphasiastrum* taxa are traditionally recognized in Europe: three (basic) species and three morphologically intermediate hybrids traditionally treated as species. Mixed populations frequently occur in Central Europe and often form apparent hybrid swarms. Species determination is quite problematic in mixed populations and especially in hybrid swarms occurring in man made habitats (eg. ski slopes, deforested strips). A number of factors complicate investigations of hybridization patterns in *Diphasiastrum*: simple morphology with few characters suitable for evaluation, high phenotypic plasticity and impossibility to accomplish hybridization experiments due to mycorrhizal gametophytes (Wilce 1961, 1965, Whittier 1977, Vogel et Rumsey 1999). The patterns of hybridization in *Diphasiastrum* have recently been addressed using two types of markers: low-copy nuclear genes and genome size. Sequences of three regions of the nuclear genome confirmed the hybrid status of *D. ×issleri*, *D. ×oellgaardii* and *D. ×zeilleri* (Aagaard et al. 2009a, 2009b). This study also indicates certain levels of recent hybridization and backcrossing within European *Diphasiastrum.* Its frequency and variation patterns in natural populations remain unknown, however. On the contrary, discrete variation in genome size in several parts of Europe indicates only primary hybridization with no hint of backcrossing (except for a few rare triploid hybrids) or introgression (Bennert et al. 2011).

## Aims of the thesis

1) To evaluate the risk of interspecific hybridization in selected rare species native to the Czech Republic

2) To assess the value of different methodological approaches (incl. karyological, phenetic and molecular techniques) for hybrid identification

3) To elucidate the human impact on the hybridization of rare plant species under investigation

## Conclusions and future directions

Although much attention has been paid to the conservation of rare species during the last decades, most studies have not taken a complex biosystematic approach. The conservation and biosystematic points of view have rarely been integrated in a single study. Fortunately, recent years have seen significant progress, which is manifested by the publication of research papers at the interface between conservation and biosystematics (eg. Hedrén et al. 2012, Moreira et al. 2013).

This thesis presents three cases of interspecific hybridization involving rare species. Hybrids coexist with parental taxa in the ***Cerastium*** *alsinifolium/C. arvense* system. The Czech serpentine endemic *C. alsinifolium* is threatened by competition from hybrids over both abiotic and biotic resources (light, nutrients and pollinators). Considering the absence of backcrosses, hybridization does not seem to severely affect the gene pool of the endemic species. Its genetic integrity will thus most likely be preserved. Nevertheless, in open sites, hybrids usually dominate over *C. alsinifolium* and may possibly outcompete it. Interspecific hybridization is much less pronounced in forest sites, which host core populations of the endemic and are therefore a conservation priority.

Flow-cytometric measurements revealed a ca 45% difference in genome sizes between ***Nymphaea*** *alba* and *N. candida*. Moreover, the genome sizes of *Nymphaea* cultivars were considerably lower than those of native species. Statistical analyses of morphological characters allowed reliable phenotypic delimitation of both *Nymphaea* species and garden cultivars. Although morphotypes with intermediate values of characters and/or a mosaic-like combination of characters have often been interpreted as interspecific hybrids, our results indicate that interspecific hybridization under natural condition is quite rare (at least in the Czech Republic), and a hybrid origin was confirmed in only eleven out of 612 analysed plants (ca 1.8%). Native *Nymphaea* species are thus not directly threatened by interspecific hybridization. An important finding is the frequent occurrence of accidentally or intentionally introduced *Nymphaea* cultivars in more or less natural habitats in the Czech Republic. It is likely that white-flowered cultivars have previously often been confused with indigenous species.

The frequency of interspecific hybridization among ***Diphasiastrum*** species and its consequences were evaluated using genome size analysis, and numerical and geometric morphometrics. Although genome sizes of basic taxa tend to differ, hybrids often form phenotypic continua. The most intricate genome size values were found in *D. ×issleri* and *D. ×oellgaardii.* The genome sizes of these hybridogenous species completely overlap even though they originated from different parental combinations. Very low genome size variation was detected in single-taxon populations. The highest variation was found in several populations that consisted of all six species, and in mixed populations comprising both *D. alpinum* and *D. tristachyum*. A similar pattern of variation was subsequently observed in both numerical and geometric morphometrics.

The origin of the hybridogenous species ***Sorbus*** *eximia* was elucidated, and a new species (*S. barrandienica*) was recognized during the biosystematic revision of the *Sorbus eximia* group in the

Bohemian Karst. Flow cytometry did not confirm the existence of two ploidy levels (di- and tetraploid) and reported agamospermy at the diploid level (Jankun et Kovanda 1988). All accessions of *S*. *eximia* and *S*. *barrandienica* turned out to be triploid. The genetic variation of both investigated species was extremely low, indicating their single origins. Long-term persistence of their populations was most likely supported by their agamospermous mode of origin.

The last decade has seen several attractive research directions in the study of hybridizing rare plant species. Although they might be methodologically challenging, they offer opportunities for gaining deeper insights into the patterns and processes of interspecific hybridization, ultimately leading to the identification of "common patterns".

Agamic complexes present a particularly promising group for the study of several microevolutionary processes. Although taxonomic studies (often resulting in the recognition of new agamospermous lineages) clearly prevailed in the last few decades (eg. in the genera *Sorbus* – Kovanda 1961, 1996, Lepší et al. 2008; *Rubus* – Lepší et Lepší 2009, Trávníček et Žíla 2011; *Taraxacum* – Trávníček et al. 2008), more recent works address ecological and microevolutionary questions (eg. in the genus *Sorbus*; Vít et al. 2012, Lepší et al. 2013, Ludwig et al. 2013). Many recent studies attempt to reveal microevolutionary mechanisms responsible for the genesis of hybridogenous species. Other attractive topics are detection of the mode of reproduction (using DNA flow cytometry and microsatellites), identification of parental taxa (microsatellites), elucidation of the direction of hybridization (chloroplast markers) and susceptibility of each parent to hybridization (using hybridization experiments).

The spread of introduced (and possibly invasive) plant taxa has been well documented. Occasionally, they can hybridize with their native counterparts, and these systems offer unique opportunities to study hybridization at its initial stages. Future studies should clarify the evolutionary consequences of hybridization on populations of native species, using a multi-method approach involving, among others, detailed ecological studies, sophisticated spatial models (Phillips et al. 2006) and historical data coupled with molecular and cytogenetic techniques. Such studies will paint a holistic picture of the patterns, processes and dynamics of interspecific hybridization.

# Interspecific hybridization between rare and common plant congeners inferred from genome size data: assessing the threat to the Czech serpentine endemic *Cerastium alsinifolium*

**Mezidruhová hybridizace mezi vzácným a hojným druhem, zjištěná na základě dat o velikosti genomu – zhodnocení ohrožení českého hadcového endemita *Cerastium alsinifolium***

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Vít P., Wolfová K., Urfus T., Tájek P. & Suda J. (2014): Interspecific hybridization between rare and common plant congeners inferred from genome size data: assessing the threat to the Czech serpentine endemic *Cerastium alsinifolium*. – Preslia 86: 95–117.

*Cerastium alsinifolium* Tausch (*Caryophyllaceae*) is an endemic species restricted to serpentine sites in the Slavkovský les Mts (western Bohemia) in the Czech Republic. Interspecific hybridization with sympatric *C. arvense* L. has long been suspected due to the substantial and continuous morphological variation observed in the field but it has never been reliably confirmed. Although both parental species share the same number of somatic chromosomes they differ considerably in the size of their monoploid nuclear genomes (~1.5-fold), which makes it easy to identify the species. Flow cytometric investigation of more than 2200 *Cerastium* samples revealed five distinct genome size categories, corresponding to the two parental species and three types of interspecific hybrids (originating via both reduced and unreduced gametes). F1 interspecific hybrids were very common (nearly 40% of the samples analysed from the Slavkovský les Mts), which indicates the barriers to breeding between the parental species are weak. However, no backcrosses were indicated by the genome size data. In contrast to a widely held view that *C. alsinifolium* mostly occurs on open serpentine outcrops, this habitat was dominated by interspecific hybrids. The endemic species occurred mainly in moist and (semi-)shaded sites, including springs in spruce forest clearings, seeps and wet margins of forest roads. Multivariate morphometrics revealed that the shape and size of cauline leaves, development of sterile axillary shoots, bract characteristics, and lengths of calyx, petals and anthers are diagnostic for the groups investigated. While the determination of *C. arvense* usually poses few problems, distinguishing *C. alsinifolium* from interspecific hybrids on the basis of morphological characters is much more challenging; reduced pollen fertility of hybrids provides the most important clue. Our results indicate that effective conservation of this important component of the Czech flora will require more emphasis on the conservation of forest sites that host core populations of *C. alsinifolium*.

K e y w o d s: *Cerastium*, conservation, Czech Republic, endemic, flow cytometry, genome size, interspecific hybridization, multivariate morphometrics, serpentine

## Introduction

Interspecific hybridization is a common and ongoing process in populations of land plants, with many important evolutionary consequences (Soltis & Soltis 2009). There are two opposing views on the role of hybridization in plant speciation. Whereas interspecific

hybridization (often connected with genome duplication) can be a source of genetic and phenotypic novelties, ultimately leading to the origin of a new species (i.e. hybrid speciation), it can also cause genetic dilution, breakdown of a species integrity and possibly species extinction. The detrimental effect of hybridization is pronounced in rare species (or species with insular-like distributions) because they usually occur at low densities and might be surrounded by larger populations of related congeners with incomplete breeding barriers. Among other factors, hybridization can contribute to the demise of rare species through the production of hybrid seeds at the expense of conspecific seeds and hybrid competition for abiotic or biotic resources (Levin et al. 1996). In addition to the demographic swamping, extensive gene flow will also influence the genetic make-up of the species by the disruption of coevolved gene complexes and affecting genetic adaptation to local environmental conditions (Givnish 2010).

One of the challenges posed by interspecific hybridization is the difficulty of recognizing it, especially when closely related species with similar phenotypes are involved. While only parental species and sterile F1 hybrids are present in some cases, complex hybrid swarms can occur in other populations, consisting of a mixture of parents, hybrids of different generations and backcrosses to parental species (Krahulcová et al. 1996, Oberprieler et al. 2010). Morphological variation in hybrid populations can span a continuum from one parent to the other, which precludes reliable determination of individual plants on the basis of phenotypic characters. Molecular markers can assist greatly in the identification of crosses and provide an insight into the dynamics of hybridization (Hegarty & Hiscock 2005). However, molecular analyses take a long time and are costly, making comprehensive population studies impractical. Cytogenetic data can also aid hybrid identification in some cases, providing the parental taxa differ in the number of somatic chromosomes or genome size (Ekrt et al. 2010, Suda et al. 2010). The field of cytotaxonomy has recently been revitalized by the advent of flow cytometry (FCM), which has made it possible to screen whole populations at large spatial and temporal scales (Kron et al. 2007, Loureiro et al. 2010, Suda & Pyšek 2010).

An illustrative example of a rare and severely threatened species in the Czech flora, whose populations have most likely been affected by interspecific hybridization, is the serpentine endemic of western Bohemia, *Cerastium alsinifolium* Tausch (Smejkal 1990, Kaplan 2012). The total area occupied by this species does not exceed 15 km$^2$, with all sites situated within the Protected Landscape Area Slavkovský les (Tájek et al. 2012). *Cerastium alsinifolium* ranks among the critically endangered plants in the Czech flora (Klaudisová & Čeřovský 1999, Grulich 2012, Kaplan 2012) and is listed as a priority species in Annex II of the European Commission Habitats Directive and in Appendix I of the Convention on the Conservation of European Wildlife and Natural Habitats (Bern Convention). It is classed as Data Deficient in the IUCN Red List of Threatened Species (IUCN 2013). Of the 74 plant taxa considered to be endemic to the Czech Republic (Kaplan 2012), *C. alsinifolium* has a prominent position because it is: (i) sexually reproducing (the majority of other endemics are apomictic microspecies), (ii) phenotypically and ecologically distinct (several other endemics are morphologically rather poorly defined taxa, often recognized at intra-specific ranks) and (iii) historically the first described (and still accepted) endemic plant, distinguished as early as 1828 (Tausch 1828).

The origin and evolutionary past of *Cerastium alsinifolium* is a matter of speculation. Although the majority of authors (e.g. Čelakovský 1873, Dostál 1989, Jalas et al. 1993) believe that *C. alsinifolium* is closely related to *C. arvense* L., Novák (1960) placed *C. alsinifolium* in the arcto-alpine *C. alpinum* agg. Currently, *C. alsinifolium* is reported from two rather contrasting types of habitat on serpentine bedrocks, namely dry open grassland on rocky outcrops and (semi)shaded springs and seeps in coniferous forests (Melichar 2005). At several sites in the Slavkovský les Mts, it cooccurs with another perennial large-flowered species, *C. arvense*, which is widely distributed in Europe and usually inhabits dry grasslands and/or semiruderal sites (Smejkal 1990). *Cerastium arvense* is relatively tolerant of soils with a high heavy metal content (Levine & Greller 2004) and in the Slavkovský les Mts it occasionally grows on outcrops of serpentine or in their immediate vicinity. Length of sepals, leaf shape and bract characteristics are considered the most important characters for the recognition of both species (Smejkal 1990, Hrouda 2002). In addition to the individuals that match the original description, there is a continuum of intermediate morphotypes in the Slavkovský les Mts, which indicate extensive interspecific hybridization (Smejkal 1990, Klaudisová & Čeřovský 1999, Rybka et al. 2004). However, the mostly quantitative species-specific morphological characters and the same number of somatic chromosomes in both species (2n = 6x = 72; Uhríková & Záborský 1980, Smejkal 1990, Měsíček & Jarolímová 1992) preclude unambiguous identification of hybrid individuals using conventional phenotype-based techniques or chromosome counting and another means of identification is needed. Despite the identical chromosome number, we found that *C. alsinifolium* and *C. arvense* differ markedly in the nuclear DNA content, which opens possibilities for a detailed examination of the structure of populations and determining the frequency of interspecific hybridization.

The main aim of this study was to assess the threat of interspecific hybridization to the survival of a rare serpentine endemic of the Czech flora, *Cerastium alsinifolium*. We utilized interspecific differences in genome size, and using DNA flow cytometry and multivariate morphometrics addressed the following questions: (i) What is the structure of the populations (proportion of hybrid individuals) at the different serpentine sites? (ii) What is the frequency of interspecific hybridization? Does data on genome size support the presence of only F1 hybrids or a more complex hybridization pattern? (iii) What are the species- and hybrid-specific phenotypic characters? Can hybrid individuals be reliably identified on the basis of morphology? (iv) Which localities host the most vigorous populations of *C. alsinifolium* and are hence of priority importance for conservation? Are the current conservation and management measures optimal for the proper protection and preservation of this endemic species or should they be revised?

## Materials and methods

### *Field sampling*

A thorough sampling of the population was done at five main localities (two open serpentine outcrops and three forest sites) in the Slavkovský les Protected Landscape Area (Electronic Appendix 1) that altogether host a substantial proportion of plants previously classified as *C. alsinifolium*. The sampling covered the entire range of microhabitats and included all the phenotypes present at these localities. A mature leaf from a total of 2086

*Cerastium* plants was collected for the FCM analysis. For comparative purposes, 136 individuals of *C. arvense* from 20 localities in the Czech Republic not in the Slavkovský les Mts were also included in this study (see Electronic Appendix 1 for locality details). A subset of 616 plants of known genome sizes, representing four recognized taxonomic groups (two parental species and two types of interspecific hybrids), was subsequently selected for morphometric analyses. Pressed flowering/fruiting shoots and flowers stored in 70% ethanol were collected for each individual. Herbarium vouchers are deposited at PRC (herbarium of Charles University in Prague).

Pollen viability was estimated for 174 individuals from the Slavkovský les Mts using the protocol detailed by Peterson et al. (2010). Anthers were collected before anthesis, fixed in Carnoy's fixative in the field and kept at room temperature until processed less than 2 months later. Two anthers per individual were then dissected on a microscopic slide, stained, and the slides were observed using a light microscope (Olympus BX41) at a 100× magnification. Two hundred pollen grains per sample were assessed. Because pollen viability was determined using different individuals than used in the morphometric analyses, this character was evaluated separately.

*Flow cytometry*

Genome sizes (2C-values; Greilhuber et al. 2005) were estimated using propidium iodide flow cytometry following the simplified two-step protocol using Otto buffers (Doležel et al. 2007). Briefly, ~0.5 $cm^2$ of intact leaf tissue per analysed plant and an appropriate volume of the internal reference standard (*Glycine max* 'Polanka', 2C = 2.50 pg; Doležel et al. 2007) were chopped up using 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 ~30 min at room temperature. The staining solution consisted of 1 ml of Otto II buffer (0.4 M $Na_2HPO_4.12\ H_2O$) supplemented with β-mercaptoethanol (final concentration of 2 μl/ml), propidium iodide and RNase II type IIA (both at a final concentrations of 50 μg/ml). After a short incubation, the samples were analysed using a Partec CyFlow instrument (Partec GmbH., Münster, Germany) equipped with a green (532 nm, 100 mW output) diode-pumped solid state laser. Fluorescence intensity of 5000 particles was recorded. Histograms were evaluated using Partec Flomax (ver. 2.4b). Only analyses with coefficients of variation for the G0/G1 *Cerastium* peaks below 4.0% were considered. Bulk samples of up to five individuals were processed during a large-scale population screening; mixed samples (i.e. consisting of plants with different fluorescence intensities) were reanalysed separately. One plant per fluorescence category from each locality studied in the Slavkovský les Mts was then selected and its absolute genome size estimated based on three replicates on different days. Differences in genome size values were tested using a general linear model (GLM, due to unbalanced data design) in the SAS 9.2 package (SAS Institute, Cary, NC, USA).

*Multivariate morphometrics*

Seventy quantitative features (incl. ratios) of vegetative and generative characters (for details see Table 1 and Electronic Appendix 2) were measured and calculated for 616 individuals, including 206 individuals of *Cerastium alsinifolium*, 191 individuals of *C. arvense* (95 and 96 from the Slavkovský les Mts and beyond, respectively), and 219

Table 1. – List of the morphological characters analysed and their contributions to the first canonical axis in canonical discriminant analyses of (i) *Cerastium arvense* vs. *C. alsinifolium* + interspecific hybrids (616 samples, 69 characters), and (ii) *C. alsinifolium* vs. interspecific hybrids with *C. arvense* (425 samples, 69 characters). Canonical correlates with the highest absolute loadings that were selected for the determination key are highlighted in bold type. Numbers in parentheses represent ranks of the strength of the correlation of each variable with the canonical axis. Character v41 was excluded from discriminant analyses because of its very strong correlation with v40.

| No. | Character description | Unit | *C. arvense* vs others | *C. alsinifolium* vs hybrids |
|---|---|---|---|---|
| Leaf and bract characters | | | | |
| v1 | Stem length (excluding inflorescence) | mm | 0.5399 (21) | –0.0404 (69) |
| v2 | Number of sterile shoots in leaf axils along the entire length of the stem | number | **0.7312 (8)** | 0.1406 (55) |
| v3 | Length of the uppermost leaf | mm | 0.6875 (12) | 0.1220 (58) |
| v4 | Width of the uppermost leaf | mm | –0.2769 (46) | –0.4246 (28) |
| v5 | Length of hairs on the margin of the uppermost leaf | mm | –0.3584 (34) | 0.0219 (67) |
| v6 | Number of hairs on the margin of the uppermost leaf (per 10 mm) | number | 0.1642 (58) | 0.3461 (34) |
| v7 | Angle of the tip of the uppermost leaf | degree | –0.5603 (19) | **–0.6278 (9)** |
| v8 | Length of the second uppermost leaf | mm | **0.7794 (2)** | 0.2827 (41) |
| v9 | Width of the second uppermost leaf | mm | –0.2796 (44) | –0.3259 (35) |
| v10 | Length of hairs on the margin of the second uppermost leaf | mm | –0.1811 (53) | 0.0904 (60) |
| v11 | Number of hairs on the margin of the second uppermost leaf (per 10 mm) | number | 0.2793 (45) | 0.5260 (20) |
| v12 | Angle of the tip of the second uppermost leaf | degree | –0.6230 (16) | **–0.6423 (7)** |
| v13 | Length of the third uppermost leaf | mm | **0.8003 (1)** | 0.4071 (31) |
| v14 | Width of the third uppermost leaf | mm | –0.2542 (47) | –0.2507 (43) |
| v15 | Length of hairs on the margin of the third uppermost leaf | mm | 0.1411 (60) | 0.2232 (45) |
| v16 | Number of hairs on the margin of the third uppermost leaf (per 10 mm) | number | 0.4659 (26) | 0.5271 (19) |
| v17 | Angle of the tip of the third uppermost leaf | degree | –0.6207 (17) | –0.5699 (12) |
| v18 | Length of the lowermost bract | mm | 0.2153 (51) | –0.1995 (49) |
| v19 | Width of the lowermost bract | mm | –0.0511 (66) | –0.2880 (40) |
| v20 | Length of the scarious margin at the tip of the lowermost bract | mm | 0.4114 (31) | 0.4881 (22) |
| v21 | Width of the scarious margin on the side of the lowermost bract | mm | 0.4827 (25) | 0.5553 (15) |
| v22 | Length of the scarious margin of the lowermost bract | mm | 0.6887 (11) | **0.6847 (2)** |
| v23 | Length of hairs on the margin of the lowermost bract | mm | –0.0950 (62) | 0.1499 (54) |
| v24 | Number of hairs on the margin of the lowermost bract (per 10 mm) | number | 0.0755 (64) | 0.3895 (33) |
| v25 | Angle of the tip of the lowermost bract | degree | –0.2847 (42) | –0.4159 (29) |
| v26 | Length of the second lowermost bract | mm | 0.3402 (37) | –0.0454 (64) |
| v27 | Width of the second lowermost bract | mm | 0.1406 (61) | –0.0376 (66) |
| v28 | Length of the scarious margin at the tip of the second lowermost bract | mm | 0.3759 (33) | 0.4153 (30) |
| v29 | Width of the scarious margin on the side of the second lowermost bract | mm | 0.4529 (29) | 0.5344 (18) |
| v30 | Length of the scarious margin of the second lowermost bract | mm | 0.6342 (15) | **0.6752 (4)** |
| v31 | Length of hairs on the margin of the second lowermost bract | mm | –0.1777 (56) | –0.0713 (62) |
| v32 | Number of hairs on the margin of the second lowermost bract (per 10 mm) | number | –0.0271 (68) | 0.0779 (61) |
| v33 | Angle of the tip of the second lowermost bract | degree | –0.1660 (57) | –0.1712 (53) |
| Flower characters | | | | |
| v34 | Calyx length (excluding the scarious margin) | mm | 0.6996 (9) | 0.4636 (24) |
| v35 | Calyx width (excluding the scarious margin) | mm | 0.4627 (27) | 0.2533 (42) |
| v36 | Length of the scarious margin at the tip of the calyx | mm | 0.3280 (39) | 0.2129 (47) |

| | | | |
|---|---|---|---|
| v37 Length of the scarious margin on the side of the calyx | mm | 0.4183 (30) | 0.2228 (46) |
| v38 Petal length | mm | **0.7718 (4)** | –0.0558 (63) |
| v39 Petal length to the notch | mm | 0.6851 (13) | –0.1356 (57) |
| v40 Petal width | mm | 0.6982 (10) | 0.2298 (44) |
| v41 Width of the lobe of a petal | mm | – | – |
| v42 Filament length | mm | 0.5434 (20) | –0.2944 (39) |
| v43 Length of anther | mm | **0.7736 (3)** | –0.0419 (65) |
| v44 Width of anther | mm | 0.4846 (24) | –0.4365 (27) |
| Fruit and seed characters | | | |
| v45 Capsule length on the convex side | mm | 0.2797 (43) | –0.6093 (10) |
| v46 Capsule length on the concave side | mm | 0.1797 (54) | **–0.6682 (5)** |
| v47 Capsule width | mm | 0.3533 (35) | –0.4601 (26) |
| v48 Tooth length of a dehiscent capsule | mm | 0.5281 (23) | 0.5766 (11) |
| v49 Tooth width of a dehiscent capsule | mm | 0.5843 (18) | 0.1916 (50) |
| v50 Calyx length at fruiting stage | mm | **0.7391 (6)** | **0.6568 (6)** |
| v51 Seed length | mm | 0.0466 (67) | 0.3107 (37) |
| v52 Seed width | mm | 0.2319 (50) | 0.1403 (56) |
| Ratios | | | |
| v53 Length / width of the uppermost leaf | | –0.6661 (14) | –0.5633 (13) |
| v54 Length / width of the second uppermost leaf | | **–0.7345 (7)** | –0.5352 (17) |
| v55 Length / width of the third uppermost leaf | | **–0.7685 (5)** | –0.5530 (16) |
| v56 Length / width of the lowermost bract | | –0.3271 (40) | –0.1803 (51) |
| v57 Width of the scarious margin on the side / total width of the lowermost bract | | 0.3980 (32) | 0.5622 (14) |
| v58 Length of the scarious margin / total length of the lowermost bract | | 0.5298 (22) | **0.6757 (3)** |
| v59 Length / width of the second lowermost bract | | –0.2382 (49) | –0.0047 (68) |
| v60 Width of the scarious margin on the side / total width of the second lowermost bract | | 0.3397 (38) | 0.4621 (25) |
| v61 Length of the scarious margin / total length of the second lowermost bract | | 0.4626 (28) | **0.6363 (8)** |
| v62 Length / width of the calyx | | –0.1782 (55) | –0.0949 (59) |
| v63 Petal length to notch / total petal length | | –0.1521 (59) | –0.1746 (52) |
| v64 Length / width of the petal | | 0.0101 (69) | 0.2947 (38) |
| v65 Calyx length / petal length | | 0.0721 (65) | –0.4843 (23) |
| v66 Length / width of the anther | | –0.1917 (52) | –0.4972 (21) |
| v67 Capsule length on the convex / concave side | | –0.3439 (36) | –0.3114 (36) |
| v68 Length / width of the capsule | | –0.0907 (63) | 0.3978 (32) |
| v69 Capsule length / calyx length at fruiting stage | | –0.3068 (41) | **–0.7758 (1)** |
| v70 Length / width of the seed | | 0.2396 (48) | –0.2039 (48) |

individuals of interspecific crosses. Missing character values were replaced by population means provided that measurements for at least 90% of the individuals from a particular population were available. The set of morphological characters analysed was selected on the basis of published determination keys, flora handbooks (Smejkal 1967, Hegi & Weber 1975, Smejkal 1990, Hrouda 2002) and our own field observations. Morphometric data were analysed using CANDISC (canonical discriminant analysis), CORR (correlation analysis), DISCRIM (classification discriminant analysis), STEPDISC (stepwise discriminant analysis with forward or stepwise selection of characters), PRINCOMP (principal component analysis) and UNIVARIATE (basic statistics) procedures in SAS

9.2 following Rosenbaumová et al. (2004). Individual plants were used as operational taxonomic units (OTUs). First insights into phenetic relationships among OTUs were gained using principal component analysis (PCA) while discriminant analyses were employed to select a set of characters that allowed for the best separation of a priori defined groups of OTUs (i.e. species and hybrids characterized by their genome sizes) and to determine the proportion of correctly classified individuals. Because the data distributions within groups were not multivariately normal (Wilks-Shapiro test), the non-parametric k-nearest neighbour discriminant function and non-parametric correlation coefficients were employed. Discriminant power was determined by cross-validation (Klecka 1980). Various modifications of discriminant analyses were performed, including all four recognized taxonomic groups (parental species, hybrids originating from unreduced gametes and those originating via reduced plus unreduced gametes), three groups (with all hybrids merged into a single category), separate analysis of the two groups of hybrids, *C. arvense* vs. *C. alsinifolium* + hybrids, *C. alsinifolium* vs. *C. arvense*, C. *alsinifolium* vs. hybrids, etc. Flowering and fruiting plants were analysed together and separately. The determination key was largely constructed on the basis of results of discriminant analyses (characters most tightly correlated with canonical axes); characters that can be easily measured/observed in the field were preferentially selected.

## Results

### *Variation in genome size*

Flow cytometric analysis yielded high-resolution histograms with distinct peaks of both the plant sample(s) and internal standard, and with little background fluorescence signals (Fig. 1). The average coefficients of variation of the G0/G1 peaks of *Cerastium* samples and the internal reference standard were 2.65% (range 1.27–3.89%) and 3.02% (range 1.55–3.84%), respectively. Estimates of genome sizes of 2222 *Cerastium* plants resulted in five distinct categories, corresponding to the two parental species and three types of interspecific hybrids (Fig. 2). Individuals morphologically matching *C. arvense* from the Slavkovský les Mts did not differ in genome size from those collected elsewhere in the Czech Republic (GLM, $F = 0.79$, $P = 0.376$). Nuclear DNA contents of the serpentine endemic *C. alsinifolium* (mean 2C-value = 4.25 pg; Figs 1, 2) was about 1.5-times greater than that of *C. arvense* (mean 2C = 2.83 pg), enabling not only the reliable recognition of both parental species but also that of their interspecific crosses, including potential back-crosses. A considerable percentage of the samples (37.4%; Table 2) from the Slavkovský les Mts had intermediate genome sizes between the putative parental species, suggesting they were of hybrid origin. In addition, a few individuals (1.3%; Table 2) had genomes markedly larger than *C. alsinifolium*, with a mean value of 4.98 pg/2C. The most parsimonious explanation for these genome values is interspecific hybridization by means of an unreduced gamete of *C. arvense*. The highest 2C-value (5.43 pg) was recorded for one individual from population Vlček (Table 2), which most likely originated via a syngamy of a 2n gamete of *C. alsinifolium* and a reduced gamete of *C. arvense* (all crosses involving unreduced gametes are further referred to as "polyploid hybrids"). Clear discontinuities between the five genome size categories (Fig. 2) indicate the lack of back-crosses.

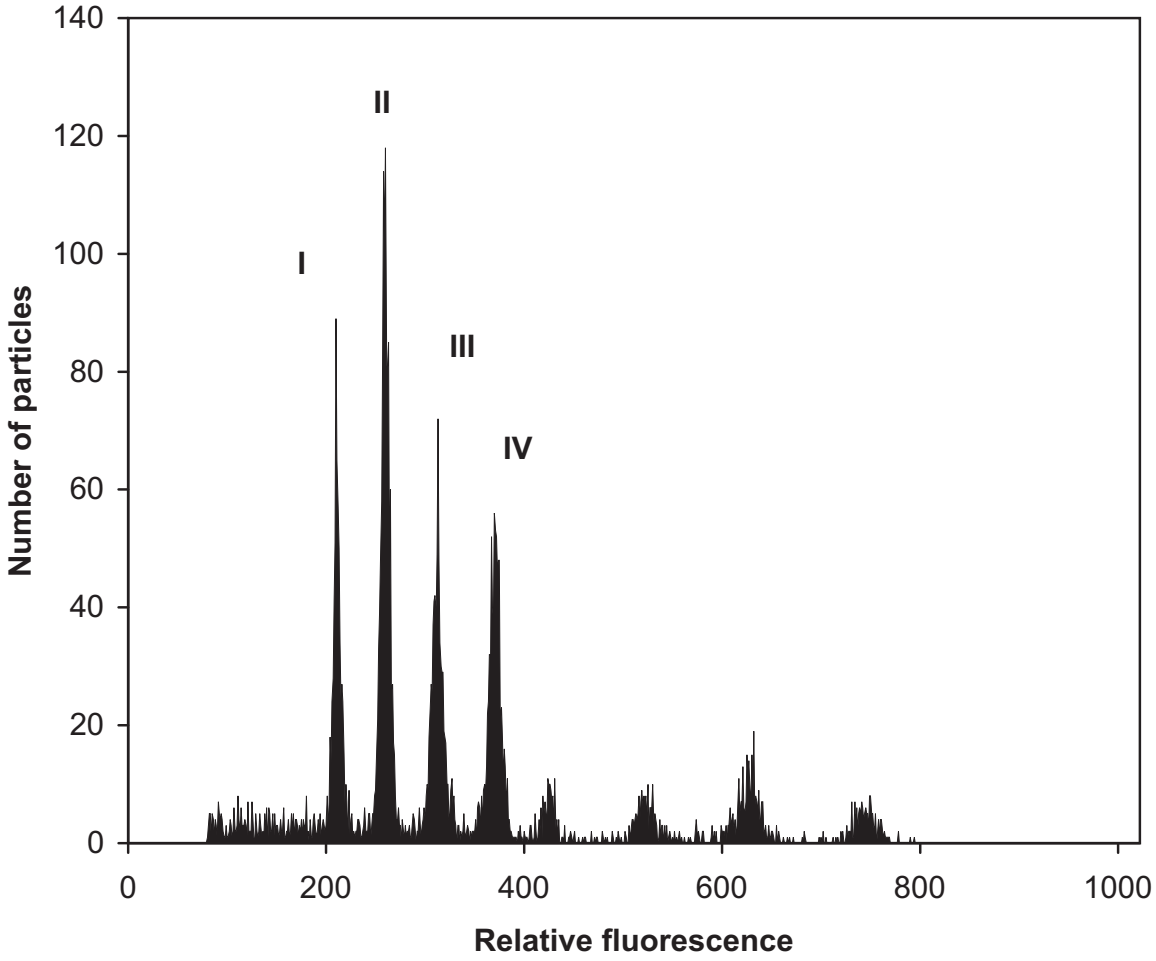


Fig. 1. – Simultaneous flow-cytometric analysis of *Cerastium* plants of four different holoploid genome sizes. Nuclei of all samples were isolated, stained with propidium iodide and analysed simultaneously. Peak designations: I – *C. arvense*; II – F1 hybrid (originating via unreduced gametes); III – *C. alsinifolium*; IV – polyploid hybrid (originating from an unreduced gamete of *C. arvense* and a reduced gamete of *C. alsinifolium*).

*Ecological preferences*

Representative samples from five serpentine localities revealed dramatic differences in the frequency of *C. alsinifolium* and interspecific hybrids in the two main types of habitat (Table 2). Whereas moist places in coniferous forests on serpentine bedrocks (spring areas in forest clearings, seeps, wet margins of forest roads, etc.) are dominated by *C. alsinifolium* (73.2–91.7% of the individuals sampled), open and dry serpentine outcrops are largely inhabited by interspecific hybrids and the endemic species only constitutes a minority of the individuals (10.6–15.7% of samples analysed). On rocky outcrops, *C. alsinifolium* clearly occurs mainly in sheltered and the most humid microhabitats, such as rock crevices covered with moss. In contrast, both hybrid plants and *C. arvense* are more heliophilous, competition- and drought-tolerant and usually occur in open short grassland.

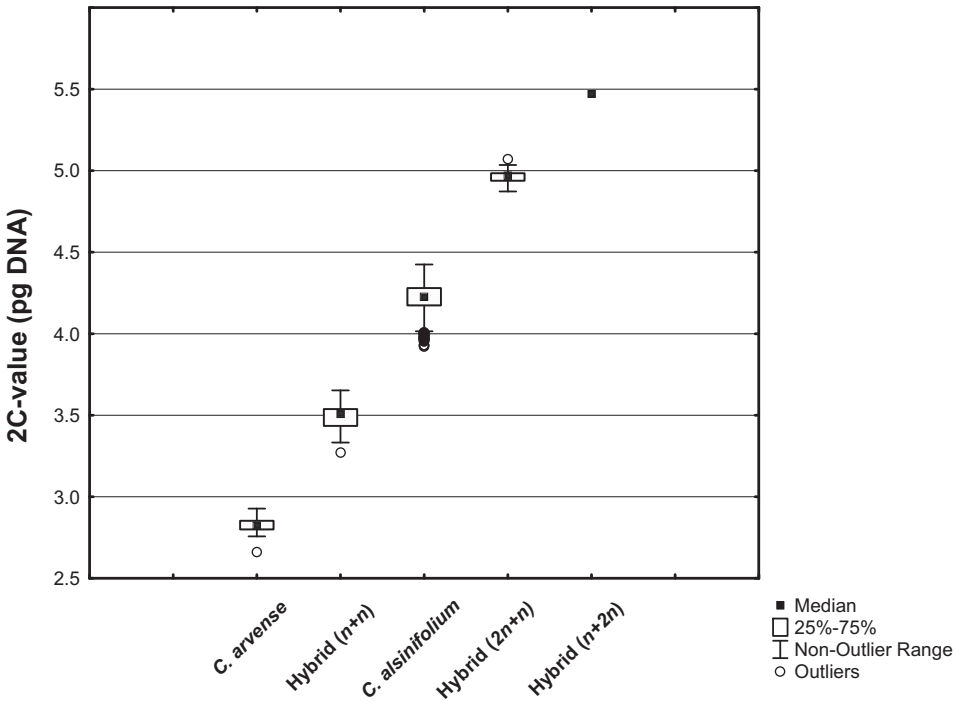


Fig. 2. – Box-and-whisker plots showing the holoploid genome sizes (2C-values) estimated for samples of large-flowered *Cerastium*, mainly from the Slavkovský les Mts. See Table 2 for number of samples in individual categories.

Table 2. – Numbers and percentages (in parentheses) of *Cerastium* samples corresponding to two parental species and three types of interspecific hybrids at five thoroughly investigated localities in the Slavkovský les Mts in western Bohemia. Habitats in coniferous forests represent moist places on serpentine bedrock.

| Locality | Habitat | *C. arvense* | F1 hybrid | *C. alsinifolium* | Polyploid hybrid (2n *C. arvense* + n *C. alsinifolium*) | Polyploid hybrid (n *C. arvense* + 2n *C. alsinifolium*) |
|---|---|---|---|---|---|---|
| Dominova skalka | open serpentine outcrops | 100 (19.4%) | 325 (63.0%) | 81 (15.7%) | 10 (1.9%) | 0 |
| Křížky | open serpentine outcrops | 94 (20.8%) | 310 (68.6%) | 48 (10.6%) | 0 | 0 |
| Pluhův bor | coniferous forest | 12 (4.5%) | 6 (2.3%) | 244 (91.7%) | 4 (1.5%) | 0 |
| Planý vrch | coniferous forest | 0 | 109 (24.9%) | 320 (73.3%) | 8 (1.8%) | 0 |
| Vlček | coniferous forest | 1 (0.2%) | 30 (7.2%) | 378 (91.2%) | 5 (1.2%) | 1 (0.2%) |

*Phenotypic variation*

Correlation analysis using Spearman coefficients revealed one tightly correlated pair of characters of petals (total petal width and width of the petal lobe), therefore the latter character (v41) was excluded from the discriminant analyses.

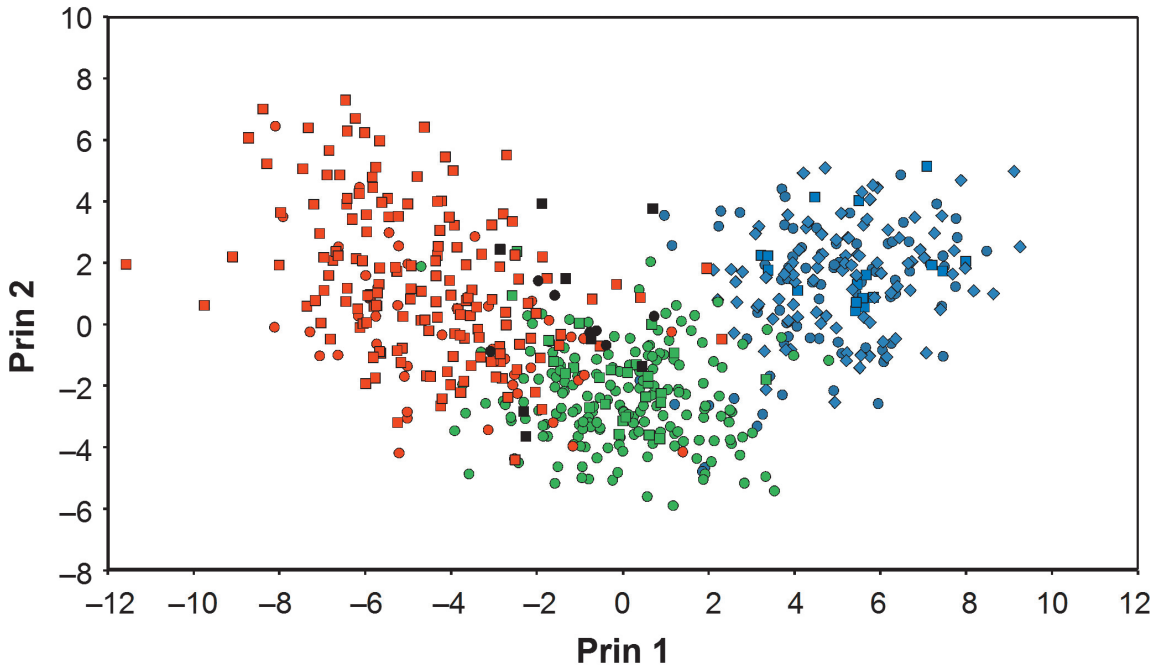


Fig. 3. – Principal component analysis of 616 *Cerastium* samples based on 70 characters (see Table 1 for character description). *C. alsinifolium* – red, *C. arvense* – blue, F1 hybrids – green, polyploid hybrids – black. Samples from open rocky outcrops and (semi)shaded forest habitats are depicted by circles and squares, respectively. Diamonds denote samples of *C. arvense* from outside the Slavkovský les Mts. The first and second PCA axes explain 26.3% and 9.8% of the total variation, respectively.

Principal component analysis of the entire data set revealed that the samples of *C. arvense* from the Slavkovský les Mts were very similar to those collected elsewhere, as were samples of *C. alsinifolium*/hybrids from different habitats within the serpentine area investigated (Fig. 3). Slight differences between plants from open rocky and (semi)shaded forest sites were mainly in stem length. Three distinct, though partially overlapping groups of characters were revealed by the PCA plot (Fig. 3). Interspecific hybrids occupied an intermediate position between their putative parents, but generally were somewhat closer to *C. alsinifolium*. Discriminant analysis of the same taxonomic groups (*C. alsinifolium*, *C. arvense* and interspecific hybrids) yielded a very similar picture (with better separated groups of OTUs; Electronic Appendix 3).

In order to follow the structure of dichotomous determination keys, we performed separate discriminant analyses on two groups of characters. Major morphological differences (characters most tightly correlated with the canonical axis) between flowering individuals of *C. arvense* and a group of *C. alsinifolium* + hybrids were revealed in the number of short sterile shoots emerging in axils of cauline leaves (character v2; see Table 1), lengths and shapes (length/width ratios) of the second and third uppermost leaves (v8, v13, v54, v55), length of the scarious margin of the lowermost bract (v22), petal length (v38) and anther length (v43) (Table 1, Fig. 4A). While the involvement of all measured and scored characters resulted in a misclassification of 14 out of 616 individuals (= 2.3%), the discrimination power was very similar when only the eight above-mentioned characters with the highest absolute canonical loadings were used (20 misclassified individuals out of 616; 3.2%). Fruiting individuals can most reliably be determined using differences in calyx length (v50), in addition to leaf and bract characters (results not shown). The taxonomic

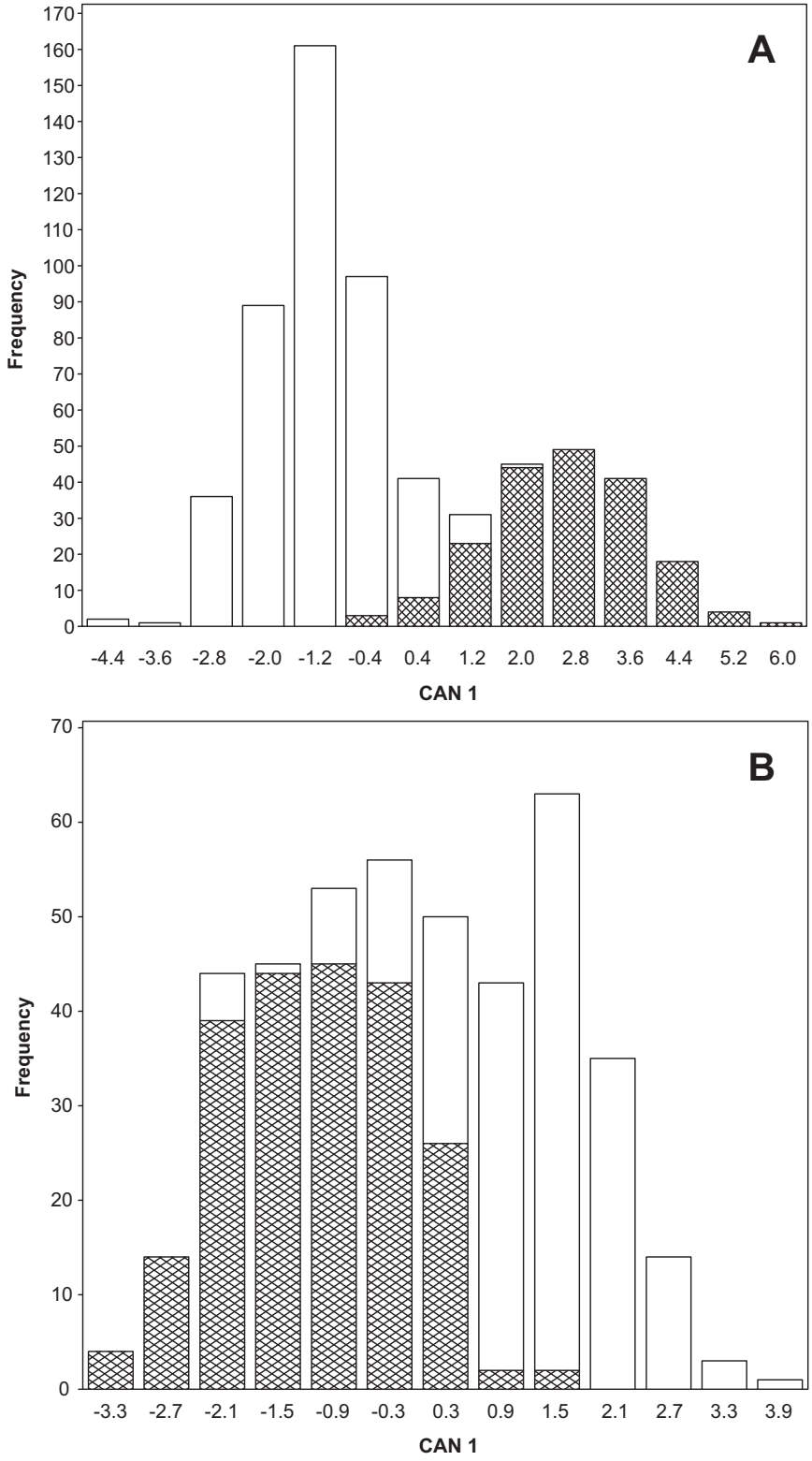


Fig. 4. – (A) Canonical discriminant analysis of a group of *Cerastium alsinifolium* + interspecific hybrids (open columns; 425 individuals) and *C. arvense* (hatched columns; 191 individuals). Eight taxonomically-important characters (v2, v8, v13, v22, v38, v43, v54, v55; see Table 1) were used for discrimination. The analysis resulted in 3.2% of the samples being misclassified. (B) Canonical discriminant analysis of *C. alsinifolium* (open columns; 206 individuals) and its hybrid with *C. arvense* (hatched columns: 219 individuals). Seven taxonomically-important characters (v22, v30, v53, v54, v55, v58, v61; see Table 1) were used for discrimination. The analysis resulted in 11.8% of the samples being misclassified.

importance of the selected characters was also confirmed in a separate analysis of parental species with all hybrids excluded; in this modified analysis, calyx length at the flowering stage (v34) was included among the diagnostic characters (results not shown).

Correct identification of *C. alsinifolium* and interspecific hybrids based on morphological characters is more challenging. Characters with the highest discrimination power and suitability in determination keys include leaf shape (= length/width of leaves; v53, v54, v55) and the size and shape of scarious margins of bracts (both absolute length and its proportion with respect to total bract length; v22, v30, v58, v61) (Table 1, Fig. 4B). There are some differences in the shape of the leaf apex (acute vs. subobtuse). The percentage of correctly classified flowering individuals reached 92.1% and 88.2% using all characters and the seven selected characters with the highest absolute canonical loadings, respectively. Calyx length (v50) and the ratio of it and capsule length (v69) are additional taxonomically-important characters that can help in the identification of fruiting individuals. Polyploid hybrids differed from their counterparts originating via crosses in which both gametes are unreduced mainly in terms of calyx (v36, v50, v69) and capsule (v48, v49) characters (Table 1) but the discrimination was unreliable and the success comparatively low due both to tiny differences and unbalanced data design.

Striking differences between parental species and their hybrids were found in pollen fertility. While the mean percentage of viable pollen grains was 95.1% (range 73.1–100%) and 97.3% (range 88.3–100%) for *C. arvense* and *C. alsinifolium*, respectively, the mean value dropped down to 25.1% (range 0–56.9%) for interspecific crosses.

## Discussion

Our study provides a detailed investigation of a phytogeographically and evolutionarily important component of the Czech flora, *Cerastium alsinifolium.* Although this species belongs among the most well-known endemic plants of the Czech Republic (Novák 1960, Smejkal 1990, Klaudisová & Čeřovský 1999, Rybka et al. 2004, Kaplan 2012), reliable data on its morphology, ecology and fine-scale distribution were missing because it is difficult to identify, in particular to distinguish it from putative hybrids with another large-flowered and sympatric species, *C. arvense*. Unlike previous studies, we used genome size as an independent marker for taxonomic decision-making and assessed phenotypic variation and ecological preferences of individuals characterized by their distinct nuclear DNA amounts.

### *Taxonomic significance of genome size data*

The last decade has seen a rise in the number of studies that acknowledge the taxonomic value of genome size data (Kron et al. 2007, Loureiro et al. 2010). The amount of nuclear DNA can vary considerably among different congeneric species irrespective of the number of chromosomes (Bennett & Leitch 2011) but is usually stable within the same species/evolutionary unit (Greilhuber 2005). Consequently, genome size can be employed as a useful character to delimit species boundaries, identify improperly developed individuals and/or resolve complex low-level taxonomies even in groups with identical chromosome numbers (Suda et al. 2007). Provided that interspecific differences are sufficiently large (dozens of percent), genome size also offers the opportunity to detect interspecific hybrids

and/or backcrosses. This promise has been recently fulfilled in several taxonomically challenging plant genera, including *Amaranthus* (Jeschke et al. 2003), *Dryopteris* (Ekrt et al. 2010) and *Elytrigia* (Mahelka et al. 2005).

Considerable karyological variation is recorded in the genus *Cerastium*. Disregarding one uncertain diploid count, all *Cerastium* species are polyploid, with chromosome numbers ranging from 2n = 4x = 36 to 2n = 16x = 144 (Hegi & Weber 1975, Goldblatt & Johnson 1979 onwards, Jalas et al. 1993, Scheen et al. 2004). Despite substantial variation in ploidy levels, aneuploidy seems to be very rare, at least among European species. Flow cytometric measurements (Boşcaiu et al. 1999, Niketić et al. 2013) reveal an approximately three-fold variation in the size of the monoploid genome in different species of *Cerastium*. A combination of chromosome numbers and nuclear DNA values led to the recognition of three different cytogenetic groups within the monophyletic section *Cerastium*, which correspond to species aggregates (Niketić et al. 2013). While the *C. arvense* agg. has the lowest genome sizes, the *C. alpinum* agg. has medium to high genome size.

Our genome size estimates for more than 2000 large-flowered *Cerastium* plants inhabiting serpentine sites in the Slavkovský les Mts revealed clear discontinuities in the amount of nuclear DNA, resulting in three main genome size categories (in addition there were a few individuals with outlying values; see Fig. 2). Disregarding the outliers, the smallest C-value category morphologically matched *C. arvense* while the medium and large categories were referred to as interspecific hybrids and the endemic *C. alsinifolium*, respectively. The estimated values for the *C. arvense* genome in our study (mean 2C = 2.83 pg) differs slightly from those previously published (2C = 2.6 pg; Boşcaiu et al. 1999), which may be due to the use of different reference standards or, perhaps more probably, of different DNA-selective fluorochromes. A more recent estimate for *C. arvense* subsp. *rigidum* (2C = 2.76 pg; Niketić et al. 2013), a taxon that has often been synonymized with the nominate subspecies, fits our data very well.

Although our FCM results were not complemented by chromosome counts, we are convinced that this limitation does not affect our interpretations and undermine the usefulness of genome size data for taxonomic purposes. According to the literature, *Cerastium alsinifolium* is octoploid (2n = 8x = 72; Měsíček & Jarolímová 1992) while tetra- (2n = 4x = 36) and octoploid (2n = 8x = 72) counts are known for the nominate subspecies of *C. arvense* (Goldblatt & Johnson 1979 onwards). Considering the genome sizes that were determined for karyologically-counted samples of *C. arvense* (Boşcaiu et al. 1999, Niketić et al. 2013) we assume that in our study we were dealing with the octoploid cytotype and this chromosome number was also shared by the majority of interspecific hybrids.

Considerable differences in the monoploid genome sizes of *Cerastium alsinifolium* and *C. arvense* (~50%) do not support their close phylogenetic relationships (e.g. placement in the same species aggregate) as suggested in some earlier works (Čelakovský 1873, Dostál 1989). Although molecular markers are necessary to elucidate the phylogenetic position of this Czech endemic, available pieces of evidence based on DNA content favour the hypothesis of Novák (1960) that *C. alsinifolium* is a descendant of some taxon from the *C. alpinum* agg. Some ancestral populations of the latter species might have reached serpentine sites in western Bohemia during climatically-driven migrations in the Quaternary and, in response to specific soil conditions, evolved there into a new taxon. After climatic amelioration and subsequent forest expansion in the Early Holocene, ancestral populations could have survived only at serpentine sites and gradually evolved into a new species.

Our FCM measurements of octoploid *C. alpinum* from Scandinavia (7 accessions), the Alps (9 accessions) and Western Carpathians (7 accessions), with mean genome size of 4.11 pg/2C, accurately matched the values for *C. alsinifolium* (mean 2C = 4.25 pg). Importantly, *C. alpinum* often inhabits serpentine sites in Scandinavia and serpentine tolerance evolved repeatedly during the postglacial colonization of Northern Europe (Berglund et al. 2003).

*Ecological requirements*

One of the most important findings of our study is a fundamental reshaping of traditional views on habitat preferences and spatial distribution of the pure *Cerastium alsinifolium* in the Slavkovský les Mts. Previous studies assumed this endemic favours open serpentine outcrops with shallow soil covered by heliophilous plant communities from where it occasionally colonizes open pine forest, heathlands and dry grasslands (Novák 1960, Smejkal 1990). Only rarely is its occurrence in wet meadows or spring areas in forest margins mentioned. However, according to our data, dry and open sites are dominated by interspecific hybrids with *C. arvense*, whereas the genuine *C. alsinifolium* mostly inhabits moist and semi-shaded sites in spruce forests, including springs in forest clearings, seeps, wet margins of forest roads, etc. The character of these localities suggests that *C. alsinifolium* is a poor competitor. This species attains its highest densities in slightly elevated sites covered by mosses with little surrounding vegetation and can be particularly common on remnants of rotten spruce trunks and along margins of forest roads, occasionally disturbed by vehicles. In forest habitats, samples corresponding to *C. alsinifolium* outnumbered by approx. 5.8-fold their hybrid counterparts, whereas the opposite was true in open rocky outcrops where the ratio of *C. alsinifolium* to hybrids was about 1: 5. Different percentages of interspecific hybrids mirror frequencies of *C. arvense* in particular habitat types. This heliophilous species is relatively common nearby serpentine outcrops (~20% of all samples from this habitat) but only rarely grows in moist sites in forests (1.2% of all samples from forests). According to our field observations, ecological requirements of hybrids seem to be intermediate between those of their parents (moist and partially shaded habitats on serpentine soils for *C. alsinifolium* vs. open and dry habitats on non-serpentine soils for *C. arvense*). However, a deeper insight into abiotic conditions of sites, associated vegetation types and the width of ecological niches of large-flowered *Cerastium* species (and their hybrids) in the Slavkovský les Mts requires further detailed study.

*Frequency of interspecific hybridization*

Reproductive barriers between species of *Cerastium* are often weak and both homoploid and heteroploid crosses are reported, especially in large-flowered perennial species growing in sympatry (Smejkal 1990, Brysting 2000, Hagen et al. 2002). For example, Brysting (2000) records plants with 90 chromosomes that originated from the hybridization of octoploid *C. alpinum* (2n = 72) and dodecaploid *C. nigrescens* (2n = 108). In sympatric populations of both species, up to 8.4% of the individuals were presumably of hybrid origin, and some back-crosses were also suspected (Hagen et al. 2002). In addition, natural hybrids between *C. arvense* and *C. alpinum* are reported on the basis of morphological characters (e.g. Richter & Gürke 1899, Hegi & Weber 1975) and the artificial crossing of both species results in vigorous hybrids (Khalaf & Stace 2000).

Although ecological optima of both *Cerastium alsinifolium* and *C. arvense* differ considerably, both species occasionally come into contact and then hybridize freely. Open serpentine outcrops provide more or less intermediate conditions with respect to the ecological requirements of parental species and are largely inhabited by interspecific crosses (645 out of the 968 samples analysed; = 66.6%). The history of hybridization is difficult to ascertain but we can speculate that *C. alsinifolium* might have been more common in suitable microhabitats on serpentine outcrops in the past. Its contact with *C. arvense* resulted in interspecific crosses that were better adapted to the ecological conditions at open serpentine sites and possibly outcompeted the endemic species (currently, *C. alsinifolium* is the least common plant on serpentine outcrops and, on average, accounts only for 13.3% of the plants studied). Hybridization might have been accelerated by natural or anthropogenic disturbances. An illustrative example is provided by the serpentine outcrop Křížky, which was used as a shooting range in the 1970s and 1980s, and this activity resulted in an increase in abundance of large-flowered *Cerastium* plants (J. Schlossar, pers. comm.). Although these were referred to as *C. alsinifolium*, they were most likely hybrids with *C. arvense.* Similarly, another such hybrid in the genus *Cerastium* seems to be *C. arvense* × *C. tomentosum*, which grows in abundance along the roads in Scandinavia (Nilsson 1977).

Spatial isolation due to ecological segregation seems to be the main prezygotic breeding barrier between *Cerastium alsinifolium* and *C. arvense*. When this barrier is overcome, extensive hybridization occurs. The overall frequency of interspecific crosses (mean 38.7%, range 3.8–68.6%) in the *Cerastium* populations studied is unusually high for any hybridizing group of vascular plants. In other plants, F1 hybrids mostly occur as single individuals within populations of parental species even in genera with frequently hybridizing species (e.g. 0–19.4% with a mean of 8.0% in the genus *Cirsium*; Bureš et al. 2010). Although backcrosses can be expected in populations harbouring substantial percentages of F1 hybrids, our FCM analyses (i.e. the uniformity in the sizes of the genomes of the hybrids and clear discontinuities between values of both parental species) refuted this assumption. The pattern of hybridization in *Cerastium alsinifolium – C. arvense* populations differs from that in other hybrid-prone genera, including *Prunus* (Wójcicki & Marhold 1993), *Senecio* (Oberprieler et al. 2010) and *Viola* (Krahulcová et al. 1996), in which complex hybrid swarms are often formed and the genetic integrity of the hybridizing species possibly threatened. Hybrid individuals of *Cerastium* have a considerably reduced pollen fertility (25.1% of stainable pollen grains on average) and their capsules contain high percentages of undeveloped seeds (our own observations), which possibly decrease the chances of further hybridization. Unfortunately, no data are currently available either on the germinability or origin of the seeds of F1 hybrids. Whether backcrosses occasionally occur at the seed stage but are later outcompeted or whether seeds produced by F1 hybrids are uniform (originating by self-pollination or cross-pollination with other hybrid individual) remains to be established. Another moot question is the direction of hybridization (i.e. the recognition of maternal and paternal parent) although our preliminary results (sequences of *psbJ-petA* inter-genic spacer and *trnG2-trnG* intron) indicate bidirectionality.

In addition to reduced gametes, unreduced gametes also participate in the origin of some hybrids (~3.5%), giving rise to presumably dodecaploid individuals. Interestingly, unreduced gametes of both parental species can enter into hybridization although those of

*C. arvense* were involved much more often (detected in 27 polyploid hybrids) than the 2n gametes of *C. alsinifolium* (detected only in one polyploid hybrid). Interspecific hybrids originating via unreduced gametes are known for example in *Elytrigia* (Mahelka et al. 2005) and *Pilosella* (Krahulcová et al. 2011).

We deliberately do not provide a name for the interspecific hybrid as the nomenclature of the group requires a separate study. The original herbarium material of I. F. Tausch consists of a mixture of *Cerastium alsinifolium* and interspecific hybrids. Considerable nomenclatural chaos resulted recently when Toman (2003) uncritically introduced several new names (e.g. *C. caesarosylvaticum*) for plants occurring in the Slavkovský les Mts.

*Phenotypic variation and species-specific characters*

The number of quantitative characters used to distinguish *Cerastium alsinifolium* and *C. arvense* (Smejkal 1990, Hrouda 2002) predisposes this group to morphometric analysis. Previously, multivariate morphometrics proved successful in the assessment of phenotypic variation and identification of species-specific characters in several taxonomically challenging *Cerastium* complexes, including *C. alpinum–C. arcticum* (Brysting & Elven 2000, Grundt et al. 2000) and *C. pumilum–C. glutinosum* (Letz et al. 2012).

According to published determination keys (Smejkal 1990, Hrouda 2002), *Cerastium alsinifolium* and *C. arvense* should mainly differ in calyx length, leaf shape, bract characteristics, development of axillary shoots and plant colour; no diagnostic characters have ever been provided for hybrids. Our morphometric analysis of 616 individuals largely confirmed the taxonomic value of the above characters. The most distinct taxon is *C. arvense*, whose determination usually poses few problems. In accordance with previous studies, leaves of this species are comparatively long and narrow (mostly at least 3.6-times longer than wide), acute at the apex, the lowermost bracts have distinct scarious margins (at least 1.7 mm long; Fig. 5C) and there are several (mostly 2–10) short sterile shoots in axils of cauline leaves (see Appendix 1 for values of the diagnostic characters discussed). The calyx of *C. arvense* is rather large as are the anthers and petals (the last character was not considered taxonomically-important by previous works). Greyish-green colour of vegetative parts can also help in the identification of *C. arvense* in the field.

In contrast, distinguishing *Cerastium alsinifolium* from interspecific hybrids with *C. arvense* on the basis of morphological characters is a more challenging task. As expected, hybrids have far fewer properly developed pollen grains (on average 25.1% vs. 97.3% in *C. alsinifolium*) although this character is only of limited value in the field. The taxonomically important morphological characters are mostly for vegetative parts and the values for interspecific hybrids are usually intermediate between those of their parents. Specifically, *C. alsinifolium* has only an indistinct scarious margin to lowermost bracts, which is mostly (in 3/4 of the individuals analysed) confined to the upper third of the bract and only rarely (in 7% of plants) reaches its bottom half (Fig. 5A). While the median value of scarious margin length/bract length was only 0.18 in *C. alsinifolium*, it was 0.53 in hybrids (Appendix 1). In plants of *C. alsinifolium* from (semi)shaded forest sites the scarious margin is often lacking. Second lowermost bracts can also be used in the identification of species although the differences are less pronounced and the scarious margin is generally more developed. Among leaves, the third uppermost leaf pair has the highest discriminating power, although there are useful characters on the upper and median cauline leaf pair

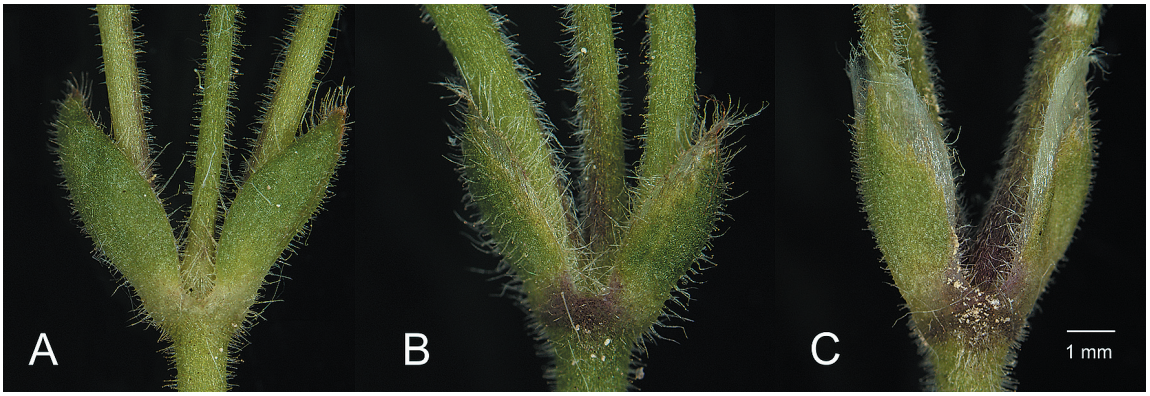


Fig. 5. – Lowermost bracts of *Cerastium alsinifolium* (A; locality Pluhův bor), *C. alsinifolium* × *C. arvense* (B; locality Pluhův bor), and *C. arvense* (C; locality Smečno). Note differences in the shape and size of scarious margins.

(most likely also on bottom pairs but these were not analysed in this study). Leaves of *C. alsinifolium* are usually less than 3.7-times longer than broad, comparatively short (4–10 mm and 5–12 mm for third and second uppermost cauline leaf pairs, respectively) and their apices are more obtuse than those of corresponding leaves of interspecific hybrids. In general, the shape and colour of cauline leaves of *C. alsinifolium* resemble those of small-flowered *C. holosteoides*. In contrast to what is cited in determination keys (Smejkal 1990, Hrouda 2002), the leaves of *C. alsinifolium* are dark (not light) green (Fig. 6). Axillary shoots in *C. alsinifolium* are lacking in about one third (35%) of individuals while there was only one in another third (29%) of the plants analysed; more than three axillary shoots occurred in 3.4% of the individuals. Hybrids were similar to *C. alsinifolium* in terms of the number of axillary shoots (24% of individuals had no axillary shoots, 31% one shoot, 38% two or three shoots and 7% four or five shoots). The floral parts measured were very similar in *C. alsinifolium* and interspecific hybrids. A clue in fruiting plants is provided by the capsules, which in *C. alsinifolium* are more exserted from a comparatively shorter calyx.

The native large-flowered *Cerastium* plants occurring in the Slavkovský les Mts can be identified using the determination key below. Values of quantitative characters are expressed as (minimum–) 5 percentile – 95 percentile (–maximum). Electronic Appendix 4 contains a modified key with added data on pollen fertility. In addition, linear discriminant functions are provided in Electronic Appendix 5.

**1a** Median and upper cauline leaves (2.5–) 3.6–10.2 (–14.7)-times longer than broad, (6.6–) 9.6–22.2 (–29.6) mm long, sterile shoots in leaf axils usually well-developed, (0–) 2–10 (–14) in number, scarious margin of the lowermost bract (1.1–) 1.7–4.2 (–4.9) mm long, calyx (excluding the scarious margin) (3.7–) 4.2–5.9 (–6.9) mm long, petals (7.0–) 7.9–11.6 (–12.6) mm long, anthers (0.6–) 0.9–1.3 (–1.4) mm long, plants of open habitats ..... ***C. arvense* L.**

**1b** Median and upper cauline leaves (1.2–) 1.7–4.7 (–7.1)-times longer than broad, (2.7–) 5.0–13.7 (–19.4) mm long, sterile shoots in leaf axils lacking or only a few [1–3 (–5)] in number, scarious margin of the lowermost bract (0–) 0.1–3.0 (–4.4) mm long, calyx (excluding the scarious margin) (2.6–) 3.4–5.2 (–5.7) mm long, petals (5.4–) 6.2–9.3 (–11.3) mm long, anthers (0.4–) 0.6–1.0 (–1.2) mm long, plants of open or (semi-)shaded habitats ..... **2**

**2a** Median and upper cauline leaves (1.2–) 1.6–3.7 (–5.3)-times longer than broad, often sub-obtuse (angle at apex usually 40–100°), scarious margin of the lowermost bract indistinct, 0–1.8 (–2.7) mm long, mostly

confined to the apical third of the bract (rarely reaches its bottom half), scarious margin of the second lowermost bract (0–) 0.2–2.2 (–2.8) mm long, usually confined to the apical half, calyx (2.6–) 3.0–4.6 (–5.1) mm long, capsule (1.1–) 1.6–3.2 (–3.6)-times longer than calyx, plants of moist and at least partially shaded habitats, usually in spring areas in spruce forests ..... ***C. alsinifolium*** **Tausch** (Fig. 6)

**2b** Median and upper cauline leaves (1.4–) 2.1–5.0 (–6.4)-times longer than broad, acute (angle at apex usually 25–65°), scarious margin of the lowermost bract usually distinct, (0–) 0.7–3.3 (–4.4) mm long, mostly reaching beyond the apical third of the bract (often up to bottom half), scarious margin of the second lowermost bract (0.2–) 0.9–2.9 (–3.6) mm long, usually reaching beyond the apical half, calyx (3.1–) 3.4–5.2 (–5.7) mm long, capsule 1.1–2.2 (–2.8)-times longer than calyx, plants of more open habitats, usually on serpentine outcrops ..... ***C. alsinifolium* × *C. arvense*** (Fig. 6)

*Implications for conservation*

The results of this study have direct and far-reaching practical implications for the protection and conservation of *Cerastium alsinifolium* in the Slavkovský les Mts. Until now, conservation measures have been selectively targeted at open serpentine outcrops, which were believed to be the main reservoir of the endemic's gene pool. Assuming a heliophilous nature of *C. alsinifolium*, the sites were regularly subjected to controlled grazing, removal of shrubs and emerging trees and/or mild disturbance. Although the presence of plants tentatively determined as *C. alsinifolium* in moist forest sites has also been known, these habitats were largely neglected by conservationists (Melichar 2005).

Paradoxically, open serpentine outcrops are mostly inhabited by *Cerastium alsinifolium* × *C. arvense* hybrids, whereas *C. alsinifolium* is relatively uncommon and is confined to suitable (moist and sheltered) microhabitats. The management practices adopted could have facilitated the establishment of hybrid seedlings. However, it is unlikely that any change in conservation measures would cause an increase in the abundance of the endemic species. These open serpentine sites (Dominova skalka, Křížky) should be viewed as natural laboratories where the contact between the two ecologically and morphologically differentiated species results in extensive hybridization. Generally, hybrids are rarer and more spatially restricted than pure *C. alsinifolium* and deserve appropriate protection. As a curiosity, most published pictures of *C. alsinifolium* actually show the hybrid, which is usually more showy than the endemic species.

Our finding that *Cerastium alsinifolium* favours moist spruce forests on serpentine bedrocks will require the development of different conservation strategies with more emphasis on forest sites. Because interspecific hybridization clearly presents a major threat to the genetic integrity of *C. alsinifolium*, spread of *C. arvense* in serpentine forest sites (along forest paths and roads, in clearings, etc.) should be controlled. The fact that only F1 crosses were encountered among mature plants *in situ* slightly diminishes the threat posed by hybridization and indicates that it is likely that the genetic integrity of the endemic species will be maintained. Due to its weak competitive ability, *C. alsinifolium* is likely to benefit from occasional disturbance. An appropriate management would seem to be an occasional passage of vehicles along forest roads. According to current knowledge, nature reserves Vlček and Pluhův bor host the most abundant and cytologically pure populations of the Czech endemic *C. alsinifolium* and these forests on serpentine bedrocks should therefore receive the highest conservation priority.

See http://www.preslia.cz for Electronic Appendix 1–5

Fig. 6. – Pictures of *Cerastium alsinifolium* (locality Vlček; top) and its interspecific hybrid with *C. arvensis* (locality Dominova skalka; bottom).

## Acknowledgements

We thank J. Kalůsková and P. Trávníček for assistance with some of the flow cytometric analyses, R. Sudová for her help in the field and Josef Brůna for preparing the map. The research was supported by the Grant Agency of the Academy of Sciences of the Czech Republic (project no. KJB601110709) and Grant Agency of Charles University (project no. 29507/2007). Additional support was supplied by long-term research development project no. RVO 67985939 (Academy of Sciences of the Czech Republic), institutional resources of the Ministry of Education, Youth and Sports of the Czech Republic for the support of science and research, and project no. 14-36079G, Centre of Excellence PLADIAS (Czech Science Foundation).

## Souhrn

Článek se zabývá mezidruhovou hybridizací endemického rožce kuřičkolistého (*Cerastium alsinifolium*) a široce rozšířeného rožce rolního (*C. arvense*) na hadcových tělesech Slavkovského lesa. Hybridizace byla studována pomocí průtokové cytometrie a mnohorozměrných morfometrických analýz (celkem bylo cytometricky zpracováno 2222 jedinců a morfometricky 616 jedinců). Navzdory stejnému počtu somatických chromozómů se oba druhy výrazně (zhruba 1,5násobně) liší velikostí jaderného genomu, což umožňuje jejich spolehlivé určení. Jejich mezidruhový kříženec vykazuje intermediární hodnoty obsahu jaderné DNA. Vzácně (28 jedinců) byly též nalezeny rostliny s nápadně většími genomy; nejpravděpodobněji se jedná o křížence vzniklé splynutím neredukované a redukované gamety rodičovských druhů. Celkově je mezidruhová hybridizace ve Slavkovském lese velice častá a na křížence připadá 38.7 % všech studovaných jedinců. Ekologické preference *C. alsinifolium* se ukázaly být velmi odlišné od informací udávaných v literatuře. Jeho ekologické optimum leží na prameništích ve smrkových lesích na hadcových podkladech (PR Planý vrch, NPR Pluhův bor, PP Vlček), zatímco na otevřených hadcových výchozech (PP Dominova skalka, NPR Křížky) převažují kříženci a vlastní *C. alsinifolium* se vzácně vyskytuje pouze ve stinných a vlhčích štěrbinách skalek. Morfometrické analýzy odhalily, že studované druhy a jejich kříženec se nejvýrazněji liší ve tvaru a délce lodyžních listů, počtu sterilních větévek vyrůstajících v úžlabí listů, tvaru a velikosti blanitého okraje listenů (zejména nejspodnějšího páru), délce kalicha, koruny a nitky. Zatímco determinace *C. arvense* většinou bývá poměrně snadná, rozlišení druhu *C. alsinifolium* a mezidruhového křížence na základě morfologických charakteristik je mnohem obtížnější; jako nejspolehlivější znak se ukázala fertilita pylu, která je u kříženců výrazně snížena. Zjištěné výsledky mají praktický význam pro druhovou ochranu významného představitele naší endemické flóry, přičemž větší pozornost bude potřeba věnovat populacím velkokvětých rožců rostoucích na lesních stanovištích, které dosud stály spíše stranou ochranářského zájmu.

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Received 6 September 2013
Revision received 19 November 2013
Accepted 20 November 2013

Appendix 1. – Values of selected taxonomically-important morphological characters for *Cerastium arvense*, interspecific hybrids and *C. alsinifolium*. Group abbreviation: H – interspecific hybrid, L – *C. alsinifolium*, R – *C. arvense*. Lengths are given in millimetres.

| | Number of sterile shoots in leaf axils (v2) | | | Length of the uppermost leaf (v3) | | | Length / width of the uppermost leaf (v53) | | | Angle of the tip of the uppermost leaf (v7) | | | Length of the second uppermost leaf (v8) | | | Length / width of the second uppermost leaf (v54) | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Group | R | H | L | R | H | L | R | H | L | R | H | L | R | H | L | R | H | L |
| min | 0 | 0 | 0 | 6.9 | 4.2 | 4.0 | 2.5 | 1.6 | 1.2 | 15 | 15 | 20 | 6.6 | 4.0 | 3.2 | 2.8 | 1.6 | 1.2 |
| 5% | 2 | 0 | 0 | 9.7 | 6.1 | 5.4 | 3.6 | 2.2 | 1.6 | 20 | 20 | 35 | 10.2 | 5.7 | 4.7 | 3.5 | 2.1 | 1.6 |
| 25% | 3 | 1 | 0 | 12.9 | 8.0 | 7.5 | 4.6 | 3.0 | 2.1 | 20 | 30 | 50 | 12.5 | 7.6 | 6.4 | 4.6 | 2.7 | 2.0 |
| 50% | 4 | 1 | 1 | 15.4 | 9.8 | 9.2 | 5.6 | 3.5 | 2.5 | 30 | 40 | 60 | 15.5 | 9.2 | 8.2 | 5.6 | 3.3 | 2.4 |
| 75% | 6 | 2 | 2 | 18.1 | 11.8 | 10.8 | 6.7 | 4.2 | 3.0 | 30 | 50 | 70 | 18.1 | 11.3 | 9.8 | 7.5 | 3.8 | 2.8 |
| 95% | 10 | 4 | 3 | 21.3 | 15.5 | 14.5 | 9.7 | 5.2 | 3.8 | 40 | 60 | 100 | 22.0 | 13.8 | 12.0 | 10.1 | 4.9 | 3.7 |
| max | 14 | 5 | 5 | 27.0 | 18.9 | 19.4 | 12.0 | 6.1 | 5.3 | 50 | 75 | 125 | 29.6 | 16.4 | 16.5 | 11.8 | 6.8 | 5.3 |

| | Angle of the tip of the second uppermost leaf (v12) | | | Length of the third uppermost leaf (v13) | | | Length / width of the third uppermost leaf (v55) | | | Angle of the tip of the third uppermost leaf (v17) | | | Length of the scarious margin of the lowermost bract (v22) | | | Length of the scarious margin / total length of the lowermost bract (v58) | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Group | R | H | L | R | H | L | R | H | L | R | H | L | R | H | L | R | H | L |
| min | 15 | 20 | 20 | 6.9 | 3.3 | 2.7 | 2.7 | 1.4 | 1.2 | 20 | 25 | 20 | 1.1 | 0.0 | 0.0 | 21 | 0 | 0 |
| 5% | 20 | 25 | 40 | 9.0 | 4.8 | 3.9 | 3.5 | 1.9 | 1.5 | 20 | 30 | 40 | 1.7 | 0.7 | 0.0 | 35 | 14 | 0 |
| 25% | 25 | 35 | 53 | 11.8 | 6.5 | 5.5 | 4.7 | 2.6 | 2.0 | 30 | 40 | 60 | 2.4 | 1.3 | 0.3 | 51 | 34 | 7 |
| 50% | 30 | 40 | 65 | 14.5 | 8.4 | 6.6 | 6.0 | 3.2 | 2.3 | 30 | 45 | 65 | 2.9 | 1.9 | 0.6 | 65 | 53 | 18 |
| 75% | 35 | 50 | 70 | 18.0 | 10.3 | 7.8 | 7.6 | 3.9 | 2.7 | 40 | 60 | 75 | 3.4 | 2.5 | 1.1 | 73 | 66 | 31 |
| 95% | 40 | 70 | 95 | 23.2 | 13.1 | 10.0 | 10.6 | 4.8 | 3.5 | 50 | 70 | 100 | 4.2 | 3.3 | 1.8 | 82 | 79 | 54 |
| max | 60 | 90 | 110 | 29.5 | 17.3 | 13.3 | 14.7 | 6.5 | 4.9 | 80 | 110 | 110 | 4.9 | 4.4 | 2.7 | 95 | 100 | 72 |

| | Length of the scarious margin of the second lowermost bract (v30) | | | Length of the scarious margin / total length of the second lowermost bract (v61) | | | Petal length (v38) | | | Anther length (v43) | | | Calyx length at fruiting stage (v50) | | | Capsule length / calyx length at fruiting stage (v69) | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Group | R | H | L | R | H | L | R | H | L | R | H | L | R | H | L | R | H | L |
| min | 0.8 | 0.2 | 0.0 | 31 | 7 | 0 | 7.0 | 5.4 | 5.6 | 0.6 | 0.4 | 0.5 | 3.7 | 3.1 | 2.6 | 1.1 | 1.1 | 1.1 |
| 5% | 1.6 | 0.9 | 0.2 | 47 | 32 | 4 | 7.9 | 6.1 | 6.4 | 0.9 | 0.6 | 0.6 | 4.2 | 3.4 | 3.0 | 1.3 | 1.1 | 1.6 |
| 25% | 2.2 | 1.5 | 0.4 | 63 | 52 | 15 | 9.3 | 7.0 | 7.3 | 1.0 | 0.7 | 0.7 | 4.6 | 3.9 | 3.3 | 1.6 | 1.4 | 2.1 |
| 50% | 2.6 | 1.9 | 0.9 | 72 | 65 | 32 | 10.1 | 7.7 | 7.9 | 1.1 | 0.8 | 0.8 | 5.0 | 4.2 | 3.6 | 1.8 | 1.7 | 2.3 |
| 75% | 2.9 | 2.3 | 1.3 | 80 | 74 | 49 | 11.0 | 8.2 | 8.3 | 1.2 | 0.9 | 0.9 | 5.3 | 4.6 | 4.0 | 2.0 | 2.0 | 2.7 |
| 95% | 3.6 | 2.9 | 2.2 | 89 | 91 | 70 | 11.6 | 9.5 | 9.1 | 1.3 | 1.0 | 1.0 | 5.9 | 5.2 | 4.6 | 2.2 | 2.2 | 3.2 |
| max | 4.8 | 3.6 | 2.8 | 100 | 100 | 100 | 12.6 | 11.3 | 10.3 | 1.4 | 1.1 | 1.2 | 6.9 | 5.7 | 5.1 | 2.6 | 2.8 | 3.6 |

# There is no diploid apomict among Czech *Sorbus* species: a biosystematic revision of *S. eximia* and discovery of *S. barrandienica*

**Mezi českými jeřáby se nevyskytuje diploidní apomikt – biosystematická revize *Sorbus eximia* a objevení *S. barrandienica***

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Vít P., Lepší M. & Lepší P. (2012): There is no diploid apomict among Czech *Sorbus* species: a biosystematic revision of *S. eximia*, and the discovery of *S. barrandienica*. – Preslia 84: 71–96.

*Sorbus eximia* Kovanda, a hybridogenous species that originated from the parental combination *S. torminalis* and *S aria* s.l., is thought to be an apomictic species, which includes diploid and tetraploid individuals. The present study confirmed the existence of only triploid individuals. A new tentatively apomictic triploid (2n = 3x = 51) species from the *S. latifolia* group: *S. barrandienica* P. Vít, M. Lepší et P. Lepší is described based on a revision of *S. eximia*. This species is assumed to have originated from a cross between *S. danubialis* or *S. aria* s.l. and *S. torminalis*. A wide palette of biosystematic techniques, including molecular (nuclear microsatellite markers) and karyological analyses (chromosome counts, DAPI flow cytometry) as well as multivariate morphometric and elliptic Fourier analyses, were used to assess the variation in this species and justify its independent taxonomic status. Allopatric occurrences of both species were recorded east of the town of Beroun in the Český kras, central Bohemia (Bohemian Karst). A distribution map of the two species is provided. *Sorbus eximia* occurs at four localities (the total number of adults and juveniles is 100 and 200, respectively) in basiphilous thermophilous oak forests (*Quercion pubescenti-petraeae*), mesic oak forests (*Melampyro nemorosi-Carpinetum*), woody margins of dry grasslands (*Festucion valesiacae*) and pine plantations. *Sorbus barrandienica* has so far been recorded at 10 localities (ca 50 adults). Recent field studies failed to verify two of these localities. It is mainly found growing on the summits of hills, usually in thermophilous open forests (*Primulo veris-Carpinetum*, *Melampyro nemorosi-Carpinetum*, *Quercion pubescenti-petraeae*) and woody margins of dry grassland. Its populations exhibit minimal genetic variation and are phenotypically homogeneous and well separated from other Bohemian hybridogenous *Sorbus* species. The epitype of *S. eximia* is designated here, and a photograph of the specimen is included. Photographs of the type specimens and in situ individuals, and line drawings of both species are presented.

K e y w o r d s: apomixis, Czech Republic, endemic, geometric morphometrics, hybridization, karyology, multivariate morphometrics, *Rosaceae*, *Sorbus latifolia* agg., SSR markers, taxonomy

## Introduction

In recent years, taxonomic research on agamospermous groups in the Czech Republic has led to the description of many new taxa. This especially applies to the genera *Taraxacum* (e.g. Øllgaard 2003, Vašut & Trávníček 2004, Vašut et al. 2005, Trávníček et al. 2008) and

*Rubus* (e.g. Zieliński & Trávníček 2004, Trávníček et al. 2005, Trávníček & Zázvorka 2005, Žíla & Weber 2005, Lepší & Lepší 2006, 2009, Žíla 2009). A recent increase in taxonomic novelties is also recorded in the genus *Sorbus* s.l. Three new species of the *S. latifolia* group – *S. milensis*, *S. albensis* and *S. portae-bohemicae* – have been described from the České středohoří hills in northern Bohemia. The finding of scattered plants recognized as *S. milensis* during extensive floristic research prompted a subsequent detailed taxonomic revision of *S. bohemica*, which in addition resulted in distinguishing two new species, *S. albensis* and *S. portae-bohemicae* (Lepší et al. 2008, 2009). Over the last few years, taxonomic research has focused on another apomictic species – *S. eximia* Kovanda.

*Sorbus eximia* was described in 1984 as a hybridogenous species endemic to the Český kras karst (Kovanda 1984). Subsequent chemotaxonomical research indicated that it most probably originated from a back-cross between the F1 hybrid *Sorbus torminalis* × *Sorbus aria* s.l. and *Sorbus aria* s.l. (Challice & Kovanda 1986). The species was subsequently studied embryologically and was presented as an apomict comprising two cytotypes: diploid and tetraploid, combined with apospory and diplospory (Jankun & Kovanda 1988). The most surprising result of that study is the discovery of apomixis at the diploid level. Apomixis in *Maloideae* is, with a few exceptions, associated with polyploidy (Campbell & Dickinson 1990). The only reported cases of diploid apomicts are some individuals of *Crataegus calpodendron* (Ehrh.) Medikus, one individual of an apple cultivar, *Sorbus eximia* and possibly *S. subfusca* Boiss. (Campbell et al. 1991). Jankun & Kovanda (1988) found diploid individuals of *S. eximia* at a single locality nearby the settlement of Koda and observed that diploids had leaf laminas more obtusely and more shallowly lobed than tetraploids.

Between 2004 and 2005, a revision of the morphological, karyological and genetic variation in *S. eximia* over its entire distribution area revealed new facts (Vít 2006) that affirmed the karyological results obtained by Jankun & Kovanda (1988). By contrast, morphometrics and molecular techniques confirmed the unique character of the Koda population (Vít 2006), which called for a new taxonomical evaluation of the species. Our additional studies of the type material indicated that the name *S. eximia* relates to the Koda morph and that the remainder of the known populations belong to a new taxon yet to be described. Detailed field work in 2009 and a revision of voucher specimens in major Czech herbaria revealed or confirmed four localities for *S. eximia* and 10 for the new apomictic taxon.

This paper presents a formal description of the newly delimited taxon based on the results of field observations, molecular analyses, karyology, multivariate morphometric and elliptic Fourier analyses. Furthermore, an epitype of *S. eximia* is designated, and its description is revised and emended here. An updated distribution map and a list of revised herbarium specimens of both taxa are also provided.

## Material and methods

### *Plant material and field work*

Mature and well developed individuals were selected for the study of phenotypic and genetic variation. For molecular analyses (nuclear microsatellite markers), 12 individuals of *S. eximia* and 10 of *S. barrandienica* were sampled. For the multivariate morphometric

analyses, 45 individuals of *S. eximia* and 19 of *S. barrandienica* were used (see Table 1 for locality details). In addition, six other hybridogenous taxa of the *S. latifolia* group occurring in Bohemia, which are closely related to the *Sorbus* species currently under study, were included in the multivariate morphometric analyses in order to assess phenotypic variation within the group and determine species-specific characters. These were *S. albensis* (84), *S. bohemica* (111), *S. gemella* (10), *S. milensis* (15), *S. portae-bohemicae* (13) and *S. rhodanthera* (12) (see Lepší et al. 2008, 2009 for locality details). Elliptic Fourier analysis was carried out to reveal species-specific characters of leaves of *S. eximia* (96 leaves analysed) and *S. barrandienica* (93) and also of the holotype of the name of *S. eximia* (see Table 1 for locality details). Specimens were collected during 2004–2009 following the recommendations of Kutzelnigg (1995) and Meyer et al. (2005), for details see Lepší et al. (2009). To describe the phytosociological affinities of *S. eximia* and *S. barrandienica*, relevés were recorded in subjectively selected plots using the Braun-Blanquet approach. The relevés are stored in the Czech National Phytosociological Database (Chytrý & Rafajová 2003) under the numbers 348308, 203571–203584. Altitudes and geographic coordinates (WGS-84) were determined using Garmin eTrex and GPSmap 60CSx instruments.

Table 1. – Details of the localities of *Sorbus* species included in the morphometric, molecular and ploidy level analyses.

| Locality | Geographic coordinates | Altitude (m a.s.l.) | Number of individuals analysed | | | |
|---|---|---|---|---|---|---|
| | | | Nuclear microsatellite markers | Classical morphometry | Elliptic Fourier analysis | Ploidy level |
| Taxon *S. eximia* | | | | | | |
| Koda hill near Srbsko | 49°56'03.6"N, 14°07'09.5"E | 360–370 | 12 | 45 | 15 | 73 |
| Kotýz prehistoric settlement near Tmaň | 49°54'57.5"N, 14°02'56.5"E | 390 | – | – | 10 | – |
| Taxon *S. barrandienica* | | | | | | |
| Paní hora hill near Bubovice | 49°57'43.1"N, 14°09'52.3"E | 410 | 3 | 8 | 5 | 5 |
| Mokrý vrch hill near Bubovice | 49°57'22.2"N, 14°09'44.2"E | 390 | – | – | 1 | – |
| Doutnáč hill near Srbsko | 49°57'23.5"N, 14°09'09.5"E | 430 | – | 1 | 3 | 1 |
| Haknová hill near Karlštejn | 49°56'15.7"N, 14°11'55.7"E | 410 | 5 | 5 | 2 | 5 |
| Plešivec hill near Karlštejn | 49°56'04.2"N, 14°11'24.5"E | 340 | 2 | 5 | – | 2 |

A taxonomic revision of the relevant *Sorbus* material kept in the following herbarium collections was undertaken: BRNM, BRNL, BRNU, CB, CHEB, CHOM, Herbarium of the Museum of Ústí nad Labem, HOMP, HR, LIM, LIT, MP, PL, PR, PRA, PRC, ROZ, SOKO and ZMT. For abbreviations of public herbaria, see Holmgren et al. (1990). Revised herbarium specimens were sorted by locality and then according to the year of collection. Information in Czech on herbarium labels was translated into English. Each locality was numbered and named. Coordinates missing on herbarium sheets were obtained using on-line maps (http://www.mapy.cz). Locality numbers were used for displaying localities on the distribution map. Names of the most frequent collectors are abbreviated: ML = M. Lepší, PL = P. Lepší, PV = P. Vít. Species nomenclature is unified according to Kubát et al. (2002) except for, *S. albensis*, *S. portae-bohemicae*, *S. milensis* and *S. latifolia*, which follow Kutzelnigg (1995) and Lepší et al. (2008, 2009). Phytosociological nomenclature follows Chytrý et al. (2001).

*Digitalization and elliptic Fourier analysis*

Detailed elliptic Fourier analysis was applied to elucidate the variation in leaf shape. Leaves for analysis were predominantly selected from the middle part of short sterile shoots, because most of the *Sorbus* studied were sterile in 2009. Several fertile short and terminal shoots were also included in the analysis to span the leaf variation of the type specimen of *S. eximia,* which only has fertile shoots. Well developed, mature and intact leaves were collected, carefully flattened and dried, and subsequently scanned at 300 dpi using Epson scan 1.11E software. The method of elliptic Fourier approximation (Kuhl & Giardina 1982) incorporated in the SHAPE 1.2 software package (Iwata & Ukai 2002) was employed to describe the variation in leaf shape of both hybridogenous species. The chain-coded contour of each leaf was approximated using the first 20 harmonics, and the elliptic Fourier descriptors (EFDs) normalized to avoid variations related to size, rotation and starting point of the contour trace. Subsequently, principal component (PC) scores for each specimen were calculated from the standardized EFDs, and the shape variation associated with each PC was visualized using the procedure described by Furuta et al. (1995).

A cross-validated linear discriminant analysis using principal component scores (from the above mentioned PCA analysis) as discriminating variables was performed in R, version 2.0.0 (R Core Development Team 2004) using the MASS package (Venables & Ripley 2002). Only the scores of selected PCA axes were used for the discriminant analysis. These axes were selected by a forward selection algorithm in the CVA analysis in Canoco (Lepš & Šmilauer 2003), using the Monte Carlo permutation test (999 permutations; only axes with P level < 0.05 were considered).

*Karyology*

Three samples each of short, two-year old branches with well-developed leaf buds of each species were collected from the type localities of *S. eximia* and *S. barrandienica* in February 2006. Actively growing vegetative tissue was pre-treated in a saturated water solution of p-dichlorbenzen (2–3 hours at RT) and fixed in ice-cold 3:1 ethanol acetic acid overnight. The maceration lasted for 30–60 s in 1:1 ethanol : HCl at 22 °C. Meristematic tissues were squashed in a drop of lacto-propionic orceine. Chromosomes were counted under a light microscope (Carl Zeiss NU, Jena, Germany) at a magnification of 1000 times.

*Estimate of the DNA ploidy level*

DAPI flow cytometry was applied to assess the variation in relative genome size and to infer DNA ploidy levels (Suda et al. 2006) in *S. eximia* and *S. barrandienica*. A group of individuals were analyzed individually, then bulked samples were analysed (i.e. five individuals simultaneously) from 73 different trees of *S. eximia* and 13 trees of *S. barrandienica*. *Bellis perennis* (2C = 3.38 pg; Schönswetter et al. 2007) was selected as a suitable internal reference standard (with genome size close to, but not overlapping that of the *Sorbus* species). Nuclei were isolated using a modified two-step procedure (Doležel et al. 2007), stained with DAPI fluorochrome and analysed following the method of Lepší et al. (2008).

*Morphometric data and analyses*

Seventeen quantitative characters were measured and scored for all of the hybridogenous apomictic *Sorbus* species studied (for a summary of the characters measured, see Lepší et al. 2008). Two new characters were included: "style length" and "length of the fused part of the style". This character set was chosen on the basis of published determination keys, floras and our own observations. The dataset was analysed using the SAS package (version 9.1; SAS Institute, Cary, NC, USA) with CANDIS and DISCRIM procedures, following the methodology described in Klecka (1980). For details see Lepší et al. (2008).

*Nuclear microsatellite markers (SSR)*

Total genomic DNA was extracted from silica-dried leaves (22 samples in total) following the CTAB protocol (Doyle & Doyle 1987) with minor modifications as described by Pfosser et al. (2005). Microsatellite primers developed for the genera *Sorbus* (Mss1, Mss5, Mss6, Ms6g and Ms14; Oddou-Muratorio et al. 2001, Nelson-Jones et al. 2002) and *Malus* (CH02D11 and CH01H10; Gianfranceschi et al. 1998) were used for the determination of intraspecific genetic variation, following the methodology provided by the original authors. For details see Lepší et al. (2008). Final visualization of fluorescently labelled fragments (NED, 6-FAM, HEX; Applied Biosystems, Foster City, CA, USA) was carried out using an automatic sequencer Avant Genetic Analyser 3100 (Applied Biosystems, Foster City, CA, USA). Based on the different ploidy levels of samples analysed (both species studied are triploids, putatively parental taxa are diploid and tetraploid), the microsatellite pattern was scored as "allele phenotypes" (Becher et al. 2000). The data set was converted to a binary matrix and analysed with procedures recommended for dominant markers (i.e. PCoA). Intraspecific variation was measured using the Arlequin ver. 3.01 computer programme (Excoffier et al. 2005), which computes the average gene diversity of all loci (AGD, Nei 1987).

## Results

*Typification of Sorbus eximia*

The type specimen of *Sorbus eximia* consists only of a fertile terminal shoot and a short fertile side shoot, both bear untypical or (partly) damaged leaves (Fig. 1). The shape of the

laminas, the shallow incision between lobes and the results of elliptic Fourier analysis indicate that the type specimen is more likely to belong to the Koda type than the second taxon. The determination, however, is not certain. Information on the label does not help much in this sense because the locality is quite broad: slopes of a hill by the village of Srbsko, which may include both the locality of the Koda population and the distribution area of the second taxon.

Because the determination of the type specimen is ambiguous we consider it advisable to select an interpretative epitype. – Holotype: Herbar. Beck., Böhmen Berghänge bei Srbsko, Kalk, leg. [Beck] 17. 8. 1918, PRC (Fig. 1). – Epitype: Bohemia centralis, distr. Beroun, pagus Srbsko (6050d): ca 300 m situ sept.-orientali a pago Koda, in rupibus in declivibus meridionalibus cotae 393 m, solo calcareo; 360 m s.m., 49°56'03.8"N, 14°07'13.6"E; disperse; leg. M. Lepší 2. 8. 2007 (**epitype designated here:** CB, No. 65278, Fig. 2).

When Kovanda described *S. eximia* he had only three specimens of the Koda taxon and had not seen it in the field. On the other hand, he observed the second taxon (*S. barrandienica* described herein) at three localities in the field and cited nine specimens clearly belonging to it. In addition, the pen drawing of a flowering shoot in the original paper (Kovanda 1984) belongs to the new species of *Sorbus*. Consequently, we can assume that the original diagnosis is based on both taxa but mainly on the formally undescribed taxon, at least for the flowers, which are not present on the specimens of the Koda type. It is apparent that a new description of *S. eximia* is needed.

*Sorbus eximia* Kovanda, Preslia 56: 170, 1984 emend. P. Vít, M. Lepší et P. Lepší
(Figs 1–4)

D e s c r i p t i o n: Trees (or shrubs) up to 16 m high. Trunk up to 1.1 m in circumference. Bark grey, smooth when young, with vertical fissures (particularly at the trunk base) at maturity, with scattered (4–) 8–11 (–16) mm long and (4–) 6–9 (–16) mm wide lenticels. Twigs brownish-grey; young shoots brown, sparsely tomentose when young and almost glabrous at maturity, with numerous elliptical or subrotund pale brown to ochraceous lenticels. Buds 6–14 mm long and 3–6 mm wide, narrowly ovoid to turbinate; scales green, with narrow brown sparsely tomentose margins. Leaves (on short fertile shoots) simple; laminas more or less broadly ovate to broadly elliptical, cochleariform to more or less flat, somewhat glossy, pale to dark green above, yellowish-greyish-green beneath, usually not undulated at margins, more or less broadly rounded acute to obtuse at apex, usually rounded or broadly cuneate and partly serrate at base, almost glabrous on upper surface, evenly tomentose on lower surface, (7.5–) 8.6–9.3 (–11.3) cm long and (5.0–) 6.7–7.5 (–9.4) cm wide, widest at (39–) 51–58 (–64)% of the lamina length (from the tip), double serrate to regularly shallowly lobed (serrate to double serrate apically); lobes serrate or doubly serrate with sharply acuminate teeth terminating the main veins, other teeth smaller, acuminate; sides of lobes more or less arcuate; the third lobe (from the base) (1.0–) 1.1–1.3 (–1.7) cm broad; incision between the second and the third lobe (0.25–) 0.40 (–0.55) cm; lobes broader than 1 cm (2–) 3–4 on each side; main veins terminating in lobes or teeth (6–) 7–8 (–9) on each side; petioles (1.5–) 1.9–2.1 (–2.6) cm long, more or less tomentose. Inflorescences with (16–) 25–42 (–70) flowers, (5.5–) 6.0–9.5 (–10.5) cm in diameter, convex; branchlets more or less tomentose. Hypanthium turbinate, tomentose. Sepals (1.7–) 2.1–2.5 (–3.0) mm long and (2.2–) 2.7–2.9 (–3.3) mm wide, triangular,

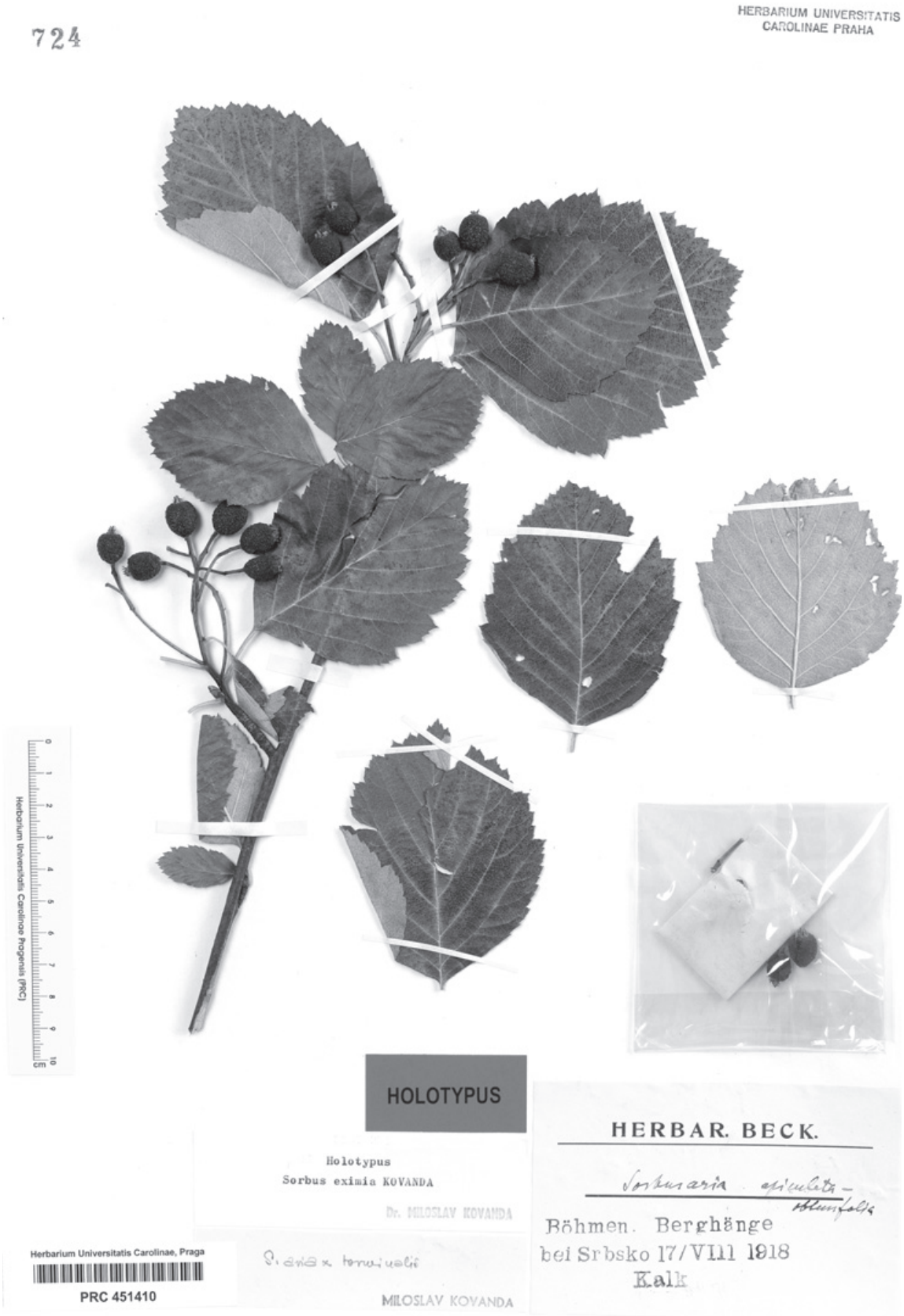


Fig. 1. – Holotype of *Sorbus eximia* Kovanda.


Fig. 2. – Epitype of *Sorbus eximia* Kovanda.

acuminate or acute, densely tomentose on both surfaces, patent, reclinate after anthesis, persistent, dry, erect. Petals (5.1–) 5.7–6.2 (–6.8) mm long and (3.8–) 4.6–4.9 (–5.2) mm wide, broadly ovate to broadly elliptical, concave, whitish, patent, sparsely hirsute at base of upper surface, with a short claw. Stamens ca 20; filaments whitish; anthers pale yellow, (1.0–) 1.2–1.4 (–1.6) mm long. Ovary semi-inferior. Styles (1–) 2, greenish-cream, (3.0–) 3.4–3.8 (–4.6) mm long, hairy at the base, connate up to (27–) 44–49 (–59)%. Stigma greenish-cream, more or less flat, 0.6–0.7 (–0.8) mm wide. Fruits (11–) 12–13 (–15) mm long and (11–) 12–13 (–15) mm wide, subglobose, often as wide as long or wider than long, orange to orange-red at maturity, glabrous or almost glabrous, glossy, with (8–) 16–32 (–64) ochraceous lenticels per 0.25 $cm^2$, mesocarp heterogeneous; endocarp cartilaginous. Seeds fuscous. Somatic chromosome number 2n = 51 (triploid). Reproduction tentatively apomictic. Flowering V.

There are pen drawings of a flowering shoot and a leaf in the Flora of the Czech Republic (Kovanda 1992). The same drawing of the leaf is used in the Key to the flora of the Czech Republic (Kubát et al. 2002).

*Diagnostic characters*

Leaf laminas are broadly ovate to broadly elliptical, (7.5–) 8.6–9.3 (–11.3) cm long and (5.0–) 6.7–7.5 (–9.4) cm wide, often cochleariform, more or less rounded acute to obtuse at apex, usually rounded or broadly cuneate at base, double serrate to regularly shallowly lobed; incision between the second and the third lobe terminating the main veins (0.25–) 0.40 (–0.55) cm long, teeth or lobe terminating the main veins sharply acuminate. Anthers are pale yellow. Styles connate up to (27–) 44–49 (–59)% of their length. Fruits are subglobose, often as wide as or wider than long, orange to orange-red at maturity (Fig. 4).

*Ecology*

*Sorbus eximia* occurs in open (woody margins of dry grasslands) and (semi)shaded habitats (forests) on base-rich soils on limestone. In forests, it usually grows in the understorey. Exceptionally it may reach the high tree layer or form monospecific stands (such as by the settlement of Koda). It is recorded on slopes of all aspects. Most individuals grow on southeast, south and southwest slopes. It inhabits mainly basiphilous thermophilous oak forests (*Quercion pubescenti-petraeae*) and mesic oak forests (*Melampyro nemorosi-Carpinetum*). It is rarely also found in narrow-leaved dry grassland (*Erysimo crepidifolii-Festucetum valesiacae*). A majority of individuals occur in semi-natural forests or grasslands with a high abundance of relic species, but it is also found in man-made habitats such as plantations of *Pinus nigra*, long-abandoned quarries (in *Sesleria* grassland – *Diantho lumnitzeri-Seslerion*) or at sites of prehistoric settlements (in species-poor dry grasslands – *Festucion valesiacae*). The species grows sympatrically with *S. aria* s.l., *S. danubialis* and *S. torminalis*. *S. aria* s.l. and *S. danubialis* are a little more heliophilous and xerothermophilous, while *S. torminalis* is a more mesophilous species.

*Geographical distribution*

*Sorbus eximia* is recorded at four localities in the Bohemian Karst between Prague and Beroun (Central Bohemia). The largest stand of this species, which includes tens of adults

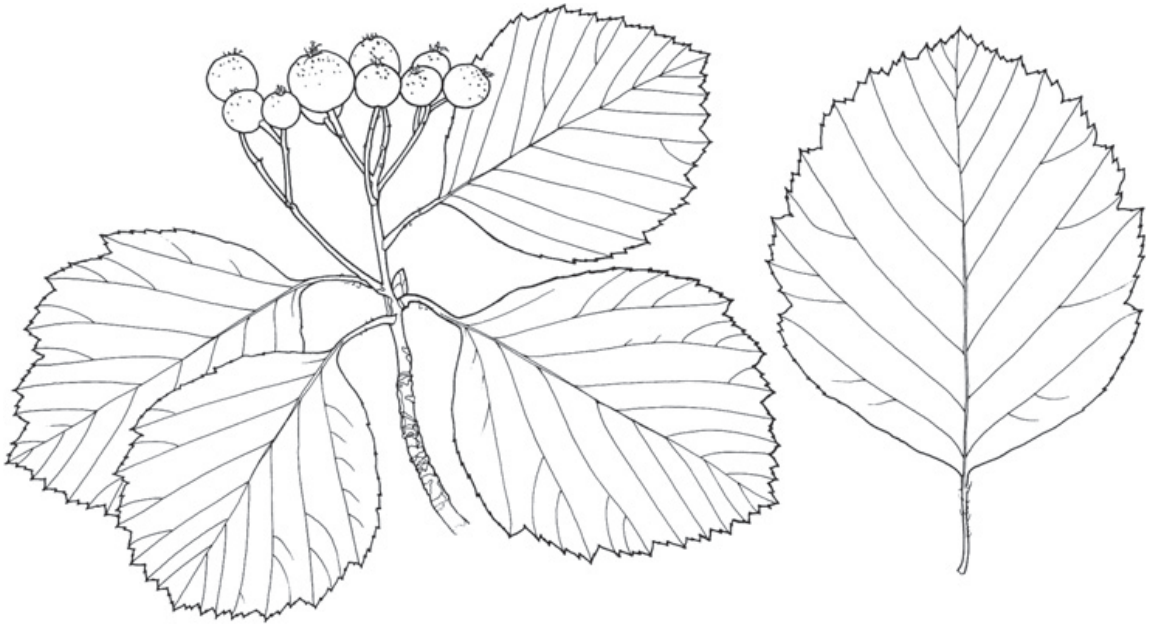

Fig. 3. – *Sorbus eximia*: short fructiferous shoot (left) and leaf from the middle part of short sterile shoot (right). Drawing by A. Skoumalová.

Fig. 4. – Fructiferous short fertile shoot of *Sorbus eximia* at the type locality (photograph taken by P. Lepší, 2009).

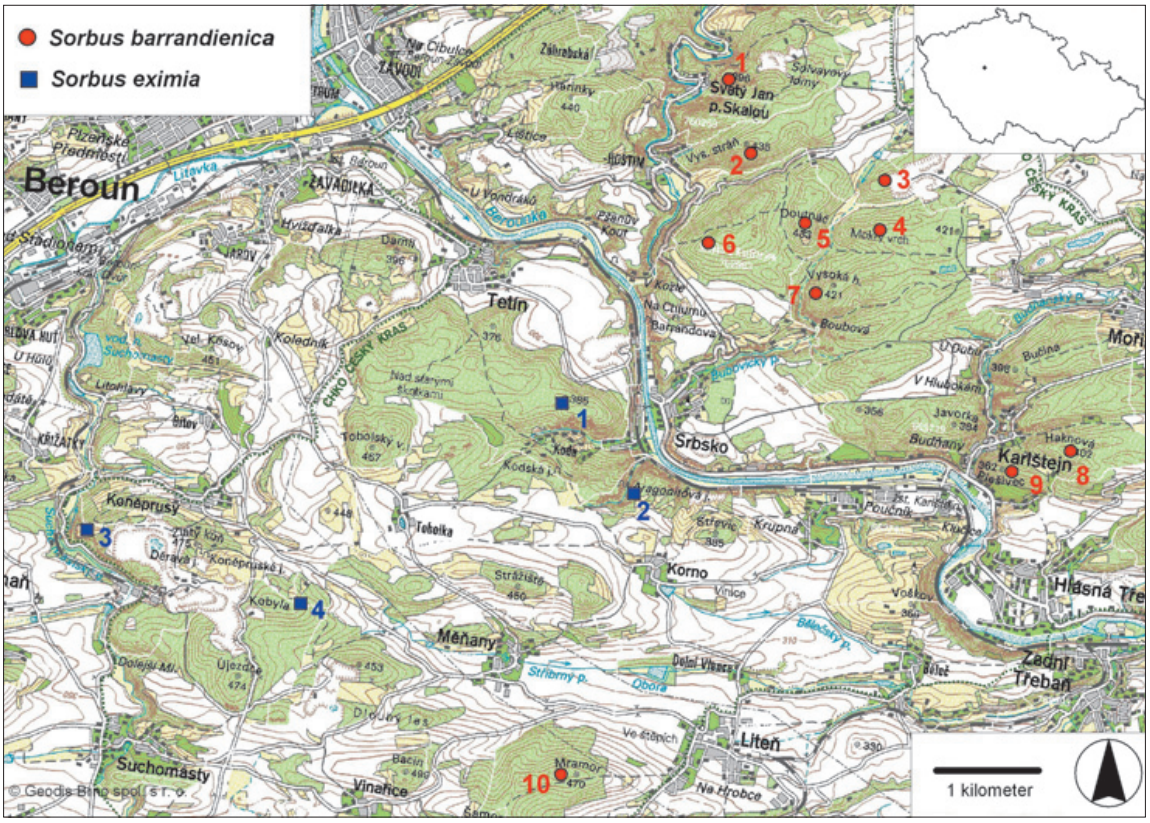


Fig. 5. – Distribution map of *Sorbus eximia* and *S. barrandienica*. The numbers on the map correspond to the locality numbers in the list of revised herbarium specimens and recorded localities (the use of the map was approved by the Ministry of Environment of the Czech Republic).

and ca 200 young trees of different ages, is located by the settlement of Koda near the village of Srbsko within the boundaries of the Koda national nature reserve and covers ca 5 ha. This locality was documented by G. Beck for the first time as far back as in 1918 (holotype in PRC). By contrast, the occurrence of this species nearby Tmaň and Koněprusy was discovered recently in 2009. These two sites harbour a distinctly smaller population, 10 individuals by the village of Tmaň and 20 by the village of Koněprusy. Both of these localities extend over a few tens of square meters. The forth locality in the Císařská rokle gorge is an ex situ conservation plot and houses 11 juveniles. The distance between the localities that are furthest apart is ca 6 km (excluding the locality Císařská rokle) (Fig. 5). *Sorbus eximia* grows in two quadrants (6050d, c) of the Central-European mapping grid (Ehrendorfer & Hamann 1965). The localities are situated in the colline vegetation belt in the phytogeographical district of Český kras (Bohemian Karst) (Skalický 1988). This species grows in a warm and moderately warm climatic region (Quitt 1971) with a mean annual temperature of about 7–9 °C and mean annual precipitation of 500–600 mm (Tolasz et al. 2007). Its altitudinal range spans from 350 m (Koda hill) to 460 m a.s.l. (Kobyla hill). The species has also been planted along the road between the villages of Řevnice and Mořina ca 8 km to the east of its nearest native occurrence (Koda hill).

Herbarium specimens and records:

**Czech Republic. Central Bohemia, Bohemian Karst: 1. Koda hill:** Herbar. Beck., Böhmen Berghänge bei Srbsko, Kalk (leg. [Beck] 17. 8. 1918, PRC, HOLOTYPE). – Böhmen auf Kalkfelsen bei Koda, nächst Srbsko, häufig (leg. B [=Beck] 1920, PRC). – Koda (leg. J. Klika 4. 10. 1942, PR). – Koda, the plateau (leg. J. Klika 11. 8. 1944, PR 174901). – The S face of the plateau of Koda hill (the reserve) (leg. J. Klika 11. 10. 1944, PR 174899). – On the plateau at the top of Koda hill above the gorge by Koda settlement, growing in association with *Quercus pubescens*, *S. cretica* [= *Sorbus danubialis*], *S. torminalis* and *Carpinus* (leg. J. Klika 5. 7. 1945, two identical specimens in PR). – The plateau of Koda hill by Beroun town (leg. [J. Klika] 2. 4. 1946, PR). – Koda settlement (leg. M. Kovanda 27. 9. 1985, PRA). – Srbsko, rocks and forest-steppe NE of Koda settlement, S slopes at this spot at a height of 393 m, scattered, 6050d: 49°56'03.2"N, 14°07'17.5"E 370 m a.s.l. (leg. ML 2. 8. 2007, PRC 65280/a, CB 65280/b), 49°56'03.8"N, 14°07'13.6"E, 360 m a.s.l. (leg. ML 2. 8. 2007, CB 65278, EPITYPE), 49°56'02.2"N, 14°07'13.6"E, 360 m a.s.l. (leg. ML 2. 8. 2007, CB 65277; leg. PL 19. 9. 2009, CB 71605), 49°56'03.6"N, 14°07'09.5"E, 370 m a.s.l. (leg. ML 19. 8. 2009, CB 71584), 49°56'03.9"N, 14°07'09.5"E, 360 m a.s.l. (leg. PL & ML 10. 5. 2009, CB 71590, CB 71592), 49°56'03.9"N, 14°07'09.6"E, 360 m a.s.l. (leg. PL & ML 10. 5. 2009, CB 71591), 49°56'04.2"N, 14°07'09.9"E, 370 m a.s.l. (leg. PL & ML 10. 5. 2009, CB 71593), 49°56'02.0"N, 14°07'13.3"E, 360 m a.s.l. (leg. PL & ML 10. 5. 2009, CB 71594). – Koda, ca 400 m WNW of the spot at a height of 390 m NE of the settlement, in a oak-hornbeam forest, 6050d, 49°56'09.5"N, 14°07'02.5"E, 375 m a.s.l., scattered (leg. PV, PL, ML & J. Mottl 19. 6. 2009, CB 71595). – Koda, ca 260 m ENE of the spot at a height of 393 m, NE of the settlement, an oak-hornbeam forest, 6050d, 49°56'17.1"N, 14°07'03.4"E, 350 m a.s.l. (leg. PL 21. 6. 2009, CB 71605). This large locality also includes Kovanda's record: Koda forest, N slope (N. of point 390 m), 360–380 m a.s.l. (Jankun & Kovanda 1988). **2. The Císařská rokle gorge:** Srbsko, ca 1 km S of the bridge across the Berounka river, on the right-hand side of the Císařská rokle gorge, 6050d, 49°55'39.5"N, 14°07'56.9"E, 360 m a.s.l., planted in lines, 11 ca 0.5 m high juveniles (leg. PV, PL, ML & J. Mottl 19. 6. 2009, CB 71596). The first record at this locality provided by Schlägelová (2006). **3. Kotýz prehistoric settlement:** Tmaň, the area of Kotýz prehistoric settlement, open thermophilous scrubland, 6050c, 49°54'57.5"N, 14°02'56.5"E, 390 m a.s.l., ca 10 individuals (leg. PL, ML & J. Mottl 19. 6. 2009, CB 71597). A new locality. **4. Kobyla hill:** Koněprusy, SE slopes of Kobyla hill, in the undergrowth of a *Pinus nigra* plantation, 6050d, 49°54'38.5"N, 14°05'05.8"E, 450 m a.s.l., ca 20 individuals of different age (not. J. Mottl 4. 8. 2009, leg. ML 20. 8. 2009, CB 71582). – Koněprusy, the wall of an abandoned quarry on N slope of Kobyla hill, 6050c, 49°54'43.6"N, 14°04'54.4"E, 460 m a.s.l., 1 young tree (leg. ML 20. 8. 2009, CB 71583). A new locality.

## *Sorbus barrandienica* P. Vít, M. Lepší et P. Lepší, **spec. nova** (Figs 6–8)

Descriptio: Arbores usque 12 m alti; foliis (in brachyblastis fertilibus) simplicibus, laminis ambitu fere ellipticis, regulariter pinnato-lobatis (lobis acuminatis, serratis), in parte superiore tantum duplicato-serratis, (8.1–) 8.8–10.3 (–11.6) cm longis et (5.4–) 6.7–7.4 (–8.9) cm latis, ad basin cuneatis usque raro late cuneatis, subintegris vel remote serratis, obscure viridibus, subtus ochro-griseo-viride tomentosis, nervis ab utroque latere (7–) 8 (–9) in numero; petiolis (1.9–) 2.1–2.4 (–2.7) cm longis; corymbothyrsis multifloris, convexis, ramis plus minusve tomentosis. Dentibus calycinis triangularibus, acuminatis usque acutis, (2.3–) 2.5–3.5 (–3.8) mm longis et (2.7–) 2.8–3.0 (–3.2) mm latis, patentibus usque reclinatis, post anthesin reclinatis, dense tomentosis, tempore fructificationis siccis, persistentibus; petalis late ovatis usque late ellipticis, breviter unguiculatis, (6.1–) 6.2–6.9 (–7.5) mm longis et (4.4–) 4.5–4.9 (–5.2) mm latis, albidis, superne ad basin sparse villosis, patentibus; staminibus ca 20, antheris pallide luteis, (1.0–) 1.2–1.3 (–1.5) mm longis; ovario semi-infero; stylis 2 (–3) ad (16–) 29–42 (–57)% coalescentibus, ad basin villosis, albo-viridis, (3.5–) 3.7–3.9 (–4.0) mm longis, stigmatibus plus minusve planis; fructibus subglobosis, (11.5–) 12.0–13.0 (–14.0) mm longis et (11.0–) 12.0–12.5 (–14.0) mm latis, maturitate aurantiacis usque rubris, glabris vel fere glabris, nitidis, cum (4–) 16–32 (–36) lenticellis parvis, ochraceis ad 0.25 $cm^2$; mesocarpio heterogeneo; endocarpio cartillagineo, seminibus atro-fuscis. Numerus chromosomatum triploideus 2n = 51. Probabiliter planta apomicta. Floret V.

Holotypus: Bohemia centralis, distr. Beroun, pagus Srbsko (6050b): in summo collis Doutnáč, in querceto, solo calcareo; 430 m s.m., 49°57'23.5"N, 14°09'09.5"E; disperse; leg. M. Lepší 2. 8. 2007; CB, No. 65274 (Fig. 6). – Isotypus: PRC, No. 65274/a.

Description: Trees up to 12 m high. Trunk up to 0.55 m in circumference. Bark grey, smooth when young, with vertical fissures (particularly at the trunk base) at maturity, with scattered (3–) 7–11 (–14) mm long and (3–) 7–11 (–14) mm wide lenticels. Twigs


Fig. 6. – Holotype of *Sorbus barrandienica* P. Vít, M. Lepší et P. Lepší.

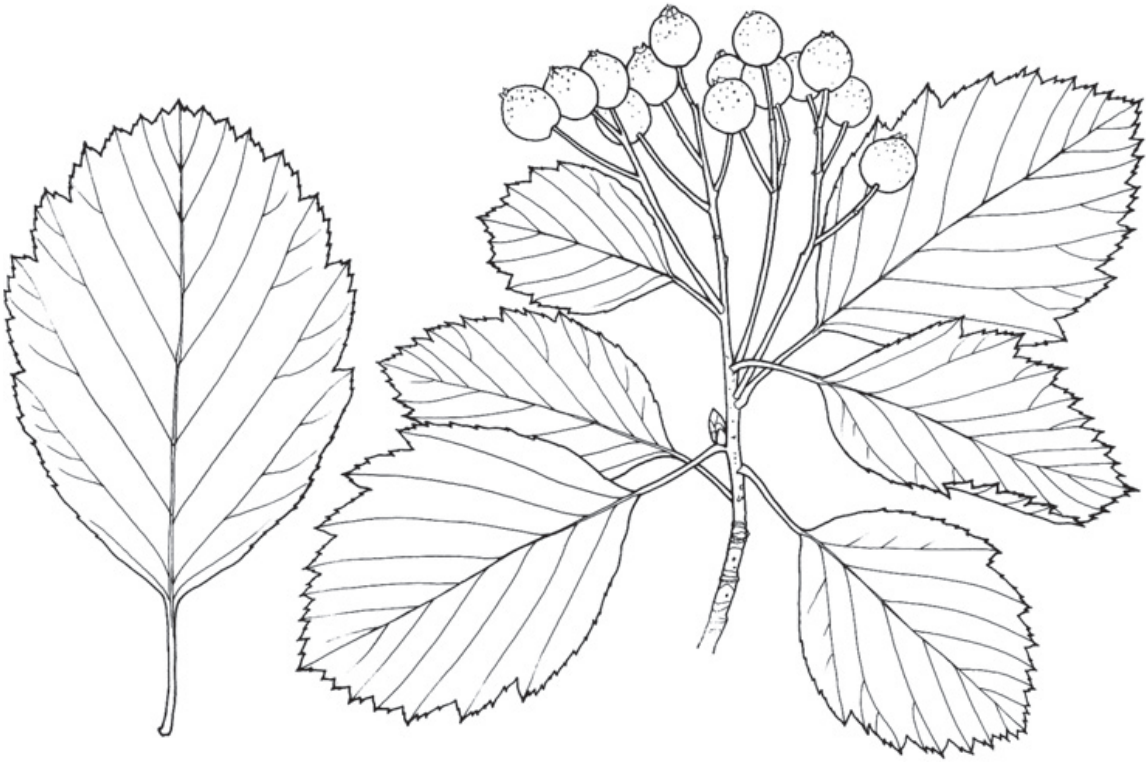

Fig. 7. – *Sorbus barrandienica*: leaf from the middle part of short sterile shoot (left), short fructiferous shoot (right). Drawing by A. Skoumalová.

Fig. 8. – Sterile shoots of *Sorbus barrandienica* growing at Doutnáč hill (photograph taken by P. Lepší 2009).

brownish-grey; young shoots brown, sparsely tomentose when young and almost glabrous at maturity, with elliptical or subrotund pale brown to ochraceous lenticels. Buds 7–14 mm long and 3–6 mm wide, narrowly ovoid to turbinate; scales green, with narrow brown sparsely tomentose margins. Leaves (of short fertile shoots) simple; laminas more or less elliptical, more or less flat, somewhat glossy, dark green above, yellowish-greyish-green beneath, usually flat at margins, more or less rounded acute at apex, usually cuneate rarely broadly cuneate and partly serrate at base, almost glabrous on upper surface, evenly tomentose on lower surface, (8.1–) 8.8–10.3 (–11.6) cm long and (5.4–) 6.7–7.4 (–8.9) cm wide, widest at (16–) 29–42 (–64)% of the lamina length (from the tip), regularly lobed (double serrate apically); lobes serrate or doubly serrate with sharply acuminate teeth terminating the main veins, other teeth smaller, acuminate; sides of lobes more or less arcuate; the third lobe (from the base) (0.95–) 1.15–1.35 (–1.80) cm broad; incision between the second and the third lobe (0.40–) 0.45–0.6 (–0.75) cm; lobes broader than 1 cm (2–) 3–4 on each side; main veins terminating in lobes or teeth (7–) 8 (–9) on each side; petioles (1.9–) 2.1–2.4 (–2.7) cm long, more or less tomentose. Inflorescences with (44–) 50–60 (–66) flowers, (7–) 8–9 (–10) cm in diameter, convex; branchlets more or less tomentose. Hypanthium turbinate, tomentose. Sepals (2.3–) 2.5–3.5 (–3.8) mm long and (2.7–) 2.8–3.0 (–3.2) mm wide, triangular, acuminate or acute, densely tomentose on both surfaces, patent, reclinate after anthesis, persistent, dry, erect. Petals (6.1–) 6.2–6.9 (–7.5) mm long and (4.4–) 4.5–4.9 (–5.2) mm wide, broadly ovate to broadly elliptical, concave, whitish, patent, sparsely hirsute at base of upper surface, with a short claw. Stamens ca 20; filaments whitish; anthers pale yellow, (1.0–) 1.2–1.3 (–1.5) mm long. Ovary semi-inferior. Styles 2 (–3), greenish-cream, (3.5–) 3.7–3.9 (–4.0) mm long, hairy at the base, connate up to (16–) 29–42 (–57)%. Stigma greenish-cream, more or less flat, (0.6–) 0.7 (–0.8) mm wide. Fruits (11.5–) 12.0–13.0 (–14.0) mm long and (11.0–) 12.0–12.5 (–14.0) mm wide, subglobose, orange to orange-red at maturity, glabrous or almost glabrous, glossy, with (4–) 16–32 (–36) ochraceous lenticels per 0.25 $cm^2$, mesocarp heterogeneous; endocarp cartilaginous. Seeds fuscous. Somatic chromosome number 2n = 51 (triploid). Reproduction tentatively apomictic. Flowering V.

There is also a pen drawing of a flowering shoot in Kovanda (1984).

*Diagnostic characters*

Leaf laminas are more or less elliptical, (8.1–) 8.8–10.3 (–11.6) cm long and (5.4–) 6.7–7.4 (–8.9) cm wide, more or less rounded acute at apex, usually cuneate, rarely broadly cuneate at base, shallowly lobed; incision between the second and the third lobe terminating the main veins (0.4–) 0.45–0.6 (–0.75) cm long, teeth or lobe terminating the main veins sharply acuminate. Anthers are pale yellow. Styles connate up to (16–) 29–42 (–57)% of their length. Fruits are subglobose, orange to orange-red at maturity.

*Etymology*

The name "*barrandienica*" derives from the Barrandien, a geologically and paleontologically conspicuous region located between Prague and Pilsen. This species is recorded growing on the Bohemian Karst, which is known as the most significant part of the Barrandien region. The authors propose the epithet "barrandienský" for the Czech name.

*Ecology*

*Sorbus barrandienica* occurs mainly on base-rich soils that develop on limestone. It occurs most frequently on the summits of hills, usually in thermophilous open forests or woody margins of dry grassland, and exceptionally in dry grassland, thermophilous scrub or rocks. In most cases, it grows in thermophilous and mesophilous oak-hornbeam forests (*Primulo veris-Carpinetum* and *Melampyro nemorosi-Carpinetum*) and in transition vegetation between these communities. It is also recorded in basiphilous thermophilous oak forests (*Quercion pubescenti-petraeae*) and their mesophilous derivatives. On one occasion, it was recorded in an acidophilous thermophilous oak forest (*Sorbo torminalis-Quercetum*). In the undergrowth of forests, it is very often sterile and does not produce fruits. When it is overshadowed by taller trees, it usually dies. It is recorded on slopes of all aspects save for eastern-facing slopes. Most localities are situated on south and southwest slopes. It does not occur in man-made biotopes. The species grows sympatrically with *S. danubialis*, *S. aria* s.l. and *S. torminalis* at most localities. *Sorbus danubialis* and *S. aria* s.l. are a little more heliophilous and xerothermophilous, while *S. torminalis* is a more mesophilous species.

*Geographical distribution*

*Sorbus barrandienica* was recorded at 10 localities in the Bohemian Karst located between Prague and Beroun (Central Bohemia). The centre of its current distribution is located close to Doutnáč hill between the villages of Srbsko and Bubovice (localities 3, 4, 5, 7). This species is not abundant and is represented by small populations in this area. At other localities there are only a few scattered individuals (localities 2, 6, 8 and 9) or the species has not been seen recently (locality 1, 10). The distance between localities that are furthest apart is ca 8 km. The easternmost locality is close to Karlštejn, both the northernmost and westernmost close to the village of Svatý Jan pod Skalou and the southernmost at a very isolated locality located on Mramor hill at the village of Liteň (Fig. 5). *Sorbus barrandienica* is recorded in five quadrants (6050b, d; 6051a, c; 6151a) of the Central European mapping grid (Ehrendorfer & Hamann 1965). The first record of this species is that of V. Krajina in 1926 near Svatý Jan pod Skalou (PRC). These localities are situated in the colline vegetation belt in the phytogeographical district of Český kras (Bohemian Karst) (Skalický 1988). This species grows in a warm and moderately warm climatic region (Quitt 1971) with a mean annual temperature of about 7–9 °C and mean annual precipitation of 500–600 mm (Tolasz et al. 2007). Its altitudinal range spans from 340 m a.s.l. (Plešivec hill) to 450 m a.s.l. (Mramor hill).

Herbarium specimens and records:

**Czech Republic. Central Bohemia, Bohemian Karst: 1. Svatý Jan pod Skalou** (49°58'10.2"N, 14°08'04.3"E): Karlštejn, Svatý Ivan (leg. Vladimír Krajina 1. 5. 1926, PRC). – Svatý Jan pod Skalou – rocks above the monastery (leg. Štěpánková 4. 6. 1962, CB). The last record for this locality was in 1985: the summit area of U Kříže hill, near Svatý Jan pod Skalou village, 396 m a.s.l. (no specimen available) (Jankun & Kovanda 1988). A locality with unconfirmed occurrence. **2. Vysoká stráň hill:** Svatý Jan pod Skalou, Vysoká stráň hill, ca 950 m NW of the summit of Doutnáč hill, in a thermophilous oak forest, 6050b, 49°57'47.1"N, 14°08'38.0"E, 390 m a.s.l., 2 fertile and 1 sterile individual (leg. ML & Karel Boublík 28. 7. 2007, CB 65276). The first record for this locality was in 1986: the summit area of Vysoká stráň hill, near Hostim village, 435 m a.s.l. (no specimen available) (Jankun & Kovanda 1988). **3. Paní hora hill:** In colle Paní hora prope pagum Bubovice haud procul ab

oppido Beroun, solo calcareo, alt. 410 m (leg. M. Kovanda 23. 6. 1964, PRA). – Bubovice, the summit area of Paní hora hill, the scrubby margins of dry grassland, scattered, 6050b: 49°57'43.1"N, 14°09'52.3"E, 410 m a.s.l. (leg. ML & PL 19. 6. 2009, CB 71600), 49°57'43.9"N, 14°09'51.3"E, 410 m a.s.l. (leg. PL 19. 9. 2009, CB 71606). **4. Mokrý vrch hill:** Karlštejn, forester's lodge N 5, Mokryj verch [= Mokrý vrch hill] (leg. B. Augstová 6. 6. 1957, PR 5900946; 24. 5. 1958, PR 590377). – Bubovice, SW slopes of Mokrý vrch hill, the woody margins of grassland, 6050b, 49°57'22.2"N, 14°09'44.2"E, 390 m a.s.l., ca 10 individuals (leg. ML 5. 5. 2009, CB 71585). – Bubovice, W slopes of Mokrý vrch hill, the undergrowth of an oak-hornbeam forest, 6050b, 49°57'24.3"N, 14°09'32.5"E, 380 m a.s.l., 1 sterile individual (leg. ML 5. 5. 2009, CB 71586). – Bubovice, ca 200 m SE of the summit of Mokrý hill, the undergrowth of an oak-hornbeam forest, 6051a, 49°57'22.6"N, 14°10'06.9"E, 420 m a.s.l., 2 old dying individuals (leg. ML 10. 5. 2009, CB 71587). – Bubovice, Mokrý vrch hill, ca 0.6 km WNW of the centre of the Malá Amerika quarry, along a forest road, 6051a, 49°57'17"N, 14°10'02"E, 400 m a.s.l. (leg. PL 20. 6. 2009, CB 71602). This locality probably includes also Kovanda's record: the forest margin in the shallow valley ca 0.8 km NW of the Amerika quarry, 300 m a.s.l. (Kovanda 1999). **5. Doutnáč hill:** Doutnáč hill by Srbsko village (sine coll. IX. 1935, PR 174743). – Distr. Beroun, in nemore ad declivia occid.-merid. montis Doutnáč supra vic. Srbsko, 370 m s.m., No 10868 (leg. Domin & Dostál 28. 6. 1939, PRC). – Beroun: in nemore ad declivia occ.-merid. montis Doutnáč supra vic. Srbsko, 370 m s.m. (leg. Domin & Dostál 28. 6. 1939, PRC). – Central Bohemia, Bohemian Karst, Bubovice, NE slopes of Doutnáč hill, above the valley at an altitude of 350 m; the margin of an open forest and stony steppe, S slope orientation (leg. R. Businský 5. 6. 1977, ROZ 31764-31772). – Doutnáč hill (leg. M. Kovanda 3. 6. 1980, October 1980, 26. 5. 1982, PRA). – Srbsko, the summit area of Doutnáč hill, an oak forest, 6050b, 49°57'23.5"N, 14°09'09.5"E, 430 m a.s.l., ca 10 individuals (leg. ML 2. 8. 2007, CB 65274, HOLOTYPE; PRC 65274/a, ISOTYPE). Some specimens listed in Kovanda (1984) (i.e. in dumetis in clivo austr. collies Doutnáč prope pagum Srbsko, leg. M. Kovanda 1963 PR; in dumetis in summo collis Doutnáč prope pagum Srbsko, leg. M. Kovanda 1964 PR, 1965 PR) are probably lost. **6. Boubová hill:** Svatý Jan pod Skalou, the SW slope of Boubová hill, 400-410 m a.s.l. [not. M. Kovanda (Kovanda 1999)]. – Boubová hill, ca 0.3 km WSW of the summit, the margin of a forest road in an oak-hornbeam forest, 6050b, 49°57'11.9"N, 14°08'17.9"E, 400 m a.s.l., one ca 0.75 m high juvenile individual (not. 19. 6. 2009 J. Mottl, ML & PL). No specimen from this locality is available. **7. Velká hora hill:** Srbsko, W slopes of Velká hora hill, ca 200 m SW of the summit, in thermophilous scrub, 6050d, 49°56'58.6"N, 14°09'24.4"E, 420 m a.s.l., 3 juvenile individuals (leg. ML 4. 7. 2009, CB 71603). – Srbsko, ca 80 m SW of the summit of Velká hora hill, near the margin of the summit plateau, the margin of a forest gap, 6050b, 49°57'01.7"N, 14°09'28.2"E, 420 m a.s.l., one ca 6 m high individual (leg. ML 16. 7. 2009, CB 71601). – Srbsko, W slopes of Velká hora hill, ca 250 m W of the summit, in thermophilous oak forest, 6050b, 49°57'03.7"N, 14°09'18.6"E, 350 m a.s.l., one ca 1.5 m high juvenile individual (not. J. Mottl 2. 8. 2009, leg. ML 29. 10. 2009, CB 71604). A new locality. **8. Haknová hill:** Karlštejn, S slopes of Haknová hill, near the summit, a thermophilous oak forest, 6051c: 49°56'15.7"N, 14°11'55.7"E, 410 m a.s.l., 1 small tree (leg. PL & ML 10. 5. 2009, CB 71588), 49°56'15.8"N, 14°11'55.9"E, 410 m a.s.l., 1 small tree (leg. PL & ML 10. 5. 2009, CB 71589). – Karlštejn, ca 100 m ENE of the summit of the Haknová hill, 6051c, 49°56'17.9"N, 14°12'01.0"E, 420 m a.s.l., one overshadowed ca 4 m high individual (leg. PL & ML 10. 5. 2009, CB 71599). The first record for this locality is 1986: the summit area of Haknová hill near Karlštejn, 402 m a.s.l. (no specimen available) (Jankun & Kovanda 1988). **9. Plešivec hill:** Plešivec hill (leg. M. Kovanda 16. 10. 1985, PRA). – Karlštejn, ca 120 m SE of the summit of Plešivec hill, the margin of steppe, 6051c, 49°56'04.2"N, 14°11'24.5"E, 340 m a.s.l., one overshadowed ca 3 m high individual (leg. ML & PL 10. 5. 2009, CB 71598). **10. Mramor hill** (49°53'54.1"N, 14°07'43.3"E): Beroun: in nemore ad declivia collis Mramor prope pag. Měňany et Liteň, 450 m s.m., s. calcareo (leg. Domin & Dostál 2. 8. 1939, PRC). The specimens listed in Kovanda (1984) (i.e. in nemore in clivo septentr. collis Mramor prope pagum Liteň, leg. M. Kovanda 1980, 1981 PR) are probably lost and are the last records for this locality. A locality with unconfirmed occurrence.

**Poorly localized specimens:** Karlštejn (leg. M. Řezáč 2001, ROZ).

### *Herbarium specimens and records not confirmed*

In 2005 (Vít 2006) and 2009, we repeatedly failed to confirm the two records cited for *S. eximia* by Jankun & Kovanda (1988) and Kovanda (1999) listed below (herbarium specimen not cited, see below). Considering the rather poor delimitation of these localities, we cannot rule out that the species was overlooked and is still present there. The distribution pattern of both species indicates that these records refer rather to *S. barrandienica* than *S. eximia*, but a field observation is needed to confirm this hypothesis. The specimen col-

lected by Hostim and mentioned below, which Kovanda (1984) referred to as *S. eximia*, we find impossible to identify with certainty. It consists of a sterile, probably epicormic shoot with lobed leaves with a greyish indumentum on the abaxial surface. Lobes are characteristic of hybrid *Sorbus* species, but *S. aria* s.l. can also have exceptionally lobed leaves, particularly on long sterile shoots. A greyish indumentum without any yellowish tinge is typical of *S. aria* s.l. A search carried out by us in the vicinity of the village in 2007 yielded many records of *S. aria* s.l., but no hybrid was recorded there.

Herbarium specimens and records:

**Unconfirmed records: 1.** Along the road from Hostim to Bubovice, 1 km from Bubovice, 350 m a.s.l. (Jankun & Kovanda 1988). **2.** The surroundings of the Králova studně spring (Kovanda 1999).
**Uncertain determination:** Böhmen, gehänge bei Hostín [= Hostim], Kalk (leg. Beck 21. 8. 1918, PRC).

### *Phenotypic variation and species-specific characters*

*Sorbus eximia* and *S. barrandienica* populations are fertile. The plants produce fully developed seeds and are morphologically homogeneous both in vegetative and generative characters. There are no records based on our field observations of morphologically intermediate types between the species (they do not occur at the same localities). The taxa belong to the *S. latifolia* aggregate (parental combination *S. aria* s.l. × *S. torminalis*). The *S. aria* group is represented by *S. danubialis* and *Sorbus aria* s.l. in this region, and both of these taxa (along with *S. torminalis*) often occur sympatrically with the species studied. Plants intermediate between the hybrid species and their putative parents have not been observed. For morphological differences between *S. danubialis*, *S. aria* s.l. and the two hybrid species, see the key in Appendix 1. *Sorbus torminalis* is not included in the key because *S. eximia* and *S. barrandienica* are apparently closer to the *S. aria* group. The species studied differ from other Bohemian members of the *S. latifolia* agg. (*S. albensis*, *S. bohemica*, *S. gemella*, *S. rhodanthera*, *S. portae-bohemicae* and *S. milensis*) in having paler (orange to orange-red) fruits and shallowly lobed leaves. All Bohemian species except for *S. gemella* have darker (orange-red) fruits. *Sorbus gemella* differs in having rhomboidal and more deeply incised laminas. In *S. eximia* the leaves often have cochleariform lamina, which is a unique character for taxa of *Sorbus* occurring in the Czech Republic.

### *Chromosome variation and ploidy level of S. eximia*

*Sorbus eximia* is cited as an example of a rare diploid apomictic species in several publications (Campbell & Dickinson 1990, Campbell et al. 1991, Nelson-Jones et al. 2002, Meyer et al. 2005, Dickinson et al. 2007, Rich et al. 2010). Our investigations have revealed that somatic cells of *S. eximia* and *S. barrandienica* have a triploid chromosome number (2n = 3x = 51). The diploid number previously reported for *S. eximia* (Jankun & Kovanda 1988) was not confirmed in the current study, not even for the populations cited by Kovanda (Koda hill near Srbsko; Jankun & Kovanda 1988). Screening the DNA ploidy levels of *S. eximia* and *S. barrandienica* using DAPI flow cytometry also did not detect any intraspecific variation. Observed sample/standard ratio for *S. eximia* was 0.60 (average CV of sample: 3.04 and standard: 1.66) and for *S. barrandienica* 0.60 (average CV of sample: 3.67 and standard: 2.35). Our recent study indicates the existence of only tetraploid cytotypes in the *S. aria* agg. in the Bohemian Karst (Lepší et al. in prep.), thus occurence

of diploid *S. eximia* is improbable. It is therefore concluded that both species studied are triploids and the existence of diploids or tetraploids as reported by Kovanda (1984) and Jankun & Kovanda (1988) must be regarded as dubious. Other hybrid species of the *S. latifolia* group in Bohemia (Jankun & Kovanda 1987, Lepší et al. 2008, 2009, P. Vít, unpubl.) also have the triploid number of chromosomes. The diploid chromosome number has thus so far only been reported with certainty for sexual species of *Sorbus*.

*Genetic variation*

*Sorbus eximia* and *S. barrandienica* showed minimal intra-specific genetic variation at seven nuclear microsatellite loci, indicating a monotopic origin (i.e., each species is a single evolutionary lineage). This phenomenon is common in several other agamospermous *Sorbus* taxa occurring in the Czech Republic (e.g., *S. albensis*, *S. portae-bohemicae*, *S. rhodanthera* and *S. milensis*; Lepší et al. 2008, 2009, P. Vít et al., unpubl.). While intra-specific variation was low, inter-specific differentiation is considerable as both of these species have distinct microsatellite patterns. Prevailing fragment length of each of the loci analysed and average gene diversity are presented in Table 2. The species differ from one another in six of the seven loci analysed. Average gene diversity of hybrid species is considerably (about tenfold) lower than that of species reproducing sexually (e.g. *S. torminalis* or *S. aria* s. str.; Vít 2006). This observation supports the independent status of each of the endemic *Sorbus* species as unique evolutionary units. The predominant, if not sole mode of reproduction of the taxa studied, which is inferred from the low morphological and genetic variation, is probably apomixis. These observations are consistent with the results for several other apomictic *Sorbus* taxa of hybrid origin (Liljefors 1953, Jankun & Kovanda 1986, 1987, 1988, Meyer et al. 2005).

Table 2. – Fragment length of each microsatellite loci (in bp) and average gene diversity over all loci (AGD) for each *Sorbus* species.

| Taxon | N | Locus Mss1 | Locus CH01H10 | Locus Mss6 | Locus CH02D11 | Locus Ms14 | Locus Mss5 | Locus Ms6g | AGD | S.E. |
|---|---|---|---|---|---|---|---|---|---|---|
| *S. eximia* | 10 | 172 | 78 | 254 | 120, 144, 178 | 128 | 112, 124 | 130 | 0.002405 | 0.003011 |
| | 2 | 172 | 78, 82 | 254 | 120, 144, 178 | 128 | 112, 124 | 130 | | |
| *S. barrandienica* | 9 | 156 | 78 | 250 | 120, 144, 168 | 120 | 112, 124 | 126 | 0.008219 | 0.008045 |
| | 1 | 152 | 78 | 250 | 120, 144, 168 | 120 | 112, 124 | 126 | | |

*Morphometric analyses*

*Sorbus eximia* and *S. barrandienica* were well separated in the canonical discriminant analysis (results not shown) from other Bohemian *Sorbus* species of the *S. latifolia* group (for details see Vít 2006, p. 55–61). In separate analysis of *S. eximia* and *S. barrandienica* (see Fig. 9), the species were also well separated. The incision between the 2nd and 3rd lobe of the leaf lamina and calyx length were the variables most tightly correlated with the first discriminant axis. When all of the characters measured are included, 62 (96.87%) of the 64 specimens of *S. eximia* and *S. barrandienica* tested were correctly classified in

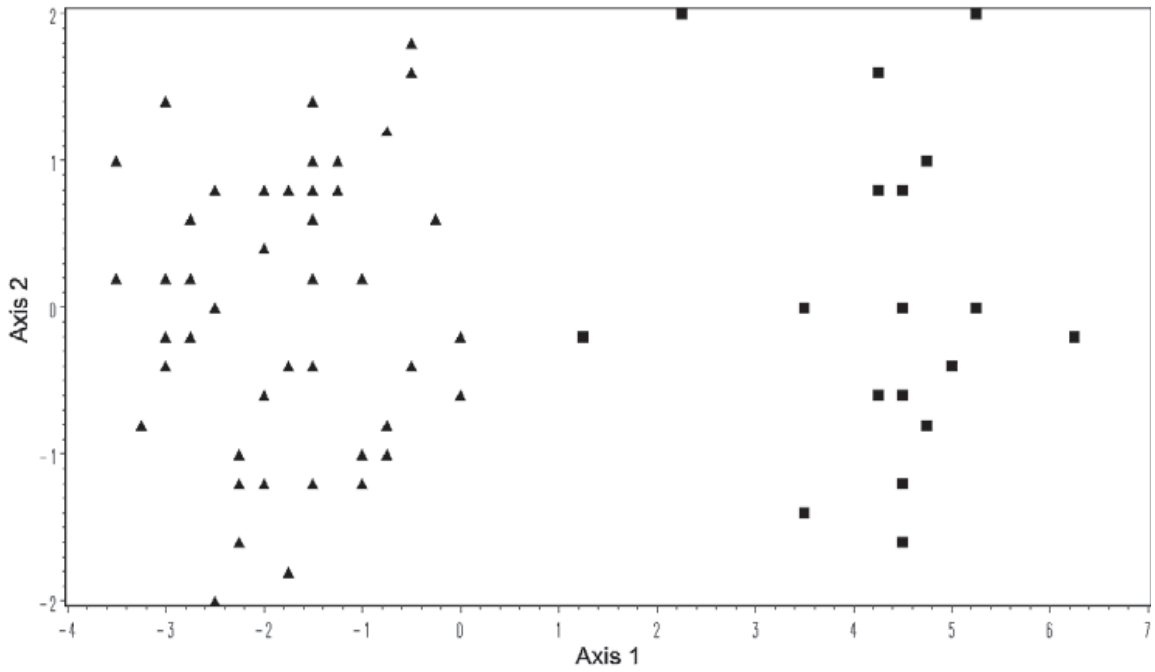


Fig. 9. – Canonical discriminant analysis of *Sorbus eximia* ▲ and *S. barrandienica* ■ using a morphometric data set of 17 characters.

a classificatory discriminant analysis (data not shown). The incorrectly classified samples may be a result of the phenotypic variation and/or problems of standardising sampling.

*Elliptic Fourier analysis of leaf laminas*

While descriptive morphometrics separated the two species on the basis of quantitative characters, an elliptic Fourier analysis allowed the separation of the two species by using the shape of the leaf lamina as a diagnostic character. Principal component analysis (PCA) performed on standardized Fourier coefficients revealed distinct differences between the species studied (Fig. 10). A morphological shape trend associated with the first principal component separates the two species based on the overall shape of the lamina. *S. eximia* has a broadly ovate to broadly elliptical leaf lamina, while that of *S. barrandienica* is more or less elliptical. Variation along the second (data not shown) and third axis demonstrated a tendency towards differentiation in the curve of the lamina base and apex. In *S. barrandienica* it tends to be rounded acute at the apex and cuneate at the base, while in *S. eximia* the apex is more or less broadly rounded acute to obtuse and the base rounded or broadly cuneate. The results of these analyses confirmed our field observations. No clear pattern was observed along the other PCA axes (data not shown). In total, seven PCA axes were found to significantly improve the discriminant power of the CVA analysis during forward selection in Canoco. A cross-validated discriminant analysis was performed on the principal component scores of these seven axes. The discriminant analysis resulted in an incorrect classification in eight of a total 194 cases (6 individuals of *S. eximia* were assigned to *S. barrandienica* and 2 individuals of *S. barrandienica* to *S. eximia*). These incorrectly classified samples were leaves untypically developed due to phenotypic varia-

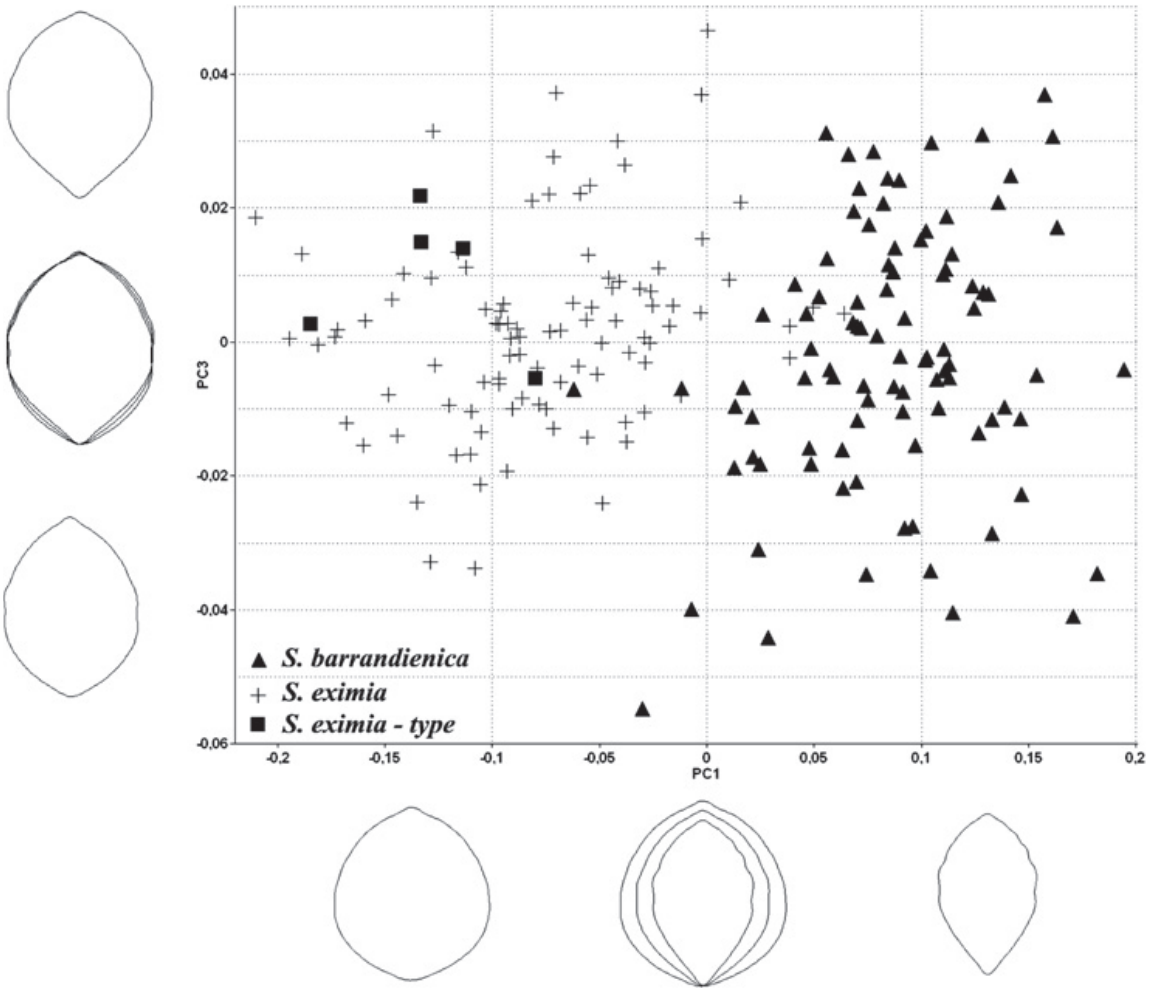


Fig. 10. – PCA of Fourier coefficients describing the total leaf lamina shape of *S. eximia* and *S. barrandienica*. The first and third ordination axes are displayed, which explain 81.8% and 2.5% of the overall variation, respectively. Reconstructed contours corresponding to the –2 and +2 SD positions on both axes are visualized along the particular axes. In the middle, these two contours are overlapped with the mean leaf shape (corresponding to the [0.0] point of the plot).

tion in this species (too overshadowed or exposed plants). All 5 leaves from the type specimen of *S. eximia* were determined as *S. eximia*. Neither the relative position nor the shape of the laminar lobes contributed to the discrimination of these two species, even when the shape of the lobes was more accurately described by using a greater (40) number of harmonics (data not shown).

*Conservation status*

All the specimens of the two taxa studied are found within the area of the Bohemian Karst protected landscape area and are (except the locality of *S. barrandienica* at Mramor hill) part of small-scale protected areas (in particular, Koda and Karlštejn national nature reserves, Kotýz national nature monument and Kobyla nature reserve). Despite this, the protection of the two endemic species is insufficient. The main threat stems from the

cessation of traditional forest management, which previously maintained open forests stands. The shady conditions that prevail in recent so-called tall forests are unfavourable for the long-term survival and regular reproduction of light-demanding *Sorbus* species. The general expansion of woods (especially of *Fraxinus excelsior*) into open (rocky and steppe) habitats represents another serious threat to these endemics.

Even before the taxonomic revision presented here, *Sorbus eximia* was regarded as a strongly endangered species (Holub & Procházka 2000). The new species *Sorbus barrandienica* should be included among the critically endangered plants of the Czech flora (C1; sensu Holub & Procházka 2000), as there are few individuals, frequent occurrence of old or dying trees and lack of juveniles at most localities. *Sorbus eximia* is considered strongly endangered (category C2) because there are considerably larger populations with lots of juveniles at its localities. Moreover, this species is able to spread into man-made non-relic biotopes, e.g. into abandoned quarries or pine plantations. Such habitats are now common in the Bohemian Karst. On the other hand, it is only recorded at three localities and the biggest one, Koda hill, is significantly affected by the spread of *Fraxinus excelsior*. Both endemic species should also be added to the list of species protected by law. According to the IUCN (2001), *S. eximia* and *S. barrandienica* rank among critically endangered species [status criteria B2b (iii) and B2b (iii,iv,v);C2a (i), respectively]. Particular attention should be paid to protecting these species in the future. Appropriate forest management (which would facilitate reproduction of these endemics) should be implemented at selected localities. Three localities are recommended: Koda hill for protection of *S. eximia* and Paní hora hill and/or Doutnáč hill for *S. barrandienica*. Supposed parental species occurring at the same localities as the endemics should also be included in the management plan, since they may generate new taxonomic diversity (by hybridization and introgression) in the future and thus play an important role in the ongoing evolutionary processes (cf. Ennos et al. 2005).

## Acknowledgements

We are grateful to J. Zázvorka for valuable comments on earlier drafts of the manuscript, J. Mottl, T. Tichý (Administration of the Český kras Protected Landscape Area) for guidance in the field and J. Mottl for information about new localities of the taxa under study. We thank V. Jarolímová for her help with chromosome counting, K. Boublík for help with elaboration of the phytosociology of the taxa, F. Kolář for help with cross-validated analysis, J. Velebil for information about the cultivation of *S. eximia* by Řevnice, and M. Ducháček, J. Hadinec and L. Kirschnerová for technical and herbarium assistance. The Herbarium of the Department of Botany (PRC), in particular J. Štěpánek, is acknowledged for permission to use the photograph of the type specimen of *S. eximia*. F. Rooks kindly improved our English. The study was supported by grant 138/2010/P from the Grant Agency of the University of South Bohemia, Institutional research support MSM-6007665801 from the Czech Ministry of Education and project AV0Z60050516 from the Academy of Sciences of the Czech Republic.

## Souhrn

V příspěvku je popsán nový apomiktický triploidní (2n = 3x = 51) druh jeřábu *Sorbus barrandienica* P. Vít, M. Lepší et P. Lepší (jeřáb barrandienský), náležející do skupiny *S. latifolia* agg. (rodičovská kombinace *S. aria* s.l. × *S. torminalis*). Byl rozlišen na základě taxonomické a chorologické revize jeřábu krasového (*Sorbus eximia*), hybridogenního druhu stejné rodičovské kombinace, který byl popsán v roce 1984 z Českého krasu (Kovanda 1984). Pomocí moderních biosystematických metod byly v rámci *S. eximia* rozlišeny dvě apomiktické linie, lišící se zřetelně morfologicky a geneticky. Průzkum ploidie pomocí průtokové cytometrie ukázal, že oba rozlišené taxony jsou triploidní. To je v rozporu s dřívějšími studiemi, kde byl u *S. eximia* zjištěn diploidní a tetraploidní stupeň

(Jankun & Kovanda 1988). Dokonce byla na diploidní úrovni pozorována i apomixie, jež je dodnes udávaná pouze v jednom nejistém případě u *S. subfusca*. Naše výsledky tyto závěry vyvracejí. Studium typového materiálu odhalilo, že jméno *S. eximia* se vztahuje k rostlinám na lokalitě v NPR Koda, populace udávané ze zbylých lokalit v Českém krasu náleží novému, zde popsanému druhu *S. barrandienica*. Terénním průzkumem bylo dodatečně zjištěno, že *S. eximia* roste na 4 lokalitách, z toho jedna vznikla výsadbou. Velikost populace je odhadována na 100 dospělých exemplářů a ca 200 juvenilních jedinců. *S. barrandienica* byl nalezen na 10 lokalitách, z toho dvě historické se nepodařilo potvrdit. Celá populace dnes čítá ca 50 exemplářů. Oba druhy nejčastěji rostou v teplomilných doubravách a dubohabřinách a v lesních lemech suchých trávníků. Vykazují malou genetickou a morfologickou variabilitu a jsou dobře diferencovaní od jeřábů vyskytujících se v Čechách. Nejvíce jsou ohroženy zánikem světlých lesů. *Sorbus barrandienica* navrhujeme zařadit do červeného seznamu taxonů ČR do kategorie kriticky ohrožený druh, *S. eximia* mezi silně ohrožené druhy (Holub & Procházka 2000). Pro přežití druhů je nutné na vybraných lokalitách zavést speciální management, k tomuto účelu doporučujeme lokality Doutnáč nebo Paní hora a Koda. K odlišení společně se vyskytujících jeřábů v Českém krasu poslouží následující klíč (čepel listů musí pocházet ze střední části fertilních brachyblastů):

**1a** Čepel listů mělce nebo pouze v horní třetině zastřihovaně dvojitě pilovitá (až mělce laločnatá), na rubu šedozelená, plody (korálově) červené ..... **2**
**1b** Čepel alespoň některých listů laločnatá, na rubu nažloutle šedozelená, plody oranžové až oranžově červené ..... **3**
**2a** Čepel listů široce eliptická až okrouhlá, mělce dvojitě pilovitá, s plochým okrajem, 6–12 cm dlouhá ..... ***S. aria* s.l.**
**2b** Čepel listů víceméně kosočtverečná až zaokrouhleně kosočtverečná, v horní třetině zastřihovaně dvojitě pilovitá (někdy až mělce laločnatá), se zvlněným okrajem, 4–10 cm dlouhá ..... ***S. danubialis***
**3a** Čepel listů víceméně eliptická, plochá, na bázi klínovitá vzácněji široce klínovitá, mělce laločnatá; zářez mezi druhým a třetím lalokem (0,40–) 0,45–0,60 (–0,75) cm dlouhý, kališní cípy (2,3–) 2,5–3,5 (–3,8) mm dlouhé, plody často delší než široké ..... ***S. barrandienica***
**3b** Čepel listu široce vejčitá až široce eliptická, často lžícovitě prohnutá, na bázi většinou zaokrouhlená nebo široce klínovitá, dvojitě pilovitá až pravidelně mělce laločnatá, zářez mezi druhým a třetím lalokem (0,25–) 0,40 (–0,55) cm dlouhý, kališní cípy (1,7–) 2,1–2,5 (–3,0) mm dlouhé, plody často stejně široké jako dlouhé nebo širší ..... ***S. eximia***

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Received 18 February 2011
Revision received 30 May 2011
Accepted 31 May 2011

Appendix 1. – Key for determining the species of the *Sorbus latifolia* agg. and *S. aria* s.l. occurring in the Český kras Karst.

**1a** Leaf lamina shallowly or coarsely double serrate (to shallowly lobed) distally, greyish-green beneath, fruits red .....2
**1b** Leaf lamina (at least some) shallowly lobed, yellowish-greyish-green beneath, fruits orange to orange-red ... 3
**2a** Leaf lamina broadly elliptical to rounded, shallowly double serrate, with flat margin, 6–12 cm long .....***S. aria* s.l.**
**2b** Leaf lamina more or less rhomboidal to round rhomboidal, coarsely double serrate (to shallowly lobed) distally, with folded margin, 4–10 cm long .....***S. danubialis***
**3a** Leaf lamina more or less elliptical, flat, cuneate rarely broadly cuneate at base, shallowly lobed; incision between the second and the third lobe terminating the main veins (0.40–) 0.45–0.60 (–0.75) cm long, sepals (2.3–) 2.5–3.5 (–3.8) mm long, fruits often longer than wide .....***S. barrandienica***
**3b** Leaf lamina broadly ovate to broadly elliptical, often cochleariform, usually rounded or broadly cuneate at base, double serrate to regularly shallowly lobed, incision between the second and the third lobe terminating the main veins (0.25–) 0.40 (–0.55) cm long, sepals (1.7–) 2.1–2.5 (–3.0) mm long, fruits often as wide as or wider than long .....***S. eximia***

# Species boundaries and hybridization in central-European *Nymphaea* species inferred from genome size and morphometric data

**Diagnostické znaky a mezidruhová hybridizace středoevropských leknínů, zjištěné na základě cytometrických a morfometrických analýz**

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Kabátová K., Vít P. & Suda J. (2014): Species boundaries and hybridization in central-European *Nymphaea* species inferred from genome size and morphometric data. – Preslia 86: 131–154.

Aquatic plants often pose considerable taxonomic problems. The genus *Nymphaea* (water lily) in central Europe is a good example of this in that their morphological similarity blurs the boundaries between species, which in addition are highly phenotypically plastic and possibly hybridize. The situation is further complicated by the occurrence of many garden cultivars. We used DNA flow cytometry and multivariate morphometrics (both distance-based and geometric) to obtain an insight into their phenotypic variation, identify taxon-specific characters and assess the frequency of hybridization in water lilies collected from 72 localities in the Czech Republic. For comparative purposes, we also included 34 garden cultivars. Flow cytometric measurements revealed a 45% difference in the holoploid genome sizes of *N. alba* and *N. candida*, which makes it easy to reliably separate them. In addition, the great majority of garden cultivars have distinctly smaller genomes than their native counterparts. Interspecific hybridization under natural conditions was quite rare (only ~1.8% of the individuals cytotyped corresponded to *N.* ×*borealis*), and involved both reduced and unreduced gametes. Discriminant analyses revealed cultivar- and species-specific morphological characters, which allow accurate determination of the samples. Gynoecium and stamen characters had the greatest taxonomic value. The recognition of *N.* ×*borealis* on the basis of morphological characters is uncertain. Our study shows that genome size may help to resolve the long-standing taxonomic complexities in this important component of the temperate aquatic flora.

K e y w o r d s: aquatic plants, Czech Republic, flow cytometry, genome size, interspecific hybridization, multivariate morphometrics, *Nymphaea*, species determination, taxonomy, water lily

## Introduction

Due to their high phenotypic plasticity and simplified morphology, many aquatic plants pose considerable taxonomic problems (Schmid 1992). Differences in water depth and chemistry, light intensity and nutrient conditions of sediment can lead to the genesis of distinct morphotypes, which are not genetically determined and change rapidly with change in environmental conditions. Formal recognition of environmentally-induced morphotypes has often resulted in a deluge of evolutionary unjustified and morphologically intergrading taxa (Kaplan 2002). Extensive geographical ranges of many species of aquatic plants (Hultén & Fries 1986) present another challenge as usually only a part of the entire distributional range can be studied. The genetic make-up of populations of aquatic plants may be greatly affected by the discrete and patchy nature of aquatic habitats and the directional transport

of propagules in running water (Barrett et al. 1993). In addition, clonal propagation supports the establishment and spread of unique genotypes and/or hybrids, further contributing to the complexity of populations.

Water lilies (*Nymphaea* L.) are among the showiest aquatic plants and have long attracted the attention of botanists, horticulturists and plant enthusiasts. About 50 species are recognized worldwide (Borsch et al. 2007), four of which are native to Europe (Tutin & Webb 1993). While *N. alba* L. and *N. candida* J. Presl are widespread in Europe, *N. tetragona* Georgi only grows in Europe in Finland, Belarus and Russia (Uotila 2009), and *N. lotus* L. is restricted to Romanian and Hungarian hot springs [as the supposedly endemic var. *thermalis* (DC.) Tuzson] (Masters 1974). *Nymphaea alba* occurs throughout most of Europe (except northern Scandinavia) and in northernmost Africa, while *Nymphaea candida* has been reported from central and northern Europe, from where it extends further eastwards (Meusel et al. 1965); its southern distribution remains a moot question (Muntendam et al. 1996, Nowak et al. 2010, Ejankowski & Małysz 2011). Native populations of both *N. alba* and *N. candida* are rapidly declining in many European countries (e.g. Tomšovic 1988, Ejankowski & Małysz 2011).

Despite the low number of indigenous European species, their high morphological polymorphism and plasticity have triggered a continuous dispute concerning the boundaries between the taxa, in particular between the widespread *N. alba* and *N. candida*. A number of species-specific morphological characters are reported, although their usefulness is often questioned. *Nymphaea alba* and *N. candida* should differ in the pattern of their leaf venation, shape of flower base (cup base), shape of the innermost stamens, shape and colour of stigma disc, number of carpellary teeth (also referred to as carpellary appendages), and pollen size and sculpture (Heslop-Harrison 1955, Tomšovic 1988, Muntendam et al. 1996, Wayda 2000, Volkova & Shipunov 2007, Nowak et al. 2010, Ejankowski & Małysz 2011). Some differences in habitat requirements are also recorded. While *N. alba* tolerates eutrophic waters, *N. candida* prefers mesotrophic conditions in central Europe (Neuhäusl & Tomšovic 1957, Szańkowski & Kłosowski 1999). The recognition of typical individuals of both species usually presents few problems, but it is the occurrence of transient morphotypes or plants with a mosaic-like combination of characters that challenge the identification of water lilies in Europe.

Morphological similarities at least partly stem from close evolutionary relationships of *N. alba* and *N. candida*. Volkova et al. (2010) show that the latter species is of allopolyploid origin, with *N. alba* and *N. tetragona* as putative parental taxa. This hypothesis, in addition to AFLP fingerprints, cpDNA and ITS sequences, is also supported by data on the size of its nuclear genome. The sum of relative nuclear DNA amounts of *N. alba* and *N. tetragona* fits very well the mean value for *N. candida*. While the authors report significant interspecific differences in genome size (~40% divergence between *N. alba* and *N. candida*), the variation at the intra-specific level is negligible, indicating that the amount of nuclear DNA is a suitable species-specific marker (Loureiro et al. 2010). The most commonly reported numbers of somatic chromosomes for European populations of *N. alba* and *N. candida* are 2n = 84 and 2n = 112, indicating hexa- and octoploidy, respectively, based on x = 14 (Májovský 1976, Pellicer et al. 2013). Several other chromosome numbers (from 2n = 48 to 2n = 160) are reported in the literature (Bolkhovskikh et al. 1969, Goldblatt & Johnson 1979 onwards, Gupta 1980). However, they must be viewed

with caution because of frequent misidentifications, different species circumscriptions and/or problems with karyological analyses (Heslop-Harrison 1955).

Individuals with intermediate morphologies are often interpreted as interspecific hybrids (Heslop-Harrison 1955, Ejankowski & Małysz 2011) although their hybrid status is rarely supported by molecular or cytogenetic markers. A few exceptions include crosses between *N. alba* and *N. candida* (= *N.* ×*borealis* Camus) at several sites in Germany and Sweden confirmed by AFLP fingerprinting (Werner & Hellwig 2006), and Indian plants originally determined as "*N. alba* var. *rubra*", which based on chloroplast and ribosomal DNA sequence data are hybrids between *N. alba* and *N. odorata* Aiton (Dkhar et al. 2012). In general, interspecific hybridization in water lilies seems to be quite extensive as indicated by the great number of horticultural crosses (Slocum 2005). The nothotaxon *N.* × *borealis* is reported from different geographic regions where both parental taxa co-occur, including the Czech Republic (Neuhäusl & Tomšovic 1957, Tomšovic 1988), Poland (Ejankowski & Małysz 2011) and Russia (Komarov 1970). In addition to unusual combinations of morphological characters, the authors also mention low pollen fertility and reduced seed set as indicators of hybrid origin (Heslop-Harrison 1955). However, considering the non-trivial recognition of parental taxa and the lack of any clear morphological discontinuities, published records of interspecific hybrids should not be accepted uncritically.

The great popularity of water lilies as ornamental plants also raises other specific issues. Long-term horticultural selection and targeted breeding have resulted in the development of several hundreds of hardy cultivars (Hříbal 1985, Slocum 2005) that are often collectively referred to as *N. hybrida* hort. Although the origin of many of these cultivars, especially the old ones, is uncertain (Conard 1905), white-flowered fragrant *N. odorata* [incl. subsp. *tuberosa* (Paine) Wiersema & Hellq.] and yellow-flowered *N. mexicana* Zucc. are among the extra-European species that were most commonly used for hybridization (Hříbal 1985). Garden cultivars were repeatedly introduced (either accidentally or intentionally) into natural habitats where they can survive for long periods and potentially interact (competition, mating interactions) with native plants. Reliable discrimination between escaped white-flowered cultivars and native species on the basis of morphological traits is difficult, if not impossible, and the occurrence of garden plants makes the study of European water lilies even more difficult.

There have been several attempts in recent years to elucidate the taxonomic composition of *Nymphaea* populations in Europe, by determining the frequency of interspecific hybridization and/or revealing diagnostic morphological characters (Muntendam et al. 1996, Volkova & Shipunov 2007, Ejankowski & Małysz 2011). Although the authors often used sophisticated morphometric approaches, a major limitation to their studies was the lack of any straightforward discriminating marker, resulting in subjective identification of the samples analysed. The findings of Volkova et al. (2010) nonetheless suggest that the amount of nuclear DNA can serve as a species-specific trait, which allows not only *N. alba* and *N. candida* but also their hybrids to be reliably recognized. We therefore built on their study and assessed the variation in genome size in water lilies occurring in the Czech Republic (incl. some garden cultivars) and subjected these cytologically-proven plants to morphometric analysis.

Specifically, we addressed the following questions: (i) What is the variation in the amount of nuclear DNA and can some distinct genome size groups be recognized?

(ii) Does the variation in genome size reflect phenotypic variation in water lilies? Which characters can be considered as species- and/or cultivar-specific? (iii) What is the incidence of interspecific hybrids and which morphological characters do the crosses share?

## Material and methods

### *Plant material*

Samples were collected in the Czech Republic during 2009–2013. More than 150 historical localities listed mainly in the Flora Database of the Czech Republic (www.florabase.cz) and identified on the basis of information supplied by local botanists were visited, and the occurrence of water lilies at 72 of them was confirmed (Fig. 1, Electronic Appendix 1). Whenever possible depending on population size and phenology, one mature leaf and one fully developed flower from each of 10 individuals (range 1–56; Electronic Appendix 1) were sampled per locality. The sampling strategy was designed to (i) include the phenotypic variation present at the localities, and (ii) collect putatively different genotypes (i.e. distantly-spaced individuals). In total, 619 *Nymphaea* individuals sampled in situ of both native species and putative garden cultivars were included to this study. This dataset was supplemented by 34 hardy garden cultivars originating from collections of the Institute of Botany, Academy of Sciences of the Czech Republic, Průhonice. Herbarium vouchers are deposited in PRC.

Plant samples were kept wet and processed within two days of collecting. Abaxial side of leaves was scanned using an A3 scanner (for large leaves or leaves with overlapping lobes, only one flank of the lamina was scanned). Flowers were dissected and pictures of individual parts (cup base, outer sepal, outer petal, innermost stamen, median section of the gynoecium) were taken, together with an appropriate ruler, under standardized conditions using a Pentax Optio W80 camera (Fig. 2). Before imaging, both sepals and petals were flattened and attached by adhesive transparent tape to a sheet of paper. Because water lilies are protogynous (Wiersema 1988), we noted the phenological stage (using a 5-point scale) of each flower in order to assess potential temporal changes in floral characters. In order to assess pollen viability, samples of pollen from selected populations (of native species, interspecific hybrids and garden cultivars) were stained following the protocol detailed by Peterson et al. (2010) and examined using an Olympus BX-61 light microscope. Stamens from three individuals per population were usually pooled, dissected and 100 pollen grains evaluated.

### *Flow cytometry*

Variation in genome size (2C-values) was estimated using DNA flow cytometry (FCM). Leaf petioles were used for isolating nuclei rather than laminas because they provided histograms of better quality (more uniform fluorescence, lower background signals). Approximately 1 cm of an upper part of the leaf petiole was chopped together with an appropriate volume of the internal reference standard using 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). *Glycine max* (L.) Merr. ‘Polanka’, 2C = 2.50 pg (Doležel et al. 2007) served as the primary reference standard (which has a similar, but not overlapping genome size with that

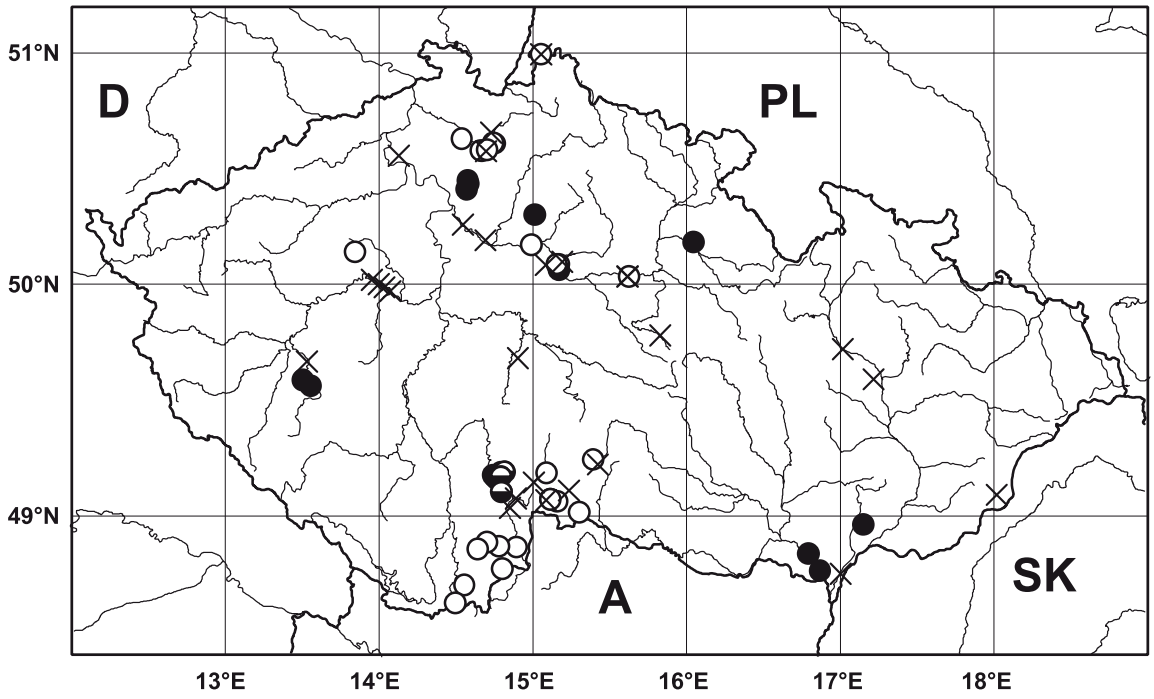



Fig. 1. – Map showing the localities where water lilies were sampled in the Czech Republic. ● *Nymphaea alba*, ○ *N. candida*, ◒ *N.* ×*borealis*, × garden cultivars.

of most samples). Garden cultivars were re-analysed using *Bellis perennis* L. (2C = 3.38 pg; Schönswetter et al. 2007) as a standard due to their similarities in genome sizes with that of *Glycine*. The crude suspension was filtered through a 42-µm nylon mesh and incubated for ~15 min at room temperature. Isolated nuclei were stained with 1 ml of Otto II buffer (0.4 M $Na_2HPO_4 \cdot 12\ H_2O$) supplemented with ß-mercaptoethanol (2 µl/ml), DNA-selective fluorochrome propidium iodide and RNase A, type IIA (both at a final concentration of 50 µg/ml). Shortly after staining, fluorescence intensities of 5000 particles were recorded using a Partec CyFlow instrument (Partec GmbH, Münster, Germany) equipped with a green diode-pumped solid state laser (Cobolt Samba, 532 nm, 100 mW output power). A subset of 147 samples was analysed using DAPI flow cytometry. In this protocol modification, the staining solution consisted of 1 ml of Otto II buffer, ß-mercaptoethanol (2 µl/ml) and AT-selective fluorochrome DAPI (4 µg/ml) and the samples were analysed using a Partec ML flow cytometer equipped with a UV diode chip set as the light excitation source. Histograms were evaluated using FloMax software, ver. 2.4d. To ensure the comparability of results, fluorescence values obtained by DAPI staining were re-calculated to propidium iodide values using a calibration set consisting of 37 individuals from all taxonomic groups that was measured using both fluorescent stains.

*Geometric morphometrics*

Six parts of plants with potential taxonomic value were subjected to a detailed analysis of the variation in their shape. On each part, several landmarks (with fixed positions) and

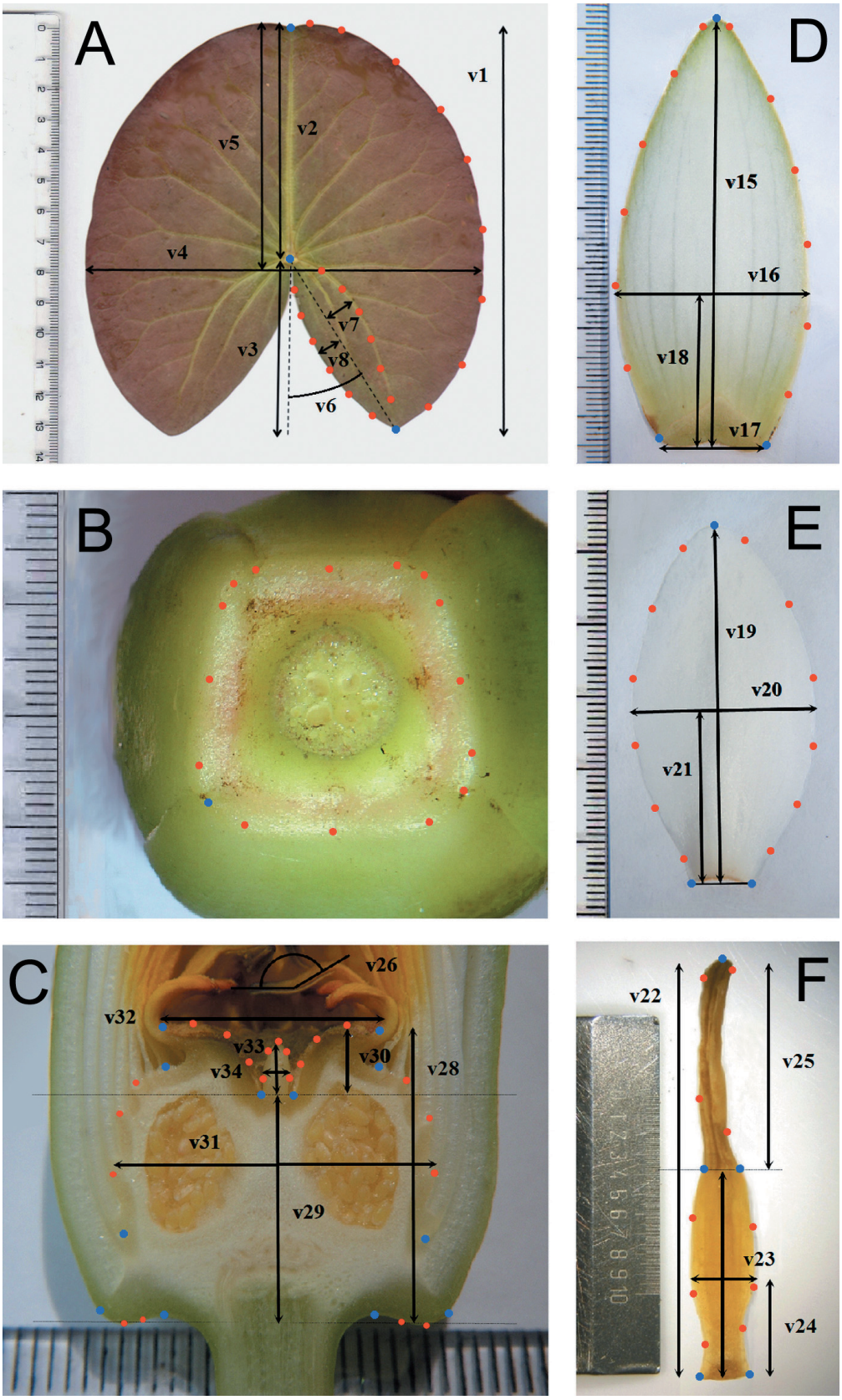



Fig. 2. – Pictures of the vegetative and generative parts used in the morphometric analysis. (A) leaf lamina, (B) cup base, (C) median section of the gynoecium, (D) sepal, (E) petal, (F) stamen. Landmarks in blue, semilandmarks in red. Variables measured in distance-based morphometrics are also shown (see Table 1 for descriptions of variables).

semi-landmarks (allowing the sliding along the abscissa connecting adjacent landmarks) were designated. Specifically, the numbers of landmarks and semi-landmarks designated on individual organs were as follows: right flank of leaf lamina – 3+21, cup base – 1+15, sepal – 3+12, petal – 3+12, innermost stamen – 3+12 and median section of the gynoecium – 12+21 (see Fig. 2). Four taxonomic groups were delimited on the basis of FCM results (i.e. *N. alba*, *N. candida*, interspecific hybrids and garden cultivars) and individual plants were used as operational taxonomic units (OTUs). Because some of the individuals cytotyped were not flowering, the numbers of OTUs for which vegetative and generative characters were analysed differed (Electronic Appendix 2).

The TPS-series software (available at http://life.bio.sunysb.edu/morph) was employed to manage the morphometric data (Neustupa et al. 2010, Viscosi & Cardini 2011). Positions of (semi-)landmarks on each plant organ were digitized in tpsDig 2.16 and the (semi-) landmark configurations were superimposed by generalized Procrustes analysis in tpsRelw 1.49. This procedure standardizes the size of the objects and optimizes their rotation and translation (Bookstein 1991). The resulting dataset was analysed using PAST 2.16 (Hammer et al. 2001). To obtain an insight into the phenetic relations among the OTUs studied, principal component analyses (PCA) were done for each plant organ. Canonical discriminant analyses and classificatory discriminant analyses of relative warp scores (i.e. deviations from the consensus shape) were performed in PAST to test for differences in shape among the a priori defined taxonomic groups and to assess the power of discrimination (i.e. the proportion of correctly classified OTUs), respectively. The deformation grids illustrating differences in shape along the discriminant axes were obtained using tpsRegr 1.38. Because of the low number of interspecific hybrids, this group was omitted from discriminant analyses.

*Distance-based morphometrics*

In total 68 quantitative, qualitative and ratio characters were measured and scored, including 11 primary leaf characters, 24 primary floral characters, 28 ratios and five colour characters (Table 1). This character set was chosen on the basis of the results of geometric morphometrics, published determination keys, flora handbooks and our own observations. Whenever possible, size variables were calculated from the digitized images using tpsDig 2.16 software.

Data were analysed in SAS 9.3 statistical package (SAS Institute, Cary, NC, USA) following the methodology of Rosenbaumová et al. (2004). Basic statistical measures, including minimum and maximum values, 5% and 95% percentiles were computed (procedure UNIVARIATE) for each character and taxon. Pearson and Spearman correlation coefficients (procedure CORR) were calculated on the pooled data matrix of all samples and on data matrices of each group to assess the relationships among variables and to identify the tightly correlated ones. Potential temporal changes in floral characters were also assessed separately in each species by analysing correlations between a phenological stage and character values. Principal component analysis (PCA) based on correlation matrices (procedure PRINCOMP), canonical discriminant analysis, CDA (procedure CANDISC) and classificatory discriminant analysis (procedure DISCRIM) were performed in order to visualize relationships among the OTUs studied, identify group-specific characters and determine the success in discriminating between OTUs. Because the

Table 1. – List of the quantitative and qualitative morphological characters analysed and corresponding contributions of individual characters to the first (Can1) and second (Can2) canonical axes in the canonical discriminant analysis. Three taxonomic groups (*Nymphaea alba, N. candida*, garden cultivars) represented by 361 individuals were analysed. Five characters with the highest absolute loadings for each axis are presented in bold. Numbers in parentheses are ranks of the strength of the correlation of each variable with the canonical axes. Ten closely correlated characters and qualitative colour characters were not included in the discriminant analysis (marked with asterisk).

| No. | Character description | Unit | Can 1 | Can2 |
|---|---|---|---|---|
| Leaf characters | | | | |
| v1 | Lamina length | cm | –0.528880 (20) | 0.153720 (35) |
| v2 | Midrib length | cm | * | * |
| v3 | Length of leaf notch (v1–v2) | cm | * | * |
| v4 | Lamina width | cm | * | * |
| v5 | Distance of the widest part of leaf lamina from lamina tip | cm | * | * |
| v6 | Angle between lobe axis and vertical axis | degree | 0.099186 (48) | 0.188051 (31) |
| v7 | Maximum distance between lobe axis and main lobe vein | cm | 0.238307 (40) | 0.168081 (33) |
| v8 | Maximum distance between lobe axis and lobe margin | cm | –0.035832 (53) | 0.494708 (5) |
| v9 | Leaf lobe width (v7+v8) | cm | 0.100678 (47) | 0.396312 (11) |
| v10 | Shape of lamina tip | 1 (sharp) – 5 (round) | –0.171442 (43) | 0.453132 (8) |
| v11 | Shape lobe tip | 1 (sharp) – 5 (round) | –0.218252 (41) | –0.214640 (26) |
| Floral characters | | | | |
| v12 | Number of air channels in flower peduncle | number | –0.284177 (34) | –0.200955 (29) |
| v13 | Number of sepals | number | –0.309134 (33) | 0.120416 (37) |
| v14 | Number of sepal veins | number | –0.102582 (46) | –0.378203 (15) |
| v15 | Sepal length | cm | –0.399280 (26) | –0.343321 (17) |
| v16 | Sepal width | cm | –0.058814 (50) | **–0.532267 (2)** |
| v17 | Sepal width at the base | cm | 0.408490 (25) | 0.246117 (23) |
| v18 | Distance of the widest part of sepal from its base | cm | –0.542271 (19) | –0.326932 (19) |
| v19 | Petal length | cm | –0.631755 (12) | –0.391256 (13) |
| v20 | Petal width | cm | –0.493601 (23) | –0.411211 (10) |
| v21 | Distance of the widest part of petal from its base | cm | * | * |
| v22 | Stamen length | mm | 0.309901 (32) | –0.256705 (21) |
| v23 | Stamen width | mm | 0.696040 (8) | 0.239937 (24) |
| v24 | Distance of the widest part of stamen from its base | mm | 0.281805 (35) | 0.430680 (9) |
| v25 | Anther length | mm | –0.326339 (30) | **0.555285 (1)** |
| v26 | Anther bending | degree | **0.897960 (1)** | –0.063472 (42) |
| v27 | Number of carpels | number | **–0.843275 (2)** | 0.055534 (45) |
| v28 | Gynoecium length (height) | cm | –0.387066 (27) | –0.135167 (36) |
| v29 | Ovary length (height) | cm | –0.337941 (29) | –0.062359 (43) |
| v30 | Stigma length (height) (v28–v29) | cm | 0.646149 (10) | –0.236619 (25) |
| v31 | Ovary (= gynoecium) width | cm | –0.593565 (15) | 0.038417 (47) |
| v32 | Stigma width | cm | **–0.732438 (6)** | 0.183488 (32) |
| v33 | Length of stigma projection | mm | –0.198461 (42) | 0.246801 (22) |
| v34 | Width of stigma projection | mm | **–0.793353 (3)** | 0.113898 (38) |
| v35 | Number of stamens potentially filling the gap at the top of the ovary | number | 0.761477 (5) | 0.012685 (52) |

| No. | Character description | Unit | Can 1 | Can2 |
|---|---|---|---|---|
| Ratios | | | | |
| v36 | Leaf lamina length/width (v1/v4) | | 0.360412 (28) | **0.530439 (3)** |
| v37 | Midrib length / leaf notch length (v2/v3) | | * | * |
| v38 | Midrib length / leaf lamina length (v2/v1) | | –0.278958 (36) | –0.018743 (49) |
| v39 | Distance of the widest part of leaf lamina from lamina tip / lamina length (v5/v1) | | –0.248325 (38) | –0.041271 (46) |
| v40 | Distance of the widest part of leaf lamina from lamina tip / midrib length (v5/v2) | | 0.058079 (51) | 0.036213 (48) |
| v41 | Maximum distance between lobe axis and main lobe vein / maximum distance between lobe axis and lobe margin (v7/v8) | | 0.091194 (49) | **–0.509065 (4)** |
| v42 | Leaf lobe width / lamina length (v9/v1) | | 0.564681 (16) | 0.203880 (27) |
| v43 | Leaf lobe width / length of leaf notch / (v9/v3) | | * | * |
| v44 | Distance of the widest part of leaf lamina from lamina tip / leaf lobe width (v5/v9) | | * | * |
| v45 | Maximum distance between lobe axis and lobe margin / length of leaf notch (v8/v3) | | 0.325765 (31) | 0.391368 (12) |
| v46 | Maximum distance between lobe axis and main lobe vein / length of leaf notch (v7/v3) | | 0.619114 (14) | 0.016986 (51) |
| v47 | Sepal length / sepal width (v15/v16) | | –0.525567 (21) | 0.354063 (16) |
| v48 | Distance of the widest part of sepal from its base / sepal length (v18/v15) | | –0.495411 (22) | –0.094882 (39) |
| v49 | Sepal width at the base / sepal length (v17/v15) | | 0.551262 (18) | –0.059793 (44) |
| v50 | Sepal width at the base / sepal width (v17/v16) | | 0.258575 (37) | **0.493132 (6)** |
| v51 | Sepal length / petal length (v15/v19) | | 0.558063 (17) | 0.078431 (41) |
| v52 | Petal length / petal width (v19/v20) | | –0.246054 (39) | 0.080753 (40) |
| v53 | Distance of the widest part of petal from its base / petal length (v21/v19) | | –0.128796 (44) | –0.201221 (28) |
| v54 | Stamen length / stamen width (v22/v23) | | * | * |
| v55 | Stamen length / anther length (v22/v25) | | * | * |
| v56 | Gynoecium width / length (v31/v28) | | –0.639304 (11) | 0.271418 (20) |
| v57 | Ovary width / length (v31/v29) | | –0.630910 (13) | 0.157981 (34) |
| v58 | Stigma width / length (v32/v30) | | –0.470322 (24) | 0.380821 (14) |
| v59 | Length/width of central stigma projection (v33/v34) | | **0.768744 (4)** | –0.002369 (53) |
| v60 | Width of stigma projection / stigma width (v34/v32) | | –0.714614 (7) | –0.018543 (50) |
| v61 | Length of stigma projection / stigma length (v33/v30) | | 0.056307 (52) | 0.476861 (7) |
| v62 | Ovary length / stigma length (v29/v30) | | 0.124951 (45) | 0.195709 (30) |
| v63 | Stigma width / ovary width (v32/v31) | | –0.693130 (9) | 0.335979 (18) |
| Qualitative colour characters | | | | |
| v64 | Colour of abaxial side of leaf lamina (0 – green, 1 – reddish, 2 – red) | | * | * |
| v65 | Colour of interior surface of sepals (0 – white, 1 – pink tinge, 2 – light pink, 3 – pink) | | * | * |
| v66 | Colour of petals (0 – pure white, 1 – pink tinge, 2 – pink) | | * | * |
| v67 | Colour of stigma (1 – yellow, 2 – reddish, 3 – red) | | * | * |
| v68 | Colour of carpellary teeth (1 – light yellow, 2 – deep yellow, 3 – orange, 4 – reddish, 5 – red) | | * | * |

distribution within the groups was not multivariate normal, non-parametric k-nearest-neighbour method was employed in the classificatory analysis. The discriminant power was determined by cross-validation. Several modifications of the discriminant analysis (e.g. all taxonomic groups, cultivars vs. native species including hybrids, parental species vs. natural hybrids, *N. alba* vs. *N. candida*) were performed. In addition to a pooled set of all morphological traits, leaf and flower characters were also analysed separately in order to discriminate sterile and fertile individuals, respectively.

## Results

*Variation in genome size*

The FCM analysis of 653 samples resulted in five distinct groups of holoploid genome sizes (Fig. 3). Disregarding two cultivars originating from the water lily collection at Průhonice ('Firecrest' and 'Virginalis') and one individual from northern Bohemia (loc. 49; Electronic Appendix 1) whose genome sizes overlapped those of *N. alba*, there were

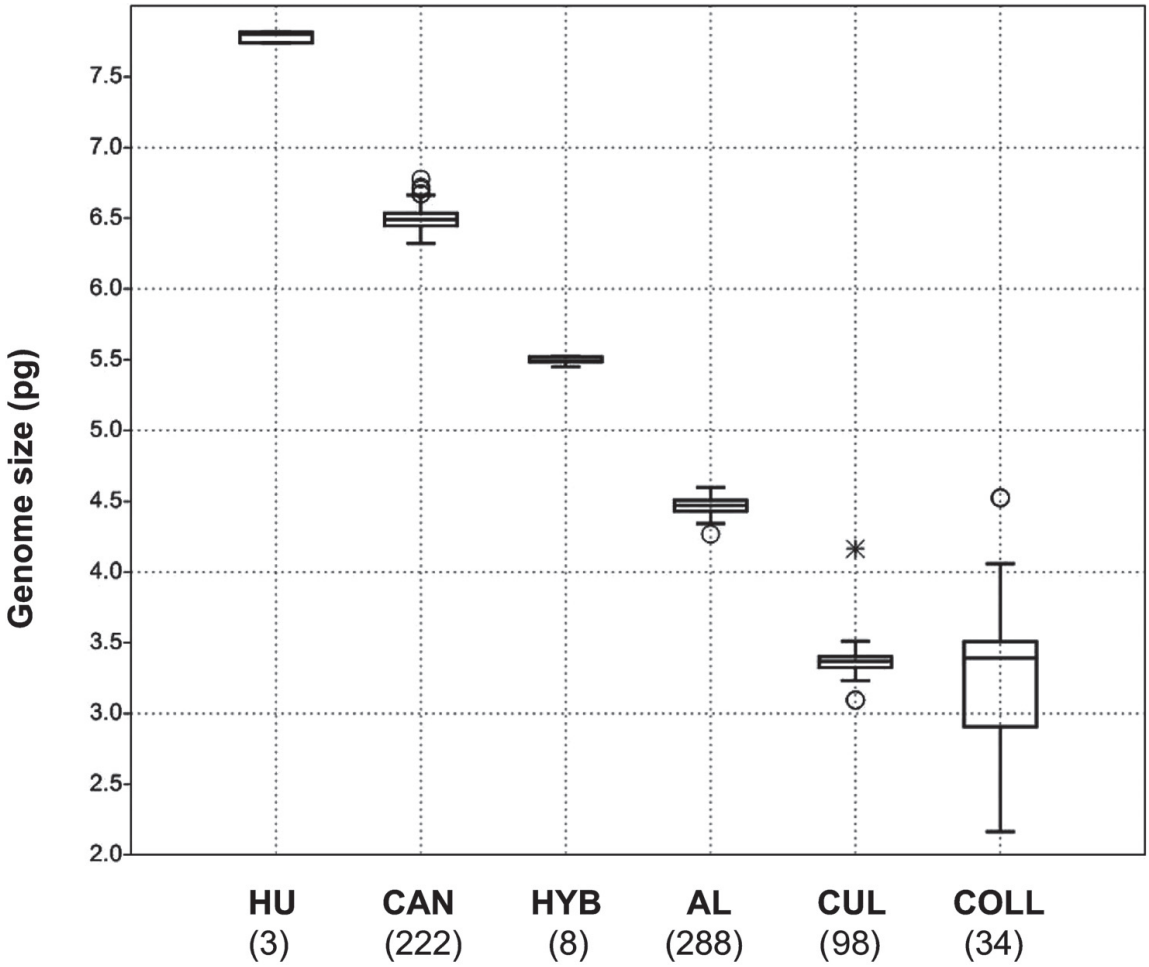



Fig. 3. – Box-and-whisker plots of the variation in 2C-values of six groups of *Nymphaea* samples, corresponding to *N. alba* (AL), *N. candida* (CAN), two types of interspecific hybrids that originated via two reduced gametes (HYB) and unreduced gamete of *N. alba* + reduced gamete of *N. candida* (HU), cultivars from natural habitats (CUL) and cultivars from a garden collection (COLL). Number of individuals analysed is given in parentheses.

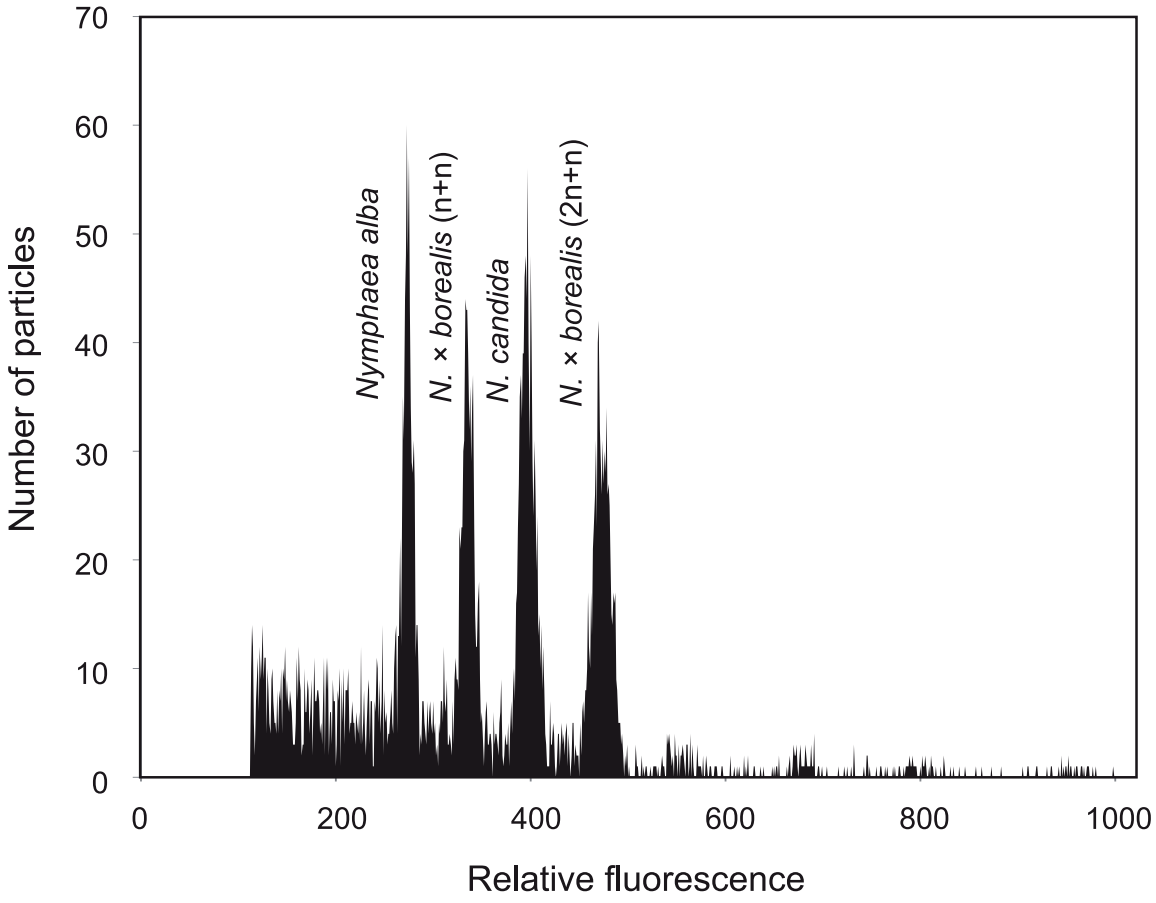
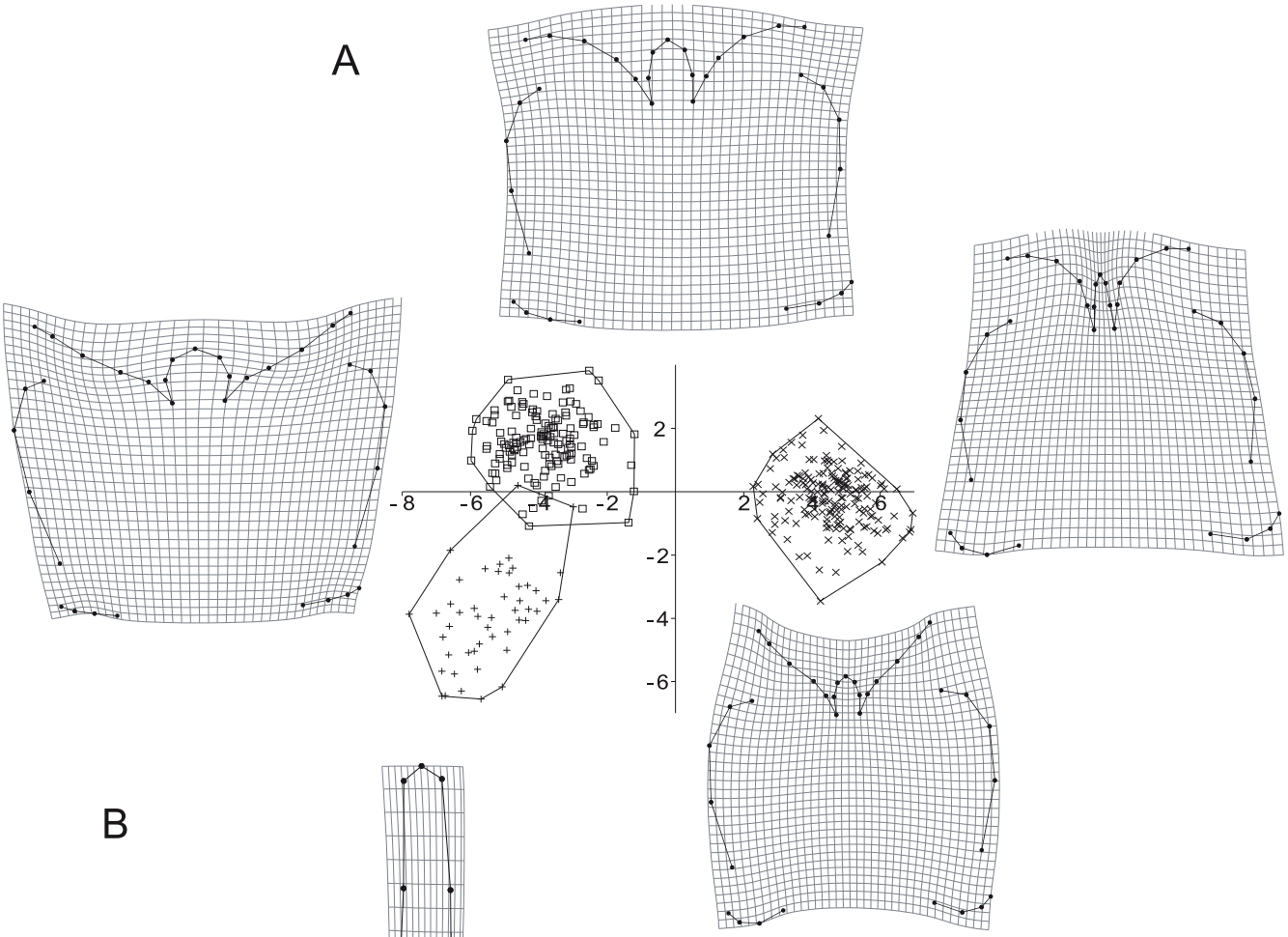



Fig. 4. – Histogram of the results of the flow cytometric fluorescence showing simultaneous analysis of DAPI-stained nuclei isolated from *Nymphaea alba*, *N. candida* and two types of interspecific hybrids (originating via two reduced gametes and 2n gamete of *N. alba* + n gamete of *N. candida*, respectively).

clear discontinuities in nuclear DNA contents. Average 2C-values of samples tentatively determined as *N. alba* (mean 2C = 4.47 pg) and *N. candida* (mean 2C = 6.50 pg) differed 1.45-fold, so both of these species could be reliably separated.

Eight samples from two sites in the Třeboň basin, southern Bohemia (ponds Fejmárek and Pohořelec; Electronic Appendix 1) otherwise occupied by *N. alba* had genome sizes intermediate between *N. alba* and *N. candida* and are classified as F1 interspecific hybrids. The greatest amount of nuclear DNA was recorded in three samples (which possibly represent only one individual) from the Skopaný pond in the same geographical region. These samples are interpreted as interspecific hybrids, originating by a syngamy of an unreduced gamete of *N. alba* and a reduced gamete of *N. candida.* While there was little variation in the 2C-values of both native species of *Nymphaea* studied and their crosses, the genome sizes of garden cultivars varied greatly, ranging from 2.16 pg/2C to 4.53 pg/2C (Fig. 3, Electronic Appendix 1). A simultaneous FCM analysis of both native *Nymphaea* species and two types of interspecific hybrids is shown in Fig. 4.

Of the 72 localities at which the occurrence of water lilies was confirmed, 17 were inhabited by *N. alba*, 25 by *N. candida* and 26 by cultivars. Sympatric growths of *N. alba* + *N.* ×*borealis* and *N. candida* + hardy cultivars were recorded at three and one site, respectively.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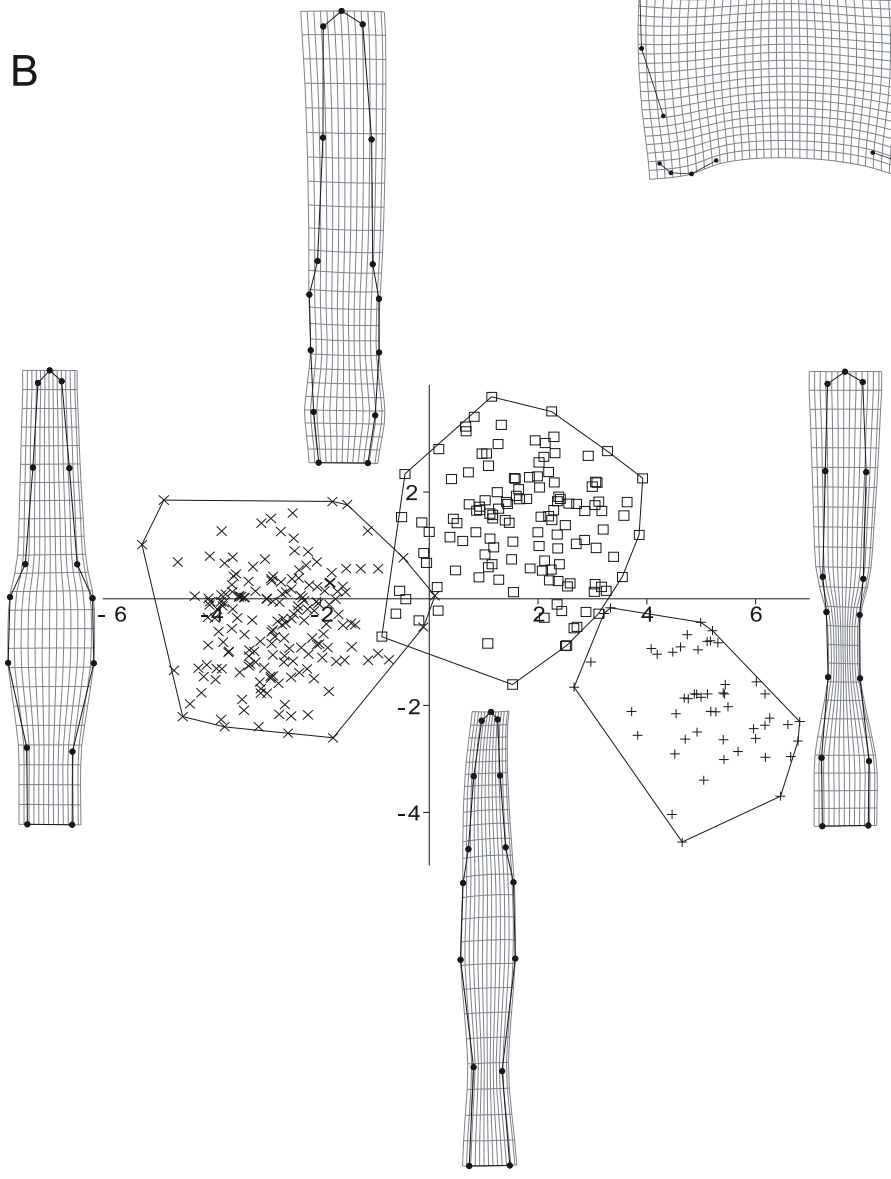
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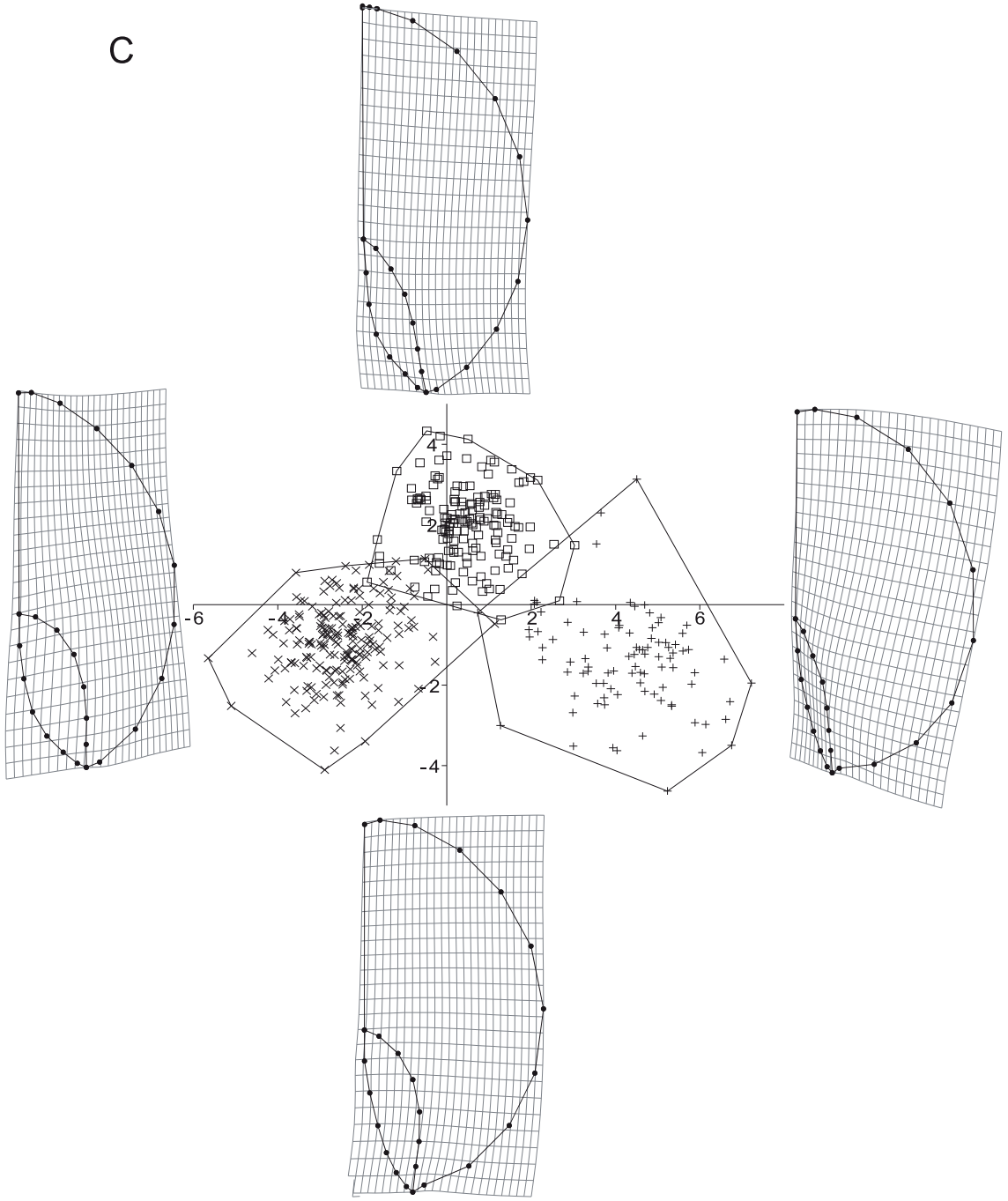


Fig. 5. – Results of the canonical discriminant analysis of the three taxonomic groups corresponding to □ *Nymphaea alba*, × *N. candida* and + garden cultivars using characteristics of the shapes of (A) gynoecium (median section), (B) stamens and (C) leaf lamina (right flank). The thin-plate spline deformation grids illustrate the changes in shape correlated with the canonical axes.

### *Geometric morphometrics*

The most pronounced differences in shape among the three groups of taxa (*N. alba*, *N. candida*, garden cultivars) were detected in the median section of the gynoecium (Fig. 5A) and the shape of inner stamens (Fig. 5B), which allowed correct classification of 99.5%

and 97.5% of individuals, respectively (Table 2). The leaf shape (Fig. 5C) had similar discriminating power (96.6% of individuals correctly classified; Table 2), whereas the shape of sepals and cup base were more similar among the taxa and their application resulted in the misclassification of about 15% of the samples analysed (Electronic Appendix 3). Of the six parts studied the shape of petals had the least taxonomic value (Electronic Appendix 3).

Table 2. – Results of classificatory discriminant analysis of *Nymphaea* samples assigned to the three taxonomic groups (two indigenous species and garden cultivars) using characteristics of the shapes of the gynoecium, stamens and leaves.

| | | Predicted group membership | | |
|---|---|---|---|---|
| Gynoecium shape (n = 365) | actual group membership | garden cultivars | *N. alba* | *N. candida* |
| | garden cultivars | 46 (95.8%) | 2 (4.2%) | 0 |
| | *N. alba* | 0 | 141 (100%) | 0 |
| | *N. candida* | 0 | 0 | 176 (100%) |
| Stamen shape (n = 355) | actual group membership | garden cultivars | *N. alba* | *N. candida* |
| | garden cultivars | 49 (96.1%) | 2 (3.9%) | 0 |
| | *N. alba* | 3 (2.3%) | 121 (93.1%) | 6 (4.6%) |
| | *N. candida* | 0 | 2 (1.1%) | 172 (98.9%) |
| Leaf shape (n = 435) | actual group membership | garden cultivars | *N. alba* | *N. candida* |
| | garden cultivars | 88 (93.6 %) | 6 (6.4 %) | 0 |
| | *N. alba* | 1 (0.7 %) | 149 (98.0%) | 2 (1.3%) |
| | *N. candida* | 0 | 6 (3.3%) | 183 (96.7%) |

The phenotype of garden cultivars was usually closer to *N. alba*, which is considered to be one of the parental species (Fig. 5, Electronic Appendix 3). Although the low number of hybrid individuals (10 for leaf characteristics and four for floral characteristics) precluded their inclusion in the discriminant analysis, PCA scatterplots indicated intermediate positions of most characters (Electronic Appendix 4). Average shapes of five taxonomically informative characters for the four groups recognized are illustrated in Fig. 6.

*Distance-based morphometrics*

Principal component analysis of 365 individuals, including 143 samples of *N. alba*, 169 samples of *N. candida*, four natural interspecific crosses and 49 garden cultivars, revealed three partially overlapping groups of OTUs (Electronic Appendix 5). Garden cultivars formed the most distinct cluster, while natural hybrids overlapped with *N. alba*. The main contributions to the first and the second PCA axes came from gynoecium and leaf characters, respectively (Electronic Appendix 5).

Discriminant analyses were employed to select a set of characters that gave the best separation of taxonomic groups, which were defined a priori on the basis of genome size data, and determine the proportion of correctly classified individuals. In total, ten characters (v2–v5, v21, v37, v43, v44, v54, v55; Table 1) were excluded from the discriminant analyses because of their high correlation (Pearson $r > 0.95$) with other characters. None of the floral characters was highly correlated with the ontogenetic stage of a flower.

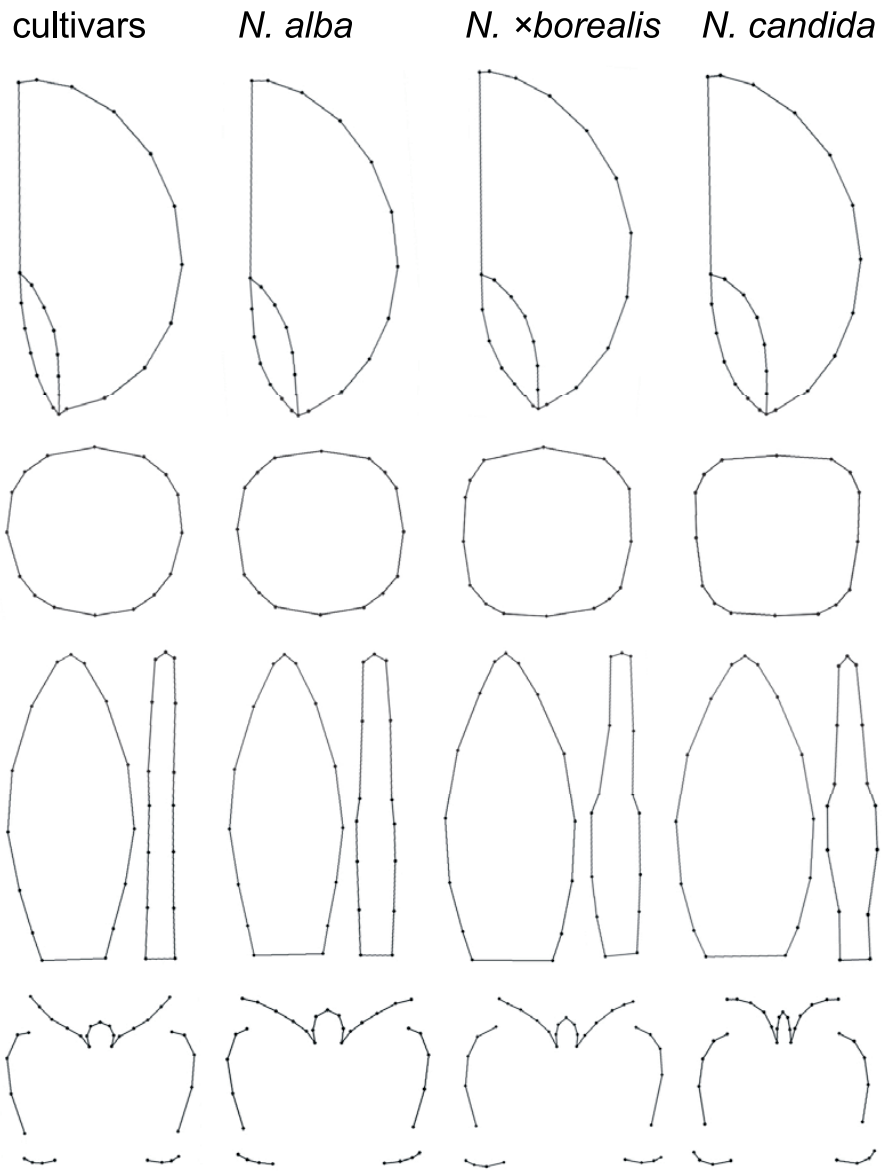


Fig. 6. – Mean shapes of five taxonomically informative characters in the four groups of *Nymphaea* samples recognized. From top: leaf lamina (right flank), cup base, sepal and stamen, median cross-section of gynoecium.

CDA of three groups (interspecific hybrids were excluded due to low number of individuals) using the remaining 54 quantitative and ratios of characters resulted in a complete separation of the groups and 100% of the individuals correctly classified (Fig. 7). Characters most closely correlated with the first canonical axis (separating *N. candida* from the group including *N. alba* and garden cultivars) were the degree of anther bending (v26), number of carpels (v27) and width of the stigma projection (v34), while anther length (v25), sepal width (v16) and leaf length/width (v36) contributed most to the division along the second canonical axis, which separated garden cultivars from *N. alba* (Table 1). The value of characters was further assessed by discriminant analyses of two groups of objects; our aim was to identify a small set of characters with the highest discrimination power and easy to use in determination keys. Major differences between native water lilies (both parental species

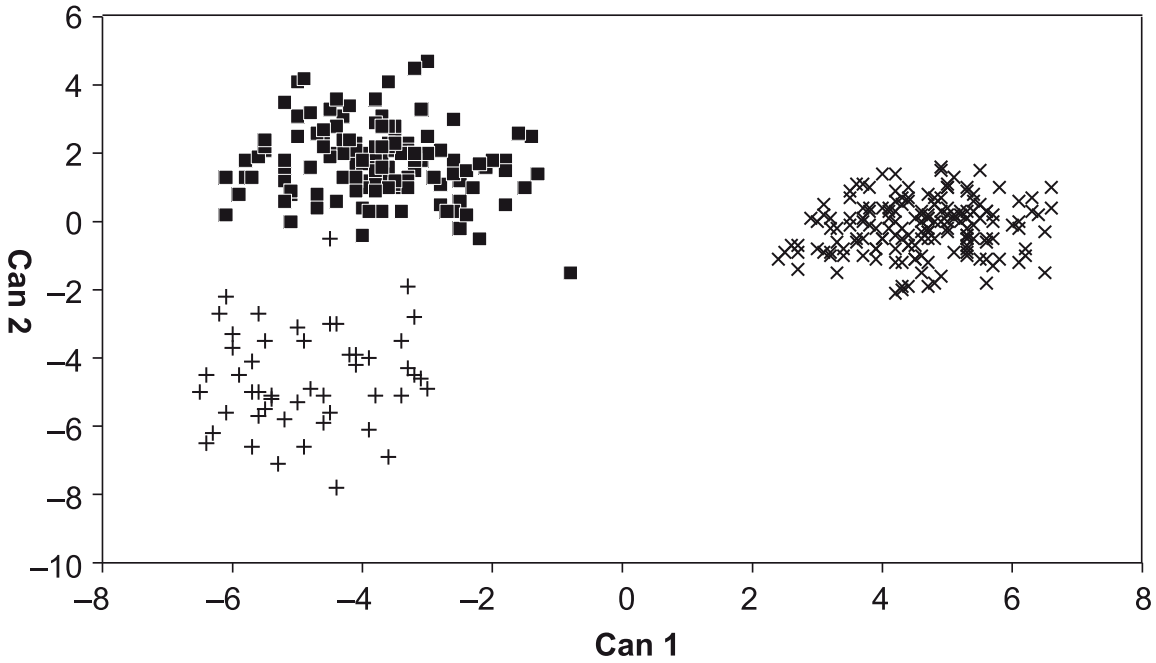


Fig. 7. – Results of the canonical discriminant analysis of the three taxonomic groups corresponding to *Nymphaea alba*, *N. candida* and garden cultivars using 53 quantitative characters (see Table 1). □ *N. alba* (n = 143), × *N. candida* (n = 169), + garden cultivars (n = 49).

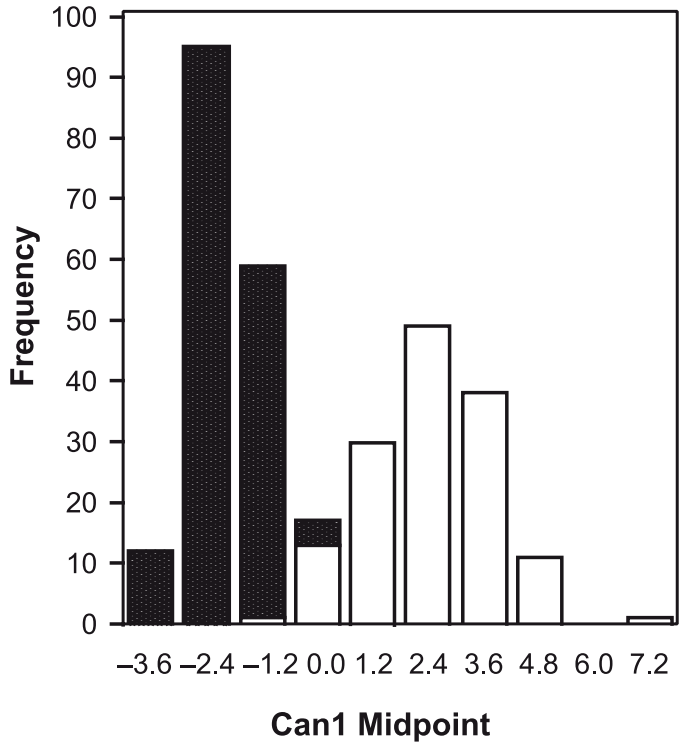


Fig. 8. – Results of the canonical discriminant analysis of *Nymphaea alba* and *N. candida* using the four quantitative characters with the highest discriminating power (degree of anther bending, number of carpels, width of the stigma projection, and stigma width). Only two individuals of *N. alba* were misclassified. □ *N. alba* (n = 143), ■ *N. candida* (n = 169).

and hybrids) and garden cultivars were found in leaf length/width (v36), petal size (both length – v19, and width – v20) and stamen width (v23); combination of these four characters resulted in 4.2% of the individuals being misclassified. Quantitative characters best discriminating *N. alba* and *N. candida* were largely similar to those identified by the CDA of three groups (see above) and included the degree of anther bending, number of carpels, width of the stigma projection and stigma width; their combination resulted in only 0.7% of the samples being misclassified (Fig. 8). Recognition of sterile individuals of *N. alba* and *N. candida* was less successful; a combination of five leaf characters with the highest discrimination power (v46, v44, v1, v42, v43; see Table 1) resulted in 7.4% of the individuals being misclassified (Electronic Appendix 6). Very small numbers of natural hybrids precluded meaningful discrimination from their parental species; nonetheless, in general, hybrids had larger sepals and petals, and their ovary and gynoecium heights were greater than those of their parental species.

### *Colours and pollen fertility*

Qualitative differences in colour are not suitable for morphometric analyses and were therefore assessed separately. Proportions of samples of a particular colour are summarized in Table 3. Colour of petals, stigma disc and carpellary teeth were identified as the most important taxonomic qualitative characters.

Pollen fertility of the garden cultivars analysed was very low (5–18%, n = 12), whereas most of the pollen of both native species was fertile (stainable) (99–100%, n = 12 and 97–100%, n = 12 for *N. alba* and *N. candida*, respectively). Pollen fertility of four individuals of *N.* ×*borealis* analysed varied considerably and ranged between 50–99%.

Table 3. – Proportions of individuals with five plant characters of a particular colour in the four taxonomic groups of *Nymphaea* recognized.

| Character | *N. alba* (n = 143) | *N.* ×*borealis* (n = 4) | *N. candida* (n = 169) | cultivars (n = 49) |
|---|---|---|---|---|
| Lamina undersurface | green (32.9%)<br>reddish (51.7%)<br>red (15.4%) | reddish (75%)<br>red (25%) | green (5.9%)<br>reddish (53.9%)<br>red (40.2%) | green (8.2%)<br>reddish (46.9%)<br>red (44.9%) |
| Inner surface of sepals | white (55.2%)<br>pink tinge (39.9%)<br>light pink (4.9%) | white (25%)<br>pink tinge (25%)<br>light pink (50%) | white (41.4%)<br>pink tinge (44.4%)<br>light pink (13.6%)<br>pink (0.6%) | white (14.3%)<br>pink tinge (26.5%)<br>light pink (34.8%)<br>pink (22.4%) |
| Petals | pure white (99.3%)<br>pink tinge (0.7%) | pure white (100%) | pure white (94.7%)<br>pink tinge (4.7%)<br>pink (0.6%) | pure white (34.7%)<br>pink tinge (24.5%)<br>pink (40.8%) |
| Stigma | yellow (100%) | yellow (50%)<br>reddish (50%) | yellow (8.3%)<br>reddish (62.1%)<br>red (29.6%) | yellow (83.7%)<br>reddish (12.2%)<br>red (4.1%) |
| Carpellary teeth | light yellow (22.4%)<br>deep yellow (77.6%) | deep yellow (25%)<br>orange (50%)<br>reddish (25%) | light yellow (0.6%)<br>deep yellow (5.3%)<br>orange (25.4%)<br>reddish (55.1%)<br>red (13.6%) | deep yellow (75.5%)<br>orange (24.5%) |

## Discussion

In this study, we assessed the variation in the morphology of water lilies occurring in the Czech Republic and identified taxon-specific characters. Unlike previous studies, which exclusively used subjective criteria for species delimitation (e.g. Muntendam et al. 1996, Wayda 2000, Volkova & Shipunov 2007, Nowak et al. 2010, Ejankowski & Małysz 2011), we used genome size, which is a more reliable way of assigning samples to a particular taxon.

### *The value of karyological data for delineating taxa*

Karyological variation is widespread in the plant kingdom and differences in ploidy level, number of somatic chromosomes and/or genome size may have detectable effects on phenotypic and/or reproductive traits (Levin 2002, Husband et al. 2013). Consequently, karyological data are often used as an important criterion guiding taxonomic delineation in plants (Stace 2000). While the accurate determination of the number of chromosomes is time- and labour-intensive and therefore impractical for large-scale population studies, genome size can serve as a proxy for chromosome numbers. The last decade has seen an increasing number of studies that used genome size data for taxonomic decision-making, including delimitation of species boundaries and detection of interspecific hybrids in both heteroploid and homoploid plant groups (Kron et al. 2007, Ekrt et al. 2010, Loureiro et al. 2010, Suda & Pyšek 2010).

Volkova et al. (2010) provide strong evidence that *N. candida* is an allopolyploid in which the genomes of *N. alba* and *N. tetragona* are combined and its relative genome size equals the sum of parental 2C-values. On average, genetically-confirmed samples of *N. alba* and *N. candida* from Russia and surroundings differ by 40% in their nuclear DNA amounts. We observed very comparable differences in genome size between typical morphotypes of both species collected in the Czech Republic. Fluorescence values of less certainly identified white-flowered water lilies usually matched genome sizes of either *N. alba* or *N. candida* and were therefore assigned to the corresponding species.

A few white-flowered individuals collected in situ possessed genomes with sizes either intermediate between those of *N. alba* and *N. candida* or substantially larger than that of the latter species. Although they were not readily identified by visual inspection in the field, FCM results clearly demonstrated their hybrid origin. We were unable to determine the exact number of chromosomes in putative natural crosses as we were unable to obtain rhizomes and our attempts to use young leaves failed. Nonetheless, we are convinced that hybridization is well supported by the genome size data as the differences between theoretical and actual 2C-values are only 0.6% and 1.2% for crosses originating by syngamy of two reduced gametes of parental species, and 2n gamete of *N. alba* + n gamete of *N. candida*, respectively. The available evidence indicate that most hybridization events are not accompanied by any dramatic changes in nuclear DNA content and genome sizes of hybrids can be simply inferred from the values for their putative parents (Kron et al. 2007, Loureiro et al. 2010). All hybrid individuals occurred as minorities in populations otherwise formed by *N. alba* in the Třeboň basin, which is one of the centres of water lily distribution (with the presence of both species) in the Czech Republic (see Fig. 1). Although the second parent (*N. candida*) has not been recently recorded at localities of *N.* ×*borealis*, it is very likely it grew there in the past (cf. floristic records of Laně 1981 and Kurka 1996).

All but two of the garden cultivars ('Firecrest' and 'Virginalis') from the water lily collection in Průhonice had distinctly smaller genomes than any native species. The cultivars with 2C-values similar to (or even overlapping) that of *N. alba*, however, were clearly recognizable on the basis of morphological characters (e.g. leaf shape and in the case of 'Firecrest' also lavender-pink flowers) and thus do not compromise the value of genome size data. The great majority of plants collected in the field for which a garden origin was suspected had small genomes (3.29–3.48 pg/2C) that fall within the range of C-values measured for cultivars. Despite the fact that the vast majority of cultivars investigated had genome sizes dissimilar to native species, our screening of garden plants was by no means exhaustive and it is possible that their variation may actually be more complex. The small genomes of garden cultivars are not surprising because exotic species that supposedly participated in their origin (e.g. *N. mexicana* and *N. odorata*) have lower 2C-values than their native European counterparts (Diao et al. 2006).

*Phenotypic variation and taxon-specific characters*

The last two decades have seen several attempts to find morphological characters that are reliable for identifying *Nymphaea* plants growing in (central) Europe (Neuhäusl & Tomšovic 1957, Tomšovic 1988, 1995, Muntendam et al. 1996, Wayda 2000, Volkova & Shipunov 2007, Nowak et al. 2010, Ejankowski & Małysz 2011). Perhaps the most comprehensive analysis of the phenotypic variation of the *Nymphaea alba-candida* complex is that done in the Netherlands (Muntendam et al. 1996). The authors report major interspecific differences in the dimension of the stigma projection, sepal width, stigma diameter, number of carpellary teeth and pollen characteristics. In addition, both species also differ in the shape of some organs, including that of fully opened flowers, cup base and/ or colour of stigma, carpellary teeth and undersurface of leaves. A morphometric study of material from the European part of Russia indicates that cup shape, filament shape of inner stamens, number of carpellary teeth and leaf position (floating or raised above the water surface) are the main diagnostic characters of *N. alba* and *N. candida*, but questions the relevance of pollen characteristics (Volkova & Shipunov 2007).

We built on these studies and assessed the value of both quantitative (using discriminant analyses) and qualitative (by calculating the proportion of samples with a particular state of the variable) morphological characters using karyologically verified samples of water lilies from the Czech Republic. In addition, we used geometric morphometrics to objectively quantify the variation in shape of particular generative and vegetative parts, an approach that has only rarely been used previously (Volkova & Shipunov 2007, Volkova et al. 2007). Our analyses confirmed the high discriminant power of gynoecium characters previously used for identifying *N. alba* and *N. candida*, including the number and colour of carpellary teeth, shape of stigma projection and dimension of stigma disc (Tables 2, 3, and Figs 5, 6). It is noteworthy that the interspecific differences in gynoecium characteristics were emphasised by J. S. Presl who described *N. candida* (Presl 1822, 1823). Shape of filaments of inner stamens (linear in *N. alba*, lanceolate in *N. candida*) is another species-specific character. Quite surprisingly, the best discriminating character in our analyses was the degree of anther bending (best seen in the median section of the flower; Electronic Appendix 7), which has never been previously considered to be taxonomically important. Determination of non-flowering plants is more challenging; our results indicate that the

most important clue is offered by the shape of the main vein of the leaf lobe, which is consistent with results of previous studies (Neuhäusl & Tomšovic 1957, Tomšovic 1988, Ejankowski & Małysz 2011). Additional support for the identification of native water lilies in the field can be provided by examining the overall habit of the plants. In accordance with some other authors (e.g. Volkova & Shipunov 2007), leaves of all the populations of *N. candida* in the Czech Republic analysed were flat and floating whereas those of *N. alba* occasionally emerged above the water surface and their margins bent upwards. The same was true for flowers (floating or partly submerged in *N. candida*, occasionally raised above the surface in *N. alba*; see also Muntendam et al. 1996, Nowak et al. 2010).

Our statistical analyses of clearly delimited species of *Nymphaea* challenged the value of some morphological characters traditionally used in determination keys. In particular, the shape of the cup base (round in *N. alba* vs rounded-quadrangular in *N. candida*) has been commonly used for distinguishing between species of *Nymphaea* (Neuhäusl & Tomšovic 1957, Tomšovic 1988, Muntendam et al. 1996, Volkova & Shipunov 2007, Nowak et al. 2010, Ejankowski & Małysz 2011). Although the cup base does show some interspecific differences in shape, these are quite difficult to grasp objectively and the incidence of intermediate morphotypes further blurs the picture. In comparison with other characters in which the variation in shape was assessed in our study (e.g. leaf venation pattern, filaments, gynoecium), the cup base yielded a distinctly lower proportion of correctly classified individuals in the discriminant analysis.

A specific challenge that accompanies the determination of *Nymphaea* plants in central Europe is a frequent in situ occurrence of garden cultivars. Although the correct recognition of cultivars can be as difficult as that of native species this issue has been completely neglected in previous studies. According to our analyses, garden cultivars can be distinguished by larger and usually distinctly coloured petals, filament dimensions and leaf shape characteristics, the most easily measurable of which is the leaf length/width ratio. Similarly to *N. alba*, leaves of cultivars occasionally emerge above the water surface. Although only a few populations of water lilies were examined for pollen fertility, this character may in some cases guide determination. The fertility of the garden cultivars analysed was dramatically low as the plants produced mostly sterile grains.

*Interspecific hybridization*

Morphotypes with intermediate values of characters and/or a mosaic-like combination of characters are usually interpreted as interspecific hybrids (Tomšovic 1988, Volkova & Shipunov 2007, Ejankowski & Małysz 2011), although this is not based on any evidence other than morphology. Some authors (e.g. Ejankowski & Małysz 2011) even report the prevalence of individuals identified as *N.* ×*borealis* over typical morphotypes of parental species. Our results, however, indicate that interspecific hybridization under natural conditions is quite rare (at least in the Czech Republic) and hybrid origin was confirmed for only eleven out of 619 cytotyped samples collected in situ (~1.8%). In addition to reduced gametes, unreduced gametes also participated in the origin of some hybrids, a situation which is not uncommon in interspecific hybridization (Mahelka et al. 2005, Krahulcová et al. 2011). The small number of natural crosses detected precluded a detailed assessment of their morphological variation. Nonetheless, on average, the floral parts of hybrids are larger, suggesting heterosis for these traits (Baack & Rieseberg 2007). Pollen fertility of

natural crosses varied considerably and were generally between the values recorded for garden cultivars and parental species. Reduced pollen fertility is also recorded in some other European *Nymphaea* populations (Heslop-Harrison 1955, Volkova & Shipunov 2007) and usually considered to indicate interspecific hybridization. Although pollen fertility seems to be taxonomically valuable, more intensive investigation using karyologically-proven samples is needed before any firm conclusions can be drawn.

*Determination key*

The following determination key is based on the results of both distance-based and geometric morphometrics; qualitative characters such as colour of plant organs were also considered. It should, however, be pointed out that the very small number of individuals of *N.* ×*borealis* included in this study makes determination of this natural interspecific hybrid uncertain. Values for quantitative characters are usually expressed as (minimum–) 5 percentile – 95 percentile (–maximum).

**1a** Filaments of the innermost stamens (4.4–) 4.6–6.6 (–7.0) times longer than wide, petals usually of different shades of pink (not pure white), (5.3–) 5.8–10.0 (–11.2) cm × (2.2–) 2.4–4.4 (–5.2) cm, leaf lamina length/width ratio (0.88–) 0.95–1.12 (–1.13) ..... **garden cultivars**

**1b** Filaments of the innermost stamens (2.1–) 2.6–4.8 (–5.6) times longer than wide, petals white or near white (rarely with a faint pinkish tinge), (2.7–) 3.8–6.9 (–8.4) cm × (1.4–) 1.6–3.2 (–3.9) cm, leaf lamina length/width ratio (0.98–) 1.04–1.21 (–1.25) ..... **2**

**2a** Sepals 7.5–9.5 cm × 3.1–4.2 cm, petals 6.0–8.4 cm × 3.2–3.9 cm, gynoecium 2.2–2.7 cm high, ovary 1.6–2.0 cm high, pollen fertility often low (usually < 75%) ..... ***N.* ×*borealis* Camus**

**2b** Sepals (2.9–) 4.3–7.9 (–9.3) cm × (1.5–) 1.9–3.5 (–4.2) cm, petals (2.7–) 3.8–6.9 (–8.2) cm × (1.4–) 1.6–3.2 (–3.7) cm, gynoecium (1.0–) 1.2–2.3 (–2.6) cm high, ovary (0.7–) 0.9–1.6 (–2.0) cm high, most pollen grains fertile (usually > 95%) ..... **3**

**3a** Anthers of the innermost stamens strongly bent [angle (83–) 89–135 (–144) degrees], their filaments with nearly parallel margins, carpellary teeth light to deep yellow, (10–) 12–22 (–24) in number, stigma disc yellow, (1.0–) 1.3–2.7 (–3.1) cm in diameter, stigma projection spherical to broadly conical, (1.6–) 2.0–5.2 (–6.3) mm wide, ovary covered up to the top by stamens (or scars of fallen stamens), primary vein on the leaf lobe only slightly bent in the proximal half, leaves and flowers occasionally emerge above the water surface .... ***N. alba* L.**

**3b** Anthers of the innermost stamens slightly bent to almost straight [angle (133–) 141–166 (–180) degrees], their filaments distinctly dilated in central part, carpellary teeth usually reddish to red, (5–) 7–13 (–15) in number, stigma disc usually orange to red, (0.6–) 0.7–1.6 (–1.9) cm in diameter, stigma projection narrowly conical, (0.4–) 0.6–2.5 (–3.5) mm wide, ovary not covered up to the top by stamens (or scars of fallen stamens), primary vein on the leaf lobe distinctly bent in the proximal half, leaves and flowers never emerge above the water surface ..... ***N. candida* J. Presl**

See http://www.preslia.cz for Electronic Appendices 1–7.

## Acknowledgement

The study was supported by Charles University in Prague (project GAUK 116710). Additional support was provided by the Academy of Science of the Czech Republic (long-term research development project no. RVO 67985939), institutional resources of Ministry of Education, Youth and Sports of the Czech Republic for the support of science and research, and project no. 14-36079G, Centre of Excellence PLADIAS (Czech Science Foundation). We thank V. Hříbal and Z. Kaplan for their valuable comments on the group under investigation, J. Neustupa and F. Kolář for introducing us to geometric morphometrics, and L. Beran, L. Hrouda, Z. Kaplan, P. Martinec, J. Prančl and J. Rydlo for their help in the field and/or providing some *Nymphaea* samples. Tony Dixon kindly improved our English.

## Souhrn

Přestože jsou lekníny (rod *Nymphaea*) ve střední Evropě zastoupeny pouze dvěma původními druhy (l. bílým – *N. alba* a l. bělostným – *N. candida*), jedná se o taxonomicky poměrně obtížnou skupinu. Jejich určování komplikuje vysoká morfologická proměnlivost v závislosti na podmínkách prostředí, časté přechodné morfotypy i předpokládaná mezidruhová hybridizace. Specifický problém představují záměrně vysazované či zplaňující zahradní kultivary. Pomocí průtokové cytometrie a mnohorozměrných morfometrických technik jsme hodnotili karyologickou a fenotypovou variabilitu 72 populací leknínů z území České republiky (pro srovnávací účely bylo do studie zahrnuto i 34 pěstovaných zahradních kultivarů). Spolehlivým determinačním znakem se ukázala být velikost jaderného genomu. Valná většina zahradních kultivarů vykazovala (výrazně) menší genomy než původní druhy. Množství jaderné DNA *N. candida* bylo v průměru 1,45násobné oproti *N. alba*. Na několika místech v jižních Čechách byly nalezeny rostliny (zhruba 1.8 % studovaných jedinců), jejichž velikost genomu odpovídala mezidruhovým hybridům (*N.* × *borealis*), přičemž na vzniku kříženců se podílely jak redukované, tak i neredukované gamety rodičovských druhů. Zpětní kříženci nebyli na základě dat o velikosti genomu zjištěni. Následná morfometrická analýza cytometricky ověřených jedinců umožnila vybrat soubor taxonomicky významných znaků. Jako nejvíce informativní se ukázal být tvar pestíku a tyčinek, určité mezidruhové rozdíly lze najít i na listech. Odlišení kříženců na základě makromorfologických znaků je problematické, nejlepším vodítkem bývá snížená barvitelnost pylu. Celkově studie odhalila vhodné determinační znaky obou původních druhů i pěstovaných kultivarů, a ukázala, že mezidruhová hybridizace je ve studovaném území poměrně vzácným jevem a nepředstavuje tedy významný ochranářský problém.

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Received 28 November 2013
Revision received 7 March 2014
Accepted 8 March 2014

# Continuous morphological variation correlated with genome size indicates frequent introgressive hybridization among *Diphasiastrum* species (Lycopodiaceae) in Central Europe

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**Abstract**

Introgressive hybridization is an important evolutionary process frequently contributing to diversification and speciation of angiosperms. Its extent in other groups of land plants has only rarely been studied, however. We therefore examined the levels of introgression in the genus *Diphasiastrum,* a taxonomically challenging group of Lycopodiophytes, using flow cytometry and numerical and geometric morphometric analyses. Patterns of morphological and cytological variation were evaluated in an extensive dataset of 561 individuals from 57 populations of six taxa from Central Europe, the region with the largest known taxonomic complexity. In addition, genome size values of 63 individuals from Northern Europe were acquired for comparative purposes. Within Central European populations, we detected a continuous pattern in both morphological variation and genome size (strongly correlated together) suggesting extensive levels of interspecific gene flow within this region, including several large hybrid swarm populations. The secondary character of habitats of Central European hybrid swarm populations suggests that man-made landscape changes might have enhanced unnatural contact of species, resulting in extensive hybridization within this area. On the contrary, a distinct pattern of genome size variation among individuals from other parts of Europe indicates that pure populations prevail outside Central Europe. All in all, introgressive hybridization among *Diphasiastrum* species in Central Europe represents a unique case of extensive interspecific gene flow among spore producing vascular plants that cause serious complications of taxa delimitation.

## Introduction

Hybridization among related taxa has a range of possible biological consequences: from the production of sterile offspring, through introgression of alleles into populations, to the formation of new entities [1,2]. According to ploidy level of participating parental accessions two types of hybridization are known – homoploid hybridization (equal ploidy level) and heteroploid hybridization (different ploidy level). Following text concerns only homoploid type.

Three different levels of homoploid hybridization may be distinguished. The most frequent is hybridization between two well delimited species (with developed hybridization barriers) producing sterile $F_1$ hybrids. On the other hand, evolutionarily younger and dynamic plant species often produce fertile hybrids that further contribute to the evolutionary dynamics of populations and lineages. The extreme form of hybridization is introgression (i.e. intense and repeated gene flow across a weak species border via numerous backcrosses), which can lead to a highly complicated situation with collapsed reproductive barriers, frequently manifested as reticulate hybrid swarms [1,3–6]. Finally, constant gene flow through introgression can result in confusing taxonomic patterns, threats of extinction of rare species *via* genetic erosion. It can, however, also lead to novel genotypes and changes in adaptive traits [7–10].

Introgressively hybridizing populations are known in many groups of angiosperms [1,11–13] but are very unusual in ferns (monilophytes), where F1 hybrids of sexual species are believed to be completely sterile in nearly all cases [12,14,15]. However, production of viable spores is thought to be relatively common in hybrids of the lycopod genera *Diphasiastrum* Holub, *Lycopodiella* Holub and *Lycopodium* L. [14,16,17]. Their hybrids are considered to be stabilized hybrids with normal meiosis. Backcrosses and introgressive hybridization have not been detected, and the stabilization of large clusters of hybrid shoots has been attributed to the strong cloning ability of these taxa [14,18]. Generally, such events may cause taxonomic confusion and numerous misinterpretations in practical determination (taxon identification) [19].

The genus *Diphasiastrum* Holub (*Lycopodium* sect. *Complanata* Victorin) with 20–30 species is the world's largest and taxonomically most complex group within the Lycopodiaceae family [17]. Its species are widely distributed across the Northern Hemisphere with several occurrences in tropical highlands. Their base chromosome number is generally accepted to be x = 23 and polyploidy (3x and 4x) is extremely rare (e.g. [14,18,20] []). Six diploid taxa are commonly recognized in Europe: three (basic) species – *D. alpinum* (L.) Holub, *D. tristachyum* (Pursh) Holub and *D. complanatum* (L.) Holub – and morphologically intermediate hybrids (formally labelled as intermediate species and currently treated as predominantly recent hybrids and, in some regions, isolated hybridogenous lineages) *D. ×issleri* (Rouy) Holub *(D. complanatum × D. alpinum)*, *D. ×oellgaardii* Stoor *et al. (D. alpinum × D. tristachyum)* and *D. ×zeilleri* (Rouy) Holub *(D. complanatum × D. tristachyum)* [18,20–24]. Especially complicated is the situation in regions where all six taxa co-occur. Mixed populations consisting of four, five or all six taxa have been detected in Central Europe, for example SE France (Vosges Mts; [23,25]), Austria (Bavarian forest; [26]), Germany ([26,27]) and the Czech Republic (Šumava Mts, Krkonoše Mts, Jeseníky Mts; [28–33]). In the rest of Europe, by contrast, *Diphasiastrum* taxa occur mostly allopatrically (e.g. [34–38]). From the point of view of practical determination, they are considered a taxonomically critical group [12,39,40]. In contrast to the thoroughly investigated populations from Western and Northern Europe, the putatively most complex Central European hybrid zone has not been studied in sufficient detail. European *Diphasiastrum* taxa are generally stress-tolerant plants that avoid high-competition habitats, especially unforested ones. Basic species occur either in tundra-type habitats (incl. alpine mountain zones) and occasionally in open forest sites [27,29,41]. On the contrary, hybrid taxa tend to occur in man-disturbed habitats such as periodically heavily disturbed ski slopes, timber storage places, forest glades, deforested strips and road margins, where basic species frequently co-occur [29]. Incidentally, all localities with sympatric occurrence of 3 and more taxa (both, basic and hybrid) are known from such type of habitat (e.g.

[29,41]). For example, *D. ×oellgaardii* has been described from a ski slope, a typical such secondary habitat, where it co-occurred with several other *Diphasiastrum* taxa [23,42].

A number of factors complicate investigations of hybridization patterns in *Diphasiastrum.* Members of this genus have a simple morphology with few discrete morphological features that can be evaluated [21,43,44]. Lycopods are also characterized by having two independent stages of life cycle – green, photosynthetic, diploid sporophytes (asexual generation) and underground, heterotrophic and long-lived haploid gametophytes (sexual generation). Sexual reproduction is restricted to gametophyte thus the hybridization is truly obscure and cryptic [16]. Also the mycorrhizal dependence of the gametophyte makes their spores difficult to germinate in controlled laboratory environments [45], and crossing experiments are virtually impossible to accomplish [24]. Large-scale *in situ* screening for various morphological, cytological or genetic traits thus seems to be the most achievable way to investigate gene flow and reproductive interactions within the group.

Various methodological approaches are available for studying introgressive hybridization (morphometrics, karyology, allozymes, microsatellites [1,4,46–49]. Flow cytometry represents a rather dated but still very efficient tool for the study of hybridization (including introgression), as it allows for rapid estimation of nuclear DNA content of large numbers of individuals [50]. DNA content is largely stable at the species level [51–59], and hybrid individuals can easily be detected by their intermediate genome size [60–64]. Although plant hybridization studies frequently employ morphometrics and flow cytometry, only a handful of them examine correlations between morphology and genome size using a large enough dataset subjected to a robust statistical evaluation [60,63,65]. Importantly, absolute genome size of diploid *Diphasiastrum* taxa has been demonstrated to be a taxonomically specific marker that allows detection of hybrid individuals [20,32,33].

The patterns of hybridization in *Diphasiastrum* have recently been addressed using two types of markers: low-copy nuclear genes and genome size. Sequences of three regions of nuclear genome (RPB2, LEAFY, LAMB4) confirmed the hybrid status of *D. ×issleri*, *D. ×oellgaardii* and *D. ×zeilleri* [18,24]. This study of a limited sample set also indicates that certain levels of recent hybridization and backcrossing exist within European *Diphasiastrum*, however, leaving unknown its frequency and variation patterns in natural populations. On the contrary, discrete variation in genome size in several parts of Europe indicates only primary hybridization with no hint of backcrossing (except for a few rare triploid hybrids) or introgression [20]. Nevertheless, as introgression leads to continuous patterns of variation in species traits (including genome size; [61], sufficiently large and carefully designed sampling is crucial for its discovery. It is thus possible that the levels of introgressive hybridization could have been underestimated because of the generally low number of individuals sampled (165), few samples per population studied (mean 1.62, range 1–9) and very limited sampling within the taxonomically most complex region of Central Europe, where all species co-occur [20].

In order to comprehensively evaluate the frequency and patterns of hybridization in the model lycopod group of *Diphasiastrum,* we conducted a study targeted at the taxonomically most challenging area of Central Europe using two independent markers for interspecific variation that allow large-scale screens: genome size and morphology (both numerical and geometric morphometrics). In one part of Central Europe (the Czech Republic and its immediate vicinity), we

exhaustively collected rich samples of all known populations. For comparative purposes, we also screened for genome size variation (and morphological features) within two other European regions (Scandinavia and the British Isles) with less complex and largely allopatric distribution of the species. We asked the following specific questions (i) Does genome size correlate with morphological variation? (ii) What is the pattern of morphological and genome size variation among *Diphasiastrum* individuals in Central Europe? Do the six taxa represent distinct morphological or cytological entities? (iii) Are populations from Central Europe uniform in their genome size and morphology, or do they rather consist of individuals that are variable in these traits? (iv) Is there any difference in the pattern of genome size variation between Central Europe and comparable areas?

## Material and methods

### *Sampling design*

We thank the administration of the Krkonoše and Šumava National Parks for granting permits to collect plants and the administrations of the Jeseníky and Beskydy National Conservation Areas for cooperation. Samples from the core Central European area were collected in 2007–2011 in the Czech Republic and adjacent countries (Supporting Information Table S1; i.e. 'Central European dataset'). In small and medium-sized populations (up to 30 individuals), all plants were sampled. In the case of three large populations (pop. no. 2, 13 and 22), a representative proportion of individuals equally covering the entire range of morphological variation was sampled. Because of high clonal ability of these taxa, we sampled 2 -5 distant plants of each morphotype. In total, 561 individuals from 57 populations (mean 10 individuals per population, range 1–56) were subjected to flow cytometric estimation of genome size. A majority of these plants (well developed and undamaged individuals) were also subjected to morphometric analyses (466/313 individuals from 55/49 populations for numerical/geometric morphometrics of Central Europe dataset and 57/51 individuals from 30/29 populations from Northern Europe, respectively). Within the Czech Republic, almost all known recent populations were sampled. As comparative material, 22 additional populations / 44 individuals were sampled in Scandinavia (Finland, Sweden, Norway) and 7 populations / 19 individuals in Scotland and Wales for estimation of genome size only (together hereafter referred to as the 'Northern European dataset'). Both datasets were treated separately in subsequent analyses.

Each population was localized using a GPS device (Garmin eTrex Legend; WGS 84). Rate of human disturbance was estimated and classified at each locality into four types: natural (sub/alpine zones, spruce forest etc.), sparsely disturbed (forest road margins), irregularly disturbed (timber storage sites) and regularly disturbed (ski slopes and other deforested strips; see also Figure 3). One well developed and intact sterile shoot was sampled per each individual. Fresh material was used for flow cytometric analyses (FCM) and numerical and geometric morphometry. Each accession was crosscheck-determined (independently confirmed by two team members) following several determination keys and floras [40,66,67]. The dimensions of ventral, lateral and dorsal leaves and their size in relation to the stem were used as the most important diagnostic characters [20,66,67]. During determination of specimens, we first identified indisputable morphotypes of basic species and then classified intermediate accessions. However, several individuals combined characters of all involved species, so their final determination must be treated as doubtful. Still, these doubtful

individuals did not influence the results because even easily determinable basic species are represented by extremely high variation both in genome size and in morphology. This taxonomic determination served only to passively display taxa in ordination diagrams and was not used in any statistical analysis.

***Flow cytometry***

Absolute genome sizes (C-values; [68]) were determined using a Cyflow SL instrument (Partec GmbH, Münster, Germany) equipped with a green solid-state laser (Cobolt Samba, 532 nm, 100 mW). For sample preparation, a slightly modified two-step procedure using Otto buffers was adopted [69]. *Pisum sativum* cv. *Ctirad* (2C = 9.09 pg; [70] was used as the internal standard. Intact shoots together with an appropriate amount of standard tissue were chopped with a sharp razor blade in a Petri dish containing 500 µl of ice-cold Otto I buffer (0.1 M citric acid, 0.5% Tween 20; [71]. The suspension was filtered through a 42-µm nylon mesh and incubated for approx. 10 min at room temperature. Finally, the suspension was stained by a solution containing 1 ml of Otto II buffer (0.4M $Na_2HPO_4 \cdot 12\ H_2O$), β-mercaptoethanol (final concentration of 2 µl/ml), propidium iodide (PI) and RNase IIA (both at final concentrations of 50 µg/ml). Samples were stained for 5 min at room temperature and run through the flow cytometer. Isolated stained nuclei were excited with a laser beam, and the fluorescence intensity of 5,000 particles was recorded. Only histograms not exceeding a 6% coefficient of variance (CV) of G0/G1 peaks were analyzed further. The reliability of FCM measurements (i.e. between-plant differences) was repeatedly confirmed in simultaneous runs of *Diphasiastrum* accessions yielding distinct fluorescence intensities (i.e. resulting in furcate double peaks in FCM histograms; [72]. In order to cover a larger spatial scale, most of the samples (566, 91%) were measured at one time point only. Nevertheless, we checked for time stability of the measurements both over a short time period (three subsequent days, 52 samples) and over a long time period (once per month over three subsequent months, 6 samples). We did not count chromosomes because chromosome numbers of *Diphasiastrum* taxa were estimated many times with identical results (2n = 46; e.g. [14,18,73,74] and our interpretation of ploidy levels is in congruent with a previous flow cytometric study [20].

***Numerical morphometrics***

In order to examine morphological variation of Central European *Diphasiastrum* (and of Northern Europe for comparison), 16 characters (Table 1) were measured. Well developed sterile shoots and fertile branchlets (if present) were directly used for morphometrics. The characters measured included traits used for the determination of taxa [12,40,66,67,75–78]. The characters measured were especially focused on the leaf proportions of fresh sterile shoots as follows: ventral leaf length (VL) and width in the widest part (VW); lateral leaf length (LL), width in the widest part (LW) and width between single leaf axillae (LD); dorsal leaf length (DL) and width in the widest part (DW). The position among different parts of ventral, lateral and dorsal leaves were measured in 9 characters: (top of the lower leaf to the top of the upper leaf (VLU), top to base of the upper leaf (VBU), top of the lower leaf to the top of the upper leaf (LLU), top to base of the upper leaf (LBU), width between bases (SW), top of the lower leaf to the top of the upper leaf (DLU), top to base of the upper leaf (DBU), width of the shoot at the widest point (DLW), width of the shoot – width of the lateral leaf (DWL). Height of the plant was not included due to its pronounced environmentally conditioned plasticity (e.g. extreme plasticity among individuals of *D. alpinum* from exposed vs. shady sites;

Supporting Information Figure 1). Basic descriptive statistical parameters were computed for each of the characters using the UNIVARIATE procedure in SAS (ver. 9.1). The correlative relationship among the characters was investigated using Pearson's correlation and the non-parametric Spearman's rank coefficients to detect high correlations (>0.95) and avoid distortion of the multivariate analysis. A principal component analysis (PCA; [79]), based on a correlation matrix, was performed to reduce the multidimensional nature of the character space using Canoco for Windows 4.5 [80]. Genome size was passively projected into PCA diagrams using a local regression (loess) model. A redundancy analysis (RDA, [81] with a Monte Carlo permutation test (999 permutations) was applied to test the association between morphological variation and genome size, using Canoco for Windows.

***Geometric morphometrics***

Photographs (RGB color images – JPG) of adult well developed parts of the stem were taken using an Olympus C-7070 digital camera mounted on an Olympus SZX12 binocular microscope) to investigate the variation in the shape and position of leaves using the thin plate spline method with sliding semilandmarks [82,83]. The shape and leaf position of the dorsal and ventral part of the branch was assessed independently. For both the ventral and dorsal part of the stem, two adjacent nodes with corresponding leaves were chosen (9 landmarks/28 semilandmarks in the dorsal part and 9/28 in the ventral part, respectively; Figure 1). Due to branch symmetry, only one half of the structure was described by landmarks and analyzed. Landmarks were digitalized using tpsDig software [84]. Individual objects were superimposed by a generalized Procrustes analysis with sliding semilandmarks in tpsRelw ver. 1.49 [84]; for the scatter of superimposed landmarks). Then a relative warp analysis (RWA) was performed also using tpsRelw (α set to 0). The RWA scores were then visualized with the PCA procedure in Canoco for Windows, and genome size was projected using a local regression model. In order to assess the level and significance of covariation between the shape (represented as 34 shape coordinates) and genome size of the investigated plants, the two-block partial least squares (PLS) method [85] incorporated in tpsPls ver. 1.18 [86] was used. The PLS method reduces the dimensionality of the data by creating new linear combinations of variables (singular axes) that are calculated to maximize the covariation between two datasets [87], i.e. morphology and genome size in our case. A permutation test (999 permutations) was used to test whether the correlation along the singular axes was higher than would be expected by chance. This procedure allows extraction of a single axis of shape change that is most significantly correlated with changes in genome size [88]. In addition, the same method was also applied to assess the levels of covariation between the two geometric morphometric datasets (ventral and dorsal branch side).

***Spore abortion percentage***

The spore abortion percentage (Ab) was estimated in order to confirm the spore fitness of individual taxa. Spores were collected from morphologically typical individuals with developed spores of each taxon (ripe strobili are found only rarely in the field). The spore abortion percentage was estimated by counting the number of aborted spores in a random sample of 100 spores per plant. Spores were considered aborted when they lacked a protoplast or were collapsed [89]. Spores were investigated under a light microscope (Olympus CH30) under 100× magnification.

**Results**

Coefficients of variance (CV) of all obtained flow cytometric histograms did not exceed 6% (range 1.22–5.78%, mean CV = 2.93%; S.D. = ± 0.64). 2C-values of the Central European samples varied between 4.76 and 7.8 pg, mean 6.19, S.D = ± 0.92. Accessions of pure (single species) populations of basic taxa varied in *D. alpinum* 6.43 - 7.68 pg and *D. complanatum* 5.24 - 5.72 pg (unfortunately *D. tristachyum* did not occur in pure populations).North European plants ranged from 5.13 to 7.33 pg, mean 6.29, S.D = ± 0.89; plants with the smallest genome sizes assigned to *D. tristachyum* were absent within this region (Table 2). Fluorescence values of replicated measurements turned out to be highly stable for samples analyzed on three subsequent days (the maximum difference was 2% in 52 triplicates) and in three subsequent months (the maximum difference among analyses did not exceed 3% in any of the six sample triplicates) and thus met the standard criteria for reproducibility of FCM genome size measurements [69]. Importantly, absolute genome size of Central European individuals increased in a continuous fashion whereas North European plants split into three groups (two of them highly distinct; Figure 2). Absolute genome sizes of particular Central European basic species (cross-check determined) tended to differ (Figure 2), even though hybrids created a continuum of genome size values. The most intricate intervals of genome sizes were found in *D. ×issleri* and *D. ×oellgaardii*, which completely overlap. Their morphology overlaps too, see below. The situation in Scandinavia and the British Isles turned out to be less difficult compared to the Central European region. Intermediate taxa are less abundant there (*D. alpinum* and *D. complanatum* dominate in Norway, Sweden and Finland). Nevertheless, *D. ×zeilleri* was found frequently in Finland.

We detected very low genome size variation in populations comprising a single taxon. These occur mostly in primary habitats and in areas with irregular or one-off disturbances, e.g. road margins and timber storage places. The highest variation in genome size was found in several populations that consisted of all six species and in mixed populations composed of *D. alpinum* and *D. tristachyum* (pop. 2, 19, 37, 13 and 22). Such populations occurred mostly at regularly disturbed sites, for example, ski slopes or other deforested strips (Figure 3 and Supporting Information Table S1).

No tightly correlated characters (i.e. with a correlation coefficient > 0.95) were found in the correlation analysis (CORR), so all vegetative characters were included in the multivariate analyses. The PCA analysis (Figure 4) revealed a different morphological trends of accessions independently assigned to the three basic species; *D. complanatum* was partly separated along the first axis (which tends to be positively correlated with VBU, LBU, LLU and also with VLU, DLU, DBU), while *D. alpinum* was well separated along the second PCA axis (which is negatively correlated with VBU, LBU and DBU but positively correlated with VL, LL and DL). The third axis partly separated *D. tristachyum* (this axis tends to be positively correlated with DLW). On the contrary, individuals assigned to hybrids overlapped with basic species or were scattered among them (Figure 4). But still mind the trend illustrative character of displayed morphologically established groups (cross-check determined). Importantly, genome size appeared to be well correlated with the second PCA axis (see the perpendicularly oriented loess curves in Figure 4). Unlike pattern (probably influenced by different habitat and taxa composition) showed PCA analysis of Northern Europe dataset (Figure 5, Supporting Information Figure 4). A significant association between morphology and genome size was further confirmed by RDA (p = 0.001, 999 permutations). The morphological characters VW, VL, DL, DW and

DL exhibited the strongest positive correlation with the canonical axis whereas the remaining characters were correlated only weakly or not at all (Figure 6).

Variation in the shape of the ventral and dorsal side of the stem, respectively, showed a very similar pattern (significant strong covariation among ventral and dorsal shape singular axes detected by PLS, r = 0.73, P < 0.001), which strongly corresponded to the results of distance-based morphometrics. Again, the basic species were well-separated from each other (showing even better separation of *D. complanatum* and *D. tristachyum* than in distance-based morphometrics), accessions of hybrids form transitions among them (Figure 7 and Supporting Information Figure S3). The first singular axis of shape change and the first singular axis corresponding to genome size were significantly correlated for both the ventral and dorsal side of the *Diphasiastrum* stem (PLS, r = 0.67; P = 0.01 and r = 0.65; P = 0.01 for the ventral and dorsal side, respectively), and this covariation was in both cases significantly higher than expected by chance (Permutation test, P < 0.001; Figure 8). A vector projection of the deviation from the mean reference (Figure 9) provides a visual demonstration of how the shape changes along the axis of maximum covariation [90]. Along the singular axis corresponding to genome size (from *D. tristachyum* to *D. alpinum*), distances between leaves become shorter, and leaves become smaller. Because the Procrustes superimposition procedure separates shape variation from size variation, the pattern of the correlation between the amount of nuclear DNA and centroid size of objects could be assessed independently. In both the ventral and the dorsal dataset, centroid size was significantly negatively correlated with genome size (p = 0.001 in both datasets); however, the correlation was very weak (r = 0.18 and 0.17 for the ventral and dorsal side, respectively).

All taxa including basic species and hybrids usually formed developed spores (Table 2). The proportion of aborted spores varied between 0 and 8% with no obvious differences among species.

**Discussion**

Our study revealed a continuous pattern in both morphology and genome size among taxa of European *Diphasiastrum*. Importantly, variation in both distance-based morphological characters and in overall shape was strongly correlated with genome size. Although genome size might have a direct or indirect effects on various plant traits including morphology e.g. size of plant cells, seed or spore size, phenology; [90–96], we interpret the correlation in *Diphasiastrum* rather as a consequence of taxonomical heterogeneity within the dataset. Firstly, the patterns in morphology and genome size clearly matched the independent cross-check determination of the taxa (i.e. genome size corresponded to the taxonomical assignment). Secondly, the rather complicated morphological differences detected (e.g. in the position and shape of ventral and lateral leaves) can hardly be explained merely by the nucleotypic effect of genome size on plant traits (e.g. [94,95]). Finally, genome size has frequently been shown to be a neutral marker within closely related taxa complexes, discriminating individual taxa or clades rather than being a factor that directly influences traits of adaptive value (e.g. [61,62,64,97]. Caution should be taken when interpreting small differences in genome size [98,99]. We are nevertheless convinced that our results are not negatively influenced by methodological artefacts (e.g. influence of secondary metabolites, DNA degradation, instrumental shifts etc.; [69]. Firstly, low coefficients of variation were achieved which are

incompatible with the presence of interfering secondary metabolites. Secondly, genome size values measured from the same 58 samples over longer time periods (52 samples measured three times in one week and 6 measured once monthly over three months) also remained stable. Finally, simultaneously analyzed plants with distinct genome size values resulted in distinct peaks (Supporting Information Figure S2), which is considered the most convincing piece of evidence for genuine differences in nuclear DNA content [68]. A possible strong influence of aneuploidy could also be ruled out based on results of morphometrics, where absolute genome size explains a major part of the observed variation. In addition, our genome size values correspond to the results of an independent flow cytometric survey of the same taxa of *Diphasiastrum* [20]. Possibility of samples with different ploidy level occurrence is highly improbable due to generally lower range of genome size values. Potential triploids were refused via confrontation of genome size values with morphology.

*Introgression mirrored by continuous genome size variation*

The continuous rather than discrete pattern of variation detected both in morphology and genome size among Central European accessions suggests frequent introgressive hybridization among the basic *Diphasiastrum* species within this area. Although the genome sizes of individuals determined as basic species tend to be distinct (except for a slight overlap of these individuals with *D. tristachyum* and *D. complanatum*), genome sizes of individuals determined as hybrids (*D. ×issleri*, *D. ×oellgaardii* and *D. ×zeilleri*) create a continuum linking these distinct values. Gene flow among the basic species thus does not result in the formation of stable hybrid zones with sterile hybrids as is usually the case in ferns [10,100,101]. Instead, it seems that the populations investigated represent reoccurring hybrid zones with fertile hybrids probably forming hybrid swarms. The hypothesis of frequent backcrossing and consequent introgression in *Diphasiastrum* has already been suggested by molecular analyses (sequence data from three low-copy regions of the nuclear genome; [18]. It may also be supported by the complete fitness of hybrid spores (Table 2 and also [102]. Frequent gene flow among species is probably facilitated by intergametophytic mating, a prevalent phenomenon among homosporous lycopods [103].

Interestingly, such a continuum has not been found in other parts of Europe where the co-occurrence of the basic species is known to be rare. Markedly discrete genome size values were detected both in the Northern European dataset (i.e. Scandinavia and the British Isles; Figure 2) and in a previous flow cytometric survey in various parts of Europe [20]. This pattern together with the low frequency of the hybrid taxa in Northern and Western Europe [36,37] suggest that the frequencies of backcrossing within these areas are generally low although Aagaard (2009) indicated backcrossing at six sites scattered in Western Europe. The low frequency of hybrids outside Central Europe might be caused by fewer suitable habitats, i.e. secondary human-disturbed sites (see discussion below). Tundra and taiga habitats suit *D. alpinum* and *D. complanatum*. *D. ×zeilleri* was repeatedly found in Finland (even without the basic species present at localities). This might explain the taxonomic confusion surrounding *D. tristachyum* and *D. ×zeilleri* in several Floras of northeast Europe [33,77,104]. It should be noted, however, that a low number of samples (e.g. four from Northern Europe in [20] and 63 in our Northern European dataset vs. 561 from C Europe) or non-random sampling (i.e. selection of typical individuals) might have contributed to the underestimation of the introgression levels within these areas. More intensive screening outside Central Europe,

particularly targeted at taxonomically intricate populations, is needed to evaluate the overall levels of gene flow within *Diphasiastrum*. At the same time to obtain accurate quantitative characteristics of introgressants (e.g. parental combination, direction of hybridization, backcrossing rate) a suitable molecular marker should be adopted.

*Origin of hybrid swarm populations*

In Central Europe, morphological and genome size variation indicates that *Diphasiastrum* is distributed in a mosaic of (i) single species populations, (ii) simple mixed populations of typically two *Diphasiastrum* species and scattered hybrids, and (iii) highly complex populations (hybrid swarms) consisting of two or all three basic species and several hybrids (pops. No. 2, 13, 22, 39 and 54; Figure 3). Whereas the first two population types tend to comprise small numbers of individuals, populations of the third type are usually composed of numerous individuals (up to a hundred). At least two of the three basic species (*D. alpinum* and *D. complanatum*) form taxonomically pure stands with negligible intra-population variation in genome size, but hybrids were predominantly found at localities where they co-occurred with basic species, resulting in populations with higher variance in genome size (Figure 3). Importantly, populations composed only of hybrids were extremely rare. A mere four of these populations (18, 24, 47 and 48), which are obviously in decline because they consisted of not more than three individuals, were detected. This pattern of distribution indicates a polytopic and probably recent origin of the hybrid taxa. Each mixed population is likely a result of an independent hybridization event. This is also supported by distinct habitat preferences of pure vs. complex populations. While pure populations of basic species prefer open subalpine habitats (*D. alpinum*) or moderately disturbed open forest patches and forest margins (*D. complanatum*), morphologically and cytologically intricate populations tend to occur in man-made secondary habitats such as timber storage places and deforested strips. *D. tristachyum* is a special case because pure populations (in boreo-continental pine forest) are extremely rare (e.g. we haven´t recorded any vital one in Central Europe) and *D. tristachyum* predominantly occurs in mixed populations.

The most complex hybrid swarms occur almost exclusively in artificial habitats such as ski slopes. Our field experience supports the connection between the high rate of hybridization (reflected by enormous morphological variation) and human-influenced habitats (followed by the highest genome size variance on; Figure 3, Table 2 and Supporting Information Table S1). It is thus possible that human-induced habitat changes in Central Europe have brought together previously ecologically isolated basic species and thus largely promoted their hybridization. Similar cases of human-induced changes that lead to secondary contact of previously separated species, promoting their hybridization, have been documented, for example, in *Prunus fruticosa* vs. *P. cerasus* [105], *Viola lutea* subsp. *sudetica* vs. *V. tricolor* [4], *Senecio hercynicus* vs. *S. ovatus* [48], *Arctium lappa* vs. *A. tomentosum* vs. *A. minus* [49].

In Central Europe, *Diphasiastrum* represents a complex, highly variable group of closely related taxa that is still undergoing evolution. The vast variation of hybrids that exist in nature may act as a "hybrid bridge" necessary for the introgression of genetic material between taxa with the potential for adaptive evolution [100]. Hybridization and introgression of single genes controlling traits with adaptive potential may cause reproductive isolation and, consequently, speciation [106].

Additional research, such as molecular study testing model populations (simplified hybrid combination of 2 basic taxa - *D. complanatum* vs. *D. alpinum* and their hybrids), is needed to shed some light on the evolutionary potential of novel genotypes generated by homoploid hybridization in *Diphasiastrum*.

*Taxonomical consequences*

Our morphometric analyses confirm that absolute genome size correlates with morphological traits of particular groups of "taxa". The most intricate pair of taxa turned out to be *D.* ×*issleri* and *D.* ×*oellgaardii*, which overlapped in all analyses (incl. the PLS analysis, which confirmed separation tendencies in other taxa groups; Figure 2 and Figure 8). Surprisingly, the absolute genome size interval of *D.* ×*oellgaardii* is shifted towards *D. alpinum* rather than being scattered around the mean value of its putative parental species. This shift may mirror more frequent backcrossing with *D. alpinum* or the participation of *D.* ×*issleri* in the hybridization (for a discussion of the possible occurrence of trihybrids, see [107]. The more complex origin of *D.* ×*oellgaardii* may be supported by the fact that *D.* ×*oellgaardii* (compared to other hybrids) never occurs without at least one of its parental taxa [22,26–28,108]. The position of *D. ×zeilleri* is less enigmatic because it does not overlap in its morphology and genome size with other putative hybrids. Moreover, its genome size is intermediate between its presumed parents although it strongly approaches that of *D. tristachyum.* Current taxonomic treatment doesn´t fully reflects the real variation in European *Diphasiastrum* group. Even though characters from recent floras and keys were used in cross-check determination, several misplaced individuals were displayed in outputs of statistical analyses. Such pattern probably reflects enormous morphological plasticity of *Diphasiastrum* (see also Supporting Information Figure S1).

The presence of backcrossing and introgression in populations blurs the delimitation of taxa in Central Europe. Additional information yielded from morphometric analyses confirms the limited applicability of determination keys. Successful determination is restricted to particular regions where taxa do not frequently co-occur (e.g. Scandinavia, the British Isles and possibly parts of Western Europe). In Central Europe, the group is immensely intricate. In agreement with molecular investigations of [18], we reckon that only the so-called basic species should remain treated at the specific level and that all hybrid plants should be regarded as recent neohybrids occurring primarily in microevolutionarily active regions.

**Acknowledgements**

We thank A. Pavlíčko and D. Půbal for collecting plant material in the field and the administrations of the Krkonoše and Šumava National Parks for granting permits to collect plants. We are also grateful to R. Štencl and M. Popelářová who collected a number of samples in the field and the administrations of the Jeseníky and Beskydy National Conservation Areas for cooperation. Our thanks also go to J. Harčarik and R. Hlaváček for providing locality information and to H. Chudáčková and J. Malíček for plant material from Northern Europe.

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**Figures:**

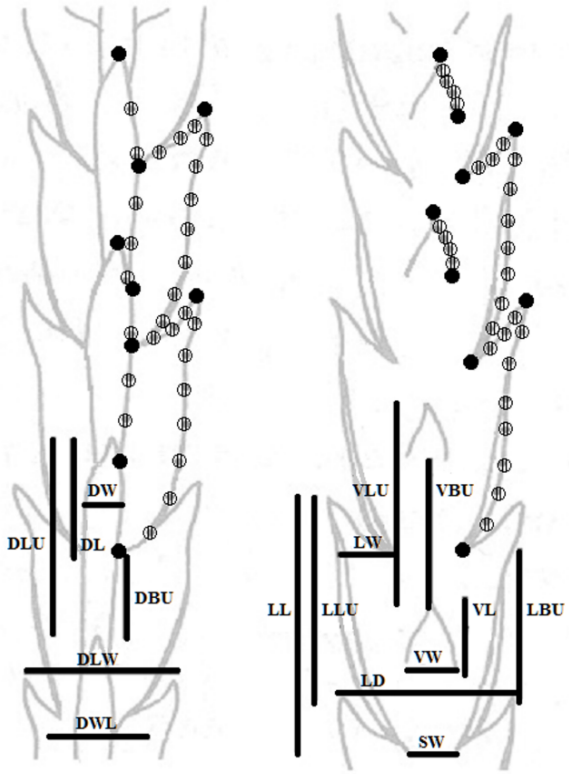


**Figure 1: Characters used in morphometric analyses.**

Characters localized on the ventral and dorsal side of the stem of *Diphasiastrum* taxa. The lines indicate variables measured for numerical morphometrics; the points denote landmarks (●) and sliding semilandmarks (◍) used in geometric morphometry.

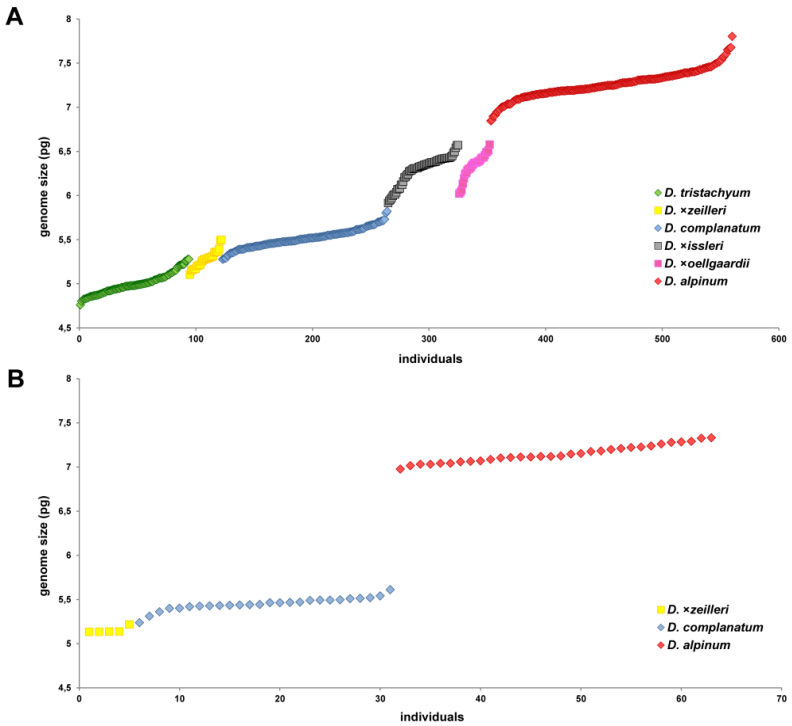


**Figure 2: Distribution of absolute genome sizes of *Diphasiastrum* samples.**

Absolute genome sizes of *Diphasiastrum* individuals assigned to six European taxa in Central (A; 561 individuals, range 4.73–7.80 pg) and Northern (B; 63 individuals, range 5.13–7.33 pg) Europe. Different colors denote species as independently cross-check determined using several regional keys and floras (i.e. a passive illustrative projection; see Methods for details).

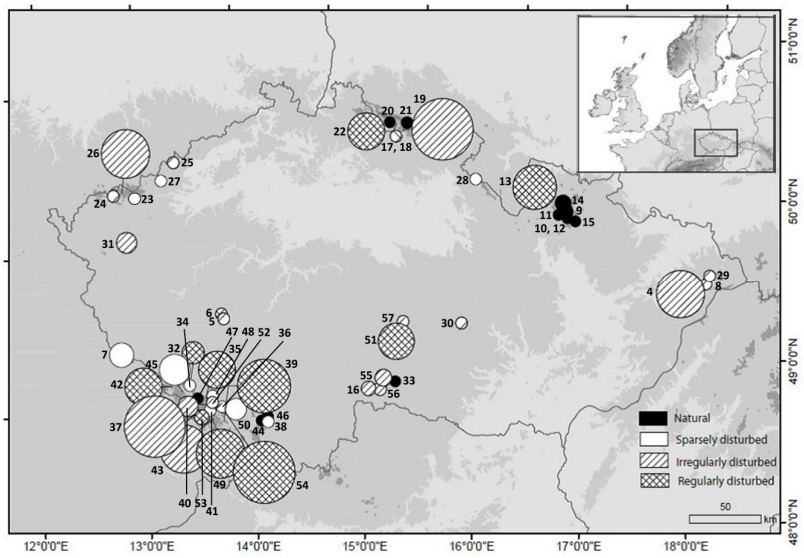


**Figure 3: Sample localities of *Diphasiastrum* taxa in eastern Central Europe.**

The size of the symbols is proportional to variance in genome size of individuals within populations (i.e. roughly corresponding to the taxonomic complexity of populations); the color pattern reflects different habitats occupied by the populations.

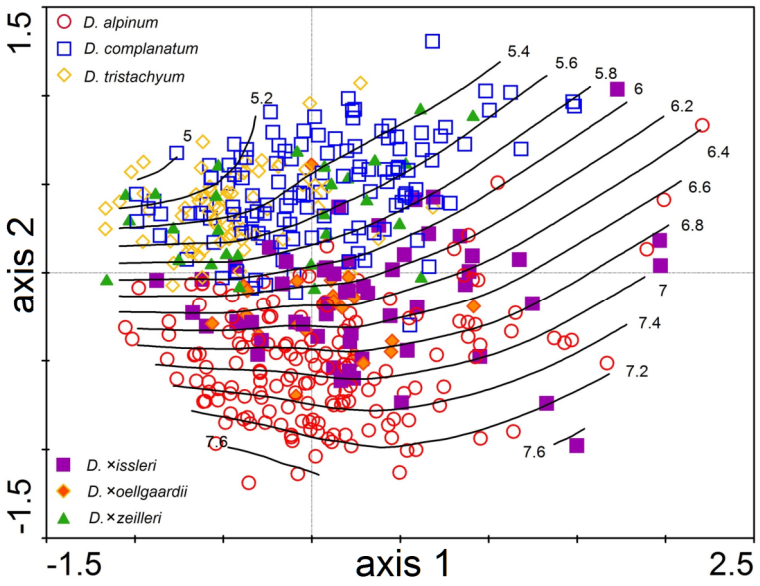


**Figure 4: Principal component analysis of *Diphasiastrum* taxa.**

PCA of 466 individuals from Central Europe based on 16 vegetative morphological characters (the first and second ordination axis explain 33.4% and 27.2% of total variation, respectively). Genome size (values in pg DNA) is passively projected in the diagram using a local regression (loess) model. Individual accessions are designated by different symbols based on their independent taxonomic determination according to regional keys and floras (i.e. a passive illustrative projection).

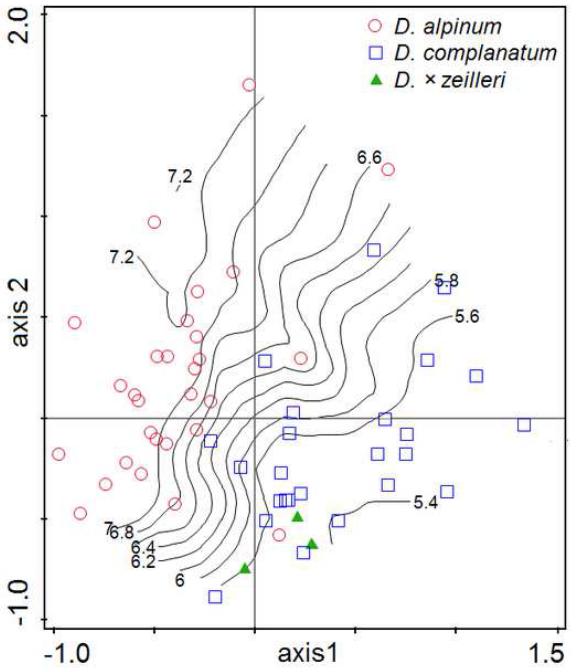


**Figure 5**: **Principal component analysis of *Diphasiastrum* taxa from Northern Europe.**

PCA of 57 individuals based on 16 vegetative morphological characters (the first and second ordination axis explain 29.0% and 23.9% of total variation, respectively) illustrates different pattern

of morphological variation in Northern Europe. Genome size (values in pg DNA) is passively projected in the diagram using a local regression (loess) model. Individual accessions are designated by different symbols based on their independent taxonomic determination according to regional keys and floras (i.e. a passive illustrative projection). Outlaying *D. alpinum* accession (0.408, 0.198) is an example of extremely shaded ecotype (see also Supporting Information Figure S1).

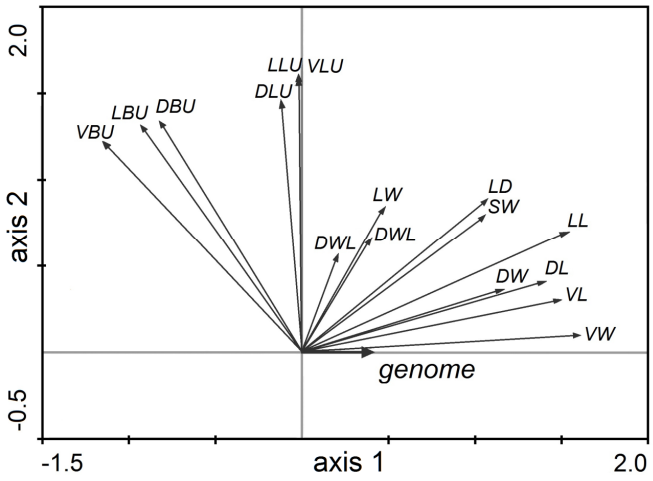


**Figure 6: Redundancy analysis.**

RDA showing the change in values of 16 vegetative morphological characters measured on 466 accessions of *Diphasiastrum* taxa along a gradient of genome size (for an explanation of the codes, see Table 1; the canonical axis (axis 1) explains 18.4%, and the first unconstrained axis (axis 2) explains 33.0% of total variation, respectively).

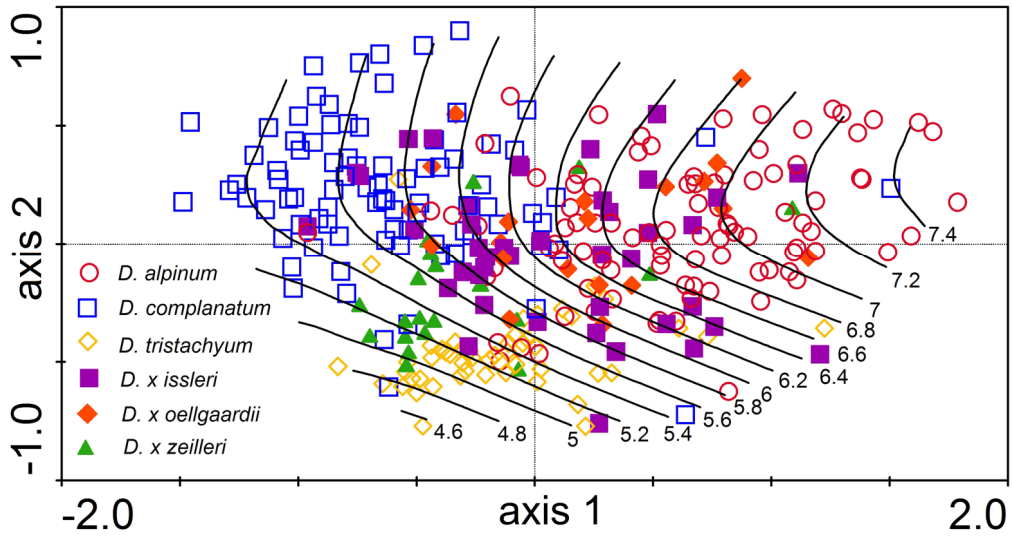


**Figure 7: Variation in the shape of the ventral side of the stem.**

Relative warp analysis of 313 *Diphasiastrum* taxa accessions based on 37 landmarks (the first and second ordination axis explain 45.7% and 12.2% of total variation, respectively). Genome size (values in pg DNA) is passively projected in the diagram using a local regression (loess) model.

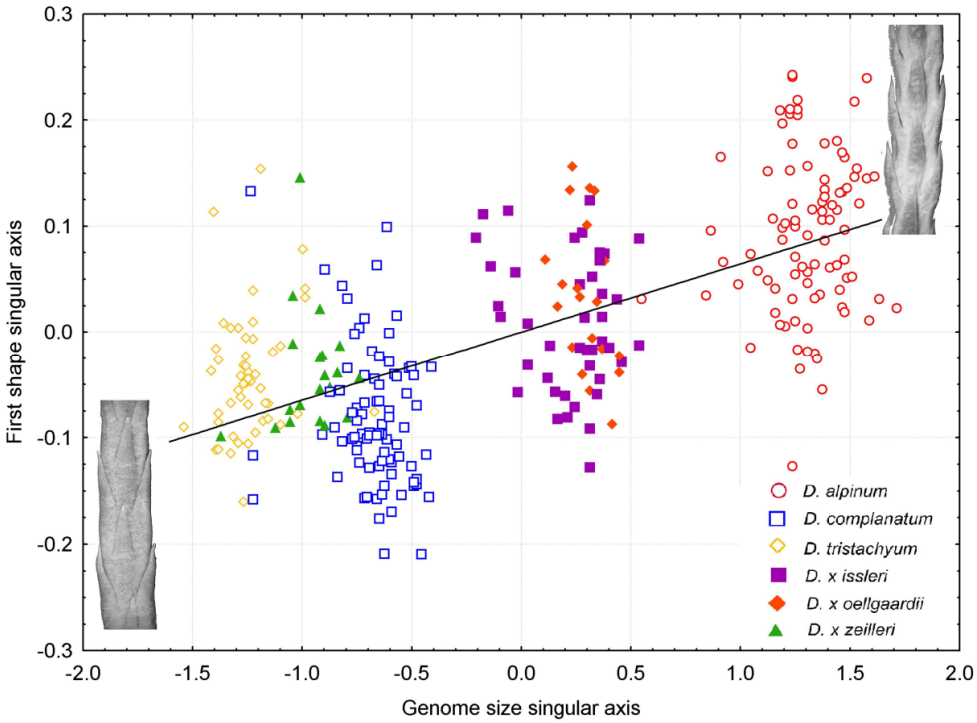


**Figure 8: A partial least squares correlation between the shape and genome size.**

PLS analysis of *Diphasiastrum* taxa (correlation coefficient is 0.67) confirmed correlation between the shape the ventral side of the stem and genome size. A taxonomic determination based on regional keys and floras is passively projected using differently colored symbols. Individual specimens are shown to highlight the shape at the upper and lower genome size extremes.

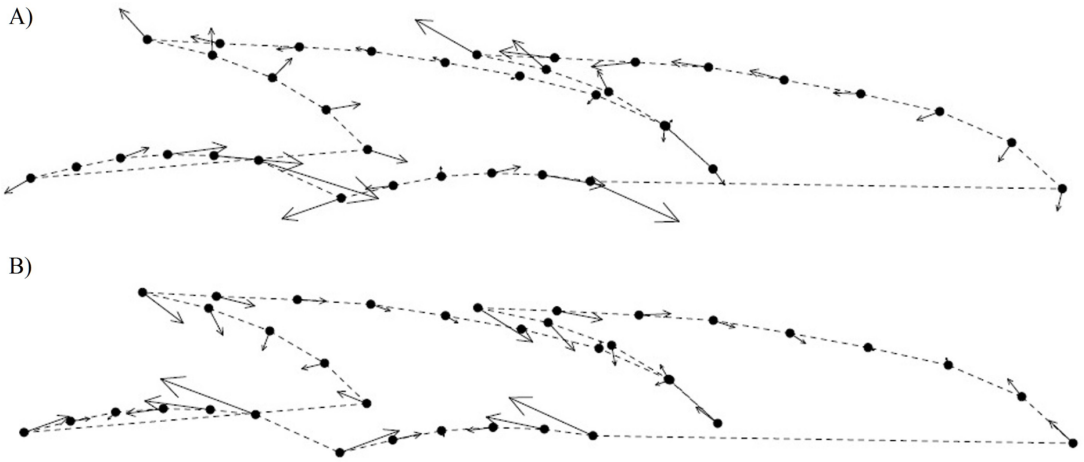


**Figure 9: Warp diagram of shape change depending on genome size.**

Warp diagram showing shape change in relation to increasing (A) / decreasing (B) genome size. Points represent landmarks of the sample with mean position on the genome size singular axis, and arrows represent the vector of shape change between the largest (A) and smallest (B) genome size values.

**Tables:**

**Table 1**: List of morphometric characters used in distance-based morphometric analyses.

| **Character number** | | **Character short** | **Character** |
|---|---|---|---|
| v1 | | GS | genome size |
| v2 | ventral leaves | VL | leaf length |
| v3 | | VLU | top of the lower leaf to top of the upper leaf |
| v4 | | VW | width in the widest part |
| v5 | | VBU | top to base of the upper leaf |
| v6 | lateral leaves | LL | leaf length |
| v7 | | LLU | top of the lower leaf to top of the upper leaf |
| v8 | | LW | width in the widest part |
| v9 | | LBU | top to base of the upper leaf |
| v10 | | SW | width between bases |
| v11 | | LD | width between single leaf axillae |
| v12 | dorsal leaves | DL | leaf length |
| v13 | | DLU | top of the lower leaf to top of the upper leaf |
| v14 | | DW | width in the widest part |
| v15 | | DBU | top to base of the upper leaf |
| v16 | | DLW | width of the shoot at the widest point |
| v17 | | DWL | width of the shoot - width of the lateral leaf |

**Table 2**: Range of absolute genome sizes with its average and spore abortion percentage of hybrids and basic taxa of *Diphasiastrum* under study.

| **Taxon** | **No. of sampled individuals** | **Average 2C value (pg) ± S.D.** | **2C values range (pg)** | **Ab (%) / GS of measured indiv. (pg)** |
|---|---|---|---|---|
| *D. tristachyum* | 95 | 5.00 ± 0.12 | 4.76–5.31 | 2/4.95; 8/5.09 |
| *D. zeilleri* (CE) | 28 | 5.27 ± 0.10 | 5.10–5.50 | 0/5.21 |
| *D. zeilleri* (NE) | 6 | 5.16 ± 0.05 | 5.13–5.24 | |
| *D. complanatum* (CE) | 142 | 5.51 ± 0.11 | 5.28–5.82 | 1/5.48; 0/5.62 |
| *D. complanatum* (NE) | 25 | 5.46 ± 0.06 | 5.31–5.61 | |
| *D. issleri* | 61 | 6.29 ± 0.16 | 5.91–6.57 | 2/6.31; 7/6.38 |
| *D. oellgaardii* | 27 | 6.33 ± 0.14 | 6.02–6.58 | 4/6.37; 2/6.46 |
| *D. alpinum* (CE) | 208 | 7.26 ± 0.16 | 6.84–7.80 | 0/7.65 |
| *D. alpinum* (NE) | 32 | 7.15 ± 0.10 | 6.98–7.33 | |

2C values - absolute genome sizes

Ab - average and spore abortion percentage
CE –Central European dataset
NE – North European dataset

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## Curriculum Vitae

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**Education**

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2006-2011: PhD student, Institute of Botany ASCR, Průhonice, Laboratory of flow cytometry (part time job)

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**Publications**

**SCI papers**

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Urfus T., Krahulec F., Vit P. & Kubešová M. (2009): Variation in Pilosella officinarum F. W. Schultz et Sch. Bip. in Central Europe: ploidy levels and their corelation with morphology. International Conference on Polyploidy, Hybridization and Biodiversity. Saint Malo, France. 17-20.5.2009.

Vít P., Suda J., Seifertová K., Kubešová M., Urfus T. (2009): Hybridization of *Cerastium alsinifolium* – caryological and morphological variation of serpentine endemic species. - International Conference on Polyploidy, Hybridization and Biodiversity. Saint Malo, France. 17-20.5.2009.

Slovák M., Vít P., Urfus T. & Suda J. (2008): Intraspecific genome size variation in *Picris hieracioides* L. - X$^{th}$ Symposium of the International Organisation of Plant Biosystematists. Vysoke Tatry, Slovakia. 2-4.7.2008.

Vít P. & Suda J. (2008): Threatened plants and management approaches – could flow cytometry help conservation programmes? - ISAC XXIV International Congress. Budapest, Hungary. 17-21.5.2008.

**Grant projects**

2007-2009: Významný český endemitní rožec *Cerastium alsinifolium* - evoluční historie, reprodukční úspešnost a důsledky křížení s *Cerastium arvense*. (GAAV, KJB601110709, project leader).

2007-2009: Introgresivní hybridizace u rožce kuřičkolistého (*Cerastium alsinifolium* Tausch): hrozí eroze genofondu význačného hadcového endemita? (GAUK, 29507/200, project leader).

**Awards**

2010: Cena Josefa Hlávky

2009: Cena Živy za nejlepší článek v kategorii 26-30 let - Endemické rostliny českých hadců 1.- 3

2007: “Purkyňova cena” – Cena časopisu Živa za nejlepší popularizační článek (kategorie nad 30 let): Endemické jeřáby – perly mezi českými dřevinami.

**Organization membership**

since 2003: Czech botanical society