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Pollen viability and natural hybridization of Central European species of *Cirsium*

Pylová viabilita a přirozená hybridizace středoevropských pcháčů (*Cirsium*)

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Pollen viability was analysed causally between and within Central European *Cirsium* species and their hybrids to determine (i) how frequently hybrids are fertile and produce viable pollen; (ii) how the pollen viability of hybrids and their parents are related and how this is affected by the genetic distance between parents; (iii) how species promiscuity relates to species pollen viability; (iv) to what extent the pollen viability of a hybrid may predetermine its frequency in nature; (v) how the pollen viability of a hybrid and sympatricity of its parental species are related; and (vii) how the frequency of females in populations of gynodioecious species may affect the observed pollen viability. Altogether, the viability of 656,363 pollen grains was analysed using Alexander's staining (1185 flowers from 301 plants from 67 field populations of 13 pure species and 1693 flowers from 345 plants from 96 field populations of 16 natural hybrids). The particular characters potentially related with pollen viability were estimated using following methods: natural hybrid frequency and species interfertility (by herbarium data), genetic distance (by AFLP), sympatricity (in local scale based on herbaria and literature data; on a global scale using the similarity between digitized maps of natural ranges). The strengths of pre- or postzygotic isolation were estimated for hybridizing species pairs using geographical data and pollen viability analyses. All hermaphrodite plants of the *Cirsium* hybrids had viable pollen, generally at lower levels than those found in pure species. The pollen viability of a hybrid generally decreased with increasing genetic distance between the parents and when the parental species had lower pollen viability. The pollen viability was decreased in frequently hybridizing species where occasionally individuals of pure species morphology may show decreased pollen viability. In some instances these might represent some unrecognized hybrid backcrosses. In populations of gynodioecious species where females co-occurred, pollen viability (in hermaphrodites) was also lower, indicating some degree of inbreeding depression. Hybrids between sympatric species exhibited higher post-pollination isolation (decrease of pollen viability), which suggests that the reproductive isolation had been increased by natural selection (effect similar to the Wallace effect). The strength of the postzygotic barrier (based on pollen viability) was generally stronger than that of the prezygotic barrier (based on distribution overlap) in studied hybridizing species pairs.

Keywords: AFLP, genetic distance, gynodioecy, homoploid hybrids, inbreeding depression, introgression, prezygotic barrier, postzygotic barrier, reinforcement, reproductive isolation, species promiscuity, Wallace effect

Introduction

Hybridization and pollen viability

Interspecific hybridization is an important mechanism of species origin (Rieseberg & Willis 2007), but at the same time, the genetic disintegration of an established species via introgressive hybridization with a more abundant congener may eventually cause its extinction (Levin 2002). The existence of separate species is therefore conditioned on the presence of effective barriers that prevent inadvertent hybridization (Arnold 1997, Rieseberg et al. 2006, Rieseberg & Willis 2007). An important postzygotic barrier is the reduction or loss of pollen viability, which reduces the fertility of hybrids, preventing further backcrossing and disintegration of the parental species (Rieseberg et al. 1999).

The pollen (or seed) viability of interspecific F1 hybrids was studied intensively in the "pre-molecular" era of plant systematics by early experimental taxonomists and pioneers of plant biosystematics (Clausen et al. 1941, Gajewski 1957, Grant 1964, Vickery 1964). The purpose of these studies was to estimate the degree of reproductive isolation in hybridizing species complexes (syngameons sensu Lotsy 1925 and Grant 1981), i.e. the extent of evolutionary differentiation they reached during gradual speciation (see Briggs & Walters 1997: 270–283, and references therein). The degree of the isolating barriers observed in experimental crossings of congeneric or conspecific taxa in previous studies (cf. Clausen 1951, Vickery 1964, Allen et al. 1983) may therefore have provided useful information analogous to the modern phylogenies of taxa based on molecular markers. However, the results of artificial crossing were only rarely compared with pollen viability in spontaneous hybrid populations (e.g. Strandhede 1966: 90), where natural selection and competition may further affect the survival and fertility of the arising hybrids. Unfortunately, in hybrids sampled in the field, it is usually not possible to discriminate between the F1 and other classes of hybrids on the basis of morphology when differences between the putative parental species are small (Hardig et al. 2000, Minder et al. 2007).

The detection of decreased pollen viability or pollen sterility in a plant is commonly used in addition to molecular markers for the identification of spontaneous interspecific hybrids or hybridogenous species (e.g. Zonneveld & Van Iren 2001: *Hosta*, Barlow-Irick 2002: *Cirsium*, Zonneveld & Duncan 2003: *Agapanthus*, Randell et al. 2004: *Rubus*, Lihová et al. 2007: *Cardamine*, Rotreklová 2008: *Hieracium* subgen. *Pilosella*). Pollen viability can also provide a measure of the reproductive fitness of hybrids, i.e. their ability to serve as pollen donors in natural populations (Mráz et al. 2005, McKenzie et al. 2008, Mao et al. 2009), and it can also serve as an evaluation criterion of the post-pollination (postzygotic) barrier between species (Rieseberg et al. 1999, Freyre et al. 2005, Jarolímová 2005, Mráz & Paule 2006, Ackermann et al. 2008).

The comparative study of pollen viability and other aspects of fitness (seed fertility, frequency of hybrids in nature, etc.) combined with the quantification of differences between taxa within complexes of closely related species (i.e. differences in their distribution, ecology, phenology, pollinators, mating systems and pollen competition) should allow for the identification of most reproductive barriers that limit spontaneous hybridization and introgressive gene flow (Ramsey et al. 2003, Nosil et al. 2005, Lowry et al. 2008).

Several methods are used for evaluating the quality of intact pollen: (i) measurement of pollen size; (ii) vital staining for the presence of cytoplasm or enzymatic activity in pollen grains; (iii) analysis of the germinability of pollen in vitro or in vivo; (iv) estimation of the proline content in pollen; and (v) assessment of the ability of pollen to effect fertilization and seed set (Dafni & Firmage 2000, Kelly et al. 2002, Dafni et al. 2005). Although the last method listed is the most accurate with regard to pollen function in sexual reproduction, the faster and easier stainability tests can also give reliable estimates of the relative differences between particular species and hybrids when: (i) one type of dye is used in all analyses; (ii) staining conditions are standardized; and (iii) pollen is prepared at the same developmental stage, e.g. from unopened flowers just before anthesis (cf. Dafni & Firmage 2000, Dafni et al. 2005).

Cirsium as a model for the study of hybridization

The genus *Cirsium* (thistle) is composed of over 300 perennial, biennial, or, rarely, annual spiny species that are distributed in the northern hemisphere, spanning the subtropical to boreal latitudes. The highest species diversity is found in the mountains of southern Europe, the Caucasus region, Central Asia, western China, Japan and western North America (Meusel & Jäger 1992). Most *Cirsium* taxa are known to form interspecific hybrids. The highest hybridization intensity is found in Central Europe, where approximately 70 different spontaneous hybrids have been observed among 17 native Central European species (Fig. 1). Less intensive interspecific hybridization has also been documented in North America (29 hybrids/62 species; cf. Keil 2006) and Japan (21 hybrids/64 species; cf. Kadota 1995). The intensive interspecific hybridization found among Central European species makes this region attractive for the study of possible factors that limit hybridization, constituting reproductive isolation between some species pairs.

Compared to other genera with frequently hybridizing species (e. g. *Carex*, *Centaurea*, *Epilobium*, *Mentha*, *Verbascum*, *Viola* and *Salix*), the apparent morphological differences among Central European *Cirsium* species facilitate the identification of the intermediate habitus of hybrids (Figs 2, 3). *Cirsium* hybrids usually occur alone or in small groups (up to 20 individuals) within populations of parental species. Typical hybrid zones and large local hybrid populations (common in the related *Centaurea*; Koutecký 2007) are rare in Central Europe (Bureš 2004). Despite the frequent hybridization, particularly within the type section of the genus, species show no evident morphological overlaps and their genetic integrity seems therefore well preserved.

Based on the frequency of spontaneous hybrids observed in Central Europe, the most promiscuous species is *Cirsium oleraceum*, followed, in decreasing order, by *C. canum*, *C. palustre*, *C. rivulare*, *C. acaule*, *C. pannonicum*, and *C. heterophyllum* (Fig. 1; Bureš 2003, 2004, Bureš et al. 2004); interspecific hybrids also seem to be frequent in the Alpine species *C. spinosissimum*, *C. erisithales*, *C. carniolicum*, and *C. montanum* (Wagenitz 1987, Bureš et al. 2004, Stöhr 2006; Fig. 1).

In Europe, Turkey, and East Asia, most *Cirsium* species are diploids with 2n = 34[1], while tetraploids (2n = 68) and hexaploids (2n = 102) occur much rarely (Talavera 1974,

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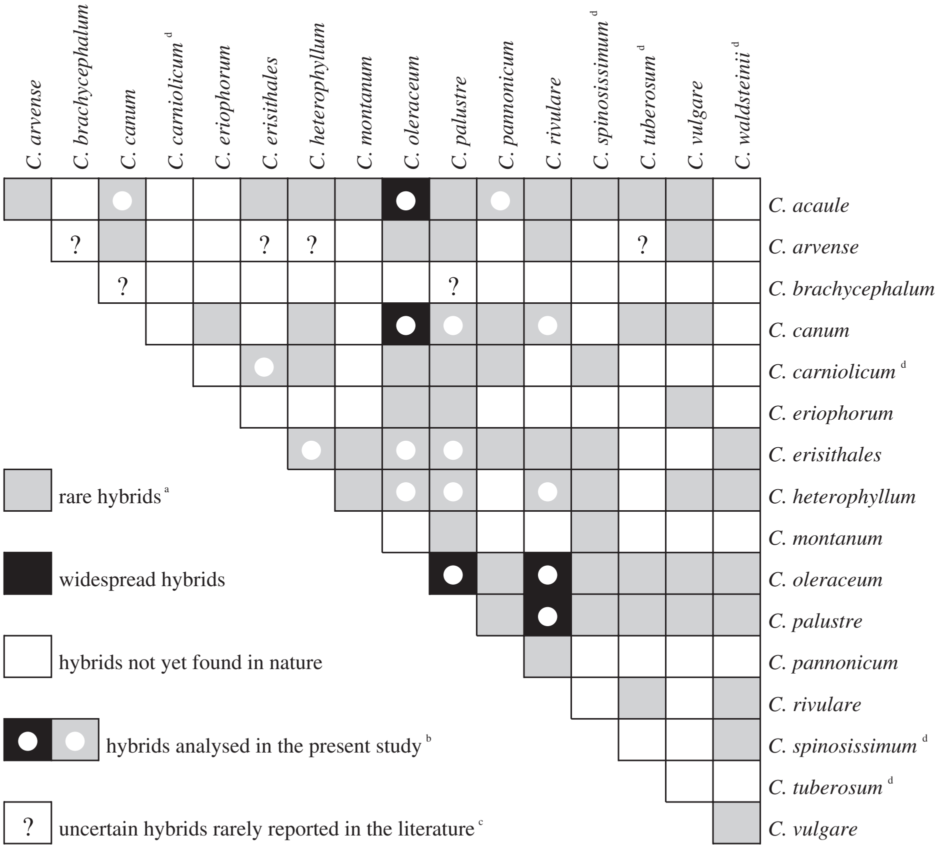
[1] In Europe, 2n = 34 has been reported in: *C. acaule*, *C. alatum*, *C. arvense*, *C. brachycephalum*, *C. candelabrum*, *C. canum*, *C. carniolicum*, *C. creticum*, *C. decussatum*, *C. dissectum*, *C. echinatum*, *C. eriophorum*, *C. erisithales*, *C. ferox*, *C. filipendulum*, *C. flavispina*, *C. glabrum*, *C. heterophyllum*, *C. heterotrichum*, *C. italicum*, *C. ligulare*, *C. monspessulanum*, *C. montanum*, *C. morinifolium*, *C. obvallatum*, *C. odontolepis*, *C. oleraceum*, *C. palustre*, *C. pannonicum*, *C. rivulare*, *C. scabrum*, *C. spinosissimum*, *C. tuberosum*, *C. valentinum*, *C. vallis-demonis*, and *C. welwitschii*; the tetraploid number 2n = 68 is rarer in Europe, reported for *C. appendiculatum*, *C. ciliatum*, *C. vulgare*, and *C. waldsteinii*.



	C. arvense	*C. brachycephalum*	*C. canum*	*C. carniolicum*[d]	*C. eriophorum*	*C. erisithales*	*C. heterophyllum*	*C. montanum*	*C. oleraceum*	*C. palustre*	*C. pannonicum*	*C. rivulare*	*C. spinosissimum*[d]	*C. tuberosum*[d]	*C. vulgare*	*C. waldsteinii*[d]
C. acaule	▨	□	▨●	□	□	▨	▨	▨	■●	▨	▨●	▨	▨	▨	▨	□
C. arvense		?	▨	□	□	?	?	□	▨	▨	□	▨	□	?	▨	□
C. brachycephalum			?	□	□	□	□	□	□	?	□	□	□	□	□	□
C. canum				□	▨	□	▨	□	■●	▨●	▨	▨●	□	▨	▨	□
C. carniolicum[d]					□	▨●	▨	□	▨	▨	▨	□	▨	□	□	□
C. eriophorum						□	□	□	▨	▨	□	□	□	□	▨	□
C. erisithales							▨●	▨	▨●	▨●	▨	▨	▨	□	□	▨
C. heterophyllum								▨	▨●	▨●	□	▨●	▨	□	▨	▨
C. montanum									□	▨	□	□	▨	□	□	□
C. oleraceum										■●	▨	■●	▨	▨	▨	▨
C. palustre											▨	■●	▨	▨	▨	▨
C. pannonicum												▨	□	□	□	□
C. rivulare													□	▨	□	▨
C. spinosissimum[d]														□	□	▨
C. tuberosum[d]															□	□
C. vulgare																▨

▨ rare hybrids[a]

■ widespread hybrids

□ hybrids not yet found in nature

■● ▨● hybrids analysed in the present study[b]

? uncertain hybrids rarely reported in the literature[c]

Fig. 1. – Natural hybridization in Central European species of *Cirsium* (according to Wagenitz 1987 and Bureš 2004; slightly modified).

[a] Parents rarely co-occur or parents co-occur frequently but hybrids are rare.

[b] Besides these hybrids, *C. erisithales* × *C. spinosissimum*, *C. heterophyllum* × *C. montanum*, and *C. pannonicum* × *C. rivulare* were also analysed, but only female plants without pollen were found in these samples.

[c] From Hungary by Soó (1970) and from Austria by Melzer & Barta (2000).

[d] Species not included in the present study. In addition, five samples of *C. carniolicum* and four samples of *C. spinosissimum* were analysed, but only female plants without pollen were found in these species.

Werner 1976, Kadota 1995, Bureš et al. 2004, Rotreklová et al. 2004, Ozcan et al. 2008). In contrast to these regions, North American species constitute almost the entire dysploid series, ranging from the predominant 2n = 34 to 2n = 18 (Keil 2006). Hybridization among Old World thistles typically occurs between diploid species and produces homoploid hybrids (Czapik 1958, Buttler 1989, Kadota 1995, Bureš et al. 2004, Rotreklová et al. 2004). Conversely, more than half of the known hybrids are between species with a different chromosome number in North America (cf. Keil 2006). Hence, the pollen viability of hybrids and frequency of natural hybridization could be also geographically determined.

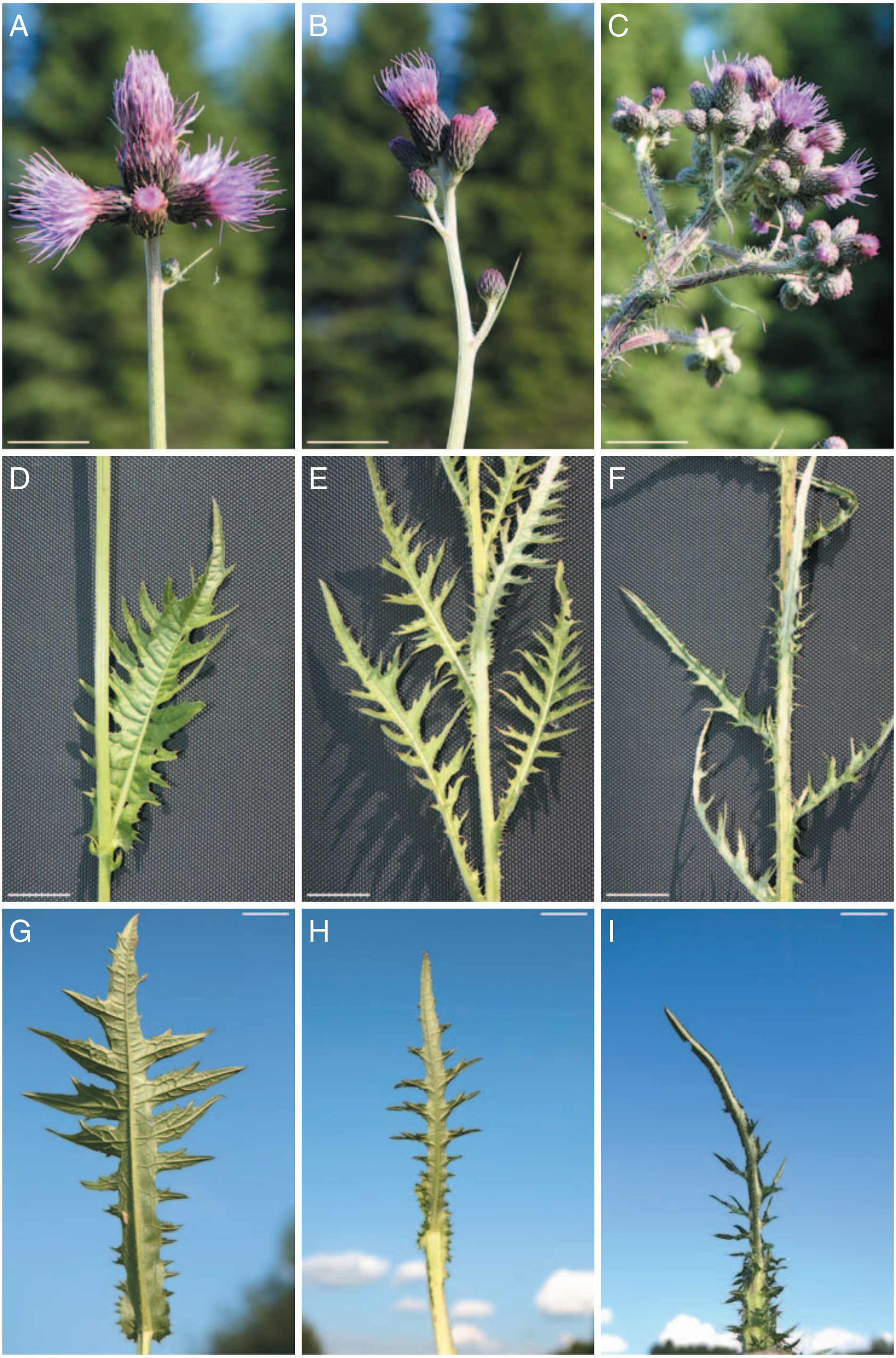


Fig. 2. – Example of morphological intermediarity of hybrid *Cirsium palustre* × *C. rivulare* (B, E, H) in comparison to its parental species *C. rivulare* (A, D, G), and *C. palustre* (C, F, I). (A-C) upper part of stem with flower heads; (D-F) middle part of stem; (G-I) backside and base of middle stem leaves. Length of scale bar equals to 2 cm.

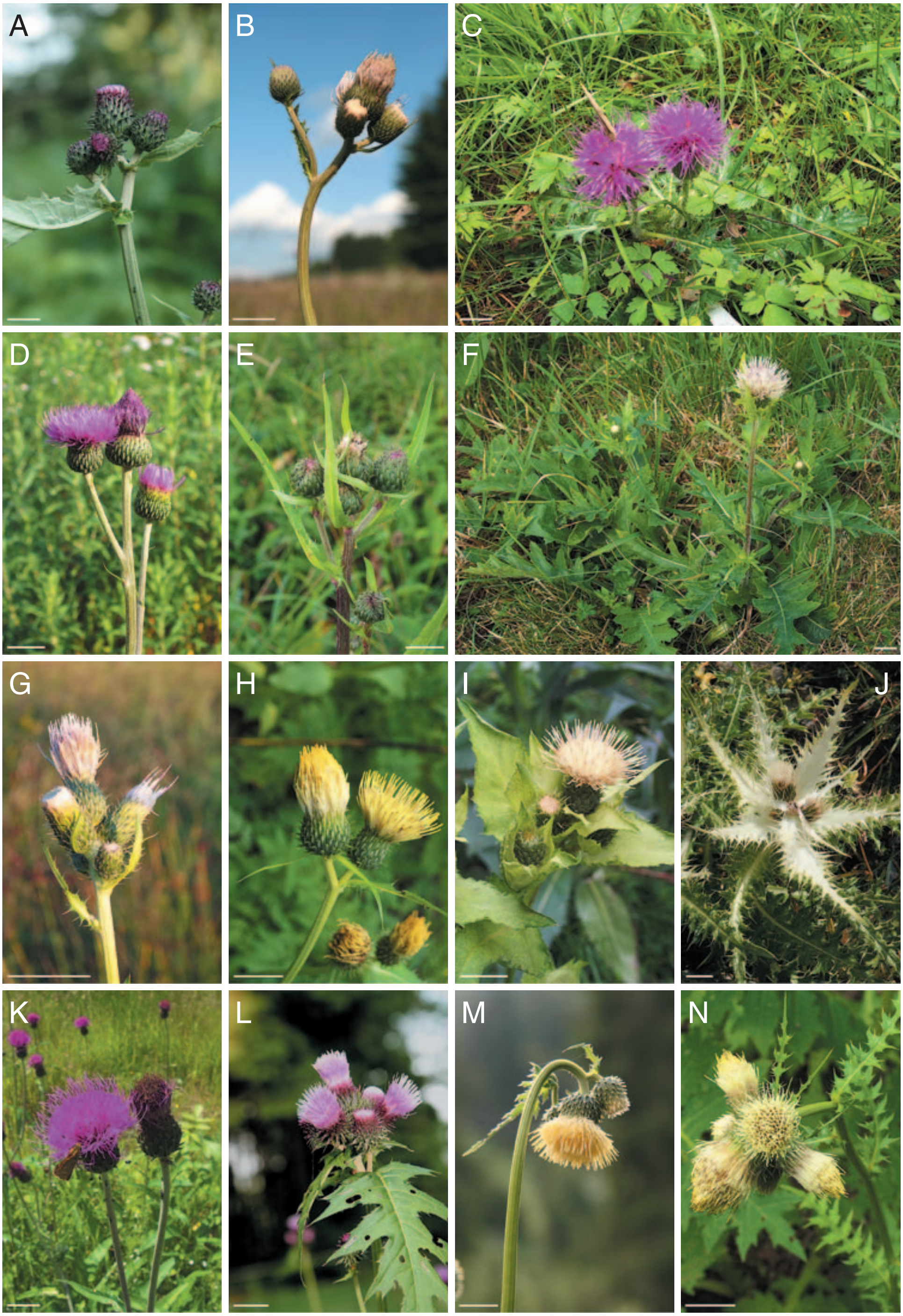


Fig. 3. – Morphological features of some selected *Cirsium* hybrids (A, B, E-H, N) in comparison to parental species (C, D, I-M): (A) *C. heterophyllum* × *C. montanum*; (B) *C. oleraceum* × *C. rivulare*; (C) *C. acaule*; (D) *C. canum*; (E) *C. canum* × *C. oleraceum*; (F) *C. acaule* × *C. oleraceum*; (G) *C. oleraceum* × *C. palustre*; (H) *C. erisithales* × *C. oleraceum*; (I) *C. oleraceum*; (J) *C. spinosissimum*; (K) *C. heterophyllum*; (L) *C. montanum*; (M) *C. erisithales*; (N) *C. erisithales* × *C. spinosissimum*. Length of scale bar equals to 2 cm.

The existence of natural triple hybrids and backcrosses has been postulated based on morphological variation (Wagenitz 1987, Bureš 2004, Stöhr 2006); however, an extensive study of fertility in spontaneous hybrids (e.g. pollen viability of F1), which is a necessary prerequisite for triple hybrid origin or backcrossing, has never been done in the genus.

Cirsium species are mostly allogamous, but self-compatibility is also frequently reported in some species: *C. acaule* (Pigott 1968), *C. dissectum* (de Vere 2007), *C. oleraceum* (Correns 1916), *C. palustre* and *C. vulgare* (Correns 1916, Mogford 1974, van Leeuwen 1981). It can be assumed that self-compatibility exists in other species across the genus because of the frequent presence of gynodioecy (Delannay 1978, 1979). *Cirsium* flower heads are protandrous and contain from 50 to 300 (rarely more) tubular florets, which are either (i) ruby or purple (*C. acaule*, *C. tuberosum*, *C. canum*, *C. eriophorum*, *C. heterophyllum*, *C. montanum*, *C. pannonicum*, *C. palustre*, *C. rivulare*, *C. vulgare*, and *C. waldsteinii*); (ii) lilac or dark pink (*C. arvense* and *C. brachycephalum*); or (iii) creamy whitish (*C. carniolicum*, *C. erisithales*, *C. oleraceum*, and *C. spinosissimum*).

Three types of reproductive strategies or breeding systems are present in *Cirsium*: (i) hermaphroditism, (ii) dioecy, and (iii) gynodioecy (Delannay 1978, 1979). In Europe, all species where the reproductive mode has been studied have been found to be gynodioecious, with the exception of the (sub)dioecious *C. arvense* (Lloyd & Myall 1976, Delannay 1978, 1979, Bureš et al., unpublished[2]).

The lengths of the florets in Central European thistles vary from 7–10 mm in *C. brachycephalum* to 25–44 mm in *C. eriophorum* (Werner 1976). The depth of the closely clustered tubular florets in the capitula limits nectar access to long-tongued insects such as *Hymenoptera* (bees or bumblebees; Pigott 1968, Klinkhamer & de Jong 1993, Kawakubo 1994, Proctor et al. 1996) and *Lepidoptera* (e.g. grass skippers and various species of *Nymphalidae*; Knuth 1898, Kawakubo 1995). Rarely, flowers may be accessed by some *Diptera* (namely *Syrphidae*) or insects from other orders (Knuth 1898, Michaux 1989, Kawakubo 1995, Ohashi & Yahara 1998, Theis 2006). The pollination distance therefore rarely exceeds 10 m (Beattie 1976, Price & Waser 1979, de Vere 2007). For bee-pollinated plants such as *Cirsium*, at least 80% of inter-flower flights are generally less than one meter in distance, and 99% are less than five meters (as summarized by Richards 1997 based on many studies).

Aims and objectives

The main objective of the present study is to evaluate the factors responsible for pollen viability in *Cirsium* with special regards to hybridization. Based on a comparative analysis of inter- and intraspecific variation in pollen viability in Central European species of *Cirsium* and their spontaneous hybrids we aim to determine (i) how frequently hybrids are fertile and produce viable pollen; (ii) how the pollen viability of hybrids and their parents are related and how this is affected by the genetic distance between parents; (iii) how species promiscuity relates to species pollen viability; (iv) to what extent the pollen viability of a hybrid may predetermine its frequency in nature; (v) how the pollen viability of a hybrid

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[2] In Europe, gynodioecy has been documented in *Cirsium acaule*, *C. brachycephalum*, *C. canum*, *C. carniolicum*, *C. ciliatum*, *C. dissectum*, *C. eriophorum*, *C. erisithales*, *C. esculentum*, *C. ferox*, *C. furiens*, *C. heterophyllum*, *C. montanum*, *C. monspessulanum*, *C. oleraceum*, *C. palustre*, *C. pannonicum*, *C. rivulare*, *C. spinosissimum*, *C. tuberosum*, *C. vulgare* (incl. *C. crinitum*), and *C. waldsteinii*.

and sympatricity of its parental species are related; and (vii) how the frequency of females in populations of gynodioecious species may affect the observed pollen viability.

The paper presents a particular analysis within the broader series of studies in *Cirsium* aimed to understand the mechanisms, principles, and consequences of natural hybridization, that is apparent and well documented in this syngameon.

Material and methods

Sampling strategy

We studied 13 species of the genus *Cirsium* native to Central Europe and their 16 spontaneous hybrids (Fig. 1: unlabeled species). Hybrids were identified in the populations of co-occurring parental species based on distinct morphological intermediarity (Figs 2, 3). The nomenclature and taxonomical treatment follow Flora Europaea (Werner 1976), with the exception of *C. heterophyllum* (L.) Hill., which is distinguished from *C. helenioides* (L.) Hill.

Samples were collected in 163 localities in the Czech Republic, Slovakia, and Austria during the late spring and summer of 2006–2009 (locations in Electronic Appendix 1). Pollen viability was studied in incompletely open terminal flower heads that were cut, including about 25 cm of the stem, and stored in watered plastic boxes in a transport refrigerator (10 °C). After transport to the laboratory, cuttings were kept at room temperature or in a refrigerator if they were to be analysed the next day. When possible, five plants were collected from populations of hybrids or species. The sampling distance in populations was at least 3 m to minimize the sampling of clonally propagated perennial (polycarpic) species. Female plants from gynodioecious or dioecious species that were identified during subsequent pollen preparation steps by the absence of pollen and abortion of anther tubes were omitted from the viability analyses, but they were used to estimate the gender ratio (female frequency) in the parallel gynodioecy study (Bureš et al., unpublished).

Pollen viability

One flower head per plant was used for pollen preparation. Pollen grains were prepared from the pre-anthesis central flowers of the capitulum that were surrounded by open marginal flowers. Five flowers were usually analysed separately in each flower head. The anther tube was placed on a slide with a drop of Alexander's stain (Alexander 1969, 1980) and lanced by a preparation needle using an Olympus SZH 10 stereo-microscope; it was then incubated for two to three hours at room temperature. Dark purple pollen grains with cytoplasm stained by acid fuchsin were considered viable, while green or whitish grains with cellulose in the cell wall, stained by malachite green, were considered non-viable (Alexander 1969). Between 10 and 14 pictures were taken from each slide (one slide per flower) using an Olympus BX 51 microscope equipped with an Olympus Color View 2 digital camera. Between 150 and 350 pollen grains were displayed on each slide and subsequently analysed (Electronic Appendices 2 and 3). The viability of the pollen grains was estimated for each flower as the sum of all viable pollen grains in all pictures divided by the sum of all pollen grains from all pictures of the given slide. Vouchers of all samples were deposited in the Herbarium of the Masaryk University in Brno (BRNU).

Hybrid frequency and species promiscuity

Hybrid frequency and species promiscuity were estimated for 11 species and their hybrids (Table 1); these species and hybrids are native to the Czech Republic and have reliable and robust data available. Estimation of absolute frequencies of spontaneous hybrids (Electronic Appendix 4) were based on the data obtained from 1612 specimens of *Cirsium* hybrids located in the Czech Republic and kept in the Czech herbaria of BRNM, BRNU, CB, GM, HOMP, HR, CHOM, LIM, LIT, MJ, MMI, MP, NJM, OL, OLM, OP, OSM, PL, PR, ROZ, SUM, VM, VYM and ZMT (abbreviations follow Thiers 2009), which were compiled during the preparation of a treatise on the genus *Cirsium* for the compendium Flora of the Czech Republic (Bureš 2004). The absolute hybrid frequency was estimated for all localities represented in the herbarium specimens, with the exclusion of duplicate locations.

Table 1. – Pollen viability and promiscuity of Central European species of *Cirsium*.

Species	Analysed populations	Analysed individuals	Analysed pollen grains	Viable pollen grains	Promiscuity[a]	Average pollen viability (%)
Cirsium acaule	5	21	14519	14005	248	96.73
C. arvense	6	15	7879	7704	12	97.16
C. brachycephalum	3	15	13720	13473	0	98.45
C. canum	6	22	18409	16971	518	93.07
C. eriophorum	4	22	13283	12953	3	97.52
C. erisithales	3	17	32806	30801	high[b]	93.67
C. heterophyllum	8	40	39425	36130	80	93.10
C. montanum	1	5	8322	7077	medium[b]	85.07
C. oleraceum	7	26	31685	29629	884	94.70
C. palustre	7	43	21395	18193	418	85.64
C. pannonicum	4	18	13156	11560	80	88.64
C. rivulare	7	32	20701	18482	375	90.31
C. vulgare	5	23	11265	11045	14	98.31

[a] Sum of all localities containing hybrids for which the species is a parent, as documented in the Czech herbaria; duplicate localities excluded

[b] Based on Wagenitz (1987)

The relative natural frequency $f(h_{AxB})$ of a particular hybrid between species A and B was estimated by correcting the absolute frequency of a particular hybrid by the extent of the distribution overlap of its parental species using the following formula:

$$f(h_{AxB}) = \frac{\sum_{i=1}^{99} (h_{AxB})_i}{\sum_{i=1}^{99} a_i b_i} \quad (1),$$

where (h_{AxB}) = the number of localities in which a hybrid between species A and B was documented in the i^{th} phytogeographical district; i.e. the numerator of Equation (1) equals the absolute frequency of a particular hybrid in the Czech Republic (Electronic Appendix 4), and a_i or b_i is the number of localities of species A or B, respectively, in the i^{th}

phytogeographical district (according to Skalický 1988)[3]. The distribution overlap $\sum_{i=1}^{99} a_i b_i$ of the parental species was estimated using the number of localities for a particular *Cirsium* species in the phytogeographical districts of the Czech Republic (Electronic Appendix 5). These data were taken from the same herbaria as the hybrids: database FLDOK (Institute of Botany, Czech Academy of Sciences, Průhonice; http://www.ibot.cas.cz/index.php?p=databaze&site=default), the Czech National Phytosociological Database (Masaryk University, Brno; http://www.sci.muni.cz/botany/vegsci/dbase.php), the Database of the Flora of South Bohemia (University of South Bohemia, České Budějovice; http://botanika.bf.jcu.cz/jpcbs), and Manuscript Documentation to the Flora of the Czechoslovak Republic up to 1953, prepared by Karel Domin (Institute of Botany, Czech Academy of Sciences, Průhonice). In total, these data for 11 Czech species represent 31,183 localities, classified into 99 phytogeographical districts (Electronic Appendix 5). The calculated approximative frequencies in particular hybrids well agree with our field experience and the data do not seem biased with the oversampling of particular hybrids in certain geographical regions or by a regional researcher.

The promiscuity of a species (Table 1) represents the sum of localities containing hybrids for which the species is a parent. In total, 1316 hybrid localities were considered in the analysis (summarized in the table in Electronic Appendix 4). In species occurring outside of the Czech Republic (*C. erisithales*, *C. montanum*), species promiscuity was estimated on a semi-quantitative scale based on field experience and data by Wagenitz (1987). The apparent discontinuity in the calculated promiscuity of species (Table 1) allows them to be divided into rarely (promiscuity ≤ 14) and frequently hybridizing (promiscuity ≥ 80; including *C. erisithales*, *C. montanum*) groups.

Distribution range similarity (sympatry)

For the studied species, we calculated the similarity of their natural ranges within Europe (digitalized from Meusel & Jäger 1992) using Jaccard similarity coefficient, i.e., the ratio between the squares of the intercept (overlap) and the union of the distribution ranges of a particular pair of parental species (Table 2).

Reproductive isolation

To estimate the reproductive isolation of particular parental species pairs of the studied hybrids, we calculated prezygotic reproductive isolation based on the geographical data on species distribution in the Czech Republic described above and postzygotic reproductive isolation based on the pollen viability of species and hybrids.

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[3] Phytogeographical division follows the compendium Flora of the Czech Republic in which the area of the Czech Republic (78,866 km^2) is divided into 99 phytogeographical districts. The delimitation of these districts is based particularly on the maximum homogeneity of flora and vegetation within these units and the maximum contrast between neighbouring units regarding these parameters (Skalický 1988; for the area of particular districts see Electronic Appendix 5). The climatic, geological, geomorphological, and pedological data and the human population density were also taken into account during the delimitation of phytogeographical districts (Skalický 1975, 1988).

Table 2. – Pollen viability of Central European hybrids of *Cirsium* and natural range similarities of their parental species.

Hybrid	Analysed populations	Analysed individuals	Analysed pollen grains	Viable pollen grains	Natural range similarity of parental species[a]	Prezygotic isolation of parental species[b]	Relationship	Postzygotic isolation of parental species[c]	Average pollen viability (%)
Cirsium acaule × *C. canum*	2	7	12190	8193	0.0202	0.024	<	0.294	67.00
C. acaule × *C. oleraceum*	11	39	56070	47638	0.1966	0.266	>	0.147	81.69
C. acaule × *C. pannonicum*	2	8	9266	7072	0.0429	–0.348	<	0.160	77.83
C. canum × *C. oleraceum*	21	84	101833	54663	0.2660	0.130	<	0.437	52.89
C. canum × *C. palustre*	3	6	3907	2432	0.1299	0.458	>	0.288	63.58
C. canum × *C. rivulare*	1	4	2690	1147	0.1755	0.051	<	0.537	42.47
C. carniolicum × *C. erisithales*	1	2	1123	833	0.0238	–		–	78.25
C. erisithales × *C. heterophyllum*	1	3	7617	7267	0.0135	–		–0.014	94.66
C. erisithales × *C. palustre*	1	2	1712	991	0.0370	–		0.350	58.25
C. erisithales × *C. oleraceum*	5	20	38923	28286	0.0428	–		0.229	72.64
C. heterophyllum × *C. oleraceum*	7	22	25222	18603	0.3836	0.248	<	0.315	64.34
C. heterophyllum × *C. palustre*	2	7	7291	3127	0.5128	–0.319	<	0.600	35.71
C. heterophyllum × *C. rivulare*	2	8	3231	1343	0.0656	0.692	>	0.276	66.37
C. oleraceum × *C. palustre*	8	13	16666	7374	0.5723	0.106	<	0.420	52.28
C. oleraceum × *C. rivulare*	14	62	75121	59999	0.2261	0.090	<	0.150	78.61
C. palustre × *C. rivulare*	11	56	46936	32048	0.1487	0.350	>	0.262	64.95

[a] Based on natural ranges within Europe (digitalized from Meusel & Jäger 1992) using Jaccard similarity coefficient

[b] Based the geographic distributions of parental species in the Czech Republic using Equation (2)

[c] Based on the pollen viability of species and hybrids using Equation (3)

Prezygotic isolation was quantified using the formula for eco-geographic isolation by Ramsey et al. (2003):

$$RI_{geogr} = 1 - \frac{\text{no. co-occurrences (natural distrib. simil.)}}{\text{no. co-occurrences (random distrib. simil.)}}.$$

The calculation of random co-occurrences was simplified because instead of randomization, homogenization of species distribution was used, retaining the total number of localities for particular species in the Czech Republic. For the calculation of geographical isolation between species *A* and *B*, the equation has the following form:

$$RI_{geogr}(A,B) = 1 - \frac{\sum_{i=1}^{99} a_i b_i}{\sum_{i=1}^{99} \frac{\left(p(phyt_i)\right)^2}{d(A)d(B)}} \quad (2),$$

where a_i and b_i are the number of localities of species A and B, respectively, in the i^{th} phytogeographical district (phytogeographical division follows Skalický 1988); $p(phyt_i)$ is the area of the i^{th} phytogeographical district; and $d(A)$ and $d(B)$ are the densities of the localities of species A and B, respectively, in the entire area of the Czech Republic: these densities equal to $\frac{\sum_{i=1}^{99} p(phyt_i)}{\sum_{i=1}^{99} a_i}$ and $\frac{\sum_{i=1}^{99} p(phyt_i)}{\sum_{i=1}^{99} b_i}$, respectively. The numerator of Equation (2) is the sum of the products of the numbers of localities for species A and B across all phytogeographical districts, which we consider to be a measure of the similarity of the natural distributions of a particular species pair in the Czech Republic. A similar sum is also in the denominator of Equation (2), but in this sum, each species is considered to be homogenously distributed throughout the whole Czech Republic; the number of all localities of a particular species is the same as in the numerator, i.e. each phytogeographical district contains as many localities for a "homogenously" distributed species as is equal to its area in the phytogeographical district divided by the ratio between the area of the whole Czech Republic $\sum_{i=1}^{99} p(phyt_i)$ and the sum of all localities of that species in the Czech Republic (i.e. in the case of species A or B, it is $\sum_{i=1}^{99} a_i$ or $\sum_{i=1}^{99} b_i$, respectively).

We quantified the postzygotic isolation using the formula of Ramsey et al. (2003):

$$RI_{postzygotic} = 1 - \frac{\text{F1 hybrid fitness}}{\text{mean parental fitness}} \quad (3),$$

where "F1 hybrid fitness" equals the pollen viability of a hybrid and the denominator of Equation (3) contains the mean of the pollen viabilities of the parental species.

Genetic distance

AFLP (Amplified Fragment Length Polymorphism) analysis of genomic DNA (Vos et al. 1995) was carried out to estimate the genetic distances between species. Forty two individuals representing 14 species (three samples per species, each from a different locality if possible) were analysed (Electronic Appendix 6).

Genomic DNA was isolated from fresh, young, visually uninfected leaves of plants cultivated in an experimental garden. These plants were transferred from field populations in the form of rosettes or adult flowering plants (in the case of polycarpic species). DNA was extracted using the Nucleospin Plant II kit (Marcherey-Nagel) with extraction buffer PL2 according to the manufacturer's instructions. The quality of the extracted DNA was checked on 1% TBE agarose gels. DNA concentration was determined photometrically with the Spectrophotometer Libra S22 (Biochrom). AFLP fingerprinting was performed according to the protocol in the AFLP^{TM} plant mapping kit (PE Applied Biosystems). Genomic DNA (300 ng) was double-digested at 37 °C for 2 h with EcoRI and MseI and ligated to EcoRI and MseI adaptors in the same reaction. The preselective amplification was done with EcoRI+A and MseI+C primers in a GenePro (Bioer) thermocycler. In the preliminary primer testing, nine different combinations of MseI primers with three selective nucleotides were tested for the production of strength AFLP signals with high and well-distinct peaks in all samples. The following seven final primer pair combinations were chosen: (FAM)-EcoRI+ACA / MseI+CAG, (JOE)-EcoRI+ACG / MseI+CTC, (NED)-EcoRI+AAC / MseI+CAA, (FAM)-EcoRI+ACT / MseI+CTC, (FAM)-EcoRI+ACA / MseI+CTA, (JOE)-EcoRI+AGG / MseI+CAG, and (NED)-EcoRI+AGC / MseI+CAT. Selective amplification products were separated on POP-4 polymer gels with an internal size standard, GeneScan-500 [ROX] (Applied Biosystems), using a Genetic Analyser ABI PRISM 310 (Applied Biosystems). Raw data were analysed with GeneScan Analysis Software version 3.7 and Genotyper DNA Fragment Analysis Software version 3.7 (Applied Biosystems).

In each of the 42 analysed plants, and for each of the 7 primer combinations, 40 of the most intensive peaks (fragments), ranging in length from 50 to 500 bp, were selected and coded as a binary presence/absence matrix. The aligned matrix, including data from all 7 primers, consisted of 42 rows (samples) and 1807 columns (peak positions). The genetic distance between samples was subsequently calculated using the Jaccard similarity coefficient in Syn-Tax 2000 (Podáni 2000). For the purposes of statistical testing of parameters among species, the pair wise distances between species (each mostly represented with two or more samples) were calculated manually from the data with Unweighted Pair Group Method with Arithmetic Mean (UPGMA; cf. Podáni 2000; Electronic Appendix 6). The available AFLP data allow the perfect separation of all species as tested with bootstrap values onto the neighbour joining or UPGMA trees made from the data (Bureš et al., unpublished, analyses not shown).

Population gender ratio

In analysed population, open flower heads of several individuals were collected (usually five per species population, all from hybrids). The distance between sampled plants was at least 3 m to avoid sampling of clonally propagated plants. The male sterile plants had aborted anther tubes with no pollen and their stigmas were usually undulated. Observations of the presence of pollen and pollen tube degeneration in prepared florets (usually five per capitulum) were made using an Olympus SZ30 stereoscopic microscope. The gender ratios (female frequency) in populations of gynodioecious species were estimated as part of a parallel study (Bureš et al., unpublished).

Datasets and statistical analyses

The basic analysis was carried out at four hierarchical levels, from individual flowers to species or hybrids. The amounts of viable and nonviable pollen from all slides were summed per flower to obtain (i) the flower pollen viability (Electronic Appendices 2 and 3). Prior to any statistical analysis the pollen viabilities of particular flowers were averaged per plant (flower head) to obtain (ii) the individual plant pollen viability (Electronic Appendices 2 and 3). For the purposes of particular analyses and graphical outputs the pollen viabilities of plants were averaged per population (locality) to obtain (iii) the population pollen viability (Electronic Appendix 1) or the population pollen viabilities were averaged per species or hybrid to obtain (iv) the pollen viability of the species or hybrid.

The differences in pollen viability between flowers, plants, populations, species, and hybridizing/non-hybridizing species were calculated using hierarchical ANOVA. Prior to the analyses the percentage viability data were rank transformed (common arcsine square root transformation was not enough to normalize the data distribution). The analogous procedures were applied to test the viability of hybrids and to test for differences in pollen viability between populations of species with and without the occurrence of hybrids and with and without the occurrence of females in the populations of gynodioecious species. To determine whether an increasing frequency of females affected the pollen viability of the respective populations across gynodioecious species, we omitted populations with no females and conducted an ANCOVA on the rank transformed pollen viability data and rank transformed female frequencies. As the data largely deviated from the normal distribution, all the correlations of characters and the tests of differences between groups were done with non-parametric methods (Spearman's Rho correlation and Mann-Whitney test, respectively). All statistical analyses were performed using Statistica 8.0 software (Statsoft Inc. 2009) or Microsoft Office Excel 2003.

Results

Altogether, the viability of 656,363 pollen grains (246,565 from pure species and 409,798 from hybrids) from 13 species (Table 1, 1185 flowers from 301 plants from 67 populations) and 16 natural interspecific hybrids (Table 2, 1693 flowers from 345 plants from 96 populations) was analysed; for the viability estimates in particular populations, see Electronic Appendices 1–3. Pollen viability in *Cirsium* was found to vary from 0 to 100% in both hybrids and species; however, the distributions differed considerably between these two subsets (Figs 4A, B). While within species, most of the analysed flowers had high

Table 3. – Hierarchical ANOVA of rank transformed data on pollen viability in Central European *Cirsium* species. The particular hierarchical levels nested each within the next higher one indicate the difference observed among individuals (level 5), among populations (level 2), between hybridizing and non-hybridizing populations (level 3), among species (level 4), and between rarely and frequently hybridizing species (level 1; Electronic Appendix 2). Level – hierarchical level in the ANOVA test; SS – sum of squares; DF – degrees of freedom; MS – mean sum of squares; F – F value; P – statistical significance; Partial η^2 – partial eta-squared, it refers to the relative importance of each level in a similar manner to the R-squared in the multiple regression analyses (it describes the proportion of total variation attributable to the factor, excluding other factors from the total non-error variation; Pierce et al. 2004).

	Level	SS	DF	MS	F	P	Partial η^2
Intercept		265827856	1	265827856	8325.605	0.000	0.907
Hybridizing species (species (hybrid populations (populations (individuals))))	1	10979590	1	10979590	343.876	0.000	0.287
Species (hybrid populations (populations (individuals)))	2	11869871	11	1079079	33.796	0.000	0.303
Hybrid populations (populations (individuals))	3	5523183	6	920530	28.831	0.000	0.168
Populations (individuals)	4	15155181	47	322451	10.099	0.000	0.357
Individuals	5	47401668	233	203441	6.372	0.000	0.634
Error		27331185	856	31929			

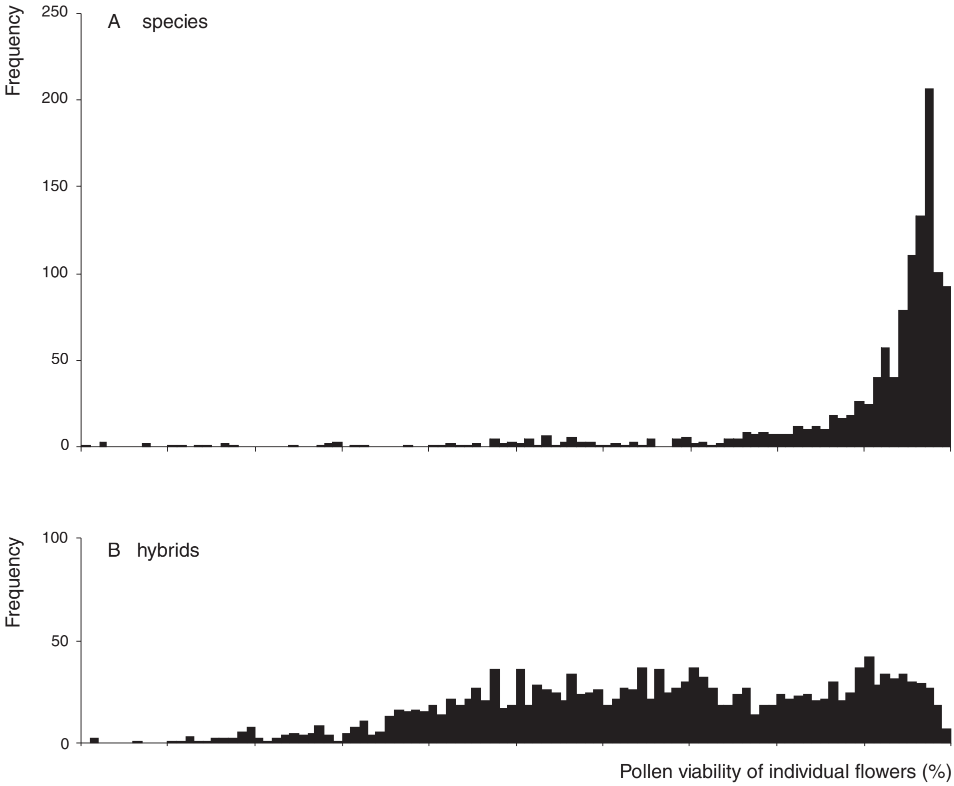

Fig. 4. – Comparison of pollen viability in species (A, total of 1185 flowers) and hybrids (B, total of 1693 flowers) of *Cirsium* (column width ~ 1%), based on the pollen viability estimated for flowers.

pollen viabilities, with peak at 97–98% (Fig. 4A), the pollen viabilities of hybrid flowers were evenly scattered between 10 and 100%, without a prominent peak (Fig. 4B).

Significant differences in pollen viability were detected at all investigated hierarchical levels, i.e. between individual plants, populations, species, and between frequently and rarely hybridizing species (ANOVA at all levels with $P < 0.001$; Table 3). Comparisons with species promiscuity further indicate an apparent decrease in pollen viability in frequently hybridizing species (Table 1, Figs 5A, B). The majority of the variation in pollen viability observed in pure species was due to the variation among individuals, followed by the variation among populations, and finally the variation among species (partial eta-squared in Table 3). Similarly, in hybrids, significant differences in pollen viability were observed at all hierarchical levels, i.e. among individual plants, populations and hybrids, with the variation in pollen viability evenly distributed among individuals, populations and particular hybrid types (ANOVA at all levels with $P < 0.001$; Table 4, Fig. 6).

The populations of species that were located in areas where hybrids co-occurred usually exhibited significantly lower pollen viabilities than those in locations where hybrids

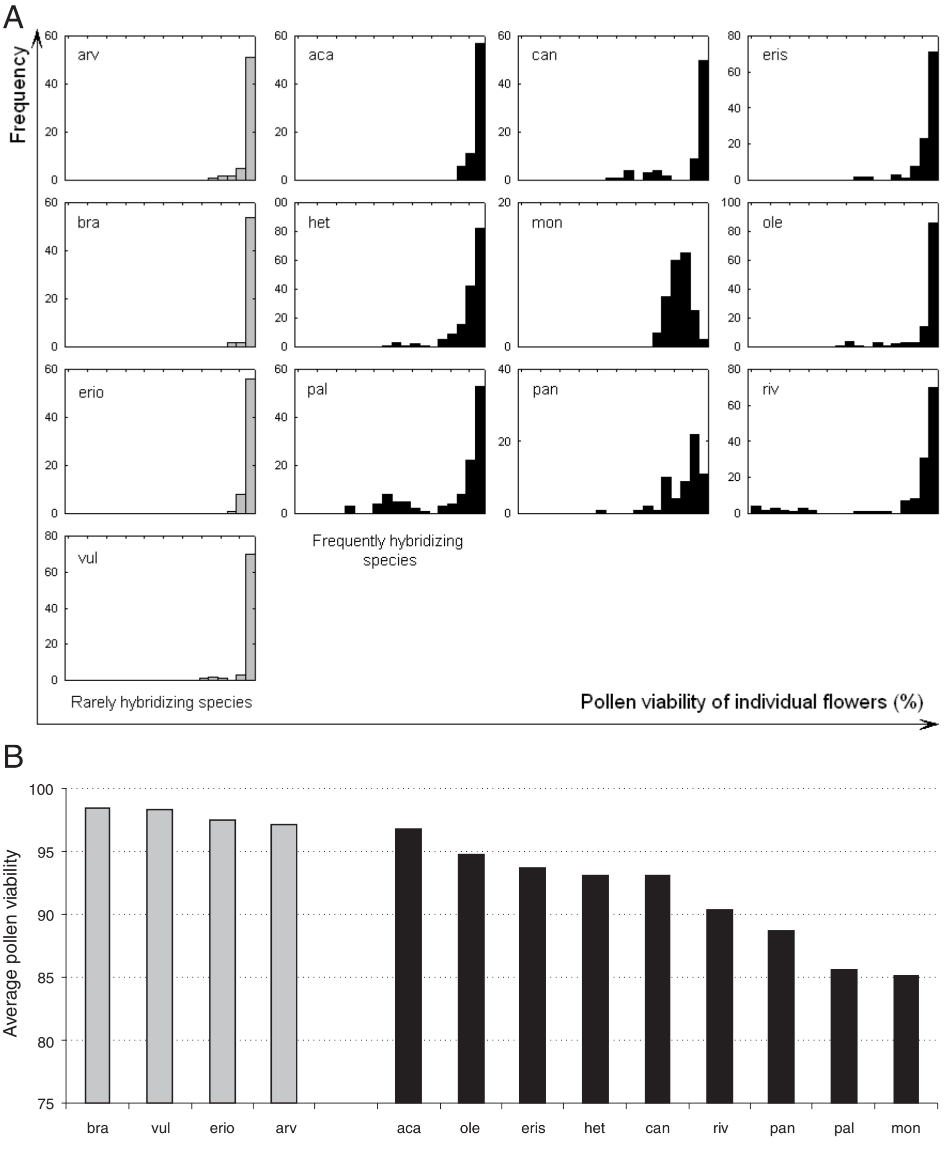

Fig. 5. – Comparison of the pollen viabilities detected in species of *Cirsium* (A) and the average pollen viabilities of species in relation to their promiscuity (B). A – pollen viability detected in individual flowers (Electronic Appendix 2); B – categories of species promiscuity (black and grey) based on Table 1; column width ~ 5%. Frequently hybridizing species (black) have lower average pollen viabilities than those hybridizing rarely (grey) – B (Mann-Whitney, P = 0.0055; Table 3); such a difference is caused by the occasional presence of individuals with low viability in populations of frequently hybridizing species – A. Species codes: aca = *Cirsium acaule*; arv = *Cirsium arvense*; bra = *C. brachycephalum*; can = *C. canum*; erio = *C. eriophorum*; eris = *C. erisithales*; het = *C. heterophyllum*; mon = *C. montanum*; ole = *C. oleraceum*; pal = *C. palustre*; pan = *C. pannonicum*; riv = *C. rivulare*; vul = *C. vulgare*.

Table 4. – Hierarchical ANOVA of rank transformed data on pollen viability in *Cirsium* hybrids. The particular hierarchical levels nested each within the next higher one indicate the difference observed among hybrid individuals (level 3), among populations of hybrids (level 2), and among particular hybrids (level 1; Electronic Appendix 3). The symbols used in the table heading follow that used in Table 3.

	Level	SS	DF	MS	F	p	Partial η^2
Intercept		460950148	1	460950148	20908.15	0.000	0.939
Hybrids (populations (individuals))	1	116052330	15	7736822	350.93	0.000	0.796
Populations (individuals)	2	102720983	78	1316936	59.73	0.000	0.775
Individuals	3	88480743	250	353923	16.05	0.000	0.748
Error		2 9740633	1349	22046			

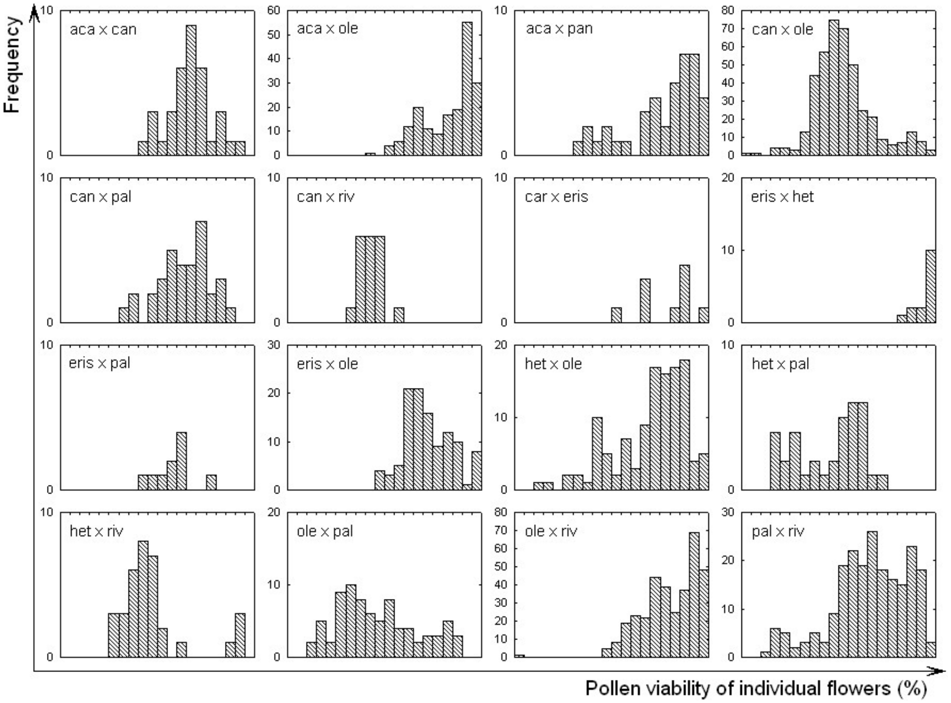

Fig. 6. – Comparison of pollen viabilities detected in hybrids of *Cirsium*. These numbers are based on the viability detected in individual flowers (Electronic Appendix 3); column width ~ 5%. The three most documented hybrids (*C. acaule* × *C. oleraceum*, *C. canum* × *C. oleraceum*, and *C. oleraceum* × *C. rivulare*) exhibit a bimodal distribution: this is thought to reflect an F_1 and a BC1. Species codes: aca = *Cirsium acaule*; can = *C. canum*; car = *C. carniolicum*; eris = *C. erisithales*; het = *C. heterophyllum*; ole = *C. oleraceum*; pal = *C. palustre*; pan = *C. pannonicum*; riv = *C. rivulare*.

were not found (Table 3, Figs 7–9). In gynodioecious species, the pollen viability of a population was also shown to be significantly decreased when females were present (ANOVA, P = 0.01; Table 5). The decrease was, however, not related to the frequency of females (ANCOVA, P = 0.878; Table 6).

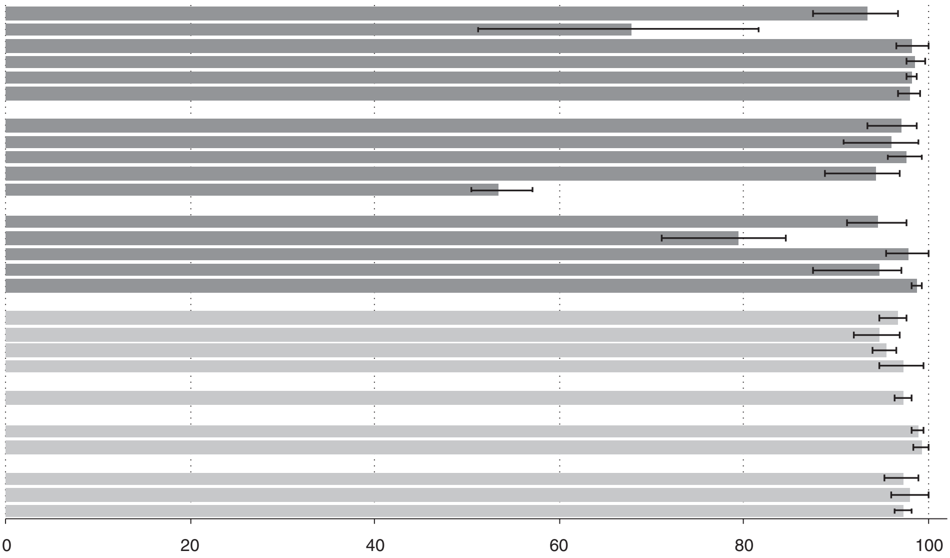

Fig. 7. – Example of the pattern seen for pollen viability in the frequently hybridizing species *Cirsium oleraceum*. Displayed is the variability among individuals (rows) and populations (separated by gaps); whiskers = minimum and maximum viabilities in flowers. Dark grey = populations in which hybrids co-occurred, light grey = populations where no hybrids co-occurred (Electronic Appendix 2). A lower pollen viability was found in some samples in populations where hybrids were also present.

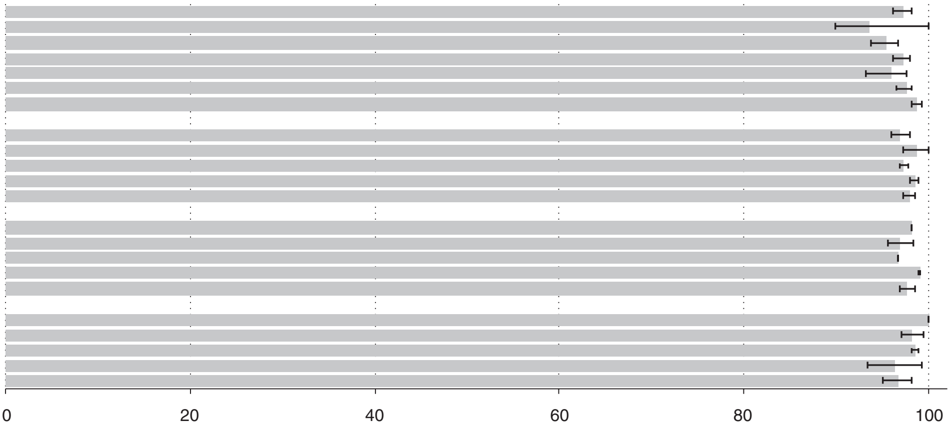

Fig. 8. – Example of the pattern of pollen viability in *Cirsium eriophorum*, a species that hybridizes rarely. Displayed is the variability among individuals (rows) and populations (separated by gaps); whiskers = minimum and maximum viabilities in flowers (Electronic Appendix 2).

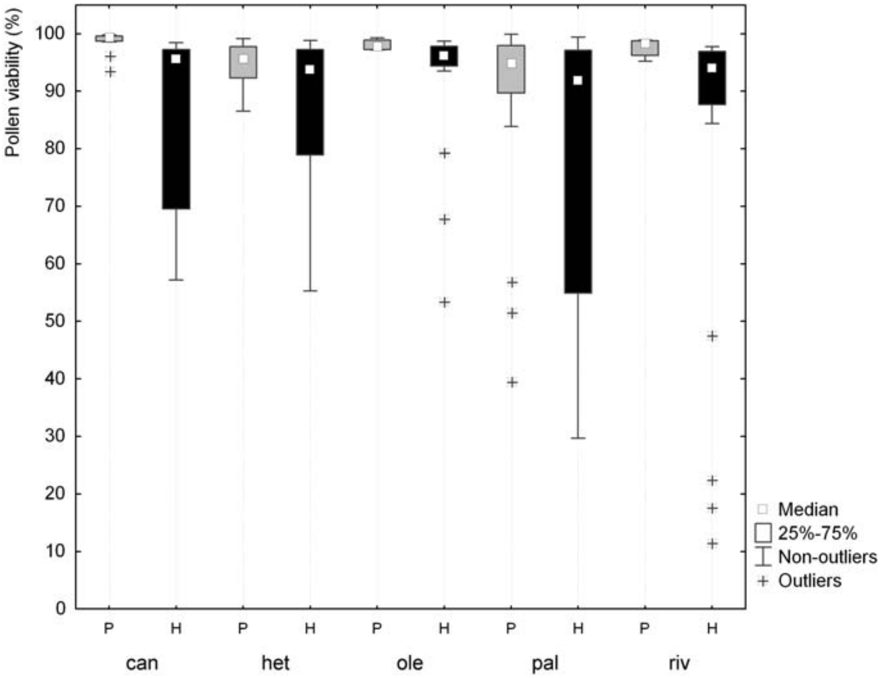

Fig. 9. – The influence of hybrid co-occurrence on pollen viability in species. P (grey boxes) = viability in populations without hybrid co-occurrence; H (black boxes) = viability in populations with co-occurring hybrids (Electronic Appendix 2); the decrease of pollen viability in species populations where hybrids co-occur likely indicates introgression. (Mann-Whitney; *C. canum*: P = 0.001; *C. heterophyllum*: P = 0.169; *C. oleraceum*: P = 0.033; *C. palustre*: P = 0.206; *C. rivulare*: P = 0.002). Species codes: can = *Cirsium canum*; het = *C. heterophyllum*; ole = *C. oleraceum*; pal = *C. palustre*; riv = *C. rivulare*.

Table 5. – Hierarchical ANOVA of rank transformed data on pollen viability of hermaphrodites (Electronic Appendix 1) and presence of females in populations of Central European gynodioecious species of *Cirsium* (Bureš et al., unpublished). Symbols in the table headings follow those used in Table 3.

	Level	SS	DF	MS	F	p	Partial η^2
Intercept		26365.57	1	26365.57	255.60	0.000	0.889
Species (presence/absence of females)	1	3680.77	8	460.10	4.46	0.001	0.527
Presence/absence of females	2	2601.66	8	325.21	3.15	0.010	0.441
Error		3300.87	32	103.15			

Table 6. – The relationship of pollen viability of hermaphrodites and frequency of females in respective populations tested in nine *Cirsium* species with ANCOVA on rank transformed data of both continuous variables (populations with no females were excluded; Electronic Appendix 2). Symbols in the table headings follow those used in Table 3.

	SS	DF	MS	F	p	Partial η^2
Intercept	323.68	1	323.6796	6.90	0.015	0.239
Pollen viability × female frequency	1.14	1	1.1377	0.02	0.878	0.001
Pollen viability among species	1620.45	8	202.5562	4.31	0.003	0.611
Error	1032.98	22	46.9536			

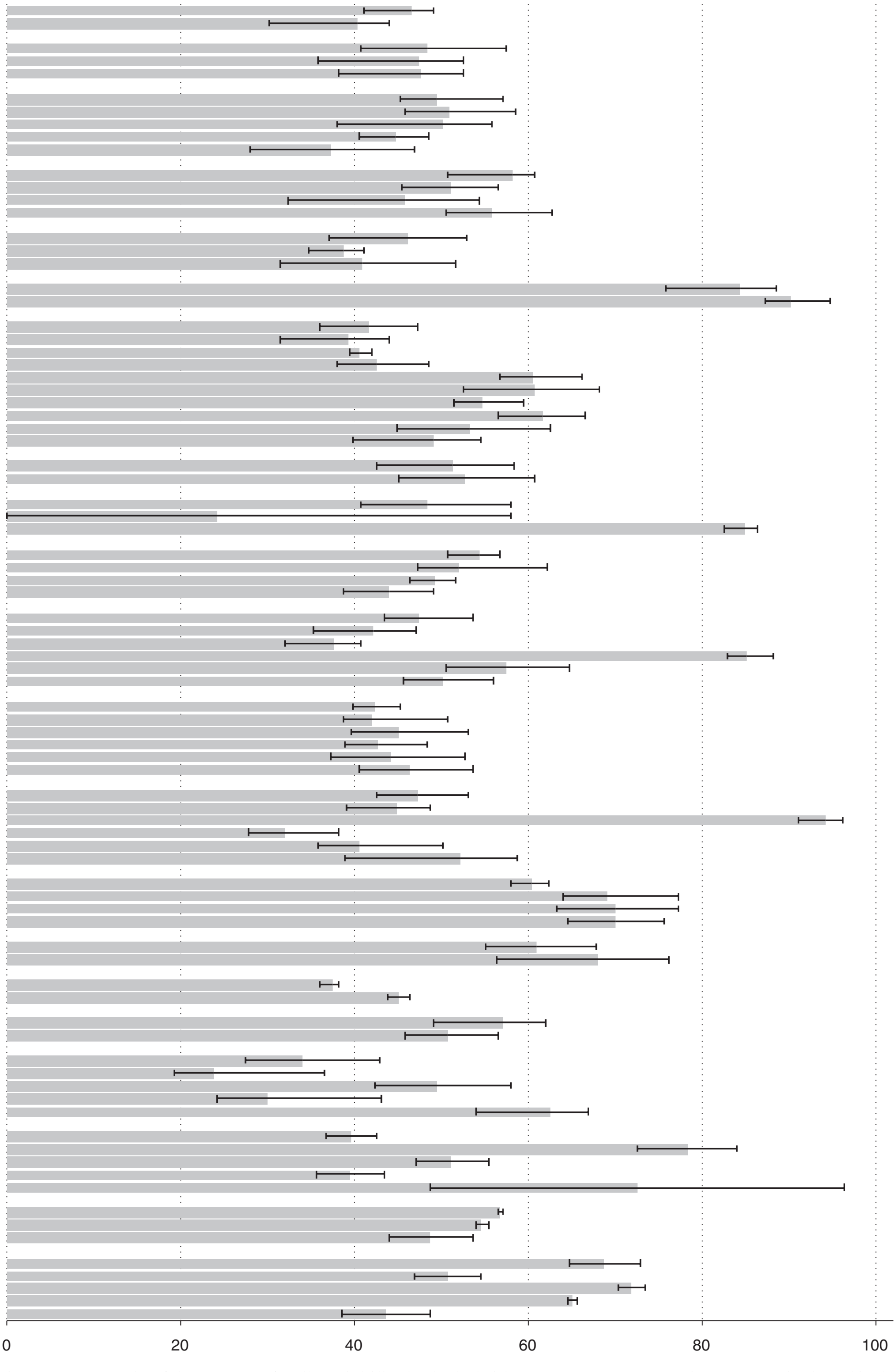

Fig. 10. – Example of the pattern of pollen viability in the hybrid *Cirsium canum* × *C. oleraceum*. Displayed is the variability among individuals (rows) and populations (separated by gaps); whiskers = minimum and maximum pollen viabilities of flowers within the sample (Electronic Appendix 3).

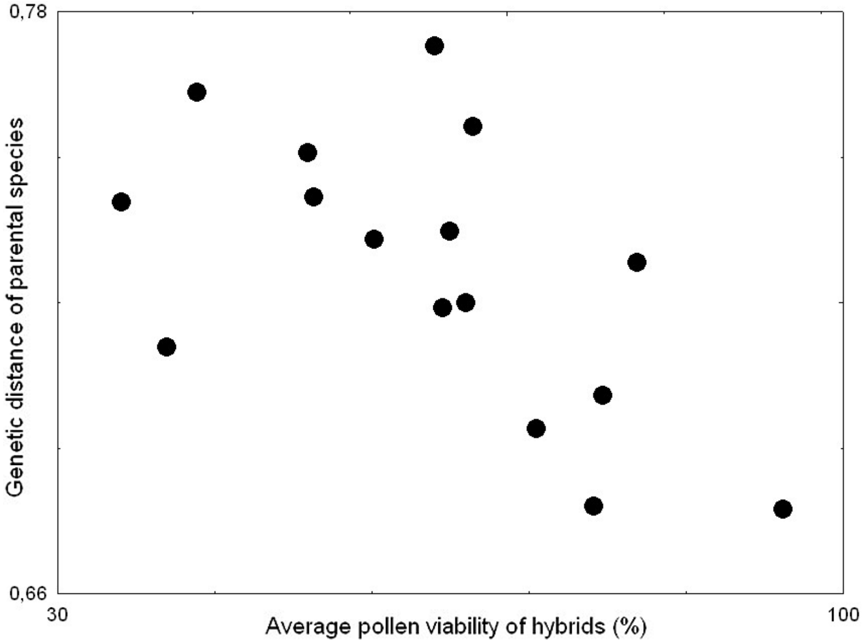

Fig. 11. – The relationship between the average pollen viabilities of *Cirsium* hybrids and the genetic distance (by AFLP) between their parental species. For a table of species genetic distances, see Electronic Appendix 6. For the average pollen viabilities of hybrids see Table 2. Phylogenetically related species produce natural hybrids with a higher pollen viability (Spearman's Rho = – 0.73; P = 0.001).

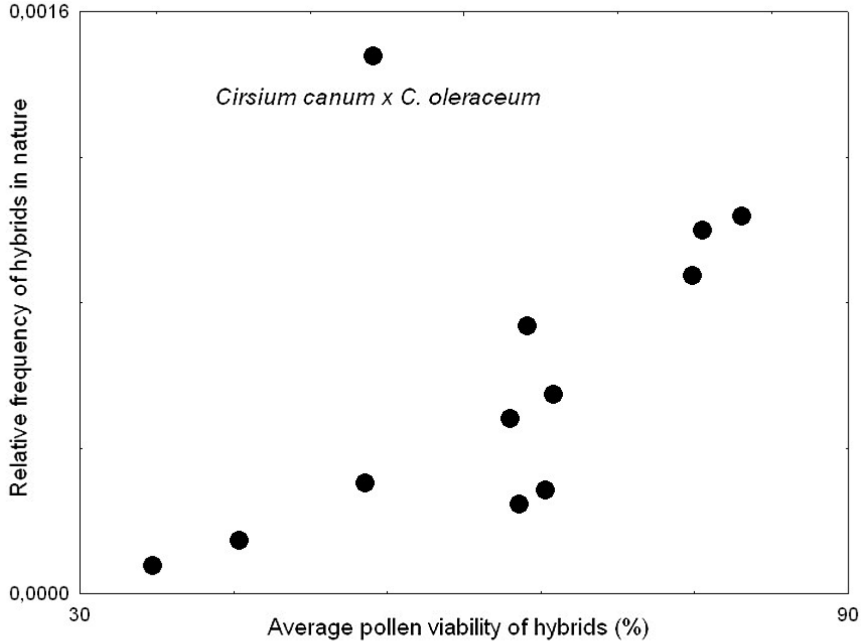

Fig. 12. – The relationship between the average pollen viabilities of *Cirsium* hybrids and their relative frequencies in nature (Electronic Appendix 4) corrected for the overlap of the geographical distributions of the parental species (Electronic Appendix 5) using Equation (1). The average pollen viabilities of hybrids are found in Table 2. Hybrids with more viable pollen occur more frequently in nature (Spearman's Rho = 0.64; P = 0.024). The most frequent hybrid, *C. canum* × *C. oleraceum,* is either over-collected (due to its presence along roads) or has a lower ability to produce backcrosses than the other frequently seen hybrids (see Fig. 4: can × ole versus aca × ole and ole × riv; see also Fig. 8).

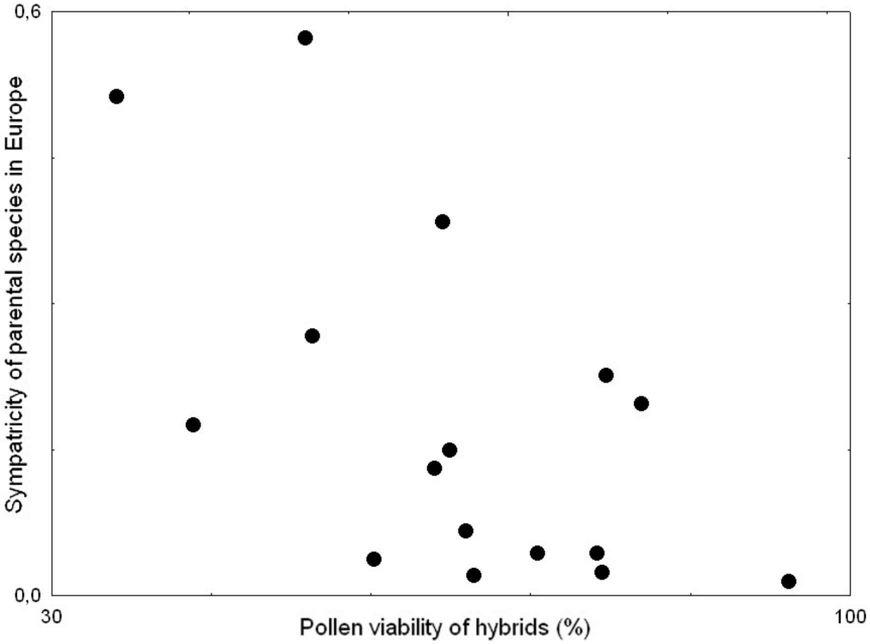

Fig. 13. – The relationship between the average pollen viabilities of *Cirsium* hybrids (i.e. post-pollination isolation of the parental species) and the sympatricity (overlap of nature ranges) of their parental species (Spearman's Rho = –0.54, p = 0.029. Crosses between sympatric species exhibit higher post-pollination isolation (decrease of pollen viability), which suggests that the reproductive isolation has been increased in more sympatric species.

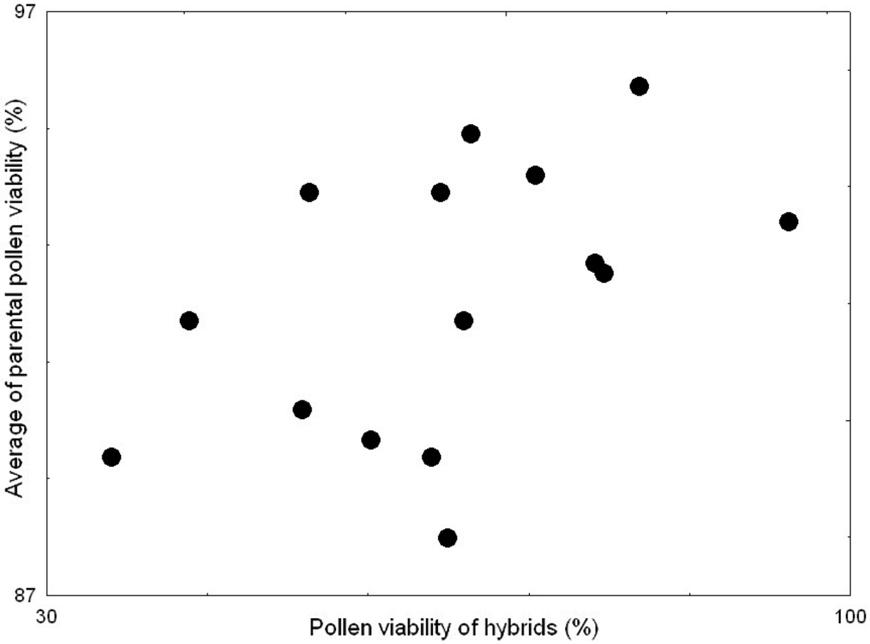

Fig. 14. – The relationship between the average pollen viabilities of *Cirsium* hybrids and the pollen viabilities of their parental species. The average pollen viabilities of species and hybrids are shown in Tables 1 and 2. Species with more viable pollen produce hybrids with higher pollen viability (Spearman's Rho = 0.56; P = 0.031; *C. carniolicum* × *C. erisithales* is excluded because of the absence of *C. carniolicum* data).

The typical pattern of pollen viability seen within hybrids is demonstrated in *Cirsium canum* × *C. oleraceum* (Fig. 10). In this hybrid, the majority of samples exhibited pollen viabilities between 35 and 65%. Only in occasional (seven) samples of this hybrid was the viability higher, i.e. around 80%. The average pollen viabilities of 16 hybrids (Table 2) significantly decreased with increasing genetic distance between parental species (Fig. 11, Spearman's Rho = – 0.73; P = 0.001). The strength of the postzygotic reproductive isolation estimated in parental species using hybrid pollen viability varied from –0.014 for the species pair *Cirsium erisithales* and *C. heterophyllum* to 0.600 for the pair of *C. palustre* and *C. heterophyllum* (Table 2). The strongest prezygotic (geographic) isolation was found between *C. heterophyllum* and *C. rivulare* (0.692), while the weakest was found between *C. acaule* and *C. pannonicum* (–0.348; Table 2).

Considering average pollen viability in particular hybrids to be a predictor of fitness, we compared the relative frequency of each hybrid in nature with its average pollen viability (Fig. 12). The resulting correlation was significant (Spearman's Rho = 0.64, P = 0.024); however, it may have been moderated by the strength of the relationship of the pollen viability of hybrids with the genetic distance of the parental species. The pollen viability of a particular hybrid was negatively correlated with the similarity of the distribution ranges of its parents in Europe (Fig. 13, Spearman's Rho = –0.54, P = 0.029) and positively correlated with the average pollen viability of its parents (Fig. 14; Spearman's Rho = 0.622, P = 0.0307).

Discussion

Pollen viability of species

In accordance with many previous studies (e.g. Krahulcová et al. 1996, Kelly et al. 2002, Jarolímová 2005, Techio et al. 2006, Kormutak et al. 2007, Zhao et al. 2007, Oyelana & Ugborogho 2008, Rotreklová 2008, Rivero-Guerra 2009), pure species in our study showed very high pollen viability when compared to hybrids, and decreased pollen viability may therefore be used as one of the supporting characteristics in the identification of *Cirsium* hybrids. Occasionally, however, some plants of a morphologically pure species may show decreased pollen viability (Fig. 7), and some hybrid individuals may occasionally approach 100% viability (Fig. 10).

The decreased pollen viability in some individuals of pure species might be due to the introgression of another species or to an ancient hybridization event that does not demonstrate enough distinctive morphological features to allow an unambiguous identification. Such individuals are more frequently found in populations containing hybrids (Figs 7, 9) and in frequently hybridizing species (Fig. 5A); in these species and populations, the pollen viability is therefore decreased when compared to populations without hybrids or species that rarely hybridize (Fig. 5B). Therefore, species promiscuity could be considered the main reason for the differences in the average pollen viability between rarely and frequently hybridizing species (Fig. 5B). At the same time, the decreased pollen viability of hybridizing species also seems to cause a decrease of pollen viability in their hybrids, as may be seen from the existence of the positive correlation between the pollen viabilities of parental species and their hybrids (Fig. 14).

The presence of “pure species” individuals exhibiting low pollen viability relative to the other individuals in populations could be also related to the presence of nuclear cytoplasmic genes widespread among species of syngameon via interspecific hybridization combined with an insufficient nuclear restoration of male fertility in such individuals (cf. Bailey & Delph 2007). Unfortunately, our sample sets are too small for tests of correlation between the frequency of such individuals and the female frequency in particular populations.

As samples were collected in the field, we cannot rule out that decreased pollen viability in some individuals relates to certain environmental stress (Iannotti et al. 2000, Parantainen & Pulkkinen 2002). The effect of the environment on pollen viability was partly eliminated by sampling pollen in unopened anthers. Moreover, the greater variability observed in hybrids (Fig. 4B) compared to parents (Fig. 4A) makes a genetic explanation of pollen viability fluctuations more likely than an environmental one.

Pollen viability of hybrids

The pollen viability of Central European *Cirsium* hybrids was higher than the low pollen viability (about 6%) reported by Bloom (1977) for North American hybrids between *Cirsium discolor* and *C. muticum.* The lower pollen viability reported by Bloom might have been due to the chromosomal rearrangements in the karyotypes of the parental species (both having 2n = 20), which would cause the meiotic irregularities observed in the resulting natural hybrids (Bloom 1977). The relatively high pollen viability of *Cirsium* hybrids would not jeopardize the genetic integrity of co-occurring species if conspecific pollen had a competitive advantage over heterospecific or hybrid pollen, particularly if the hybrids rarely occur in the large populations of over-dominating parental species (Carney et al. 1994, Arnold 1997, Campbell et al. 2003). Unfortunately, no data about interspecific pollen competition or about a competitive disadvantage in hybrid pollen are available for *Cirsium.*

Two peaks of pollen viability were observed in the most represented hybrid, *Cirsium canum* × *C. oleraceum* (Figs 6, 10). The hybrid individuals with unexpectedly increased pollen viabilities were found namely in two of the other most frequently sampled hybrids, i.e. *C. acaule* × *C. oleraceum*, and *C. oleraceum* × *C. rivulare* (Fig. 6). They may be perhaps putative backcrosses (BC1) and/or F2s in addition to the recognized F1 hybrids in these samples. Such an increase of pollen viability in the F2 and BC1 plants has been reported by various authors, e.g. Sweigart et al. (2006). These authors found the peak of pollen viability in the F1 hybrid of *Mimulus guttatus* × *M. nasutus* to be about 50%, while the peaks of pollen viability in the F2 and in BC1 were shifted toward the parental (higher) pollen viability.

Pollen viability and inbreeding depression

In addition to ongoing hybridization, the decrease of pollen viability in some populations may also be associated with an increase in inbreeding depression (Husband & Schemske 1996, Carr & Dudash 1997, Melser et al. 1999, Goodwillie 2000, Kelly 2003). Inbreeding depression in hermaphrodite progeny is one of the important forces promoting and maintaining the presence of females, which provide a higher number of obligatory out-crossed heterotic seeds, in the populations of gynodioecious species (Charlesworth & Charlesworth 1987, McCauley & Bailey 2009), and gives a reasonable explanation for the observed decrease in pollen viability in gynodioecious populations containing females (Table 5). An analogous negative relationship between the pollen viability of hermaphrodites and the restoration of cytoplasmic male sterility was documented by Dufay et al. (2008) in natural populations of the gynodioecious *Beta vulgaris* subsp. *maritima* in France. Similarly, Asikainen & Mutikainen (2003) found a negative correlation between the relative fitness of hermaphrodite seeds and the frequency of females in natural populations of the gynodioecious *Geranium sylvaticum* in Finland, and Sun & Ganders (1986) reported that the frequency of females was positively correlated with the selfing rate in hermaphrodites of Hawaiian gynodioecious species of *Bidens*.

As the level of inbreeding depression (and consequently the female frequency) in populations can be substantially influenced by environmental factors (Ashman 2006, Landergott et al. 2009, McCauley & Bailey 2009, Smith 2009; in *Cirsium* populations, the female frequency is substantially affected by altitude: Bureš et al., unpublished), by the size and spatial structure of population (Dudash & Fenster 2000, Caruso & Case 2007), it seems though difficult to use pollen viability as a direct predictor of the degree of inbreeding depression (or female frequency) in populations of gynodioecious species of *Cirsium* (Table 6). However, the presence of females in populations where hybrids and putative backcrosses have occurred supports the idea that interspecific hybridization may account for the origin and maintenance of females in hybridizing gynodioecious species (Bureš et al., unpublished).

Although the possible genetic relation between the lower pollen viability and the female frequency both in gynodioecious species and their hybrids is still not empirically supported, there are some theoretical models of gynodioecy evolved from partial nuclear male sterility (Schultz 2002) or from threshold trait of multiple nuclear restoration in nuclear-cytoplasmic male sterility (Bailey & Delph 2007). We found no relation between higher female (male sterile) frequency and lower pollen viability (partial male sterile) individuals detected in species and hybrids which could support these models. Tests of such relation at the population level have not been possible regarding small sample sets.

Pollen viability in relation to the genetic distance and sympatry of parental species

Pollen viability is generally viewed as a measure of species crossability because it plays a key role, along with other components of postzygotic barriers, in reproduction (Dobzhansky 1937, Lowry et al. 2008). Because of the assumption of the gradual evolution of postzygotic barriers (cf. Briggs & Walters 1997), it was previously assumed that the strength of such barriers would increase with the increasing genetic distance of crossed species (Clausen 1951, Vickery 1964, Allen et al. 1983). However, this assumption, confirmed in various animal models (Coyne & Orr 1997, Sasa et al. 1998, Presgraves 2002, Price & Bouvier 2002), was only rarely successfully observed in experimental plant hybrids (in *Silene*: Moyle et al. 2004, and in Mediterranean food-deceptive orchids of *Anacamptis*, *Neotinea* and *Orchis*: Scopece et al. 2008) and was apparently never confirmed in spontaneous hybrids. Indeed, the present data on spontaneous *Cirsium* hybrids clearly indicate the existence of a strong negative relationship between the pollen viability and the genetic distance of parental species, as previously documented in animals and the above-mentioned plant genera (Fig. 11).

The negative correlation between hybrid pollen viability and the similarity of the natural ranges (degree of sympatry) of the hybrid's parents in Europe (which includes substantial parts of the entire natural range for most studied taxa) may result from the fact that hybrids between sympatrically occurring taxa generally show a much higher post-pollination isolation (as measured by pollen viability) than do allopatric species (Fig. 13), as shown in *Gilia* (Grant 1966). The documented lower pollen viability of hybrids between thistles exhibiting a higher similarity of their natural ranges somewhat resembles the Wallace effect (or reinforcement; Dobzhansky 1937, Noor 1997); however, in the case of *Cirsium* this pattern is based on increasing of postzygotic barriers, not the prezygotic, as in the case of true Wallace effect. The Wallace effect was demonstrated in some animal taxa, but is considered to be very rare among plants (Moyle et al. 2004, Silvertown et al. 2005); probably the best documented evidence of reinforcement among plants was described between *Costus pulverinus* and *C. scaber* by Kay & Schemske (2008).

Reproductive isolation

Our analyses of the relative hybrid frequency, Equation (1), are based on a correction for the absolute number of observed hybrids on the distribution overlap of the parental species. A similar principle was used to calculate the prezygotic reproductive geographic isolation of the parental species, Equation (2), where the actual distribution overlap was divided by a calculated distribution overlap that assumed a homogenous (density of) distribution for the compared species. Our calculation was based on two assumptions. The first was the relative homogeneity of floral composition in phytogeographical districts: we assumed an equal probability of the occurrence of hybrid events within the whole district, which results in dependence on only the abundances of the parental species. This presumption follows from the principles of phytogeographical division (cf. Skalický 1988). The second assumption was that particular *Cirsium* species were not selectively sampled by field botanists in different phytogeographical districts. This can be safely assumed apart the fact that some *Cirsium* species could be less attractive for field botanists than others because the attractiveness of a particular species seems consistent across all phytogeographical districts for particular species. Nevertheless, the knowledge of regional flora in the districts certainly differs. Although geographical isolation may be a very important barrier in plant speciation (Ramsey et al. 2003, Lowry et al. 2008), its estimation is rarely included in studies of reproductive barriers because it requires the tedious collection of species distribution data from the literature and herbaria or in the field (Schemske 2000, Ramsey et al. 2003, Husband & Sabara 2004, Kay 2006, Lowry et al. 2008). Our calculation of geographic isolation could be successfully applied to all other cases of hybridizing complexes where the distribution data follow a phytogeographic division, and it could easily be modified if the distribution is in a grid map form. Such data are available for most species in most European countries that have a long tradition of field botanical research (cf. Kaplan 2010).

The present correction of the relative hybrid frequency in nature is still very simplistic and eliminates just one of the components that affect the potential fitness of the hybrid under natural conditions. Because the individual components of reproductive barriers act sequentially, the earliest-acting geographical isolation contributes more to total isolation than do the later-acting ones (Ramsey et al. 2003, Nosil et al. 2005, Lowry et al. 2008).

Three other potential components of prezygotic isolation should be taken into the account to make the correction more reliable: (i) the flowering time overlap of crossed species (Martin & Willis 2007), which may differ substantially between species and may be complicated by the reflorescence of late spring species (Fajmon & Bureš, unpubl.); (ii) the divergence in ecological preferences of different species, which may substantially increase the necessary pollination distance even when species occur sympatrically; and (iii) the competition of conspecific and heterospecific pollen (Arnold 1997, Alarcón & Campbell 2000), or pollen mass effect, which could play a substantial role, especially when the abundance ratio of co-occurring species varies on the local scale. Although there are other potential prezygotic barriers such as (iv) pollinator specificity and (v) immigrant inviability, their roles seem to be less important than those previously mentioned. However, some butterfly species have been found to prefer purple thistles to whitish ones, as observed by Kay (1978): such a preference could bias gene flow via interspecific pollination, depending on the species-specific colours of flowers (Levin & Anderson 1970).

Among the usually studied post-pollination reproductive barriers (F1 pollen viability, F1 seed formation, F1 seed germination, and F1 seed set), two others could also be important in *Cirsium*: the competitiveness of hybrid pollen (cf. Campbell et al. 2003) and the species/hybrid selectivity of seed predators (cf. Münzbergová 2005, Skuhrovec et al. 2008).

The relative contributions of (relationship between) pre- and postzygotic reproductive isolating mechanisms vary substantially between particular pairs of hybridizing *Cirsium* species (Table 2). A similar situation has been documented in various models by Lowry et al. (2008). By summarizing the roles of pre- and postzygotic isolation across the whole genus by averaging the values from Table 2 (following Lowry et al. 2008), we obtained mean strengths of 0.15 and 0.32 for pre- and postzygotic barriers, respectively. This is somewhat surprising because recent studies have suggested that prezygotic barriers are stronger than postzygotic ones (Ramsey et al. 2003, Nosil et al. 2005, Kay 2006, Martin & Willis 2007, Lowry et al. 2008). This difference is probably biased by the limited numbers of studied reproductive barriers in comparison with analyses documented in literature. Also, the particular comparison of the mean strengths of reproductive isolation based purely on geographic isolation and pollen viability showed a much greater importance of geographic isolation (using data presented by Lowry et al. 2008).

In general, our analyses indicated that (i) hybrids of related *Cirsium* species characterized by high pollen viability (i.e. relatively high reproductive fitness) originate less frequently because of the lower sympatricity of their parental species and (ii) the most frequent hybrids of promiscuous species are characterized by low pollen viability (i.e. relatively low reproductive fitness). These patterns of pollen viability observed in species and hybrids seem to contribute to the preservation of species genetic integrity in the *Cirsium* syngameon.

See http://www.preslia.cz for Electronic Appendices 1–4.

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Souhrn

Zkoumali jsme životaschopnost pylu 13 druhů a 16 spontánních hybridů rodu *Cirsium* ve střední Evropě. Srovnávací analýza zahrnovala celkem 656 363 pylových zrn, reprezentujících 2878 květů z 646 rostlin sbíraných ve 163 populacích (Electronic Appendix 1). Ve srovnání s druhy, které měly životaschopnost (viabilitu) pylu často téměř stoprocentní, měli hybridi viabilitu rozprostřenou od 10 do 100%. V rámci druhů byli vzácně zjištěni jedinci morfologicky nehybridní, avšak se sníženou viabilitou, a to častěji na lokalitách, kde se nacházeli také skuteční hybridi. To může potvrzovat probíhající introgresi, kterou naznačuje i bimodalita v rozložení viability u nejvíce vzorkovaných hybridů. Často hybridizující druhy měly viabilitu nižší než druhy hybridizující vzácně. Pylová viabilita hybridů korelovala negativně s genetickou vzdáleností rodičovských druhů, což je zdánlivě samozřejmé, avšak, jak dokládají recentní výzkumy, u rostlin to zdaleka vždy neplatí na rozdíl od živočichů, kde viabilita gamet hybrida takový trend zpravidla sleduje. Kříženci druhů, jejichž areály se více překrývaly, vykazovali nižší viabilitu pylu, což může znamenat, že postzygotická bariéra, daná sníženou viabilitou pylu, se vyvinula až poté, co se původně alopatrické druhy znovu setkaly. Tento jev poněkud připomíná evolučními biology často diskutovaný Wallaceův effect, který je však založen na druhotném zesílení prezygotických bariér u sympatrických (populací různých) druhů. Jelikož jsme v populacích gynodioecických pcháčů, kde se vyskytovaly samičí rostliny, zaznamenali nižší viabilitu, považujeme ji za indikátor inbrední deprese, jež je faktorem udržujícím samičí rostliny v populacích gynodioecických druhů spolu s hermafrodity. Abychom mohli srovnávat frekvenci hybridů v přírodě, dělili jsme u každého hybrida počet jeho herbářově doložených lokalit z ČR překryvem rozšíření jeho rodičovských druhů, kterým byl součet 99 součinů počtů lokalit jeho rodičovských druhů v jednotlivých okresech fytogeografického členění ČR. Při stanovení geografické izolace jsme srovnávali skutečný překryv dané dvojice druhů s jejich hypotetickým překryvem, předpokládajícím homogenní hustotu jejich lokalit v rámci ČR, při které na každou lokalitu daného druhu připadá v rámci celé ČR stejná průměrná plocha (tj. plocha ČR dělená počtem jeho lokalit); tím je dán i počet lokalit na každý fytochorion (= plocha fytochorionu dělená průměrnou plochou daného druhu v rámci ČR). Z těchto počtů lokalit při homogenizovaném rozšíření spočteme hypotetický překryv pro příslušné druhové páry výše popsaným způsobem a podělíme jím překryv skutečný, čímž získáme míru vzájemné geografické izolace.

Podobně by bylo možné kvantifikovat podobnost rozšíření jakýchkoli dvou druhů, pro něž máme data o počtech lokalit ve fytochorionech k dispozici, a získat tak např. cestu k objektivnímu vymezení „regionálních fytochorotypů“. Při uvážlivém postupu by k takovým analýzám bylo možno použít dokonce transformovaná semikvantitativní data, která poskytuje pro většinu druhů v odstavci „Rozšíření v ČR“ Květena České republiky.

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