Rare occurrence of reciprocal hybridization in a sympatric population of the Czech stenoendemic *Dianthus arenarius* subsp. *bohemicus* and widespread *D. carthusianorum*

Sporadická obousměrná hybridizace mezi českým stenoendemitem *Dianthus arenarius* subsp. *bohemicus* a široce rozšířeným *D. carthusianorum*

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Hybridization between rare and common plant congeners can pose a serious threat to the rare species through gene swamping, production of hybrid seed at the expense of conspecific seed and/or hybrid competition for abiotic or biotic resources. Assessing the frequency and dynamics of interspecific hybridization is therefore of paramount importance for conservation purposes. Here we investigate, using DNA flow cytometry, multivariate morphometrics and chloroplast DNA sequencing, the frequency and direction of interspecific hybridization between the critically endangered Czech endemic psammophyte *Dianthus arenarius* subsp. *bohemicus* and its sympatric congener *D. carthusianorum* (*Caryophyllaceae*) in a single population in central Bohemia. Flow cytometry allowed unambiguous identification of both parental species, based on differences in the amounts of nuclear DNA and revealed a few individuals (< 1.1% of the samples analysed) with intermediate genome sizes that corresponded to F1 hybrids. Clear discontinuities in estimated genome sizes and a low variation within recognized taxonomic groups make backcrossing to parental species or introgression unlikely. Interspecific hybrids were considerably less fertile, producing largely aborted pollen grains and no seed. Analysis of chloroplast haplotypes provided evidence for reciprocal hybridization (both species served as maternal and paternal parents). Length of the lowermost pair of cauline leaves, calyx length and petal length (incl. separate lengths of petal claw and petal limb) were taxonomically the most informative characters, allowing reliable identification of both parental species and their hybrids. The results indicate that interspecific hybridization has only a minor effect on the genetic integrity of the endemic *D. arenarius* subsp. *bohemicus* in its last remaining natural population. Nonetheless, we recommend periodic monitoring especially as the recent controlled large-scale disturbances (mechanical removal of the vegetation cover) in the locality may promote the establishment of interspecific crosses.

K e y w o r d s*:* conservation, Czech Republic, *Dianthus*, endemic, flow cytometry, haplotype, interspecific hybridization, multivariate morphometrics, polyploid

Introduction

Interspecific hybridization, with or without subsequent introgression from one species into another, occurs commonly in different groups of organisms. On average, it is estimated that around 25% of plant and 10% of animal species are able to hybridize with at

least one other species (Mallet 2005). The evolutionary role of interspecific hybridization is still a matter of controversy. It can be viewed as a creative force that may enhance the probability of evolutionary transition and ultimately lead to the formation of a new species (Coyne & Orr 2004, Seehausen 2004, Hegarty & Hiscock 2005, Wissemann 2006). Hybrid speciation is particularly relevant in the context of the formation of allopolyploid species: recent estimates indicate that up to one third of plant species within genera have originated by this polyploid speciation mechanism (Wood et al. 2009). On the contrary, others emphasize the harmful effect of interspecific hybridization because it could erode established gene pools and blur species boundaries (reviewed in Coyne & Orr 2004). The evolutionary outcome of hybridization largely depends on the rate of hybridization and hybrid fitness (Chapman & Burke 2006). If hybrids are inviable or sterile, the hybridizing species remain genetically intact because no further gene flow can occur. Alternatively, under persistent gene flow, hybridization may drive one (in case of asymmetric hybridization) or both parental species to extinction, possibly leading to the establishment of a hybrid swarm in their place. Hybridization is a significant conservation issue especially for rare species that come into contact with their more abundant congeners (Kothera et al. 2007, Field et al. 2009, Vít et al. 2014). Reproductive interactions may lead to the extinction of a rare species through genetic or demographic swamping (Levin et al. 1996, Rhymer & Simberloff 1996, Soltis & Gitzendanner 1999, Wolf et al. 2001). Hybrids may compete with parental species for habitat or resources and limit the population growth of the rare species. In addition, the ability of a rare plant to reproduce may be negatively affected by the production of hybrid seed at the expanse of conspecific seed. When interspecific mating barriers are weak, the size of hybrid population will increase and the rare congener may ultimately be assimilated into a more common species.

Assessing the threat of interspecific hybridization to a rare species and the development and implementation of appropriate conservation measures requires the accurate identification of hybrid individuals, which is not always an easy and straightforward task. Early attempts to determine hybrids relied upon the intermediate nature of the morphological characters of putative parents (Mallet 2005). However, it is often difficult to distinguish hybrids from purebred individuals based on their phenotypic variation (e.g. Řepka et al. 2014) because hybrids may express a mosaic of parental phenotypes or show similarities to progenitor species, especially when backcrossing is involved (Rhymer & Simberloff 1996). The advent of molecular tools has greatly advanced the field because molecular markers have provided a much more accurate and environmentally independent means of identifying hybrids (Soltis & Gitzendanner 1999, Hegarty & Hiscock 2005). Karyological data can aid identification of hybrids in groups with species-specific ploidy levels and/or number of chromosomes (Ekrt et al. 2010, Kabátová et al. 2014). However, both molecular and karyological techniques are time and labour intensive, and therefore impractical for large-scale population studies. A valuable tool for exploring the pattern of variation in heteroploid groups is DNA flow cytometry, which can process large numbers of samples and provide data (e.g. intermediate values of nuclear genome size) indicating hybrid states (Kron et al. 2007, Loureiro et al. 2010).

One of the ploidy-variable genera with easily hybridizing species is *Dianthus* L. (pink, carnation). This second largest genus of the family *Caryophyllaceae* contains around 300 species distributed mainly in Eurasia (Tutin & Walters 1993). Interspecific hybridization, even across different sections and ploidy levels, based on the analysis of morphological

variation, is frequently reported (Carolin 1957, Tutin & Walters 1993). Geographical isolation and ecological segregation are assumed to be the major barriers preventing gene flow under natural conditions.

The section *Plumaria* (Opiz) Asch. et Graebn., as defined by Novák (1927), includes tufted perennials with many sterile basal leaf rosettes and fragrant flowers with laciniate petal limb. The species have a dimorphic breeding system (gynodioecy) in which female individuals coexist in populations with hermaphrodite plants (Kovanda 1990). Kovanda (1982) recognized about 30 species in this section, occurring from western Europe to the Himalayas, and extending to northern Africa. While several species have limited distributions (e.g. *D. lumnitzeri* Wiesb. and *D. serotinus* Waldst. et Kit.), *D. arenarius* L. is a widely distributed white-flowered psammophyte, ranging from Scandinavia and the Baltics through Poland, Germany and the Czech Republic to Ukraine, Belarus and western Russia (Jalas & Suominen 1986). In most taxonomic treatments (Novák 1927, Meusel & Mühlberg 1978, Kovanda 1990, Tutin & Walters 1993), this species is split into five more or less geographically vicariant subspecies, although the taxonomic value of the diagnostic morphological characters (e.g. colour and height of flowering stems, and number and size of flowers) is uncertain and the group is in the need of revision using molecular tools.

Dianthus arenarius subsp. *bohemicus* (Novák) O. Schwarz is the most narrowly distributed subspecies and the only one native to the Czech Republic (Kovanda 1990, Danihelka et al. 2012). Currently, it occurs at a single site in central Bohemia: the National Nature Monument Kleneč (Kaplan 2012). The actual population size, incl. young seedlings, was estimated to be around 2000 individuals (censuses in 2010 and 2011; A. Šlechtová, pers. comm.) although the taxon was on the verge of extinction in 1980s when roughly 200 plants remained and no seedlings were recorded (Bělohoubek 2008). Historically, the second and more abundant population occurred by the neighbouring village of Vražkov but it was destroyed by tree planting in the second half of the last century (Kovanda 1990). In the late 1980s, a few plants grown from seeds collected at Kleneč were planted near to the village of Kyškovice, where a very small population still survives (Bělohoubek 2008). Due to its local occurrence and limited population size, *D. arenarius* subsp. *bohemicus* ranks among the critically endangered plants in the Czech flora (Čeřovský & Abtová 1999, Grulich 2012, Kaplan 2012). It 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).

In addition to its small population size, the genetic integrity of *D. arenarius* subsp. *bohemicus* may be threatened by interspecific hybridization with sympatric deep pink-flowered nominate subspecies of *D. carthusianorum* L. (section *Carthusianorum* Boiss.). The existence of interspecific crosses has long been suspected based on the occurrence of plants with petal colour between both putative parents, including tinged pink, light pink or white with pink spots or markings (Novák 1915, 1927, Kovanda 1990, Kováč 1996, Bělohoubek 2008). However, these hybrids were not confirmed by using molecular or cytogenetic methods. Because both taxa differ in the number of somatic chromosomes (*D. arenarius* subsp. *bohemicus* is tetraploid with 2n = 60, *D. carthusianorum* is diploid with 2n = 30; Kovanda 1984, 1990), it is possible to use karyological characteristics as an independent marker to distinguish parental plants as well as their hybrids.

In the present study, DNA flow cytometry together with multivariate morphometrics and sequencing of maternally-inherited chloroplast DNA were used to: (i) assess variation in nuclear genome size in a sympatric population of *D. arenarius* subsp. *bohemicus* and *D. carthusianorum* and quantify the proportion of interspecific hybrids, (ii) assess phenotypic variation of recognized karyological groups and identify species- and hybrid-specific morphological characters, and (iii) infer the direction of hybridization (i.e. identify maternal and paternal parents).

Material and methods

Plant material

Plant material was collected at the single natural site near the village of Kleneč in central Bohemia (50°23'23.0" N, 14°15'25.0" E; 215 m a.s.l.; Electronic Appendix 1). This locality was repeatedly visited from May to August in 2009–2011 and the following were collected: (i) one leaf per individual for flow cytometric estimation of nuclear genome size (1023 individuals in total, both mature plants and young seedlings were included), (ii) flowering plants of all the recognized genome size groups for morphometric analysis and assessment of pollen fertility (108 individuals in total, including 57 samples of *D. arenarius* subsp. *bohemicus*, 40 samples of *D. carthusianorum* and 11 hybrids; both hermaphrodite and female plants of each group were collected and attempts were made to include all the morphotypes present at the locality), (iii) silica-dried leaf tissue for molecular analysis (34 individuals in total, including 12 samples of *D. arenarius* subsp. *bohemicus*, 11 samples of *D. carthusianorum* and 11 hybrids). Individuals not bearing pure white flowers were given particular attention because these morphotypes are supposed to have arisen via interspecific hybridization (Novák 1915, 1927, Kovanda 1990, Bělohoubek 2008). Herbarium vouchers of both parental taxa and their hybrids are deposited in PRC. The artificially established small population near the village of Kyškovice was not included in this study.

Flow cytometry

DNA ploidy levels (Suda et al. 2006) were inferred from relative fluorescence intensities of DAPI-stained nuclei following the simplified two-step protocol detailed in Doležel et al. (2007). *Bellis perennis* L. (2C = 3.38 pg; Schönswetter et al. 2007) served as an internal reference standard. Briefly, a piece of intact leaf (about 0.5 cm in length) of an analysed plant was chopped together with an appropriate volume of internal reference standard with a sharp razor blade in a Petri dish containing 0.5 ml of ice-cold Otto I buffer (0.1 M citric acid, 0.5% Tween-20). The suspension was filtered through a 42-µm nylon mesh and then incubated for about 5 min at room temperature. After incubation, 1 ml of staining Otto II solution (0.4 M $Na_2HPO_4 \times 12\ H_2O$, 2 µl/ml of ß-mercaptoethanol and 4 µg/ml of DAPI) was added and the samples were kept for a short period at room temperature. Measurements were made using a Partec PA II flow cytometer equipped with a UV mercury arc lamp. Fluorescence intensity of 5000 particles was recorded and the resulting histograms evaluated using a Partec Flomax (ver. 2.4b). Up to 10 *Dianthus* individuals were pooled for the large-scale ploidy screening. Our previous experiments showed

that a minority cytotype can be reliably detected using flow cytometry even if it occurs at low frequencies (< 10%). Each plant was analysed separately if mixed samples were found or if the quality of resulting histograms was not sufficient (i.e. coefficient of variation of G0/G1 peak of *Dianthus* sample above 3%).

Absolute genome sizes (C-values) were estimated using propidium iodide flow cytometry in 16 plants, including four individuals of *D. arenarius* subsp. *bohemicus*, three individuals of *D. carthusianorum* and nine interspecific hybrids. The same protocol as above was adopted but the fluorochrome DAPI was replaced by an intercalating dye propidium iodide + RNase IIA (both at final concentrations of 50 µg/ml). Samples were analysed using a Partec CyFlow cytometer equipped with a green (532 nm, 100 mW output) diode-emitted solid-state laser. Each sample was reanalysed three times on different days. Differences in the monoploid genome size (Cx-values; Greilhuber et al. 2005) were tested using a generalized linear model (GLM) in the SAS 9.1 package.

Multivariate morphometrics

Eleven quantitative and four ratio characters were assessed on 108 individuals (Table 1). Measurements (in millimetres) of epicalyx scales, calyx, petals and the lowermost pair of cauline leaves were done on separated parts that were individually attached by a transparent adhesive tape to a white sheet of thick paper immediately after collection. Missing character values were replaced by a group mean (primary values were available for > 90% of individuals in each category). The data were analysed using UNIVARIATE (basic statistics), CORR (correlation analysis), PRINCOMP (principal component analysis), CANDISC (canonical discriminant analysis, CDA), and DISCRIM (classification discriminant analysis) procedures in the SAS 9.1 package. In general, the analyses followed Rosenbaumová et al. (2004). In discriminant analyses, individual plants were the operational taxonomic units (OTUs) and taxonomic groups (parental taxa and their hybrid) were defined based on DNA ploidy levels estimated using flow cytometry. Because the data distributions within the groups were not multivariate normal, the Spearman rank correlation coefficient and the k-nearest-neighbour discriminant function were used. The discriminant power was determined by cross-validation (Klecka 1980).

Chloroplast DNA sequencing

DNA from silica-dried leaf tissue of 34 samples was isolated using an Invisorb Spin Plant Mini Kit (Invitek). The concentration of DNA was determined spectrophotometrically and the samples were diluted to a final concentration of 5 ng/µl. Ten universal chloroplast primers (Taberlet et al. 1991, Shaw et al. 2005, 2007) were tested for variation: *atp*I-*atp*H, *ndh*F-*rpl*32, *psb*J-*pet*A, *rpl*32-*trn*L, *trn*G-*trn*2G, *trn*H-*psb*A, *trn*L-*trn*F, *trn*Q-*rps*16, *trn*V-*ndh*C, and *ycf*6-*psb*M. Each 20-µl PCR reaction contained 11.9 µl of sterile Milli-Q water (Millipore), 2 µl of AmpliTaq Gold® 360 buffer l0× (Life Technologies), 4 µl of $MgCl_2$ (25 mM), 0.4 µl of deoxyribonucleotide triphosphate mix (10 mM, Fermentas), 0.25 µl of each primer (25 nM, Sigma Aldrich), 0.2 µl of AmpliTaq Gold® 360 DNA polymerase and 1 µl of DNA (5 ng/µl). PCR amplifications were done in a Mastercycler (Eppendorf) using the following programme: initial denaturation at 94 °C for 10 min, followed by 35 cycles of 30 s at 94 °C, 30 s at annealing temperature ranging from 53 to 57 °C, 2 min at 72 °C and a final extension of 10 min at 72 °C. Successfully amplified PCR products were

Table 1. – List of morphological characters and their contributions to the first (Can1) and second (Can2) canonical axes in the canonical discriminant analysis. Three taxonomic groups (*Dianthus arenarius* subsp. *bohemicus*, *D. carthusianorum* and their hybrid) represented by 108 individuals were analysed. Four characters with the highest absolute loadings for the first axis are in bold. Character v8 was not included in the discriminant analyses because of its strong correlation with v7.

No.	Character description	Can1	Can2
v1	Length of the lowermost cauline leaf	**0.904**	0.222
v2	Inner epicalyx scale length	0.841	0.190
v3	Inner epicalyx scale width	–0.256	0.210
v4	Calyx tube length	**–0.932**	0.261
v5	Calyx tube width	0.561	0.399
v6	Apical calyx tooth length	–0.518	0.008
v7	Petal length	**–0.886**	0.290
v8	Petal claw length	*	*
v9	Petal limb length	–0.826	0.251
v10	Petal width	–0.730	0.135
v11	Length of the undivided part of the petal limb	–0.427	0.313
v12	Inner epicalyx scale length / width (v2/v3)	0.858	0.074
v13	Calyx tube length / width (v4/v5)	**–0.945**	0.099
v14	Petal length / width (v7/v10)	–0.466	0.230
v15	Depth of limb laciniation (petal limb length / length of the undivided part of the petal limb; v9/11)	–0.834	0.010

purified using a GeneElute PCR Clean-Up Kit (Sigma) and directly sequenced by Macrogen Inc. (Seoul, Korea) using the original PCR primer sets in both directions. Sequences were edited using Chromas Lite (Technelysium) and manually aligned in BioEdit (Hall 1999). DNA sequences of the variable *psb*J-*pet*A chloroplast region are available in GenBank (accession numbers KP096403, KP096404, KP096405, and KP096406)

Pollen and seed fertility

The viability of the pollen grains of 18 individuals of *D. arenarius* subsp. *bohemicus*, 9 of *D. carthusianorum* and 3 hybrids was determined using modified Alexander staining (Peterson et al. 2010). Because most hybrids did not develop or aborted their anthers (they were possibly females), pollen of only three samples were analysed. Anthers were collected at anthesis and stored in paper bags at room temperature for a few weeks. Anthers were then fixed in Carnoy's fixative (96% ethanol : chloroform : acetic acid in a ratio of 6 : 3 : 1) for at least two hours, transferred onto a microscope slide and gently dissected with a needle in a drop of staining solution. The staining stock solution consisted of 54.5 ml distilled water, 25 ml glycerol, 10 ml 96% alcohol, 4 ml glacial acetic acid, 5 ml acid fuchsin (1% water solution), 1 ml malachite green (1% solution in 96% alcohol) and 0.5 ml orange G (1% water solution). The slide was slightly heated over a weak flame and then covered with a cover slip. Samples were observed under an Olympus BX41 optical microscope at 100× magnification. Several photographs were taken using an Olympus C-7070 digital camera, and the viability of 400–1000 pollen grains per sample was assessed. Pollen grains with a purple-stained cytoplasm were considered viable, while green or whitish grains were considered non-viable.

Seed set was assessed in situ for labelled individuals of all three taxonomic groups. The numbers of well-developed and aborted seeds were determined in 144 ripe capsules originating from ten hybrid individuals. In each parental species, seed set was visually inspected in approximately 100 capsules.

Results

Flow cytometric measurements provided high-resolution histograms with distinct peaks of analysed samples and a low background signal for the samples analysed (Fig. 1). The analysis of 1023 plants yielded three non-overlapping groups of fluorescence intensities, corresponding to *D. carthusianorum* (mean fluorescence ± SD relative to internal reference standard, *Bellis perennis*, with a unit value 0.324±0.008, n = 40), interspecific hybrid (0.510±0.005, n = 11) and *D. arenarius* subsp. *bohemicus* (0.685±0.007, n = 972). Although the hybrid status of 39 individuals was suspected because of the characteristics of their flowers, it was confirmed by flow cytometry only in 11 of them. Nuclear DNA C-values of both parental taxa and their hybrid are summarized in Table 2. Monoploid genome sizes of the three taxonomic groups differed significantly (GLM, $F = 71.88$, $P < 0.001$) although the absolute differences were quite small.

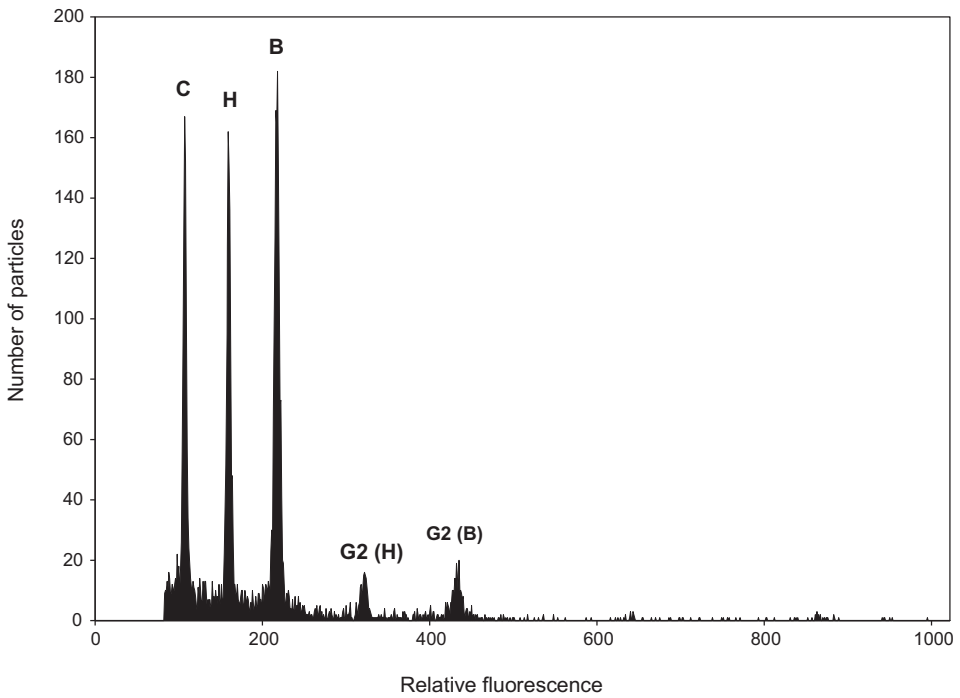

Fig. 1. – Fluorescence histogram of DAPI-stained nuclei showing simultaneous analysis of *Dianthus carthusianorum* (C), *D. arenarius* subsp. *bohemicus* (B) and their F1 hybrid (H).

Table 2. – Holoploid (mean 2C-values ± SD) and monoploid (mean Cx-values) genome sizes (in pg of DNA) of the two *Dianthus* taxa and their F1 hybrid.

	Mean 2C-value ± SD	Mean Cx-value	N
D. arenarius subsp. *bohemicus*	2.17 ± 0.02	0.54	4
D. carthusianorum	1.01 ± 0.01	0.51	3
hybrid	1.58 ± 0.02	0.53	9

Table 3. – Species-specific variation in the *psb*J-*pet*A chloroplast region. Hybrids share the haplotypes with both parents.

	position in the *psb*J-*pet*A alignment			N
	126–127	422	853	
D. arenarius subsp. *bohemicus*	–	A	G	12
hybrid	–	A	G	8
hybrid	GG	C	C	3
D. carthusianorum	GG	A	C	11

Six of the ten primer pairs amplified successfully and one of them (*psb*J-*pet*A) showed species-specific variation between *D. arenarius* subsp. *bohemicus* and *D. carthusianorum*. Analysis of interspecific crosses provided evidence for reciprocal hybridization: three hybrids shared the *D. carthusianorum* haplotype and eight the *D. arenarius* subsp. *bohemicus* haplotype (Table 3).

Principal component analysis based on 15 phenotypic characters revealed a distinct group of OTUs corresponding to *D. carthusianorum* while interspecific hybrids were only slightly different from that of the group *D. arenarius* subsp. *bohemicus* (Fig. 2). In all the taxonomic groups recognized, female individuals occupied a marginal position with respect to their overall morphology. The origin of hybrids (i.e. the identity of the maternal and paternal parent) had no major effect on their phenotypic appearance.

The length of petal claw (v8; Table 1) correlated strongly (Spearman coefficient > 0.95) with the total petal length (v7) and as a result, the former character was not included in the discriminant analyses. CDA of the three taxonomic groups using the remaining 14 characters resulted in a near-complete separation of all groups along the first canonical axis (Fig. 3) and correct classification of 97.2% of the individuals. Only three plants (two of them being females) of *D. arenarius* subsp. *bohemicus* were misclassified as hybrids. Calyx characteristics (length and length to width ratio; v4, v13), petal length (v7), and the length of the lowermost pair of cauline leaves (v1) were the characters most closely correlated with the first canonical axis (Table 1). CDA using the two primary characters with the highest discriminatory power (v4 and v1) yielded a highly comparable classification success (96.3%) as that with all 14 characters. In this modification, three plants of *D. arenarius* subsp. *bohemicus* and one plant of *D. carthusianorum* were erroneously classified as hybrids. Basic descriptive statistics of all characters studied (incl. mean, median, minimum and maximum values, 5% and 95% percentiles) for the three taxonomic groups are summarized in Electronic Appendix 2.

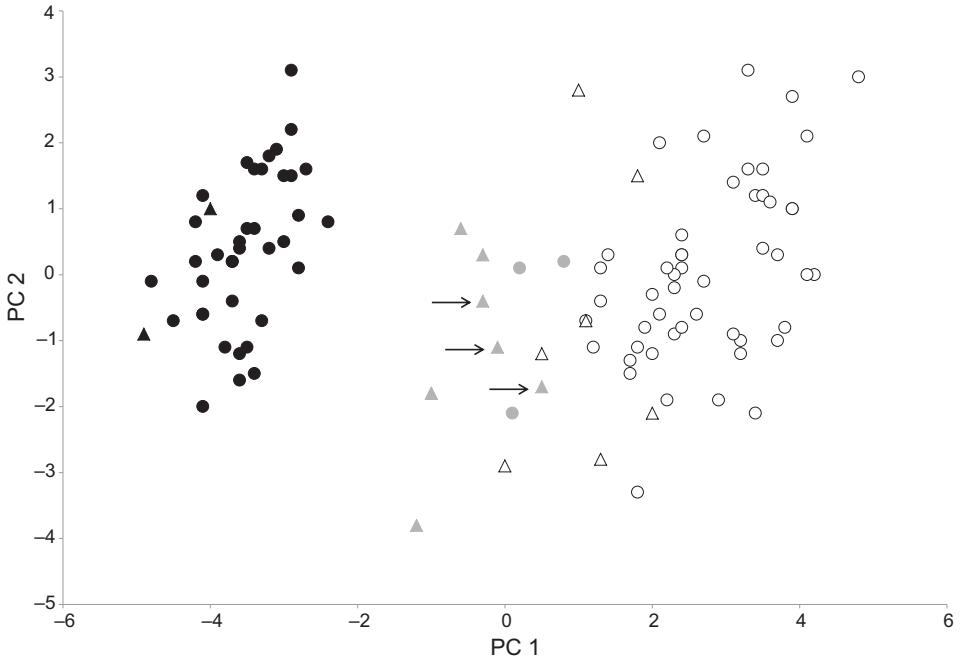

Fig. 2. – Principal component analysis of 108 *Dianthus* samples based on 15 morphological characters (see Table 1 for character descriptions); *D. arenarius* subsp. *bohemicus* – white symbols, *D. carthusianorum* – black symbols, hybrid – grey symbols. The first and second ordination axes explain 59.5% and 13.3% of total variation, respectively. Hermaphrodite and female individuals are shown as circles and triangles, respectively. Arrows indicate hybrids with *D. carthusianorum* as maternal parent.

Fertility of hybrid individuals was considerably reduced. Pollen fertilities of the three hermaphrodite hybrids were 0%, < 1% and ~25%, whereas the proportion of potentially viable pollen grains in parental species always exceeded 90% (ranges for *D. arenarius* subsp. *bohemicus* and *D. carthusianorum* were 91.2–99.2 % (n = 18) and 90.4–98.4 % (n = 9), respectively; Fig. 4). There were only aborted seeds in capsules of hybrid individuals.

Discussion

This study aimed at resolving the long-standing debate (e.g. Novák 1915, 1927, Kovanda 1990, Kubát et al. 2002, Bělohoubek 2008) concerning the rate and evolutionary consequences of interspecific hybridization between the Czech stenoendemic *D. arenarius* subsp. *bohemicus* and its widespread common congener *D. carthusianorum* in the last remaining natural population of the former taxon. In contrast to previous studies that subjectively identified putative hybrids on the basis of morphological characters (colour of petals in particular), we used differences in ploidy level of both parental species and recognized interspecific crosses using DNA flow cytometry, based on intermediate values of the amount of nuclear DNA.

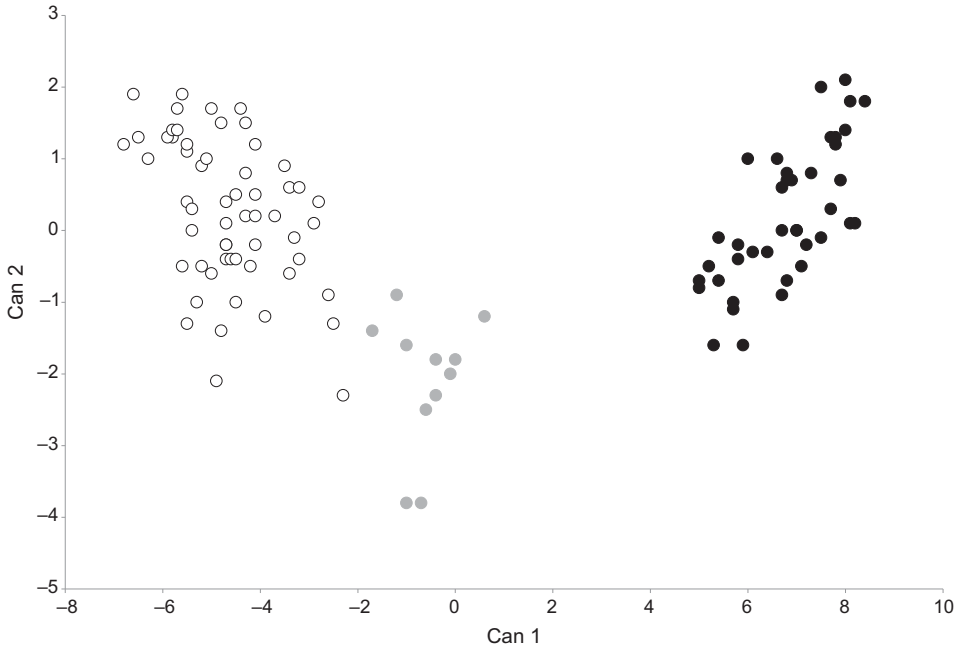

Fig. 3. – Canonical discriminant analysis of 108 *Dianthus* samples based on 14 morphological characters (see Table 1 for character descriptions). *D. arenarius* subsp. *bohemicus* – white symbols, *D. carthusianorum* – black symbols, hybrid – grey symbols. The first and second ordination axes explain 98.3% and 1.7% of total variation, respectively.

Difficulties in hybrid identification

Although both parental species are morphologically distinct (they belong to different sections of the genus), reliable identification of hybrid individuals is not easy. In particular, published studies mention weak boundaries between *D. arenarius* subsp. *bohemicus* and putative hybrids (Novák 1915, 1927). The authors emphasize pink colour or pink tinge of flower petals, either over the entire surface or in the form of streaks or spots, as diagnostic characters of hybrids. However, a rather continuous variation in petal colour and pattern precludes any firm taxonomic conclusions and made accurate estimates of the incidence of interspecific hybridization impossible.

Heteroploid hybrids can be reliably identified by intermediate numbers of chromosomes or genome sizes intermediate between the values of putative parents (e.g. Bureš et al. 2003, Rosenbaumová et al. 2004, Suda et al. 2004, Ekrt et al. 2010, Koutecký et al. 2011, Kabátová et al. 2014). We followed these studies and performed a comprehensive flow cytometric screening of nuclear DNA amounts in all mature (flowering) individuals and most seedlings present at the site of *D. arenarius* subsp. *bohemicus*. Mature individuals with at least partly pink-coloured petals and seedlings growing in their vicinity were of particular interest. In total, 39 morphotypes were thought to be hybrids. However,

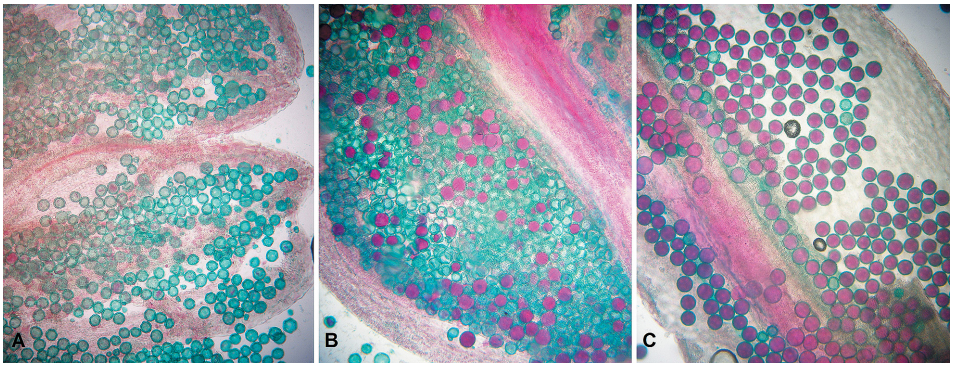

Fig. 4. – Differential staining of aborted (green) and viable (purple) pollen grains. (A) Hybrid with fully aborted pollen; (B) hybrid with partially viable pollen; (C) *Dianthus arenarius* subsp. *bohemicus* with mainly viable pollen.

most of them had the same nuclear genome size as 4x *D. arenarius* subsp. *bohemicus* and intermediate C-values were only recorded for 11 individuals (~1.1% of all the samples analysed). Low intra-group variation and clear discontinuities in the amount of nuclear DNA between interspecific hybrids and their parents indicate that backcrossing is very unlikely. Interspecific hybrids may also originate via unreduced gametes of one or both parents (e.g. Koutecký et al. 2011). In our particular case, syngamy of an unreduced gamete of diploid *D. carthusianorum* and a reduced gamete of tetraploid *D. arenarius* subsp. *bohemicus* would yield tetraploid hybrids with a holoploid genome size only slightly different (by ~3% lower) from that of the tetraploid parent and therefore hard to distinguish with the aid of flow cytometry. However, we consider this hybrid scenario unlikely because all the tetraploid individuals with (partly) pink-coloured petals analysed grouped together with pure white plants of *D. arenarius* subsp. *bohemicus* in the PCA analysis, and were fertile. Moreover, both pure white and pink flowers can occur on the same plant (Fig. 5F) and variation in petal colour is reported in several populations of other subspecies of *D. arenarius* (J. Vítová & P. Vít, unpubl. observations). Based on our experience, a pink tinge to the petals of *D. arenarius* often becomes more apparent as the flowers age. In addition, it seems that the production of unreduced gametes by *D. carthusianorum* is very low because our ploidy screening of samples from across central Europe revealed only one triploid (originating via a fusion of reduced and unreduced gametes) among the 732 plants analysed (unpubl. data).

Although we have not determined the exact number of chromosomes in hybrid individuals for obvious reasons (rarity and sterility of hybrids), we argue that they are most likely triploid (based on x = 15). Triploids seem to originate only rarely in natural populations of *Dianthus* species. In addition to our unpublished record of DNA-triploidy in *D. carthusianorum*, a few triploids in an otherwise diploid population of *D. broteri* are reported (Balao et al. 2009). There are several triploid cultivars of the ornamental *D. caryophyllus* (Yagi et al. 2009).

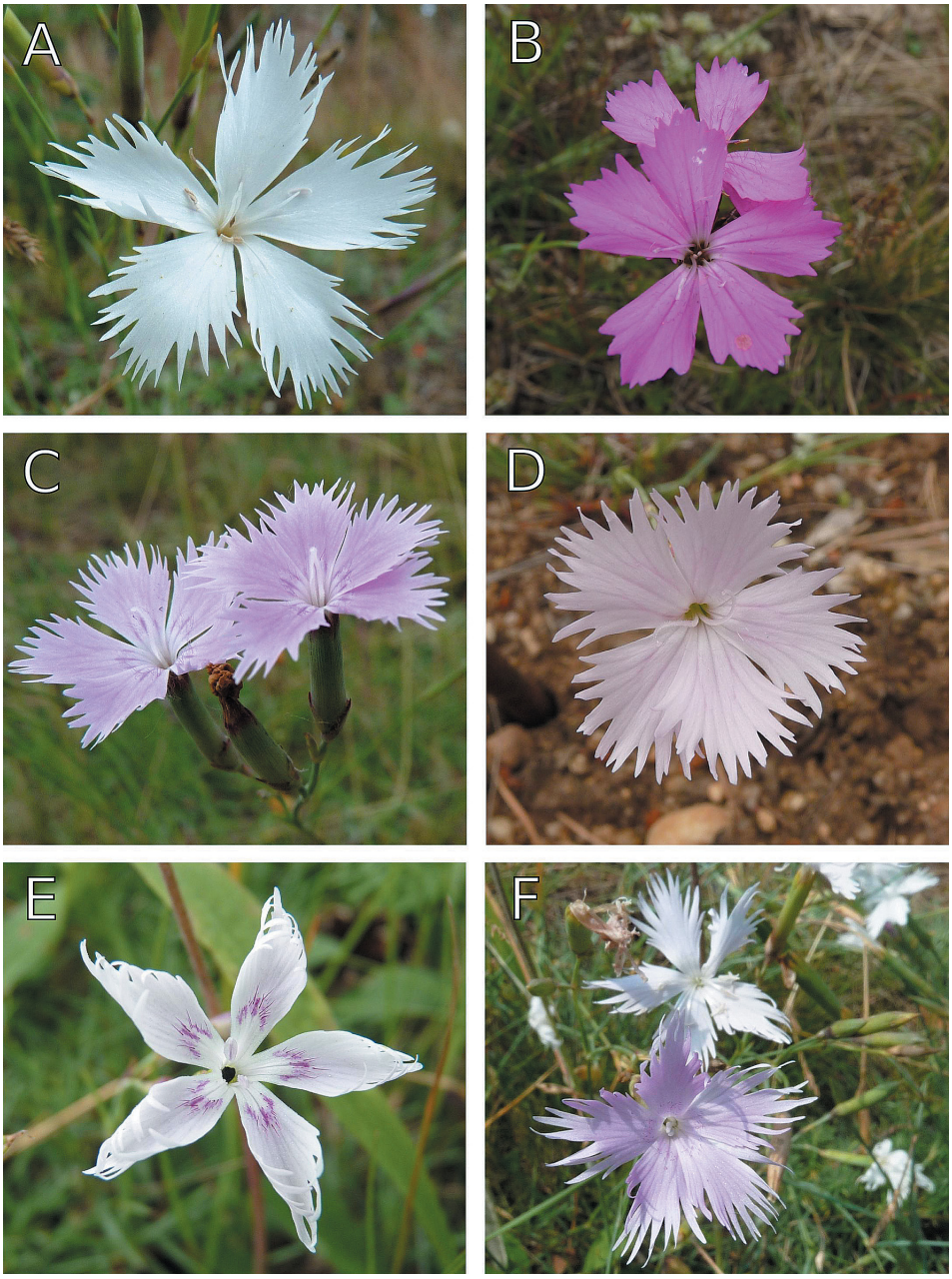

Fig. 5. – Variation in flower colour and pattern recorded at the Kleneč site. (A) Typical *Dianthus arenarius* subsp. *bohemicus*; (B) typical *D. carthusianorum*; (C) typical hybrid with an intermediate flower colour; (D) pale-flowered hybrid; (E) *D. arenarius* subsp. *bohemicus* with pink spots and hairs on the petal limb; (F) a single tuft of *D. arenarius* subsp. *bohemicus* with both white and light pink flowers. Note that (E) and (F) may be misidentified as hybrids and (D) erroneously identified as an endemic taxon.

Phenotypic signatures and direction of interspecific hybridization

Analysis of chloroplast DNA revealed that interspecific hybridization at the Kleneč site is reciprocal. Most hybrids share their plastid haplotype with *D. arenarius* subsp. *bohemicus* and the *D. carthusianorum*-haplotype was recorded in only three hybrid individuals. Actually, there was a one-base difference between the latter type of hybrid and local *D. carthusianorum* (see Table 3), which may indicate the involvement of a now extinct or unsampled genotype of the diploid parent. Indirect support for reciprocal hybridization comes from the spatial distribution of hybrids – while some occurred close to *D. arenarius* subsp. *bohemicus*, others occurred in patches of *D. carthusianorum*. Most other published incidences of reciprocal heteroploid hybridization are based on experimental studies (e.g. Hayashi et al. 2007) whereas to the best of our knowledge, the co-existence of genetically-proven reciprocal interspecific hybrids in a single natural population has yet to be reported.

Irrespective of their origin, all of the interspecific *Dianthus* hybrids studied had considerably reduced reproductive fitness, expressed in terms of pollen fertility (not exceeding 25% in the three hybrids with developed stamens) or seed set (the lack of fully developed seeds). In contrast, pollen fertility in both parental species exceeded 90% and their capsules largely contained fully developed seeds. The triploid block recorded in many other plant species (e.g. Greiner & Oberprieler 2012) is widely acknowledged as a major breeding barrier preventing inter-ploidy gene flow (Köhler et al. 2010). Maternal plant of all three hybrids with developed stamens (i.e. hermaphrodite individuals) was *D. arenarius* subsp. *bohemicus*; nevertheless, this is considered coincidental due to the very limited sample size. The proportion of functionally female individuals in hybrids considerably exceeded their frequencies in either parent (Fig. 2). However, it is currently not possible to distinguish whether the lack of anthers in hybrids is due to conventional gynodioecy or a consequence of hybridization-driven male sterility. Interestingly, hybrids were all mature plants, while relative genome sizes of all the seedlings studied corresponded to one or the other parent. Possibly, the hybrids established within a narrow time frame in the late 1990s when large gaps with reduced competition were created by mechanical disturbance as a part of site management practice (Bělohoubek 2008).

Most of the vegetative and floral characters of hybrids studied showed intermediate values between those of their parents (Electronic Appendix 2). Although the colour of petals has a limited taxonomic value, hybrids can be identified by using a combination of cauline leaves, calyx and petal characteristics. Flowers of hybrids are born solitary or in lax, few-flowered inflorescences, similar to *D. arenarius* subsp. *bohemicus*. In contrast to this parental species, hybrids have a shorter calyx and smaller petals. Petals of hybrids are usually more shallowly incised and have a uniform pink shade although only faintly coloured flowers were also encountered. We admit that the number of individuals (108 in total) subjected to morphological analyses was rather low but we consider the sampling sufficiently representative. In particular, we included all recognized hybrids and attempted to sample the entire morphological variation of both parents present at the locality (especially with respect to the colour, pattern, size and laciniation of petals). Moreover, the stenoendemic status of *D. arenarius* subsp. *bohemicus* precludes more extensive sampling due to conservation concerns. Last but not least, the group sizes in discriminant analysis should not differ dramatically (Klecka 1980).

Conservation consequences and determination key

Our results have practical conservation applications as the knowledge of the extent and rate of interspecific hybridization is essential for effective conservation measures of this Czech endemic taxon in its last remaining population. The low number and limited fertility of F1 hybrids together with the lack of later-generation hybrids indicate that the genetic integrity of *D. arenarius* subsp. *bohemicus* is currently not seriously threatened. Moreover, identification of hybrid individuals using a set of taxonomically informative morphological characters (see below) is quite straightforward, allowing timely intervention if the size of hybrid population increases. Despite these promising findings, there is need for caution because recent dramatic disturbances at the site (i.e. the large-scale removal of the vegetation cover) as a part of the management practice may enhance the establishment of hybrid individuals in the near future.

The taxa investigated can be determined using the following key. Values of quantitative characters are in terms of their (minimum–) 5 percentile – 95 percentile (–maximum) values. For practical purposes, we rounded the values to multiples of 0.5.

1a Flowers many, in a compact head, petals deep pink, limb shallowly incised (to less than 1/4 of its length), calyx (12.0–) 12.5–16.0 (–17.0) mm long, lowermost cauline leaves (37–) 43–115 (–122) mm long ..... ***D. carthusianorum* L.** (Fig. 5B)

1b Flowers solitary or in lax, 2–3-flowered inflorescences, petals white to pink, limb laciniate (incised to more than 1/4 of its length), calyx 14.5–26.0 (–27.5) mm long, lowermost cauline leaves (8–) 9–33 mm long **2**

2a Calyx (18–) 20.5–26.0 (–27.5) mm long, petals (21.5–) 27.0–43.0 (–45.0) mm long, claw (7.5–) 17.5–28.0 (–29.5) mm, limb (8.5–) 11.0–15.5 (–17.5) mm, pollen of hermaphrodite individuals fertile, most seeds fully developed. Plants forming relatively dense tufts, petals deeply incised, usually pure white (but occasionally tinged pink and/or with pink spots or hairs on the limb base) ..... ***D. arenarius* subsp. *bohemicus* (Novák) O. Schwarz** (Fig. 5A, E, F)

2b Calyx 14.5–20.0 mm long, petals 22.0–31.5 mm long, claw 13.5–20.0 mm, limb 8.5–11.5 mm, pollen fertility of hermaphrodite individuals greatly reduced, seeds aborted. Plants forming loose to relatively dense tufts, petals more shallowly incised, usually with a pink tinge, rarely almost white ..... ***D. arenarius* subsp. *bohemicus* × *D. carthusianorum*** (Fig. 5C, D)

See http://www.preslia.cz for Electronic Appendix 1–2.

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Souhrn

Vážné riziko pro přežití některých vzácných druhů rostlin může představovat mezidruhová hybridizace s jejich široce rozšířenými hojnými příbuznými. Ohrožení spočívá v genetické erozi, snížené tvorbě vlastních semen na úkor hybridních i v kompetici o abiotické a biotické zdroje. Při podezření na mezidruhové křížení je proto nezbytné spolehlivě stanovit četnost hybridů i charakter hybridizace. Naše studie se zabývá podrobným zhodnocením hybridizace mezi kriticky ohroženým endemickým hvozdíkem českým písečným (*Dianthus arenarius* subsp.

bohemicus) a sympatrickým hvozdíkem kartouzkem (*D. carthusianorum*) na jediné původní lokalitě ve středních Čechách (NPP Kleneč). Oba rodičovské druhy byly jednoznačně odlišeny průtokovou cytometrií na základě rozdílů v množství jejich jaderné DNA (hvozdík kartouzek je diploidní, zatímco hvozdík písečný tetraploidní). Přibližně 1% analyzovaných jedinců vykazovalo intermediární velikost genomu, která odpovídala F1 hybridům. Zpětné křížení nebo introgrese nebyla na základě cytometrických dat potvrzena. Hybridní jedinci mají výrazně sníženou fertilitu: jejich pyl je z velké části abortovaný a tvorba semen nebyla pozorována. Srovnání chloroplastových haplotypů odhalilo, že hybridizace probíhá obousměrně (oba rodičovské druhy mohou fungovat jako mateřský i otcovský jedinec). Mezi nejdůležitější morfologické znaky umožňující spolehlivé určení rodičovských druhů i jejich kříženec patří: délka spodních lodyžních listů, délka kalicha a délka korunních lístků (vč. délky nehtu a délky čepele). Získané poznatky ukazují, že mezidruhová hybridizace v současné době nepřestavuje výrazné nebezpečí pro přežití endemického hvozdíku písečného českého. S ohledem na nedávné ochranářské zásahy na lokalitě (velkoplošné stržení drnu) je však potřeba provádět občasnou kontrolu populační struktury, neboť rozsáhlé disturbance mohou napomáhat uchycení hybridních jedinců.

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