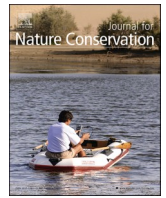




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journal homepage: www.elsevier.com/locate/jncPotential risk of interspecific hybridization in *ex situ* collectionsSissi Lozada-Gobilard^{a,b,*}, Hana Pánková^a, Jinlei Zhu^{a,c}, Bojana Stojanova^e,
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ABSTRACT

Spontaneous hybridization in *ex situ* conservation facilities could potentially contaminate endangered plant species destined for the reintroduction into the wild. Despite the general recognition of the importance of such danger, studies exploring the consequences of hybridization including subsequent performance of the hybrids in comparison to the original species are rare. The aim of the present study was to assess the potential consequences of hybridization between the endangered endemic serpentinophyte species, *Minuartia smejkalii*, and its congener allopatric species, *M. caespitosa*. We performed controlled inter-specific crosses and compared seed production, germination, growth, seed morphology and dispersal ability between *M. smejkalii* and the hybrids. Our results showed that *M. smejkalii* and *M. caespitosa* can hybridize and produce viable seeds. Number of seeds per capsule produced by *M. smejkalii* and the hybrids did not differ but germination rates were higher in the hybrids. In addition, hybrids produced a higher number of flowers than *M. smejkalii* in open sites and serpentine soils, indicating that the hybrids perform better in the extreme serpentine conditions than the original serpentine species. Our simulation results showed a higher dispersal potential in hybrids compared to *M. smejkalii* despite their higher terminal velocity. Our results provide evidence that hybrid individuals from *ex situ* collections might outcompete the endemic *M. smejkalii* in the wild, if they are introduced by mistake. Therefore, for conservation and reintroduction purposes, *ex situ* hybridization events should be considered as a potential threat and treated carefully. We recommend establishing an adequate management of potentially crossable species and cautiously selecting individuals to be used for restoration.

1. Introduction

Plant conservation can be achieved through *in situ* or *ex situ* management. *In situ* conservation refers to protection of plant species in their natural habitats, while *ex situ* focuses on conservation of species outside their habitats in artificial facilities (e.g. botanical gardens, arboreta, Mounce, Smith, & Brockington, 2017; Raven, 2004). *Ex situ* conservation plays an important complementary role to *in situ* conservation and reintroduction programs to ensure survival of species in the wild (Abeli et al., 2020). To achieve a successful reintroduction of plant species from *ex situ* collections to the wild, careful phenotypic and genotypic assessments of the material should be implemented (Heywood, 2011; Raven, 2004). Therefore, a lot of attention was given to the quality and reliability of *ex situ* collections for revegetation and restoration purposes

due to negative phenotypic and genetic effects of plant cultivation (Ensslin & Godefroid, 2019). For example, previous studies documented maladaptive changes in life-history traits, fitness, genetic diversity and adaptation in plants from botanic gardens compared to their wild counterparts (e.g. Ensslin, Sandner, & Matthies., 2011; Ensslin, Tschöpe, Burkart, & Joshi., 2015; Ensslin, Van de Vyver, Vanderborght, & Godefroid, 2018; Schröder & Prasse, 2013b).

Ex situ collections in botanical gardens or arboreta can also create new opportunities for hybridization bringing together geographically isolated taxa (Ye, Yao, Zhang, Kang, & Huang, 2006). Hybridization is the interbreeding of individuals from genetically distinct populations regardless of their taxonomic status (Haig & Allendorf, 2006). Spontaneous hybridization can cause negative effects in *ex situ* collections contaminating open-pollinated seed or seedlings destined for

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reintroduction (Maunder, Hughes, Hawkins, & Culham, 2004). If hybrids possess the same or higher fitness than their parental species, and are used as restoration material, they could outcompete the parental species and cause its local extinction in the wild (Hegde, Nason, Clegg, & Ellstrand, 2006; Laguna, Navarro, Pérez-Rovira, Ferrando, & Ferrer-Gallego, 2016; Maunder et al., 2004).

For this reason, it is very important that *ex situ* plant material is not mixed with related species prior to reintroduction programs. Previous studies reported negative effects of hybridization of threatened and endangered populations in the wild (Hegde et al., 2006; Macková, Vít, & Urfus, 2018; Wolf, Takebayashi, & Rieseberg, 2001), but only a few focused on the effect of hybridization in *ex situ* living collections. For example, changes in life history traits such as germination were reported where hybrids presented a shorter germination time and higher germination rate than the wild parents (Schröder & Prasse, 2013a). In addition, absence of reproductive isolation in naturally allopatric species constitutes a potential risk of genetic introgression and loss of genetic diversity when grown together in *ex situ* facilities (Ye et al., 2006).

Most studies on hybridization focus on fitness, but changes in traits related to dispersal of pollen and seeds, can also cause negative effects on populations. For example, Zhang, Ye, Yao, and Huang (2010) found longer pollen dispersal distances in interspecific hybrids of the extinct in the wild *Sinojackia xylocarpa* with its congener *S. rehderiana*, compared with their parental species in a botanic garden. In seeds, changes in morphology could lead to increased dispersal (Zhu, Liu, Xin, Liu, & Schurr, 2019) and colonization abilities of the hybrids increasing their invasive character in the natural populations. More efficient dispersal and colonization traits combined with a higher and faster germination could allow hybrids to outcompete the wild populations. For example, hybridization of *Spartina maritima* with *S. alterniflora* resulted in both a sterile hybrid (*S. x townsendii*) and later a fertile hybrid (*S. anglica*) that caused the extinction of the parents and now the hybrids have expanded their range in Western Europe (e.g., Ayres & Strong, 2001; Gray & Benham, 1990; Nehring & Hesse, 2008; Salmon, Ainouche, & Wendel, 2005). Nevertheless, to the best of our knowledge, no studies have focused on seed dispersal-related traits and abilities of hybrids compared to their congener species in *ex situ* collections.

Wild plants are well adapted to their natural environments because they have acquired the most efficient traits through natural selection. Species adapted to extreme environmental conditions are particularly vulnerable to any kind of disturbance by the introduction of hybrids, as they have developed extreme adaptation to their environment (Schröder & Prasse, 2013a). These species are usually endemic because they are well adapted to very hard conditions (i.e. high temperature, water deficit, heavy metals) presenting a shift in their life-history traits (i.e. earlier flowering, faster fruit development and seed production) compared to their generalist relatives (Brady, Kruckeberg, & Bradshaw, 2005; Wu, Lowry, Nutter, & Willis, 2010). Therefore, hybridization with close relatives could cause loss of the abilities to grow in these extreme habitats and thus have strong negative effects in these highly adapted species.

In the last decade, conservation efforts including creation of *ex situ* collections have been initiated in *Minuartia smejkalii*, an herbaceous serpentinophyte species endemic to the Czech Republic. *Minuartia smejkalii* populations are declining due to habitat fragmentation related to anthropogenic activities since 1960s (Pešout, 2001). Small remnant populations highly specialized to serpentine soils make this species particularly vulnerable to extinction. In the last decade, *M. smejkalii* populations have been grown in *ex situ* facilities. Two other species of *M. verna* agg. occur naturally in allopatry within the Czech Republic: *M. corcontica*, occurring in rocky soils, and *M. caespitosa*, which grows in sandy soils. *Minuartia caespitosa* has a larger distribution than *M. smejkalii*, while *M. corcontica* has a very limited distribution, with even smaller total area than *M. smejkalii*. All three species are categorized as endangered in the Czech Republic and are therefore grown in *ex situ* facilities. Due to the allopatry of *M. smejkalii* with its congener species, it is very likely that breeding barriers were not established and

therefore they might hybridize if given the chance. In *ex situ* conservation facilities, spontaneous hybridization with congener species might occur, and if not treated adequately, it might cause negative effects in natural populations. Therefore, understanding the potential risks and consequences of *ex situ* hybridization is essential for successful conservation and reintroduction of *M. smejkalii* into its natural habitat.

In the present study, we investigated the possible consequences of hybridization of *M. smejkalii* with *M. caespitosa* by comparing the hybrids with the *M. smejkalii* individuals. Specifically, we asked the following questions: (i) What are the effects of hybridization on plant fitness (seed production, germination), seed morphology and dispersal ability? (ii) What is the effect of hybridization on species adaptation to its natural environment? We hypothesize that hybrids will possess a higher fitness (Schröder & Prasse, 2013a), as well as different seed morphology and dispersal traits (seed release height and terminal velocity) with potential longer seed dispersal distances, but they will lose the ability to adapt to serpentine habitats.

2. Methods

2.1. Studied species

Minuartia smejkalii is an endemic species of the Czech Republic adapted to serpentine soils. Serpentine soils are patchily distributed rocky soils (Fig. 1B) characterized by low Ca/Mg ratios, low levels of nutrients (potassium, phosphorous, nitrogen), and elevated levels of toxic heavy metals such as nickel, chromium, magnesium and cobalt (Brady et al., 2005). This, together with low water retention and high temperature and erosion levels creates strong selective pressures on plants growing on serpentine soils resulting in low rates of population regeneration, small populations and high endemism (Brady et al., 2005).

Since the 1960s, some populations of *M. smejkalii* went extinct and others declined due to anthropogenic activities related to mining, construction of a motorway and a dam and agricultural expansion. Currently, *M. smejkalii* occurs in two regions (Želivka and Hrnččre) distributed over a total area of 500 km² (Fig. 1A). In Hrnččre, *M. smejkalii* occurs in one small population, while in the Želivka region, there are six geographically distinct populations (Z1-6) with a total demographic count between 400 and 1200 individuals in the last 13 years (Pánková, pers.obs). Despite the population size reduction caused by habitat fragmentation, levels of genetic diversity in this species are high with only moderate regional differentiation (Stojanova et al., 2020). This might be explained by a mainly outcrossing mating systems of the species, but might also constitute a transient state right after a bottleneck that will not last long without conservation measures (Stojanova et al., 2020). Even though *M. smejkalii* is self-compatible, self-pollination is not favored and smaller seeds are produced from selfing flowers compared to open pollinated flowers (Pánková and Stojanova, pers. obs.). *Minuartia smejkalii* has been classified as “critically endangered” in the Czech Republic and included in Appendix I of the Convention on the Conservation of European Wildlife and Natural Habitats and the IUCN international Red list (Bilz, Kell, Maxted, & Lansdown, 2011) and belongs to the species of priority European interest according to Habitats directive 92/43/EEC, Annex II.

Minuartia caespitosa has broader distribution, from northern Czech Republic, through north-western Germany to eastern Belgium (Hejný & Slavík, 1990). This species occurs on small, unstable sand dunes with sporadic vegetation at the bottom of sandstone rocks (Sádlo, Petřík, Boublík, Rychtařík, & Šimová, 2011). *Minuartia caespitosa* is classified as endangered in the Czech Republic. Although, *M. smejkalii* and *M. caespitosa* do not co-occur in nature, they can meet in *ex situ* facilities and potentially hybridize.

Due to the difficulty that represents manipulating the tiny flowers (size ~6–8 mm, Hejný and Slavík, 1990) (Fig. 1C) for artificial crossings and since *M. smejkalii* is the main conservation focus for the present study, we hybridized *M. smejkalii* × *M. caespitosa* with *M. smejkalii*

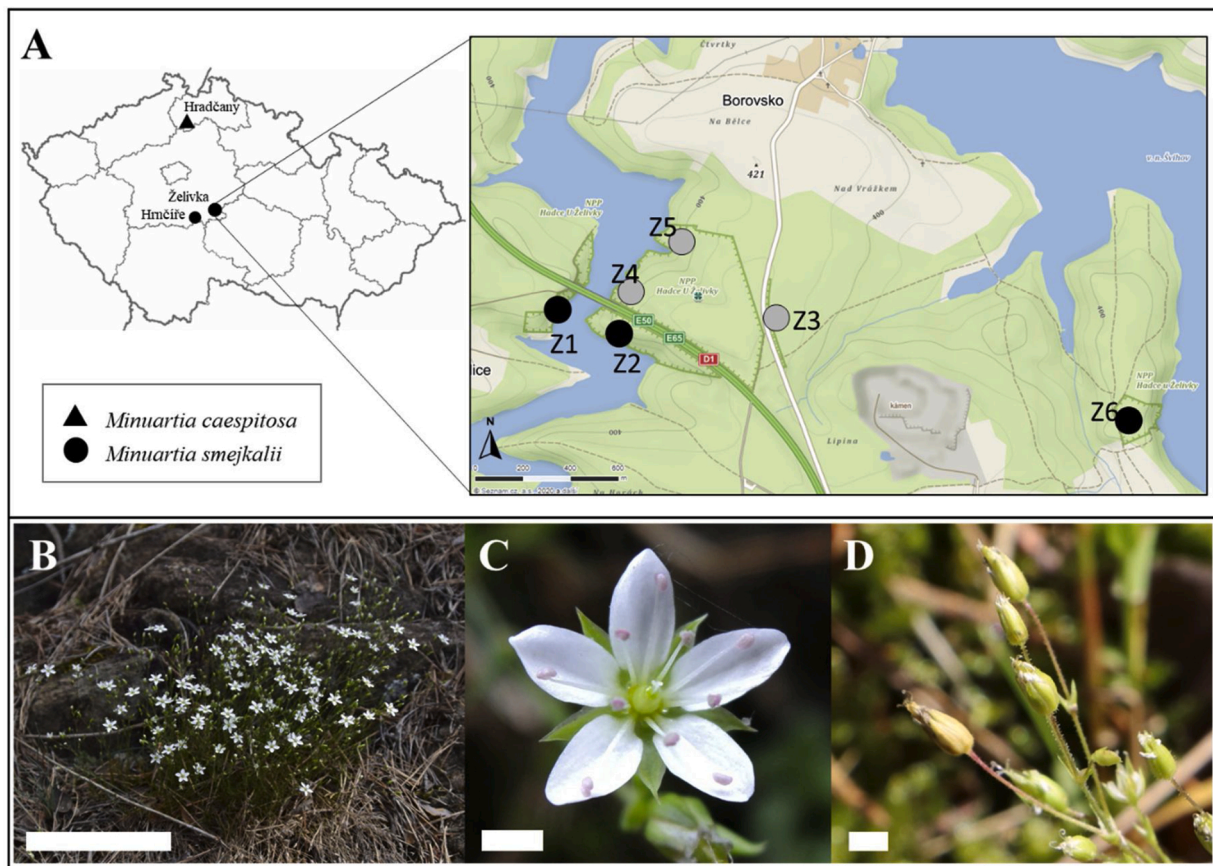


Fig. 1. Distribution of the populations of *Minuartia smejkalii* and *Minuartia caespitosa* and natural populations of *M. smejkalii*. (A) *Minuartia smejkalii* occurs in two regions: Želivka and Hrnčfře (in circles). In the Želivka region, six total populations occur (Z1–Z6). Black circles represent the populations where seeds were collected from for the experiments of the present study (Z1, Z2, Z6). *Minuartia caespitosa* seeds were collected from the Hradčany region located in the north of the Czech Republic (triangle). (B) Individual of *M. smejkalii* in a natural population growing in rocky serpentine soils (scale = 10 cm). Detailed flower (C) and fruit (D) of *M. smejkalii* (scale = 2 mm). Photographs taken by H. Pánková (B, C) and K. Hrušková (D).

always used as the maternal plants and compared it only to *M. smejkalii* crossed with itself. For the purposes of this study, we used *M. smejkalii* seeds coming from populations Z1, Z2 and Z6 from the Želivka region and *M. caespitosa* seeds from the Hradčany region, the latter being the only one with occurrence of *M. caespitosa* within the Czech Republic (Fig. 1A).

2.2. Crossing experiment

To assess whether *M. smejkalii* produces hybrids with *M. caespitosa*, we conducted a crossing experiment in August and September 2017 in a greenhouse of the Botanical Institute of the Czech Academy of Science in Průhonice. Plants of *M. smejkalii* were grown in serpentine soil and watered when necessary. Flowers from 16 *M. smejkalii* maternal individuals from the three populations received pollen *M. smejkalii* flowers from individuals coming from the same population and from *M. caespitosa*. When plants from populations Z1 and Z2 were used as pollen donors, we avoided using the same pollen donor multiple times on a single pollen receiver, while this was not always possible for population Z6 due to the low number of plants available for the experiment. For each maternal *M. smejkalii* plant, approximately half of the produced flowers were crossed with *M. smejkalii* pollen of the same corresponding population, and the other half were pollinated by *M. caespitosa*. This resulted in a total of 317 hand pollinations corresponding to 157 inter-specific and 160 intra-specific crossings (Table S1). The average number of flowers produced by plant is 421 ± 413 (Mean \pm SD, Stojanova, pers. com.).

Since the greenhouse was not insect-proof, plants used for the crosses were kept under $120 \times 80 \times 60$ cm cages covered with a double layer of

fine mesh fabric which allows light and wind to go through the cage and were only uncovered when performing the controlled crosses. Pollinations were performed daily between 8 and 12 a.m. Flowers designated to receive pollen were marked and castrated (removal of stamens) to avoid self-pollination. When the stigma became receptive, it was hand pollinated between 24–72 hours after castration. One to three anthers of the pollen donor flowers were delicately removed from a single flower and gently rubbed against each of the three ends of the receptive stigma. Pollen deposition on the stigma was verified with a magnifying glass.

No extra precautions were made to prevent pollen contamination after the hand pollinations, because intact castrated flowers did not develop seeds (Stojanova, pers. obs.). Mature capsules were collected and stored in a dry place at room temperature until counted and afterwards used for germination assays and seed morphology tests (see below).

2.3. Seed morphology

Seed morphology might have an effect on dispersal and also is a key trait to distinguish the *Minuartia* species (Dvořáková, 1990). Seed morphology was analyzed using a scanning electron microscope FEI Quanta 200 ESEM (Environmental Scanning Electron Microscope; FEI, Thermo Fisher Scientific) in seeds from the crossing experiments and from one natural population (Z2). Due to the destructive nature of this imaging technique, we limited our photography to 12 seeds of *M. smejkalii* compared to 12 of the hybrids, 10 seeds of *M. smejkalii* collected from natural populations and additional 10 seeds of *M. caespitosa*. All *Minuartia* seeds were scanned in their natural stage

(without coating) using low vacuum mode of the microscope (300×). Stability testing of the seeds in the microscope showed that they remained stable throughout the scanning process without tendency to artifact creation. From the images, we measured seed area and perimeter using ImageJ software following standardized protocol (Schneider, Rasband, & Eliceiri., 2012). We calculated adjusted seed perimeter as $\text{Perimeter} / \sqrt{\text{area}}$.

2.4. Germination assay

Seeds from each maternal plant and pollen donor type combination were sown in a separate dish on two layers of filter paper (Table S1). When no new germinated seeds were recorded for at least 7 days, we added gibberellic acid to stimulate germination and obtain maximum germination rates and evaluated seed viability for the following 14 days. Seedlings were counted at the end of the assay and germination rate was calculated as the number of germinated seeds relative to the numbers of sown seeds. Due to high mortality of seedlings from *M. smejkalii* × *M. smejkalii* crossings (less than 10 individuals left in total), extra seeds of *M. smejkalii* from nature were germinated at the same time and in the same conditions as the hybrids (Fig. S1). These seedlings, together with those from *M. smejkalii* × *M. caespitosa* crossings, were grown and used for further experiments as described below.

2.5. Plant performance under different conditions

Seedlings of *M. smejkalii* × *M. caespitosa* from the crossing experiments, plus seedlings of *M. smejkalii* from seeds coming from natural populations of the germination assays were transplanted into individual pots and grown for six months in standard common garden initial soil. After six months, ~3 cm in diameter plants were transplanted into serpentine or sandy soils and exposed to two different light conditions: shade (30 % light transmitted) and open (light) in a full factorial and balanced design (Fig. S1). Serpentine and sandy soils resemble the natural conditions where *M. smejkalii* and *M. caespitosa* respectively occur. The shading and open treatments simulated two different growing conditions: open resembles the primary conditions on open rocks, while shade represents sites overgrown with self-seeded trees. We measured plant size and number of flowers in 120 individuals, 60 *M. smejkalii* and 60 hybrids (Table S1). Plant size was calculated as the total area occupied by the individual based on an ellipse area equation ($\text{Area}_{\text{ellipse}} = \pi \times \text{length}/2 \times \text{width}/2$), where length was the longest distance of occupied space of the individual and width as the distance orthogonal to the length. Total number of flowers were counted from 10 randomly chosen stems per individual. Number of flowers per individual was calculated multiplying the total number of flowering stems by the mean number of flowers per stem.

2.6. Seed dispersal ability

We measured two dispersal traits to estimate seed dispersal ability: seed release height and terminal velocity. Seed terminal velocity is defined as the constant speed of a falling seed in still air and is inversely related to dispersal distance (Tackenberg, 2003; Zhu, Liu, Xin, Zhao, & Liu, 2016). Terminal velocity was measured in 31 seeds from the crossing experiment: 15 hybrids and 16 seeds from *M. smejkalii*. A total of 158 measurements were evaluated: 77 of hybrids vs. 80 of *M. smejkalii* (Table S1). Terminal velocity was automatically measured using a self-developed apparatus at the Institute of Landscape and Plant Ecology, University of Hohenheim, Germany. The apparatus consists of an automatic seed release device, a high-speed camera (acA1920-155um, BASLER) at a fixed speed of 130 frames per second with a lens (LM8HC, 1" 8 mm/F1.4, Kowa Optical Products Co., Ltd), and a customized operating software. We used this apparatus to shoot videos of a falling seed from two perspectives, and we used ImageJ (Schneider et al., 2012) to extract the seed's three-dimensional coordinates over time from the

videos. We then regressed the vertical coordinates against time, and calculated seed terminal velocity as the slope of the regression line. Detection of seeds by the apparatus was sometimes very low due to the small size of the seeds and therefore, we released each seed multiple times until getting 2–15 valid measurements per seed. In a few cases only one measurement was detected.

A total of 55 individuals were measured to assess seed release height: 40 *M. smejkalii* individuals and 15 hybrids (Table S1). Per individual plant, we chose 2–20 flowering stems covering a range of different heights (whenever possible). The height was measured from the soil until the flower (Fig. S1). All measured individuals belonged to the treatment group of light and serpentine soil.

Seed release height and terminal velocity were used to simulate seed dispersal distance with the WALD model (Katul et al., 2005), in combination with wind profile and height of the surrounding vegetation. Wind data was obtained with month time resolution from a type IV climatological station in Hulice of the Czech hydro-meteorological institute, occurring 4 km from the Zelivka populations of *M. smejkalii*. Dispersal was simulated under ideal conditions (flat terrain, homogeneous vegetation, no wind turbulence during dispersal) to assess potential dispersal distances of *M. smejkalii* and the hybrids. The model was run 10 times with 1 million simulated seeds per run for each treatment and population.

2.7. Statistical analyses

We tested our response variables comparing *M. smejkalii* vs. the hybrid *M. smejkalii* × *M. caespitosa*, referred to as the two species hereafter. We tested whether all response variables: number of seeds per capsule, germination rate, seed morphology (size and perimeter) and dispersal-related traits (seed release height and terminal velocity) differ between species. In the plant performance experiment, the effect of species as well as soil and shading effects and their interactions were tested on plant size and number of flowers. Unless stated otherwise, we used Generalized Linear Mixed Model (GLMMs) with maternal plant code as a random effect and species, maternal population and their interaction as predictors.

2.7.1. Seed production and germination

For number of seeds per capsule, we used GLMMs with a quasi-Poisson distribution due to overdispersion of the residuals caused by zero-inflation of the data. Zero-inflation of the number of seeds can be a result of a biological process (such as seed abortion) but also can be caused by experimental artefacts (e.g., effect of the experimenter, weather conditions) in the hand pollination assays. To account for this, we also analyzed two further models where the data was encoded as 0 and 1: one model using a quasi-Poisson distribution of non-null entries only, and the second using a quasi-binomial distribution (at least one seed germinated yes/no) considering all entries. As these two tests did not bring any additional insights, they are not reported further. To evaluate germination rate, we used a Generalized Mixed Model with a quasi-binomial distribution for proportional data due to overdispersion. To include a "quasi" families in our mixed model, we applied the `glmer.nb` function from the "lme4" package.

2.7.2. Seed morphology

Area and perimeter of the seeds were log transformed to achieve normality of residuals. We compared these values between *M. smejkalii* and the hybrids using a Generalized Linear Model (GLM). Including maternal plant as a random effect was not possible in this case due to the low number of replicates. In a separate test, the area and perimeter of *M. smejkalii* and hybrids from crossing experiments were compared with *M. caespitosa* and *M. smejkalii* seeds collected from natural populations. Seeds of *M. caespitosa* were collected from Hradčany, while *M. smejkalii* seeds were collected from only one maternal population (Z2).

2.7.3. Seed dispersal ability

Seed dispersal traits included seed release height and terminal velocity. Seed release height was log transformed and a linear model was applied to achieve normality and homogeneity of variances. Seed release height, terminal velocity, vegetation height and wind data were used in a mechanistic model to estimate dispersal distances of hybrids and *M. smejkalii*. We calculated maximal and 99 % quantile of dispersal distance as relevant measurements for long-distance dispersal and population spread (Clark, Lewis, & Horvath., 2001; Hemrová, Bullock, Hooftman, White, & Münzbergová, 2017; Nathan, 2005). The model was run 10 times and each run was taken as a replicate. We tested the effect of species and population on the maximal and 99 quantile dispersal distances (Table S2).

2.7.4. Plant performance

To compare species performance in different environments, we tested the effect of species, soil and shading; and their interactions on plant size and number of flowers. Since hybrids were from the previous crossing experiments but *M. smejkalii* individuals from natural populations due to high seedling death rate, we considered mother populations as a fixed factor and not as random effect. Plant size was log transformed to fulfill assumptions of normality of residuals and homogeneity of variances. A linear model was applied to plant size and a Generalized Mixed Model with a Poisson distribution to number of flowers due to lack of normality and homogeneity of variances after transformation.

An overview of the methodology and detailed number of individuals used in each experiment are presented in Fig. S1 and Table S1. Generalized Linear Mixed Models were performed using the “lme4” package. All analyses were performed using R version 3.6.2 (R Core Team, 2019).

3. Results

3.1. Seed production and germination

From a total of 157 crossings within populations, 92 flowers produced capsules (58.6 %), while from the 160 crossings with *M. caespitosa* 77 flowers (48.1 %) produced capsules. There were no significant differences in number of seeds per capsule between *M. smejkalii* and the hybrids (Mean \pm SD: 2.6 ± 3.7 ; 2.2 ± 3.5 , respectively, Fig. 2A). Species \times maternal populations interaction showed a difference in number of seeds among the species in population Z2 only (Fig. S2). Germination rate significantly differed between species (Table 1, Fig. 2B) being higher in hybrids in all maternal populations (Mean \pm SD: 0.51 ± 0.3 vs 0.79 ± 0.3 , Fig. S2).

3.2. Seed morphology

Seed morphology did not differ significantly between *M. smejkalii* and hybrids from crossing experiments (Table 2). However, there was a significant difference between *M. smejkalii* seeds compared to *M. caespitosa*. Seeds of *M. smejkalii* and *M. caespitosa* have a circular shape and both possess appendages on their surface which are clearly distinguishable between species. *Minuartia caespitosa* has three-armed appendages compared to a simple, taller appendage in *M. smejkalii* (Fig. 3A–D). This difference was reflected in the (adjusted) perimeter where *M. caespitosa* possess significantly smoother contour (lowest value), than *M. smejkalii* (Fig. 3). Interestingly, there was a significant difference in perimeter between *M. smejkalii* seeds resulting from the crossings experiments compared with those that were collected from natural populations (Fig. 3F), however, these differences were not reflected in seed area (Fig. 3E). Seeds from *M. smejkalii* from natural populations possess a significantly higher perimeter with taller, larger, more differentiated appendages on the surface than seeds of *M. smejkalii* produced in the experiments, and compared to hybrids and *M. caespitosa* (Fig. 3).

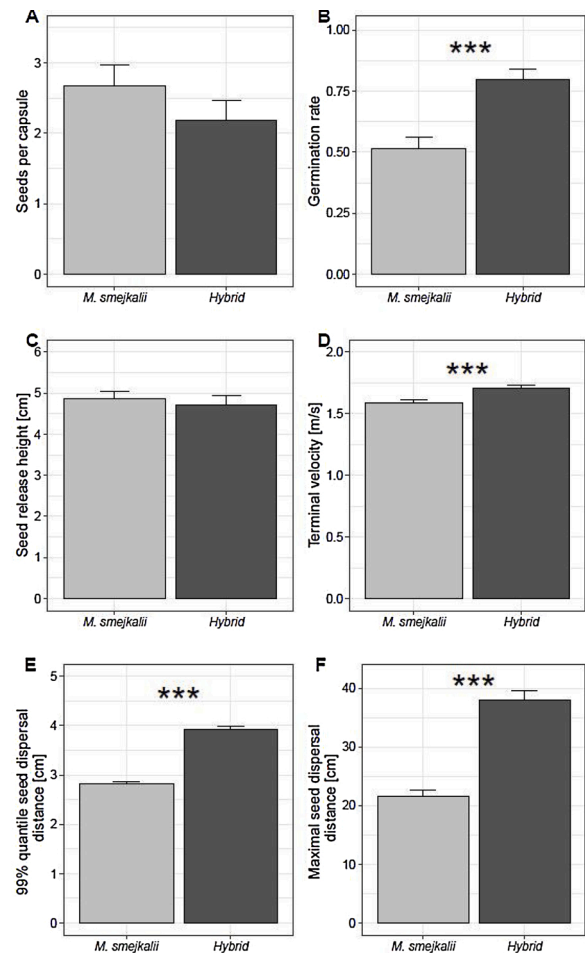


Fig. 2. Fitness, seed dispersal traits and seed dispersal distances in hybrids compared to *M. smejkalii*. Number of seeds per capsule did not differ between *M. smejkalii* and the hybrids (A), but hybrids had a significantly higher germination rate (B). Seed dispersal traits: Seed release height did not differ between *M. smejkalii* and the hybrids (C) but terminal velocity was higher in hybrids (D). Estimation of seed dispersal distances showed a higher distance in hybrids in both 99 % quantile (E) and maximal distances (F). Bar plots show mean values with Standard Error bars of *Minuartia smejkalii* in light gray and hybrids in dark gray. Significance values $p < 0.001$ ***, $p < 0.01$ ** , $p < 0.05$ * of Wilcoxon tests for small datasets.

3.3. Seed dispersal

Seeds of hybrids had higher terminal velocity (Table 1, Fig. 2D) than seeds of *M. smejkalii*. Simulation results showed higher potential seed dispersal distance in the hybrids (37.9 ± 0.06 cm) compared to *M. smejkalii* (21.6 ± 0.03 cm, Fig. 2E-F). Hybrids had significantly higher 99% quantile and maximal dispersal distance than did *M. smejkalii*. However, the difference was small, and dispersal distance was mainly limited to half meter (Table S2, Fig. S3).

3.4. Plant performance under different conditions

For plant size and number of flowers, there was a significant effect of species, type of soil and the interaction between species \times soil \times maternal population (Table 3). Light treatment did not have any significant effect on plant size (Table 3), and hybrids were larger in both soil treatments (Fig. 4A). Maternal populations, light treatment, light \times species and soil \times species interactions had a significant effect on number of flowers only (Table 3), with hybrids producing more flowers under the open site treatment and less flowers in shade (Fig. 4B). In particular,

Table 1

Effect of species: *Minuartia smejkalii* or hybrids, on fitness (seed number per capsule, germination) and dispersal traits (seed release height, terminal velocity).

	Df	Fitness				Dispersal			
		Seed number		Germination		Seed release height		Terminal velocity	
		F value	P-value	F value	P-value	F value	P-value	F value	P-value
Species	1	1.31	0.218	45.48	<0.001	0.23	0.389	49.9	<0.001
Population	2	0.73	0.483	0.97	0.615	1.37	0.232	1.2	0.212
Species × Population	2	4.92	0.085	3.90	0.142	0.79	0.410	1.6	0.607

Table 2

Comparison of seed morphology between *M. smejkalii* and hybrids from controlled crosses (upper part), and *M. smejkalii* and *M. caespitosa* from natural populations (lower panel). For details of the model see materials and methods.

Seed morphology:	Df	Seed area (log)		Seed perimeter (log)	
		F value	P-value	F value	P-value
Species	1	0.03	0.856	2.75	0.114
Population	2	0.07	0.928	2.90	0.081
Species × Population	2	0.71	0.708	0.06	0.942
Including <i>M. smejkalii</i> and <i>M. caespitosa</i> from nature					
Species	3	0.90	0.456	20.33	<0.001

M. smejkalii had significantly more flowers than the hybrids in sand soils, whereas in serpentine soils hybrids had significantly more flowers (Fig. 4B).

4. Discussion

In the present study, we explored the possible dangers of interspecific hybridization in *ex situ* conservation facilities using the endemic species *Minuartia smejkalii* as a model. Our results showed differences in fitness and dispersal-related traits between *M. smejkalii* and its hybrids with *M. caespitosa*. We found higher germination rate, seed perimeter and potential seed dispersal distance in hybrids compared with *M. smejkalii*. In addition, under different soil and light conditions, hybrids produced higher number of flowers (fitness) when grown on serpentine soils and full light, while *M. smejkalii* produced more flowers when grown on sandy soils in shade. These results suggest a higher performance of hybrids to serpentine soils and open sites only compared to parental *M. smejkalii* individuals.

4.1. Germination and seed production

Contrary to our expectations, seed production did not differ between *M. smejkalii* and the hybrids, but a higher germination rate in hybrids

was found (Table 1, Fig. 2A-B). Similar results were reported in *Plantago lanceolata* and *Lotus corniculatus*, where hybrids of cultivated varieties showed higher germination rates than their wild relatives (Schröder & Prasse, 2013a). In *Dianthus* spp., interspecific hybrids were less fertile with high abortion of pollen grains and no seed production in F1 (Vítová, Vít, & Suda, 2015). Increased fitness of hybrids has been described as heterosis (Lippman & Zamir, 2007). Usually, the first hybrid generation, F1, is fitter than the parents, but higher fitness can also be maintained in subsequent generations (e.g., Willi, Van Kleunen, Dietrich, & Fischer, 2007). It is very important to understand the effects of heterosis in the long-term because if fitter hybrids are introduced into wild populations of *M. smejkalii* they may cause an alteration of *M. smejkalii* life-history strategies acquired by natural selection. Here, we focused only on the F1 generation. The results indicate that the

Table 3

Plant size and number of flowers of *M. smejkalii* and hybrids under different light conditions: open and shade, and soil types: sand and serpentine.

	Df	Area (log)		Number of flowers (log)	
		F value	P-value	Deviance	P-value
Species	1	18.91	<0.001	344.24	0.016
Shading	1	1.74	0.189	289.07	<0.001
Soil	1	17.53	<0.001	283.44	0.018
Population	2	0.99	0.376	281.11	0.312
Species × Shading	1	3.35	0.070	234.09	<0.001
Species × Soil	1	0.02	0.886	228.95	0.023
Shading × Soil	1	0.09	0.753	223.09	0.015
Species × Population	2	1.09	0.339	222.92	0.921
Shading × Population	2	1.09	0.337	222.70	0.893
Soil × Population	2	0.55	0.577	219.40	0.192
Species × Shading × Soil	1	1.87	0.174	219.29	0.748
Species × Shading × Population	2	0.91	0.40	218.78	0.772
Species × Soil × Population	2	3.67	0.029	200.67	<0.001
Shading × Soil × Population	2	1.47	0.234	197.27	0.182
Residuals	96				

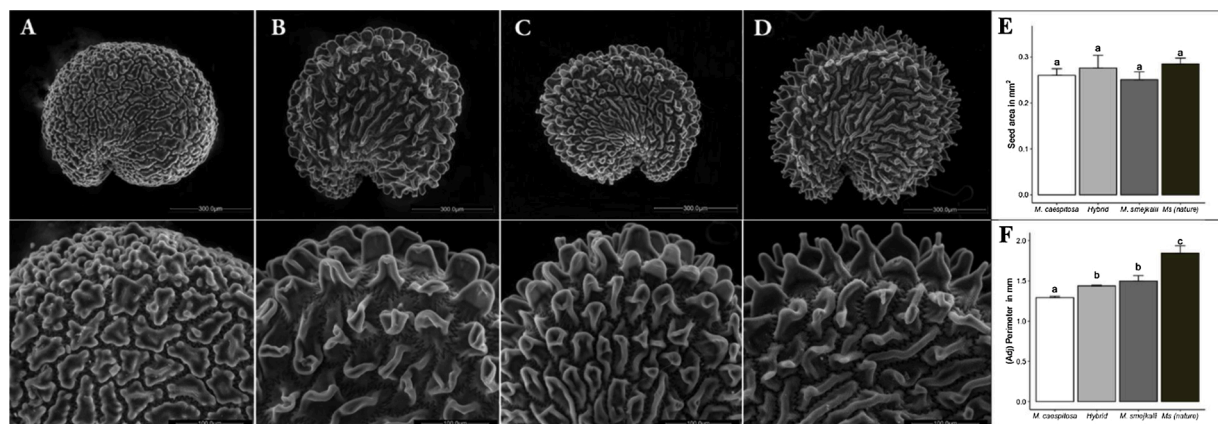


Fig. 3. Comparison of EM images of seed surface and appendages between *M. caespitosa* (A), hybrids (B), *M. smejkalii* (C), and *M. smejkalii* from natural populations (D) as well as calculated seed area (E) and perimeter (F). Bar plots show mean values with Standard Error bars and different levels showed differences after Tukey Post-Hoc tests. Figure includes individuals from population Z2 only. Photographs taken by Jiří Machač.

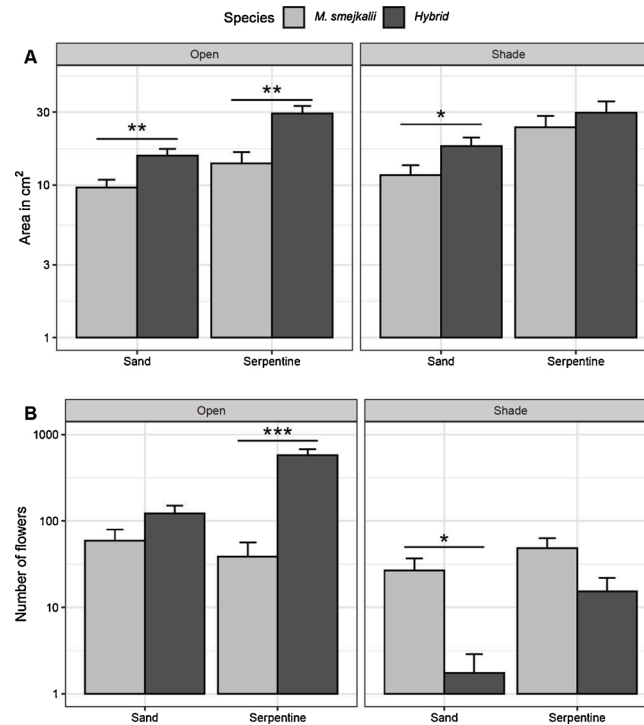


Fig. 4. Effect of type of soil and light conditions on plant size and fitness of hybrids compared to *M. smejkalii*. Plant size was measured as the total area occupied by one individual and the fitness as the total number of flowers produced by individual plant. Types of soil are sand and serpentine and light conditions are open sites and shade. Hybrids were larger than *M. smejkalii* in both types of soils and light conditions (A). Higher number of flowers in hybrids was only in open light treatment, while in the shade the hybrids produced less flowers, in particular in sandy soils (B). Bar plots show mean values with Standard Error. Significance values $p < 0.001$ ***, $p < 0.01$ ** , $p = 0.05$ (*) of Wilcoxon tests for small datasets.

M. caespitosa genes could easily spread in *M. smejkalii* populations if introduced by mistake. Whether this change of fitness will be transferred to next generations requires further studies focusing on fitness in hybrids compared to *M. smejkalii* in subsequent generations.

4.2. Seed morphology and dispersal

External environmental factors could have an effect on seed morphology and dispersal traits. For example, it is well known that artificial selection for yield, fruit size or palatability in domesticated crop species, causes changes in seed morphology related to seed coat color, surface texture and thickness (Fuller & Allaby, 2009). We found higher roughness of the seed coat of *M. smejkalii* seeds collected from natural populations compared with those obtained from hand crossing experiments (Table 2, Fig. 3), suggesting an effect of the environment on seed morphology. We speculate that this might be caused by epigenetic changes (reversible heritable genetic marks) caused by stressful conditions (such as cultivation) or hybridization events (Bräutigam et al., 2013; Mondoni et al., 2014). These epigenetic changes are known to affect plant development and phenotypic traits affecting plant responses to the environment (Bräutigam et al., 2013; Mondoni et al., 2014). Therefore, we recommend observing seeds of *M. smejkalii* in *ex situ* collection carefully in order to avoid a quality decrease of plants reintroduced into wild populations. On the other hand, assuming that *M. smejkalii* possesses orthodox (desiccation tolerant) seeds as other *Minuartia* species (see the Seed Information Database <http://data.kew.org/sid/>), reintroductions should preferably be implemented with seeds collected in the wild and stored in seed banks. This methodology requires a brief propagation phase of the species in *ex situ* cultivation, a few months to produce sufficiently robust plants (sub-adults not yet reproducing) which can then be reintroduced into the wild without any risk of altering the integrity of the taxon.

Furthermore, changes in dispersal might also potentially constitute a threat if unwanted species e.g. hybrids, “escape” from botanical gardens

into the wild. Seed dormancy is known to be reduced in *ex situ* collections compared to their wild counterparts (Ensslin et al., 2011, 2018), but other dispersal traits related to time and space (e.g. dispersal distance), have not been previously considered. The reason for this might be that in general estimation of seed dispersal distances is a difficult task, but more complicated models to estimate seed dispersal in a more accurate way considering different traits are constantly being developed (e.g. Soons, Heil, Nathan, & Katul., 2004; Schurr, Bond, Midgley, & Higgins., 2005; Zhu et al., 2019).

At its basis, seed dispersal distance is a function of mean wind speed, mean seed release height and mean terminal velocity (Katul et al., 2005). All else being equal, higher seed terminal velocity should cause shorter seed dispersal distances. In our study, seed release height did not differ between *M. smejkalii* and hybrids (Fig. 2C), but seed terminal velocity of hybrids was higher than that of *M. smejkalii* (Fig. 2D). Our simulations showed that hybrids had significantly higher maximal and 99 % quantile dispersal distances than did *M. smejkalii* (Fig. 2D-E, Table S2). The seemingly surprising results could be caused by the larger variance in seed release height and terminal velocity in hybrids than in *M. smejkalii*, and thus the maximal dispersal distance should be interpreted carefully due to the stochasticity of simulations and to the small difference in length in terms of cm.

It was rare events of 1 per million that caused higher maximal dispersal distance in hybrids: 37.9 ± 8.9 cm compared to 21.6 ± 0.4 cm in *M. smejkalii* (Fig. 2E). Although the difference in dispersal distance is small, it may have a significant effect on population spread over a longer time periods (Clark et al., 2001; Marco, Montemurro, & Cannas, 2011). This is especially the case for *M. smejkalii* because it is perennial, and the height of adult plants is very short. However, it should be noticed that wind dispersal distance was critically limited to very short distances both in *M. smejkalii* and hybrids (<0.5 m, Table S2). In addition, their seeds, except being dusty, lack obvious appendages or structures that could facilitate wind dispersal (Fig. 3). Therefore, other dispersal vectors such as ants could play a more important role than does wind in

M. smejkalii, as well as in the closely related species *M. caespitosa* (Pladías, 2020).

4.3. Plant performance

In general, one could expect hybrids to be fitter than the parents on average due to heterosis effect (Lippman & Zamir, 2007). But at the same time, since *M. smejkalii* is adapted to growth on serpentine soils, one could also expect these individuals to perform better in such conditions compared to the hybrids. Hybrids were larger and produced more flowers on serpentine soils, and fewer flowers when grown on sand compared to *M. smejkalii*. These results suggest that hybrids are generally fitter than the parental *M. smejkalii* when grown on serpentine soils. Because we found no or an opposite pattern in the sand (original habitat of *M. caespitosa*), this result cannot be attributed to general heterosis effect. Higher number of flowers in hybrids might also be interpreted as a response to stress (Takeno, 2016; Wada & Takeno, 2010) induced possibly by the toxicity of serpentine soils. This would mean that the plants are not fitter, but are trying quickly to escape from the unsuitable conditions and will thus die within the next few years. However, while stress may increase the rate of flowering, it does not explain the higher size of the hybrids on the serpentine soils. The hybrids also performed better in open sites but significantly worse (number of flowers) under shade (Fig. 4B). As open serpentine habitats are the primary habitats of *M. smejkalii*, these unexpected results suggest that hybrids have high potential to spread in natural *M. smejkalii* populations if introduced by mistake and outcompete the local *M. smejkalii*. One important stress factor on the serpentine habitats, not accounted for in our study, is drought. It is thus possible that the hybrids would be less fit under extreme drought conditions and populations with prevailing hybrids could collapse under these circumstances, but also drought stress could induce a flowering response (Riboni, Galbiati, Tonelli, & Conti., 2013; Yang et al., 2015). Further studies are needed to prove this hypothesis.

4.4. Hybrids as a conservation tool?

Hybridization can be a double-edged sword (Haig & Allendorf, 2006). On one hand, hybrids can be a threat for rare species where parental or invasive species could replace endemics in their natural environment (Allendorf, Leary, Spruell, & Wenburg, 2001). On the other hand, hybridization is a driver of speciation. Preserving closely related species that hybridize might thus promote long-term survival of taxonomic lineages (Becker et al., 2013), and might be used as a conservation measure to preserve the genetic pool of otherwise doomed species (e.g., Clark, Schlarbaum, Saxton, & Hebard., 2016; Hamilton, Royauté, Wright, Hodgskiss, & Ledig, 2017). However, using hybrids to preserve a lineage should only be considered after a careful evaluation of possible genotypic and phenotypic effects on the local population (Allendorf et al., 2001; Chan, Hoffmann, & van Oppen, 2019), and as a last resource when the preservation of the focal species is beyond our capabilities.

In general, hybridization can have negative consequences as in the case of *M. smejkalii*. The results presented in this study suggest that in order to achieve a successful restoration of *M. smejkalii* in the wild, hybridization events should be avoided in *ex situ* conservation facilities. We recommend to keep *M. smejkalii* collections separated from their congener species in *ex situ* collections due to lack of information about pollen dispersal distances; establishment of a seed bank and a close examination of putative *M. smejkalii* individuals selected for restoration purposes.

5. Conclusion

Our results showed higher germination rates in seeds of hybrids compared to *M. smejkalii* as well as increased hybrid performance in serpentine, but not in sandy soils. These results suggest that hybrids might be fitter than their parents but only in the habitat of *M. smejkalii*

and not in the habitat of *M. caespitosa*. This counterintuitive result requires further exploration and should be confirmed in subsequent generations. In any case, these results indicate that the hybrids have the potential to overtake *M. smejkalii* wild populations in the serpentine habitats if introduced by mistake. In addition, simulation results showed a two times higher maximal dispersal distance in hybrid seeds compared to *M. smejkalii* seeds suggesting they have higher colonization abilities. Finally, similarities in size and seed morphology between hybrids and *M. smejkalii* individuals make them particularly difficult to distinguish. Therefore, in order to achieve a successful reintroduction of *M. smejkalii* into the wild, hybridization in *ex situ* facilities should be avoided and reintroduction candidate individuals carefully scrutinized if grown nearby other *Minuartia* taxa.

Authors' contributions

BS, HP and ZM designed the study. BS and HP performed the experiments. JZ performed the seed dispersal simulations. SLG analyzed the data and lead writing of the manuscript. All authors contributed critically to the drafts and gave final approval for publication.

Declaration of Competing Interest

The authors report no declarations of interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:<https://doi.org/10.1016/j.jnc.2020.125912>.

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