

## RESEARCH PAPER

# Increasing the germination percentage of a declining native orchid (*Himantoglossum adriaticum*) by pollen transfer and outbreeding between populations

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## Keywords

Conservation; cross-pollination; dry grasslands; isolation; population size.

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## ABSTRACT

- The declining native orchid *Himantoglossum adriaticum* H. Baumann is a European endemic of priority interest (92/43/ EEC, Annex II). Northern Italian populations of *H. adriaticum* are small and isolated, with depressed seed set. Given the important implications for plant population conservation, we tested the hypothesis that artificial pollen transfer (hand-pollination) and outbreeding between populations increases fruit set and seed germination percentage.
- The background fruit set and *in vitro* germination rates were determined for ten reference populations. An artificial cross-pollination experiment included (a) pollen transfer from one large population to two small and isolated populations; (b) pollen transfer between two small but not isolated populations; (c) within-population pollen transfer (control). All seeds were sown on a modified Malmgren's medium and cultured in a controlled environment. Germination percentage was compared using a Kruskal-Wallis ANOVA.
- The background fruit set (mean = 18%) and germination (<5%) rates were consistently low across populations. Fruit set after hand-pollination was consistently 100%. Pollen transfer from the largest population to smaller populations resulted in an increase in total germination ranging from 0.9% to 2.9%. The largest increase in germination occurred between small-sized and less isolated populations (from 1.7% to 5.1%).
- The results of pollen transfer between the small populations are particularly encouraging, as the mean increase in germination was almost four times that of the control. Outbreeding can be considered a valuable tool to increase genetic flow and germination in natural populations, limit the accumulation of detrimental effects on fitness driven by repeated breeding with closely-related individuals, thereby increasing the possibility of conservation of rare or endangered species.

## INTRODUCTION

The declining native orchid *Himantoglossum adriaticum* H. Baumann is a European endemic species of priority interest (Directive 92/43/ EEC, Annex II), suffering population declines in many European countries (Dostalova *et al.* 2013). Its range comprises Austria, Bosnia-Herzegovina, Croatia, Czech Republic, Hungary, Italy, Slovakia and Slovenia (Dostalova *et al.* 2013). In Italy, it is mostly found in dry grasslands and nearby ecotonal meso-xeric scrubland/woodland patches (Slaviero *et al.* 2016), but it can also occur in secondary habitats such as roadsides and vineyards (Fekete *et al.* 2017).

In common with other orchid species, its vulnerability stems from particular requirements for germination, seedling establishment and reproduction (*e.g.* Slaviero *et al.* 2016; Fantinato *et al.* 2017; Magrini & De Vitis 2017). Pollen transfer is mainly carried out by pollinating insects (wasps, bees, bumblebees, butterflies, flies and night-flying insects; Fantinato *et al.* 2017) that are attracted through a sophisticated mechanism.

*Himantoglossum* species are non-rewarding, since there is no evidence of nectar in the spur and they are believed to lure pollinators with a male goat smell (Carey & Farrell 2002; Fantinato *et al.* 2017). The pollination system of food deceptive orchids, which do not offer any food reward to their pollinators, is particularly problematic and has been thought to determine a low visitation rate, since pollinators learn to avoid deceptive flowers, resulting in a low fruit set due to pollination limitation (Pellissier *et al.* 2010).

Although often being species- and life stage-specific, seed germination and growth of orchid seedlings or adult plants rely on symbiosis with mycorrhizae (Rasmussen 2002; Pecoraro *et al.* 2013). Seeds are extremely small and light, contain few nutrient reserves, and may have morphological and morphophysiological dormancy, often imposing a low germination percentage (Baskin & Baskin 2014). Indeed, the reproductive success of *H. adriaticum* is generally low, being characterised by low fruit set (between 5 and 45%; Biró *et al.* 2015) and low germination percentage (Gilián *et al.* 2018).

The persistence of populations is also threatened by habitat destruction and fragmentation due to land-use change (Fischer & Lindenmayer 2007; Buffa *et al.* 2018; Fantinato *et al.* 2019). In particular, the increase in human settlements and infrastructure, the spread of industrialised agriculture and the abandonment of low-intensity agricultural regimes, such as grazing and mowing, have been evoked as the main causes of dry grassland decline (Janišová *et al.* 2011; Fantinato *et al.* 2016a). Habitat fragmentation creates small and/or isolated populations, often found in residual patches which frequently lack suitable environmental conditions for the proliferation of rare and endangered species with specific ecological requirements.

Small and isolated populations exhibit low fecundity, which results in a negative feedback on the population size (Hens *et al.* 2017). Compared with large populations, small and isolated populations have increased extinction risk because of reduced gene flow, which leads to a shift in allele frequencies and a reduction in alleles and genotypes (Oostermeijer 2003). Small and isolated populations also suffer from inbreeding (Angeloni *et al.* 2011), which often results in reduced reproduction and offspring survival (Charlesworth & Charlesworth 1987; Keller & Waller 2002; Lienert 2004) because of increasing homozygosity.

Individuals in small populations may also experience reduced viability and reproduction for demographic reasons. By limiting pollinator movement among patches, fragmentation may also reduce the abundance and richness of pollinator populations and alter their foraging behaviour (Aguirre & Dirzo 2008; Fantinato *et al.* 2019). Small and isolated plant populations thus likely receive fewer flower visits and smaller pollen loads, thereby suffering pollen limitation, reduction in reproductive success and low seed quality (Jacquemyn & Brys 2015).

The negative effects described above can be alleviated through gene flow and outbreeding, which consist of the transfer of genetic material between different populations, either in the form of seeds, plants or pollen. Cross-pollination has been proven to enhance offspring fitness, increasing fruit set, fruit size and mass (Hill *et al.* 2006; Spinardi & Bassi 2012). This technique has been widely used historically in agronomy to improve crops (Goldman 1998). Cross-pollination has also been tested for other orchid species (Pierce *et al.* 2010; see Tremblay *et al.* 2005 for review), demonstrating that orchids that were given supplemental pollination had higher levels of fruit set than those pollinated naturally. In conservation biology, artificial cross-pollination may thus represent an important tool for the conservation of rare and endangered species, especially those characterised by a complex life cycle and often by fragmented populations (Hill *et al.* 2006).

In light of these considerations, the aim of this work was to test the germination capacity of *H. adriaticum* and test the efficacy of artificial pollen transfer (hand-pollination) and outbreeding between populations in order to increase fruit set and germination percentage. The hypothesis tested was that individuals of *H. adriaticum* from isolated and smaller-sized populations fertilised with pollen originating from larger, non-isolated populations would exhibit higher fruit set and germination capacity under controlled conditions than when pollinated using pollen from within the population.

## MATERIAL AND METHODS

### Study area and population selection

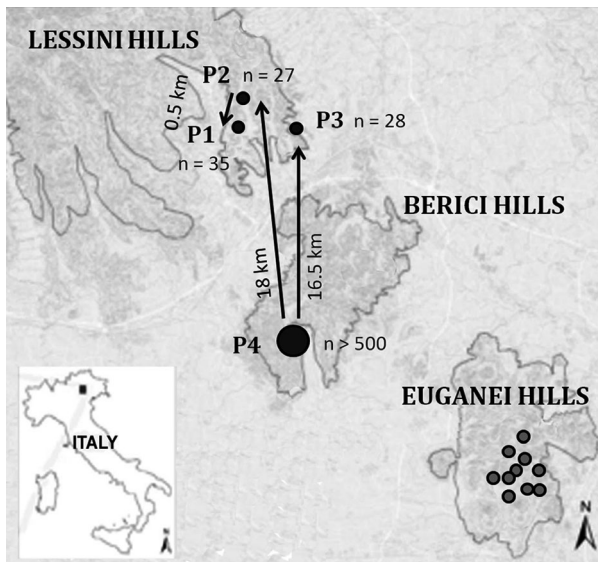
The study was carried out in northern Italy, in the Veneto region, specifically in the Lessini, Berici and Euganei Hills. Mean annual temperature is 13 °C, and annual rainfall range from 850 to 1200 mm ([www.arpa.veneto.it/bollettini/htm/dati\\_idrometeo.asp](http://www.arpa.veneto.it/bollettini/htm/dati_idrometeo.asp)).

Dry grasslands develop on mid-altitude limestone slopes (47–443 m a.s.l.) with an average pH of 7.5 (Fantinato *et al.* 2016b, 2018). They have established and been maintained by traditional human management until a few decades ago, when drastic changes in the local socio-economic patterns triggered the abandonment of mowing, haymaking and pasturing practices, thereby causing surface reduction, fragmentation and species loss (Buffa *et al.* 2016). However, the area still represents a significant hotspot for dry grassland orchid diversity (Rizzieri Masin & Tietto 2006).

We selected ten populations of *H. adriaticum* growing in the most suitable habitat conditions and used these as reference populations to test germination percentage of the species in the study area (Fig. 1). The population size varied between ten and 100 shoots, with a mean  $\pm$  SE of  $42.5 \pm 13.7$ . To estimate the reproductive fitness of each population, we measured the fruit set by calculating the fruit/flower ratio. We marked ten individuals for each population and counted the number of flowers. At fruiting, we counted the number of fruits on these marked individuals. We then selected three small populations consisting of approximately 30 shoots (a size comparable with those of the reference populations) and one large population (>500 shoots) to test the effect of outbreeding on fruit set and germination percentage. Two of the small populations (P1 and P2) were only 0.5 km apart from one another, separated by an agro-forest matrix (consisting mainly of olive groves and maize fields). The other small population (P3) was separated from the others by a larger distance (~5 km) and by an urban matrix. The large population (P4) was selected at a much longer distance (~20 km) from the others, separated by an urban matrix. According to our experimental design, a small chance of naturally occurring cross-pollination is possible only between P1 and P2, which are the only populations separated by an agro-forest matrix within a short distance. Seed dispersal is also unlikely, as 99% of orchid seeds fall within 1 m of the parent plant (Jersáková & Malinová 2007), with long-distance dispersal possible only during extremely rare climate events.

### Pollen transfer

To test the effectiveness of outbreeding in increasing fruit set and total germination, pollen was artificially transferred (hand-pollination) from: (i) the large population (P4) to the two small and isolated populations (P2 and P3); (ii) between the two small but not very isolated populations (from P2 to P1); (iii) within-population (on the small populations, P1, P2 and P3), used as a control. Considering the small size of the recipient populations, pollen transfer was made on one quarter of the shoots of *H. adriaticum* in order to avoid excessive artificial manipulation of the population. The fruit/flower ratio was calculated after hand-pollination for all crosses.



**Fig. 1.** Study area and sampling design. The arrows indicate the pollen transfer for the cross-pollination experiment, while 'n' indicates the number of shoots in each population. Dark grey populations were used for the outbreeding experiment; light grey populations were used as the reference populations to investigate the background germination capacity of *Himantoglossum adriaticum*.

Ripened pollinia (dark grey in colour) were collected from 10 to 50 (depending on the population size) healthy *H. adriaticum* shoots at the peak of the flowering season in 2016 (*i.e.* third week of May) using toothpicks. To prevent desiccation before use, pollinia were placed in 9-cm diameter Petri dishes, sealed in the field with paper adhesive tape and kept in a cool-bag ( $15 \pm 2$  °C) until returning to the lab facilities, where they were stored in the refrigerator (4 °C). The day after collection, pollinia were used to pollinate target shoots of the target population.

Healthy shoots selected for hand-pollination were marked with a transparent plastic label. To avoid accidental autogamy, all pollinia were removed and used for within-population pollination (control). Also, flowers that had already been pollinated were removed from the target shoots. Tagged shoots were monitored weekly after pollination to check for fruit development and maturation. Ripened capsules were harvested in the last week of June. All fruits were cleaned by hand in the lab, and stored in airtight vials in a freezer for 3 months, after dehydration at 15 °C and 15% RH following the FAO international standard (FAO 2014).

### Germination tests

Seeds were soaked in a solution of 0.8% NaOCl (equivalent to 0.5% active chlorine) for 15 min, mainly for surface sterilisation (Wilson 1915) but also to weaken the hard structure of the seed testa, which is likely to hinder germination due to low water permeability (Rasmussen 1995). Successively, seeds were sown in a sterile environment on agar medium in 6-cm diameter ventilated Petri dishes under a laminar flow hood (Mod. 1200 FLO; Permax Srl, Milano, Italy). Malmgren's orchid medium (Malmgren 1996) was used, modified with the addition of

0.5 g<sup>-1</sup> peptone and 0.1 mM 6-benzylaminopurine (BAP), since these compounds are proven to be effective in aiding germination of several terrestrial orchid species (Pierce *et al.* 2010, 2015; Pierce & Cerabolini 2011).

Seed samples were sown in a growth chamber (Snijders Economic Deluxe; Thermo-Lab, Codogno, Italy) at 20/10 °C day/night for 6 months. Eleven to 20 replicates were prepared according to the amount of seed available, resulting in 195 Petri dishes for the reference populations and 262 Petri dishes for the within- and between-population crosses. Petri dishes were checked monthly for evidence of germination. Seeds were checked using a stereomicroscope and were considered germinated when the embryo swelled sufficiently to split the seed coat and form a white protocorm (*i.e.* the seed entered developmental phase II, following Butcher & Marlow 1989). Total germination was recorded 6 months after sowing, when no further germination was observed.

### Data analyses

To explore the reproductive capacity of *H. adriaticum*, we compared fruit set, the proportion of fertilised and unfertilised seed and the germination percentage of the fertilised seed for the ten reference populations. Then we compared fruit set and germination percentage of within- and between-population pollination to test for the effect of outbreeding. We used Kruskal-Wallis ANOVA, with multiple comparisons, because data distribution was not normal, even after transformation (arcsin, log + 1, or square root transformations). Analyses were performed using the Statistica 8 (2007; Statistica, Hamburg, Germany) software package.

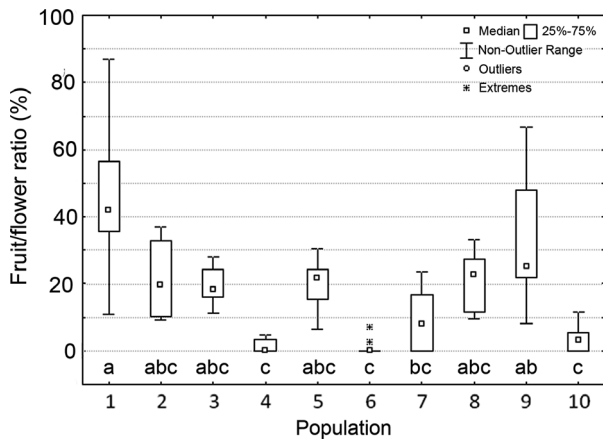
## RESULTS

*Himantoglossum adriaticum* showed a low percentage fruit set and extensive variability across populations (mean  $\pm$  SD:  $18\% \pm 14\%$ ; Fig. 2; Kruskal-Wallis test for the fruit set percentage:  $H = 52.4$ ,  $P < 0.0001$ ). Overall, the number of unfertilised seeds was high ( $30.0\% \pm 12.1\%$ ) although very variable across populations (Fig. 3A; Kruskal-Wallis test:  $H = 143.3$ ,  $P < 0.0001$ ), while the germination percentage was very low, consistently  $<5\%$  (Fig. 3B; Kruskal-Wallis test:  $H = 45.5$ ,  $P < 0.0001$ ).

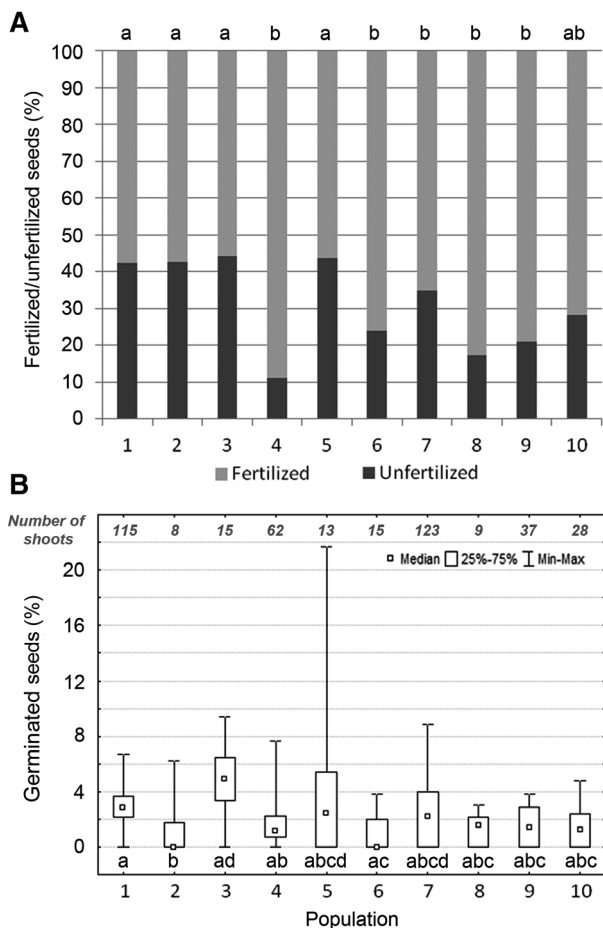
Pollen transfer had the same effect for both control and treatment groups, resulting in a fruit/flower ratio of 100% (Fig. 4A). Pollen transfer produced a significant increase in total germination percentage between all control and all treatment medians (Kruskal-Wallis test:  $H = 43.64$ ,  $P < 0.0001$ ; Fig. 4). Pollen transfer from the largest to the smaller populations produced a positive effect only for population P3 (small and isolated population), whose median total germination increased from 0.9% to 2.9% (multiple comparisons:  $z = 3.22$ ,  $P = 0.019$ ; Fig. 4B), while P2 had no significant variation (from 0.8% to 1.6%;  $z = 2.16$ ,  $P = 0.46$ ). Cross-pollination between small-sized populations produced the largest increase in total germination (P1; from 1.7% to 5.1%,  $z = 3.11$ ,  $P = 0.028$ ; Fig. 4).

## DISCUSSION

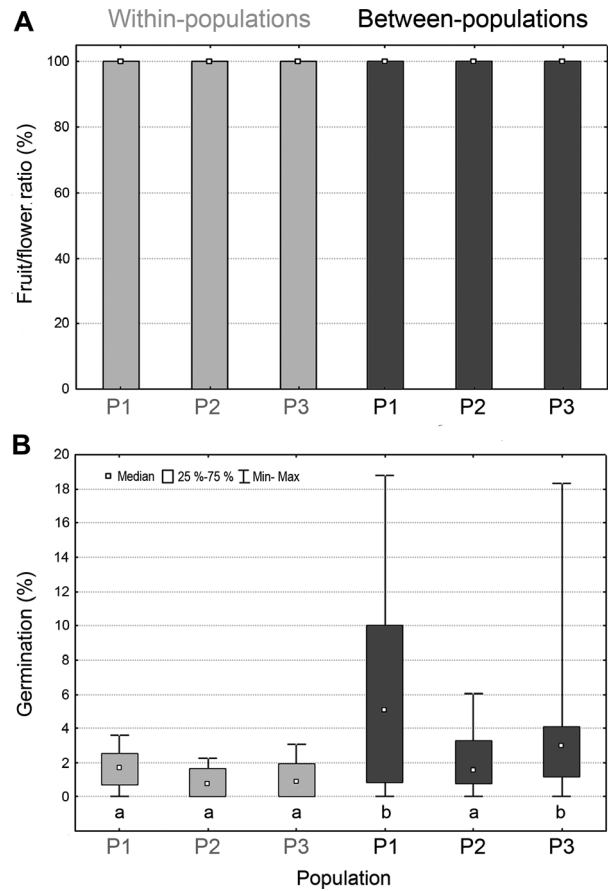
*Himantoglossum adriaticum* consistently exhibited low fruiting and germination percentages across populations, often  $<5\%$  in



**Fig. 2.** Fruit set proportion, expressed as percentage of the fruit/flower ratio of the ten reference populations of *Himantoglossum adriaticum*. Boxes with different letters are significantly different according to the multiple comparison test.



**Fig. 3.** Proportion of fertilized seeds (A) and germination percentage (B) of the ten reference populations of *Himantoglossum adriaticum*. Boxes with different letters are significantly different according to multiple comparison test.



**Fig. 4.** Fruit/flower ratio (A) and germination percentage (B) of within-population (control) and between-population (treatment) pollen transfer. Boxes with different letters are significantly different according to multiple comparison test. P4 (large population) was pollen donor for P2 and P3. P2 (small population) was the pollen donor for P1.

the reference populations, and also in the out-crossing experiment. Although species germination percentage can vary according to the site or the breeding process (e.g. Del Vecchio *et al.* 2012, 2018; Caruso *et al.* 2015), the drastic increase in both fruiting rate and germination capacity of *H. adriaticum* after hand-pollination suggests that pollination limitation may be a widespread phenomenon among small and isolated populations of this orchid. As a consequence of the abandonment of traditional management practices (e.g. mowing, haymaking and pasture), dry grasslands in the study area have undergone a significant decrease in surface area. Nowadays, they survive as small to medium-sized patches (0.2–2.0 ha), surrounded by woodland, areas under cultivation (particularly vineyards), villages and industrialised areas, and the majority of orchid populations are small and exhibit fragmented distributions (Slaviero *et al.* 2016; Fantinato *et al.* 2018). In small, isolated populations, genetic flow decreases, while the chances of inbreeding increase, resulting in inbreeding depression (Frankham 2015). Detrimental effects of inbreeding can be observed at different stages of the plant life history (e.g. low production of fruit and viable seed, low total seed production per fruit, high production of empty seeds per fruit), but are often most evident at the

germination stage, resulting in a reduced germination percentage (Ferdy *et al.* 2001; Smithson 2006; Sletvold *et al.* 2012).

The germination rate of different orchid species is highly variable (Nadarajan *et al.* 2011; Ponert *et al.* 2011; Pierce *et al.* 2019) and contrasting results can be obtained from laboratory and field experiments (Gilián *et al.* 2018). However, our results are consistent with previous research showing a very low germination percentage in the laboratory for *H. adriaticum* (1.3%; Gilián *et al.* 2018). Our results are also in line with the findings of Carey & Farrell (2002) for the congeneric species *H. hircinum*, in which a long-term experiment demonstrated a similar germination percentage in the field (~5% in the first year, even with mycorrhizal infection). Although further research should be devoted to investigating the germination percentage in the field after hand-pollination, our study demonstrated that artificial between-population pollen transfer can enhance the germination capacity of *H. adriaticum* and thus represents a useful tool for conservation of this species, contributing to mitigation of the negative effects of small population size and isolation on genetic mechanisms (Ferdy *et al.* 2001; Pierce *et al.* 2010; Sletvold *et al.* 2012; Hens *et al.* 2017).

The results were particularly encouraging for the smallest but closest populations, since the germination percentage was increased almost four-fold, with respect to the control, following artificial out-crossing. In contrast, out-crossing performed on the small population using pollen from the largest but most distant one had a lesser effect (despite a doubling of germination with respect to the control). The beneficial effect of outbreeding on fitness has already been highlighted, with results being consistent across taxa (invertebrates, vertebrates and plants) and benefits increasing in stressful conditions (Frankham 2015). Case studies from natural populations have shown that gene flow from other populations reversed the detrimental effects of inbreeding over the short term (Arrenda *et al.* 2004) by increasing both fitness and population size.

Several parameters have been suggested as good predictors of plant performance for the transfer of genetic material between different populations: similarity of habitats (Bischoff *et al.* 2006) or overall ecological similarity of the two sites (Smith *et al.* 2009), geographic distance between the donor and the recipient population (*e.g.* Joshi *et al.* 2001; Becker *et al.* 2008) or climate difference between the donor and the recipient site (Weissshuhn *et al.* 2012). However, there is still substantial debate and recently Caruso *et al.* (2015) and Bucharova *et al.* (2017) found that although both geographic and climate distances were correlated with species fitness traits, there was no general pattern supporting the hypothesis that climate or geographic distance would be better predictors of plant performance than habitat similarity.

In our study we tested the effects of cross-pollination between populations growing under the same ecological condition (*e.g.* temperature, water availability, soil and habitat type),

but differing in the geographic distance between donor and recipient population. Although it is difficult to make general predictions from small numbers of extremely rare species, our results are in agreement with the hypothesis that the distance between donor and recipient population may be a key factor in the success of artificial pollen transfer. Although contrasting results have been found (Baskin & Baskin 2015), our results also agree with the existence of an optimal out-crossing distance for *H. adriaticum*, at which the positive and negative effects of outbreeding are balanced. The optimal distance varies according to the species, but when the individuals are too close (separated by only a few metres), the likelihood of inbreeding is very high, while crossing between very widely spaced individuals or populations increases the probability of outbreeding depression; consequently, the optimal outbreeding rate is obtained at an intermediate distance (Paschke *et al.* 2002; Billingham *et al.* 2007).

In our work, the closest populations (where the highest success of outbreeding was observed) were also the least functionally isolated, being separated by 0.5 km of an agricultural matrix. As has previously been demonstrated for other species, the success of outbreeding can be a function of the extent of isolation, having positive effects in less isolated and genetically similar populations (Hufford & Mazer 2003; Raabová *et al.* 2007; Weissshuhn *et al.* 2012; Yardeni *et al.* 2016). In plants, gene flow occurs *via* migration of vegetative propagules, seeds or pollen and often depends on the foraging distances of pollinators. The most frequent pollinators of *H. adriaticum* are beetles and solitary bees (Fantinato *et al.* 2017), which do not travel long distances, but this orchid can also be occasionally visited by other pollinators, such as *Apis mellifera*, which can cover longer distances of approximately 1 km (Beekman & Ratnieks 2000; Fantinato *et al.* 2017). We can thus assume that partial gene flow can occur between these two populations and this supports the hypothesis of higher outbreeding success in relatively similar populations.

Although our experiment did not cover the full spectrum of cross-pollination possibilities, our study has important implications in the context of restoration ecology, in particular for the choice of the most suitable areas to be selected as sources of pollen to reinforce endangered populations in restoration plans. Specifically, our results suggest that pollen for cross-pollination projects should come from the closest populations, with vicinity used as a proxy for ecological similarity.

The restoration of plant communities is often realized through the introduction of seedlings obtained from non-local populations, with the risk of introducing inappropriate alleles into locally adapted populations (Broadhurst *et al.* 2008; Hölzel *et al.* 2012). Therefore, we underline the importance of empirical studies to investigate the possible role of outbreeding in reducing this risk. Such studies would provide a practical instrument for the conservation of endangered species.

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