The impact of hybridization on long-term persistence of polyploid *Dactylorhiza* species[1]

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PREMISE OF THE STUDY: Hybridization may pose severe threats to the long-term survival of the parental taxa through introgression and the formation of hybrid swarms. However, when the resulting hybrids show strong male and female sterility, backcrossing and introgression are unlikely to occur, but the parental species may suffer from reduced male and female fitness.

METHODS: We assessed the impact of hybridization on the long-term persistence of two food-deceptive orchids in the genus *Dactylorhiza* (the common *Dactylorhiza maculata* and the rare *D. sphagnicola*). The extent of hybridization was investigated using both molecular markers and morphometric measurements. To determine the strength of postmating reproductive isolation, hand pollinations were conducted between pure and hybrid individuals. Finally, fruit set and seed viability of open-pollinated plants were determined in sympatric and allopatric populations to investigate the impact of hybridization on the reproductive output of the pure parental species.

KEY RESULTS: Our results showed that postmating reproductive isolation was weak and that hybridization occurred frequently within the studied sympatric population. Although hybrids were characterized by very low female fitness, mainly because of strongly reduced seed viability, backcrossing appeared to occur and was asymmetric toward the rare *D. sphagnicola*. Fruit set and seed viability of open-pollinated plants were also significantly lower in the sympatric population than in the allopatric populations, indicating that hybridization and ongoing introgression incurred fitness costs in the pure parental species.

CONCLUSIONS: Overall, our results suggest that extensive hybridization can affect the long-term viability of the parental species through the combined effect of introgression following interspecific hybrid fertilization and reduced fitness of the parental species.

KEY WORDS hybridization; introgression; long-term population viability; plant fitness

Hybridization and introgression are complex processes that may increase genetic diversity within species, transfer genetic adaptations between species, and break down or reinforce reproductive barriers between closely related groups (Barton and Hewitt, 1989; Abbott, 1992; Rieseberg, 1997). As such, hybridization has been shown to play an important role in the evolution of plant taxa, and it can even lead to the emergence of new ecotypes or species and thus affect speciation (Abbott et al., 2003; Rieseberg, 2006; Mallet, 2007; Rieseberg and Willis, 2007; Thomas, 2015; Vallejo-Marín and Hiscock, 2016). On the other hand, hybridization may also incur severe fitness costs and lead to the extinction of populations or even

species (Levin et al., 1996; Rhymer and Simberloff, 1996; Ellstrand et al., 1999) and therefore have important conservation implications, especially when rare species are involved (Allendorf et al., 2001; Stronen and Paquet, 2013, Čertner et al., 2015; Fitzpatrick et al., 2015; Piett et al., 2015; Wayne and Shaffer, 2016). Moreover, the chances of widespread hybridization and introgression are likely to increase as a result of increased habitat modification and introduction of nonnative species (Vallejo-Marín and Hiscock, 2016), which, in turn, may lead to accelerated extinction of the parental species (Ellstrand and Elam, 1993; Rhymer and Simberloff, 1996).

When the first-generation hybrids are fertile and can easily mate with the parental species, genetic mixing and widespread introgression may occur, which may lead to genetic erosion of the parental gene pool and, eventually, to extinction of one or both parental species (Allendorf et al., 2001; Wolf et al., 2001). When F_1 hybrids appear to show lower fertility or complete sterility, backcrossing and introgression will evidently be limited, but the parental species may

suffer from reduced male and female fitness (Rhymer and Simberloff, 1996). Pure pollen of the parental species that are deposited on hybrid plants may not lead to viable seeds, but at the same time they are no longer available to pollinate flowers of conspecific plants. Similarly, flowers of the parental species that are pollinated by nonviable hybrid pollen will most likely produce very few or no viable seeds and are therefore no longer available to be pollinated by pollen of conspecific plants. The combination of both processes can be expected to lead to pronounced reductions in fitness and, therefore, affect the long-term viability of the parental species.

Recent investigations in a range of European (*Orchis* L., *Anacamptis* Rich., *Dactylorhiza* Neck. ex Nevski) and Neotropical (*Epidendrum* L.) food-deceptive orchids have shown that reproductive barriers are far from strict and that hybridization may frequently occur (e.g., Cozzolino et al., 2006; Moccia et al., 2007; Pellegrino et al., 2010; Pinheiro et al., 2010; De Hert et al., 2011, 2012; Jacquemyn et al., 2012a, b; Moraes et al., 2013; Scopece et al., 2013; Marques et al., 2014). Comparative analyses of premating and postmating barriers have revealed that, in contrast to sexually deceptive species, premating isolation barriers in food-deceptive species are mostly weak (Scopece et al., 2007; Marques et al., 2014). This is because food-deceptive orchid species are frequently pollinated by generalist pollinators, most often bees and bumblebees, and many of these orchid species show considerable overlap in their pollinator community. Postzygotic barriers, on the other hand, appeared to be more important (Scopece et al., 2007, 2008, 2013) but were, in many instances, insufficient to impede hybridization. Although introgression and the formation of hybrid swarms have been observed in some cases (Jacquemyn et al., 2012a), in most species pairs hybridization was limited to the F_1 generation. This suggests that the evolution of intrinsic postzygotic isolation strongly contributes to maintaining species boundaries among food-deceptive orchids (Scopece et al., 2008, 2013) and that neither introgression nor the formation of hybrid swarms represents a major threat to the long-term survival of most of these species. However, the possibility that hybridization in orchids reduces the fitness of the parental species has only rarely been considered (but see Cozzolino et al., 2006).

We investigated the fitness consequences of hybridization between two food-deceptive *Dactylorhiza* species: *Dactylorhiza maculata* (L.) Soó and *D. sphagnicola* (Höppner ex Soó) Aver. The genus *Dactylorhiza* consists of a large group of species that are widely distributed across the boreal and temperate zones of Europe, Asia, North America, and northern parts of Africa (Delforge, 2006; Devos et al., 2006; Pillon et al., 2007). Its taxonomic status is quite complex, due to high morphological variation of many taxa and the numerous intra- and inter-genus hybrids (Pillon et al., 2007; De Hert et al., 2011, 2012; Stace et al., 2015). Some studies found indications of backcrossing and showed that hybridization can lead to introgression and the formation of hybrid swarms (Aagaard et al., 2005), whereas others found evidence of introgressive gene flow between ploidy levels (Nordström and Hedrén, 2008; Ståhlberg, 2009), suggesting the occurrence of introgression and, hence, of some hybrid fertility. However, little is known about the extent of hybridization between *D. maculata* and *D. sphagnicola* (but see Hedrén 1996, 2003) and whether hybridization affects fitness and, ultimately, the long-term viability of the parental species. Nonetheless, this information is particularly relevant for *D. sphagnicola*, given its limited distribution and its frequent co-occurrence with other species in the genus *Dactylorhiza*. More specifically, we addressed the following questions:

(1) Is there any evidence of hybridization between *D. maculata* and *D. sphagnicola*?
(2) If hybridization has occurred, what is the degree of introgression in natural populations of *D. maculata* and *D. sphagnicola*?
(3) Does hybridization result in fitness costs in the pure parental species?

MATERIALS AND METHODS

Study species—*Dactylorhiza maculata* and *D. sphagnicola* are food-deceptive, herbaceous, perennial, polyploid orchid species. *Dactylorhiza maculata* is an autotetraploid species (Devos et al., 2005) that is widely distributed across Europe and can locally attain high abundances (Delforge, 2006). It occurs in a wide range of habitats, including moist to wet meadows, woodland edges, forest clearings, and the edges of peat bogs. *Dactylorhiza sphagnicola* is an allotetraploid that includes one subgenome similar to that of *D. maculata* (Devos et al., 2003; Hedrén, 2003). It shows an Atlantic distribution, occurring from the French Ardennes, Belgium, north to Scandinavia. It occurs predominantly on damp to wet, acidic substrates, including raised bogs and swampy deciduous or coniferous woodland (Delforge, 2006), where it can co-occur with *D. maculata*. In comparison with *D. maculata*, *D. sphagnicola* is much rarer and strongly declining, due to major losses of its prime habitat.

Like other species in this genus, the two study species do not produce any reward and are pollinated by generalist pollinators, including bumblebees (*Bombus terrestris*, *B. pascuorum*, *B. pratensis*, *B. hortorum*), cuckoo bumblebees (*Psithyrus barbutellus*, *P. globosus*), dance flies (*Empis livida*, *E. pennipes*), hoverflies (*Eristalis arbustorum*, *E. horticola*), and halictid bees (*Halictus calceatus*) (Claessens and Kleynen, 2011). The two species differ in phenology, with *D. sphagnicola* flowering about three weeks earlier than *D. maculata*. However, because flowering lasts for more than four weeks, there is at least one week of temporal overlap in flowering periods, offering the opportunity for interspecific pollen transfer. Both species depend on mycorrhizal fungi for seed germination and seedling establishment (Rasmussen, 1995). Molecular analyses indicate that the two species share a considerable number of mycorrhizal fungi (most often Tulasnellaceae and, to a lesser extent, Ceratobasidiaceae; Jacquemyn et al., 2012c), suggesting that mycorrhizal fungi do not limit establishment of hybrid plants.

Sampling—The study was conducted at one sympatric site where *D. maculata* and *D. sphagnicola* co-occur and where signs of hybridization have been observed (D. Tyteca, personal communication) and at two allopatric sites (one for each species). Both the sympatric and the allopatric sites were located on the Plateau des Tailles in the Belgian Ardennes, which consists of a series of acidic fens occurring within forests and agricultural land. The sympatric site consisted of a large acidic fen near Regné (Belgium) (50°15′N, 5°47′E; 556 m a.s.l.) where the two species grew next to each other along with other rare species such as *Menyanthes trifoliata*, *Potentilla palustris*, and *Polygonum bistorta*. The two allopatric sites were part of a series of populations that occurred at a nearby site called Pisserotte (50°13′N, 5°47′E; 562 m a.s.l.). The habitats where both allopatric populations occurred were very similar to the one where the sympatric population was found and consisted of wet, acidic bogs surrounded by pine forest.

In May 2012, a total of 121, 72, and 56 plants were sampled for molecular and morphometric analysis in the sympatric and allopatric *D. maculata* and *D. sphagnicola* populations, respectively. In all populations, plants were randomly selected and two leaves were collected from each plant for amplified fragment length polymorphism (AFLP) analysis.

Molecular analysis—DNA extraction and AFLP analysis followed the same protocol as described in De Hert et al. (2012). Briefly, leaf material was freeze dried for 48 h and homogenized with a mill (Retsch MM 200) to fine powder. Total DNA was extracted from 30 mg of freeze-dried leaf material using the Nucleospin 96 Plant Kit (Machery-Nagel). DNA concentrations were estimated using a Nano-Drop ND-1000 spectrophotometer (running software 3.0.1; Nano-Drop Technologies) following the manufacturer's instructions.

AFLP analysis (Vos et al., 1995) was carried out according to De Hert et al. (2012), using commercial kits and following the protocol of Roldán-Ruiz et al. (2000). The enzymes EcoRI and MseI were used for DNA digestion. Each individual plant was finger-printed with two primer combinations: EcoRI-AGG/MseI-CTGG and EcoRI-ACTG/MseI-CCC. Fragment separation and detection took place on an ABI Prism 3130xl capillary sequencer. GeneScan 500 ROX-labeled size standard (Applied Biosystems) was used for fragment sizing. The fluorescent AFLP patterns were scored using GeneMapper version 3.7 (Applied Biosystems). We scored the presence or absence of each marker in each individual plant. Monomorphic markers were excluded from all further analyses. To assess reproducibility, three independent DNA extractions were carried out for 10 individuals, and AFLP fingerprints were generated for each replicate (a total of 60 AFLP fingerprints). Mean reproducibility values (calculated as the percentage of markers that were identical in the three repeats) were high (>98.5%).

The extent of hybridization in the sympatric population was assessed using a principal coordinate analysis (PCoA) and two Bayesian clustering analyses. In all analyses, the data from the allopatric populations were included as a reference. First, a binary data matrix was constructed based on the presence/absence of AFLP bands. This matrix was used to calculate an individual pairwise genetic distance matrix in GENALEX (Peakall and Smouse, 2012), which was then subjected to a PCoA analysis. Bayesian clustering was performed using STRUCTURE (Pritchard et al., 2000; Falush et al., 2007). STRUCTURE uses a model-based clustering method to assign individuals to groups in which deviations from Hardy–Weinberg equilibrium and linkage equilibrium are minimized. Individuals assigned to two sources with nontrivial probabilities were considered putative hybrids. STRUCTURE was run 10 times at $K = 1$–6, assuming no prior population information, with correlated allele frequencies and admixture, 200,000 burn-in cycles and 1,000,000 MCMC. The value of K that best fits our data were selected using the ΔK statistic (Evanno et al., 2005). Additionally, NEWHYBRIDS (Anderson, 2008) was used to classify the genetic composition of the hybrids into six different classes: F_1, F_2, backcross to each parental species, and pure parental species. Again, a burn-in of 200,000 steps followed by run lengths of 1,000,000 steps was used.

Morphometric measurements—To assess differences in morphological trait expression between parental species and putative hybrids, 14 vegetative and 14 flower characters were used (Appendix S1; see Supplemental Data with the online version of this article) (Dufrêne et al., 1991). All vegetative characters were measured in the field using a digital caliper for length and width measurements. Because *D. maculata* has spotted leaves (hence its name), whereas leaves of *D. sphagnicola* have no spots, we also recorded the presence of spots on the leaves on a 1 to 3 scale (1 = no spots, 2 = intermediately spotted, and 3 = heavily spotted), yielding 29 characters in total. To obtain floral characters, one flower was harvested per plant and brought to the laboratory. All flowers were harvested at the same position within the inflorescence. Afterward, each flower was dissected (Appendix S1), and a digital photograph was taken of each flower. Finally, the image analysis software IMAGEJ 1.33 (Rasband, 2011) was used to measure all floral characters presented in Appendix S1.

A principal component analysis (PCA) was conducted to get a general overview of the quantitative variation in morphological traits and to describe the overall differences in flower morphology among individuals from the allopatric populations and individuals from the sympatric population. Both vegetative traits and floral traits were used in the analysis. Because the first two axes represented almost 50% of the total variation, only PCA scores of the first two axes were plotted. Additionally, a multivariate analysis of variance was used to test whether the overall morphology differed between plants from the allopatric and sympatric populations. Plants from the sympatric population were assigned to either pure *D. maculata*, pure *D. sphagnicola*, or hybrid plants based on the posterior probabilities (Q) derived from the Bayesian clustering analysis. All plants with a Q value between 0.05 and 0.95 were considered as hybrids. All floral and vegetative characters were included in the analysis, and plant type (allopatric and sympatric *D. maculata* and *D. sphagnicola* and hybrids) was used as fixed factor. Afterward, univariate analyses of variance were conducted to test which floral or vegetative character differed between the five plant types. Because multiple tests were conducted, a Bonferroni correction was applied to avoid the possibility that some of the differences arose purely by chance. Additionally, post hoc tests using Tukey's multiple comparison test were applied to see which characters differed between hybrid, allopatric, and sympatric *D. maculata* and *D. sphagnicola* plants.

Assessment of reproductive isolation—To assess the strength of postmating reproductive isolation, hand pollination experiments were conducted between individuals of the same species (intraspecific crosses) and between individuals of different species (interspecific crosses). All pollinations were reciprocally conducted on six individuals of each species. Per plant, three flowers were pollinated with intraspecific pollen and three flowers with interspecific pollen. Pollinia were removed with a toothpick and gently scrubbed against the back of the gynostemium of the receptive flower. At the same time, all treated flowers were marked, and afterward each plant was covered with nylon mesh to avoid further pollination by insects. When fruits were ripe, all plants were revisited. All treated flowers were closely inspected to see whether capsules had formed, and fruit set was determined afterward.

For each plant, all fruits resulting from the pollination treatments were harvested, and for each fruit the proportion of viable seeds was determined using the tetrazolium method described in Van Waes and Debergh (1986). In this case, only viable embryos are stained red after the treatment. Because the tetrazolium method can overestimate the real germination rate, the seed viability percentages should be treated with caution. However, for comparative purposes this method is sufficient to investigate differences in seed

quality resulting from pure and hybrid pollinations. To obtain a percentage of viable seeds of each of these fruits, we counted a subsample of 200–300 seeds from each capsule and calculated the ratio of the number of colored seeds to the total number of seeds.

Using methods outlined in Scopece et al. (2007) and Marques et al. (2014), two measures of postmating reproductive isolation were calculated. First, postmating prezygotic isolation was estimated as the proportion of fruits formed after interspecific crosses in relation to the proportion of fruits formed after intraspecific crosses:

$$\text{Post}_{\text{prezygotic}} = 1 - \text{(average fruit set after interspecific crosses) / (average fruit set after intraspecific crosses)}$$

Second, postmating postzygotic isolation was calculated as the proportion of viable seeds from interspecific crosses in relation to the proportion of viable seeds obtained from intraspecific crosses:

$$\text{Post}_{\text{postmating}} = 1 - \text{(percent of viable seeds formed after interspecific crosses / percent of viable seeds formed after intraspecific crosses)}$$

Finally, to test whether naturally established hybrids show reduced female fertility, hybrid plants were hand pollinated with pollen of the pure species, and seed viability was assessed using the same methods as outlined above. To test whether seed viability differed between the different pollination treatments, we used a generalized linear model with logit link function and pollination treatment as independent variable and the proportion of viable seeds as dependent variable. A post hoc test using Tukey's multiple comparison test was applied to see which pollination treatments differed significantly from each other in terms of seed viability.

Fruit set and seed viability of open-pollinated plants—After fruit maturation, natural fruiting success was determined for each sampled plant as the ratio of the number of fruits to the number of flowers. Per plant, three fruits (or all fruits if fewer than three fruits were produced) were harvested and brought to the laboratory to assess the viability of the seeds using the same methods outlined above. To test whether fruit set and seed viability differed between plants from both allopatric and the sympatric populations, a generalized linear model with logit link function and post hoc tests using Tukey's multiple comparison procedure were used to see whether proportional fruit set and the proportion of viable seeds differed significantly between plant type (allopatric and sympatric *D. maculata* and *D. sphagnicola* and hybrids). All statistical analyses were conducted using IBM SPSS Statistics 20 (IBM, 2011).

RESULTS

Hybridization—The two primer combinations generated a total of 129 polymorphic bands, of which 74 and 96 were polymorphic in the sympatric and allopatric populations, respectively. Each individual displayed a unique banding pattern. The PCoA clearly separated the two species of the allopatric sites, whereas a large number of intermediate plants was observed at the sympatric site (Fig. 1). Bayesian admixture analyses using STRUCTURE yielded similar results. The likelihood (Ln $P(D)$) increased greatly between $K = 1$ and $K = 2$, but less so after $K = 2$, which, together with the fact that ΔK
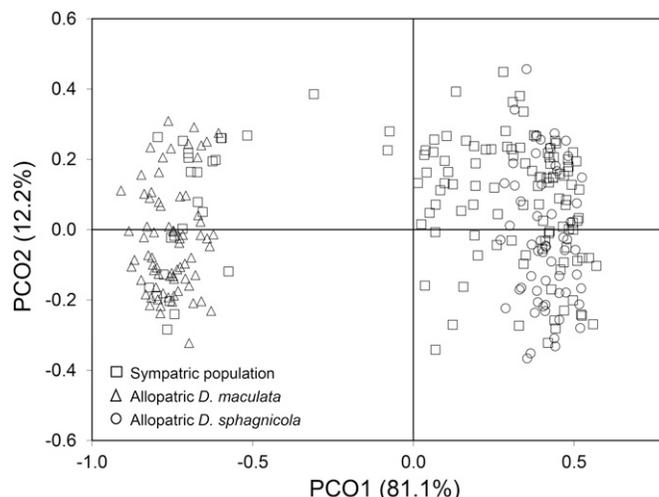

FIGURE 1 Principal coordinate plot based on individual genetic distances calculated with 129 AFLP markers for a sympatric population and two allopatric populations of *Dactylorhiza maculata* and *D. sphagnicola*.

reached its maximum at $K = 2$, indicates the existence of two clusters. In the allopatric populations of *D. sphagnicola* and *D. maculata*, all individuals had $Q > 0.95$, and 85.7 and 86.1% of the individuals were assigned almost exclusively ($Q > 0.99$) to one of the two clusters, indicating clear genetic divergence between the two species (Fig. 2A). By contrast, the sympatric population contained a large number of individuals with mixed ancestry, indicating substantial hybridization. Admixed individuals formed a continuous spectrum of genetic variation from highly admixed individuals (most likely early-generation hybrids) to late-generation hybrids/relatively pure *D. sphagnicola* individuals. The pattern of admixture was therefore clearly asymmetric, with ancestry proportions varying gradually toward *D. sphagnicola*, but not toward *D. maculata*. These results were confirmed by analyses using NEWHYBRIDS, which also revealed a high number of individuals with admixed ancestry, mainly between backcrosses toward *D. sphagnicola* and first-generation hybrids (Fig. 2B).

Morphometric results—The morphometric analyses provided further evidence for substantial hybridization. The first two axes of the PCA explained 30.4% and 16.9% of the total variation (Fig. 3) and clearly separated the two allopatric populations, but the distinction between the two species was less clear in the sympatric population. In this population, there were a lot of individuals that were intermediate in morphology or showed a morphology that was different from that of individuals in the allopatric populations (Fig. 3), suggesting substantial hybridization and, in some cases, heterosis. The first PCA axis was correlated mainly with variables related to the shape and size of flowers, whereas the second axis was mainly related to plant size (plant height) and the number of spots on the leaves. Results of a multivariate analysis of variance showed that the overall morphology was significantly different (Wilks's $\Lambda = 0.056$, $F = 7.98$, $P < 0.001$) between individuals of *D. maculata*, *D. sphagnicola*, and their hybrids. Univariate analyses of variance investigating each morphological character independently confirmed the strong morphological difference between the allopatric populations of the two species, but the distinction was less clear in the sympatric populations. However, in most cases, traits of the hybrids showed
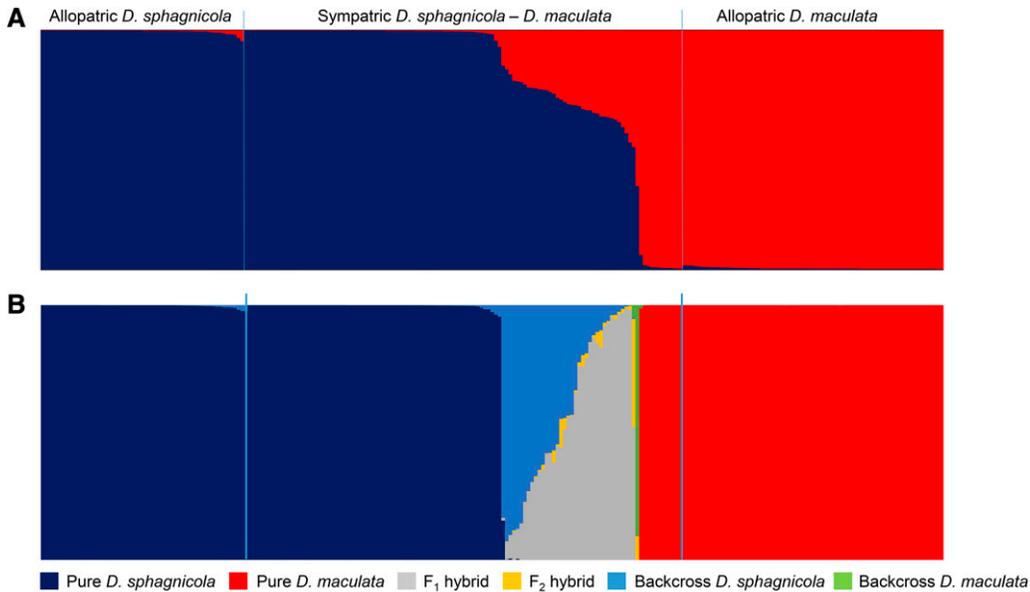
FIGURE 2 Bayesian clustering analyses for one sympatric and two allopatric populations of *Dactylorhiza maculata* and *D. sphagnicola*. (A) Assignment of sampled individuals using STRUCTURE. Each individual is represented by a column, with the colors representing the proportion of their genome assigned to the $K = 2$ inferred clusters in the model-based admixture analysis. (B) Assignment of sampled individuals using NEWHYBRIDS. The proportion of color in each bar represents an individual's assignment probability with respect to each genotype class (pure parental species, F_1 and F_2 hybrids, and respective backcrosses).

an intermediate position between pure plants of both species or overlapped with those of one of the parental species (Table 1).

Reproductive isolation—All experimental crossings resulted in successful fruit set, resulting in no postmating prezygotic isolation at this stage ($Post_{prezygotic} = 0$). For flowers that developed into a fruit, the proportion of viable seeds differed significantly ($F_{5,25} = 101.5$, $P < 0.001$) between the different treatments that were applied (Fig. 4). For both species, intraspecific crosses resulted in high percentages of viable seeds (on average, 78.1% and 75.8% of all seeds contained a
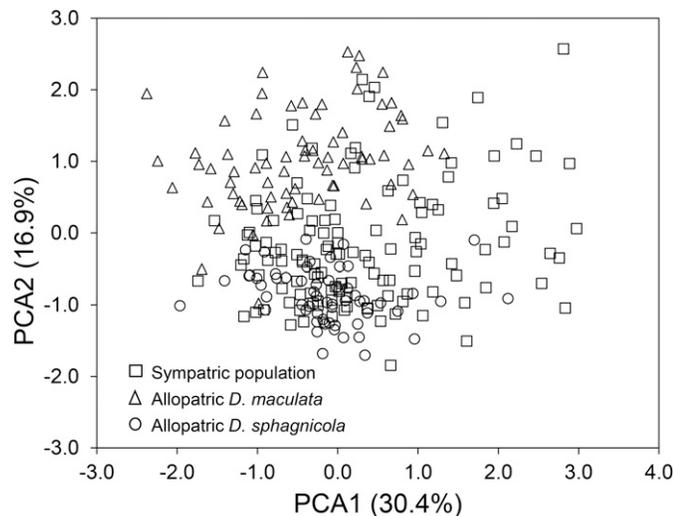

FIGURE 3 Principal component plot of the first and second axis for 29 morphological characters measured in allopatric and sympatric populations of *Dactylorhiza maculate* and *D. sphagnicola*.

viable embryo for intraspecific crosses of *D. maculata* and *D. spagnicola*, respectively). First-generation interspecific crosses resulted in a slightly lower proportion of viable seeds (Fig. 4), resulting in relatively low barrier strength ($P_{postmating} = 0.17$ and 0.26 for *D. maculata* and *D. sphagnicola* as pollen receptor, respectively). By contrast, backcrosses resulted in very low numbers of viable seeds, and most seeds lacked a well-developed embryo.

Fruit set and seed viability—Fruit set of open-pollinated plants differed significantly ($F_{4,147} = 15.25$, $P < 0.001$) between sites and species. Fruit set was, on average, significantly higher in *D. maculata* than in *D. sphagnicola*. Fruit set was also significantly higher in the allopatric populations than in the sympatric population. Plants of *D. maculata* produced, on average, 13.2 fruits (28.9% fruit set) in the sympatric population and 19.5 fruits (51.2% fruit set) in the allopatric population, whereas the average number of fruits in *D. sphagnicola* varied between 4.75 (12.4% fruit set) in the sympatric population and 8.24 (39.9% fruit set) in the allopatric population (Fig. 5A). Hybrids produced, on average, 10.0 fruit (27.8% fruit set). Seed viability was generally higher in *D. sphagnicola* (26.8% and 29.3% in the sympatric and allopatric population, respectively) than in *D. maculata* (20.1% and 22.4%, respectively) (Fig. 5B), but post hoc tests showed that this difference was not statistically significant ($P > 0.05$). Seed viability of hybrids, on the other hand, was much lower (3.3%) and statistically different ($P < 0.001$) from that of the parental species.

DISCUSSION

The extent of hybridization—We used both molecular markers and morphometric analyses to assess the extent of hybridization in a sympatric population of *Dactylorhiza maculata* and *D. sphagnicola* and to investigate the consequences of hybridization on reproductive success and fitness of the parental species and their resulting hybrids. In the sympatric population, a substantial number of individuals were of hybrid origin, whereas no such hybrids were observed in the allopatric populations. Morphologically, the hybrids were not always easy to discern from the parental species in the sympatric population, and for many traits there was overlap between hybrid plants and pure species. However, patterns of genetic variation indicated that hybrid plants were genetically more related to *D. sphagnicola* than to *D. maculata*, suggesting that they most likely represented early-generation hybrids and backcrosses toward *D. sphagnicola*. Only one individual could almost certainly be considered as a later-generation hybrid toward *D. maculata*, most likely a backcross toward *D. maculata*. These results suggest

TABLE 1. Results of univariate analyses of variance to test for differences in vegetative and floral traits between allopatric and sympatric individuals of *Dactylorhiza maculata* and *D. sphagnicola* and their hybrids. Letters accompanying means indicate the outcome of Tukey's multiple comparison test; means that share the same letter within a row do not differ significantly ($\alpha = 0.05$).

Trait	*D. sphagnicola*: allopatric	*D. spaghnicola*: sympatric	Hybrid	*D. maculata*: sympatric	*D. maculata*: allopatric	Significance [a]
Vegetative traits						
Plant height from soil level (cm)	33.4[a]	34.9[a]	41.2[b]	47.4[c]	45.5[bc]	***
Number of cauline leaves	5.09[a]	5.99[b]	6.45[b]	6.54[b]	6.57[b]	***
Lowermost leaf length (cm)	11.29	11.63	10.74	12.79	10.67	ns
Lowermost leaf maximum width (cm)	1.38[a]	1.56[ab]	1.83[bc]	2.09[c]	1.92[c]	***
Length of second leaf (cm)	13.75[a]	14.63[a]	13.06[a]	14.73[a]	13.12[a]	**
Maximum width of the second leaf (cm)	1.44[a]	1.75[b]	1.84[b]	2.02[b]	1.80[b]	***
Position of the second leaf greatest width (cm)	6.03[a]	6.08[a]	6.03[a]	7.52[b]	6.34[a]	*
Uppermost leaf length (cm)	4.45[c]	4.23[bc]	3.57[ab]	3.63[ab]	3.32[a]	***
Uppermost internodium length (cm)	5.11[ab]	4.69[a]	5.46[ab]	5.65[bc]	6.42[c]	***
Stem diameter under inflorescence (mm)	44.36[b]	51.07[b]	45.00[b]	37.40[a]	37.15[a]	***
Stem diameter above lowermost leaf (mm)	55.66[abc]	64.47[c]	58.86[bc]	54.23[ab]	49.46[a]	***
Number of flowers	18.55[a]	33.14[b]	37.76[bc]	43.62[c]	36.99[bc]	***
Inflorescence length (cm)	7.49	8.72	8.15	8.37	8.44	ns
Length of inflorescence axis between the bract insertion points of first and fifth flowers (cm)	2.44[b]	2.087[a]	1.93[a]	2.05[a]	2.00[a]	***
Floral traits						
Bract length	1.958[bc]	2.177[c]	1.874[b]	1.875[b]	1.437[a]	***
Bract width	0.434[c]	0.407[c]	0.353[b]	0.348[b]	0.275[a]	***
Ovary length	1.223[c]	1.229[c]	1.102[b]	1.089[b]	0.98[a]	***
Lateral sepals length	0.863[ab]	0.927[b]	0.896[ab]	0.904[ab]	0.83[a]	***
Lateral sepals width	0.269[ab]	0.291[c]	0.282[c]	0.264[ab]	0.237[a]	***
Petals length	0.701[ab]	0.747[b]	0.714[b]	0.709[ab]	0.652[a]	***
Petals width	0.294[b]	0.291[b]	0.292[b]	0.269[ab]	0.242[a]	***
Labellum length	0.858	0.881	0.869	0.897	0.844	ns
Labellum lateral lobes length, from base	0.79[a]	0.809[ab]	0.858[bc]	0.893[c]	0.851[abc]	***
Labellum median lobe length	0.239[a]	0.284[b]	0.247[ab]	0.217[a]	0.226[a]	***
Labellum width	1.067[a]	1.134[ab]	1.221[b]	1.23[b]	1.147[ab]	***
Labellum median lobe width, at base	0.265[a]	0.294[ab]	0.306[b]	0.276[ab]	0.270[a]	**
Spur length	0.877[b]	0.911[b]	0.903[b]	0.851[b]	0.764[a]	***
Spur diameter, at base	0.260[c]	0.252[c]	0.252[c]	0.218[b]	0.177[a]	***

Note: *** $P < 0.001$; ** $0.001 \leq P < 0.01$; * $0.01 \leq P \leq 0.05$; ns = nonsignificant.

that interspecific gene flow was asymmetric and occurred primarily from *D. maculata* toward *D. sphagnicola*. Asymmetric hybridization is not unusual in nature (Tiffin et al., 2001) and has been observed in other food-deceptive orchid taxa as well (e.g., *Orchis*; Jacquemyn et al., 2012a) and *Epidendrum* (Marques et al., 2014).

Our finding that admixture proportions varied almost continuously from ~60% to 100% *D. sphagnicola* ancestry suggests that reproductive barriers are far from complete between the study species. Although flowering phenology differs largely between the two species, there is still some phenological overlap that allows interspecific pollen transfer. Furthermore, the results of our interspecific hand pollinations showed that postmating barriers (RI < 0.26) were also weak. Only when pollen of pure species were experimentally deposited on flowers of hybrid plants did this result in the production of a very low proportion of viable seeds, but even then the effect was not strong enough to prevent the formation of later-generation hybrids. The latter finding is confirmed by our observations on natural hybrids that were exposed to open pollination, as these hybrids also produced some viable seeds. These results thus suggest that even with limited opportunities for backcrossing, a significant signature of introgression can be observed (Sambatti et al., 2012). Similar results have been shown in a natural hybrid zone of *Mimulus nasutus* Greene and *M. guttatus* DC. in which a significant proportion of hybrid plants was found, mostly resulting from backcrossing to one of the parental species (Sweigart and Willis, 2003; Kenney and Sweigart, 2016). Surprisingly, it was shown before that, due to

pronounced differences in mating system, premating reproductive barriers reduced hybrid formation to 1% of the offspring between both species, and that postmating isolation was also commonly acting between these species (Martin and Willis, 2007).

In our study population, directional backcrossing most likely resulted from repeated pollen transfer between *D. sphagnicola* and hybrid plants. Although this was not explicitly investigated, field observations have indicated that the flowering phenology of hybrid plants most likely showed a stronger overlap with that of *D. sphagnicola* than with that of *D. maculata*. Differences in flowering patterns were also proposed to explain directional hybridization in food-deceptive *Epidendrum* species (Marques et al., 2014). Besides, *D. sphagnicola* flowers were also more abundant in the study population when the hybrids were flowering; therefore, differences in local abundance may also have contributed to the observed pattern of asymmetric introgression (Ellstrand and Elam, 1993). Finally, our field observations also suggest that in the studied population, habitat isolation may have been less pronounced between the hybrids and *D. sphagnicola*. Both tended to occupy the most central and wettest part of the investigated bog, whereas *D. maculata* mainly occurred at the edges of bog, where environmental conditions were somewhat drier. Clearly, more research is needed to test these hypotheses.

Individual plant fitness in hybrid and pure populations—Our results further showed marked differences in female fitness (fruit set and seed viability) between plants growing under sympatric and
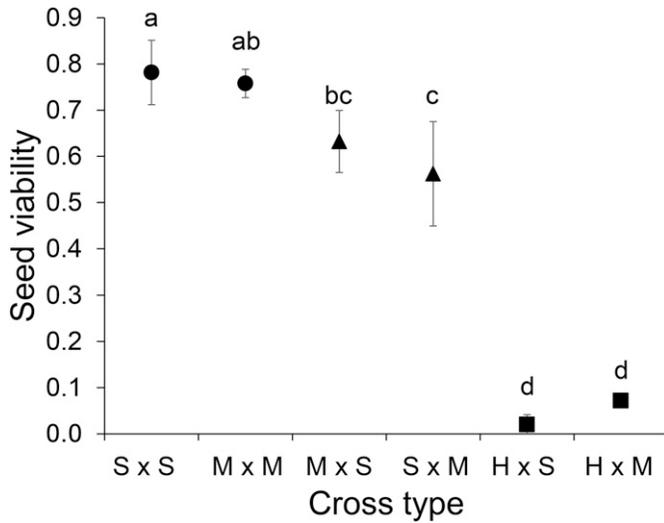
FIGURE 4 Differences in seed quality resulting from intraspecific and interspecific crossings between *Dactylorhiza maculata*, *D. sphagnicola*, and their hybrids. Letters accompanying means indicate the outcome of Tukey's multiple comparison test; means that share the same letter do not differ significantly (α = 0.05).

FIGURE 5 Differences in fruit set and seed viability between hybrid plants and pure individuals in sympatric and allopatric populations of *Dactylorhiza maculata* and *D. sphagnicola*. Letters accompanying means indicate the outcome of Tukey's multiple comparison test; means that share the same letter do not differ significantly (α = 0.05).

allopatric conditions, suggesting that extensive hybridization may have a significant impact on the reproductive success of the parental species. Both *D. maculata* and *D. sphagnicola* showed significantly lower fruit set in the sympatric population, which contained a relatively high proportion of hybrids (~30%), than in the allopatric populations. Given that the hybrid plants were intermediate in size and number of flowers, competition for pollinator services may have led to the strong decrease in fruit set in the parental species, especially in *D. sphagnicola*, which was smaller and produced fewer flowers than hybrid plants. Moreover, mixing of hybrid pollen in the total pollen pool may further have lowered female reproductive success in the parental species and therefore affected their long-term viability. Interspecific mating between pure parental plants may have contributed to the lower seed viability in the sympatric population, although the reductions in seed viability appeared to be lower than those following backcrossings with putative hybrids. These results are in contrast with findings of Cozzolino et al. (2006), who showed that parental species had higher or similar fitness in sympatry than in allopatric populations of two *Orchis* species: *Orchis mascula* (L.) L. and *O. pauciflora* Fisch. ex Lindl.

Our results also showed that *D. sphagnicola* consistently set fewer fruits than *D. maculata*, and that this effect was most pronounced in the sympatric population. On the other hand, fruits of *D. sphagnicola* contained more viable seeds than fruits of *D. maculata*. Nonetheless, the difference in fruit set was much more pronounced than the difference in seed viability, indicating that the rare *D. sphagnicola* may suffer more from extensive hybridization than the more common *D. maculata*. Mixing of hybrid plants with parental plants can therefore be considered an important factor that influences the long-term viability of the parental species. On the other hand, site differences between the allopatric and sympatric populations or differences in local pollinator communities may have contributed to the observed differences in fruit and seed set as well. However, since all populations were close to each other and part of the same hydrological system (Plateau des Tailles),
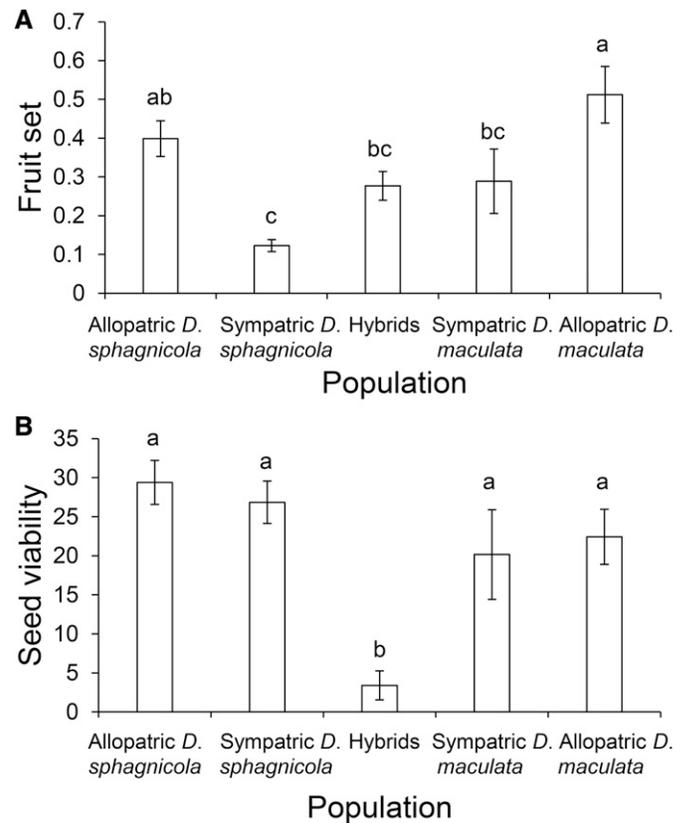
differences in site conditions were probably less important. Nonetheless, to fully exclude the role of environmental variables in determining patterns of fruit and seed set, a common garden experiment with controlled hand pollinations should be performed, but setting up such an experiment would be very challenging for species that typically grow in bogs.

Implications for conservation—Our detailed study at Plateau des Tailles, where *D. sphagnicola* and *D. maculata* populations come into secondary contact, has shown that hybridization can strongly influence population genetic diversity and plant fitness of the parental species. Given that *D. sphagnicola* is a species that shows a very localized distribution and is extremely rare in Belgium and elsewhere in Europe (Delforge, 2006), the species may be vulnerable to any disturbance affecting its population viability, including hybridization. For example, De Hert et al. (2012) mentioned that in a dune slack where three *Dactylorhiza* species and their putative hybrids occurred, individuals of one of the pure species, *D. praetermissa* (Druce) Soó, had disappeared. Although the exact reasons for the disappearance were not further specified, their findings indicate that parental species can go locally extinct and that hybridization may be a serious threat to the long-term survival of hybridizing plant species, especially when one of the parental species is rare. Our results indicated that interspecific gene flow occurred primarily from *D. maculata* toward *D. sphagnicola*.

Therefore, to protect the genetic integrity of both species in the short term, the chances of interspecific gene flow should be minimized. Given that both species prefer different edaphic conditions and occupy different locations within sympatric populations, some simple management interventions that aim at spatially segregating both species may already be helpful for avoiding hybridization and maintaining the integrity of both species. Our data from the allopatric populations suggest that the presence of physical barriers such as small hedges or patches of trees can be sufficient to effectively reduce the probability of hybridization between the two species. In the long term, however, ongoing hybridization and introgression may introduce novel alleles that increase species survival, particularly in rare species with a low level of standing genetic variation.

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