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A CASE STUDY OF MODIFIED INTERACTIONS WITH SYMBIONTS IN A HYBRID MEDITERRANEAN ORCHID[1]

BERTRAND SCHATZ[2,5], ALEXANDRE GEOFFROY[2], BENJAMIN DAINAT[3], JEAN-MARIE BESSIÈRE[4], BRUNO BUATOIS[2], MARTINE HOSSAERT-MCKEY[2], AND MARC-ANDRÉ SELOSSE[2]

[2]Equipe Interactions Biotiques, Centre d'Ecologie Fonctionnelle et Evolutive (CEFE), UMR CNRS 5175, 1919 route de Mende, F-34293 Montpellier Cedex 5, France; [3]Zentrum für Bienenforschung, Eidgenössisches Volkswirtschaftsdepartement EVD Forschungsanstalt Agroscope Liebefeld-Posieux ALP, Schwarzenburgstrasse 161 CH-3003 Bern, Switzerland; and [4]Laboratoire de chimie appliquée, Ecole Nationale Supérieure de Chimie de Montpellier, 8 rue de l'Ecole Normale 34296 Montpellier Cedex 5, France

- *Premise of the study*: Most studies on orchid hybrids examine separately the effects of hybridization on interactions with pollinators or with mycorrhizal fungi. Here, we simultaneously investigated both interactions in the mediterranean food-deceptive *Orchis simia*, *O. anthropophora*, and their hybrid (*O.* ×*bergonii*) and tested a possible breakdown of coevolution using a multidisciplinary approach.
- *Methods*: We compared leaf growth, seed viability, emitted scent, and mycorrhizal fungi (species and rate of infection) among these three taxa.
- *Key results*: We show that leaf surface is greater in adult hybrids than in the parental species, suggesting a heterosis effect for vegetative growth. We demonstrate that flowers of the two parental species emit well-differentiated bouquets of volatile organic compounds, while hybrids emit larger quantities, accumulating most compounds of the two parental species. However, hybrids fail to attract pollinators and have a 10 times lower fruit set. We determined that closely related Tulasnellales are mycorrhizal in the three taxa, suggesting that the mycorrhizal partner does not impair hybrid survival. We propose an interpretative model for *O.* ×*bergonii* compared with its parents.
- *Conclusions*: In hybrids, carbon resources normally devoted to reproduction may be reallocated to the mycorrhizal symbiosis as a result of the disruption of the pollination interaction in hybrids. Higher mycorrhizal infection may in turn enhance vegetative growth and scent emission. Such interplay between the two obligate biotic interactions yields new insights into hybridization among orchids.

Key words: chemical ecology; hybridization; mediterranean orchids; mycorrhizae; pollination biology; Orchidaceae; *Orchis simia*; *Orchis anthropophora*; southern France.

Hybridization is a major mechanism in plant evolution (Waser, 2001; Hegarty and Hiscock, 2005). A significant fraction of flowering plants are of hybrid origin (Ellstrand et al., 1996; Rieseberg et al., 1999), and at least a quarter of plant species are involved in hybridization and potential introgression with other species (Mallet, 2005). Whenever parental and hybrid taxa co-occur and bloom during overlapping periods, they may share common pollinators and similar soil preferences, i.e., biotic and abiotic factors (Arnold, 1997; Waser, 2001; Mallet, 2005; Cozzolino et al., 2006). Flowering plants possess various reproductive isolation mechanisms, acting before or after pollination or even in combination (Cozzolino et al., 2004; Moccia et al., 2007; Raguso, 2008; Stökl et al., 2008), which limit hybridization. For example, divergence in floral traits (different pollination syndromes) leads to attraction of different pollinators and hence to reproductive isolation between species such as *Iris* spp. (Hodges et al., 1996), *Penstemon* spp. (Castellanos et al., 2004), *Mimulus* spp. (Ramsey et al., 2003), and numerous orchid species (van der Cingel, 1995; Cozzolino et al., 2004; Moccia et al., 2007; Stökl et al., 2008).

Orchids, the largest angiosperm family, are well known for their extraordinary floral diversity associated with exquisite relationships with pollinators, which act as a driving force in their diversification (Cozzolino and Widmer, 2005). Most orchids emit characteristic bouquets of volatile compounds, widely varying among species in their composition. Each orchid species has a restricted range of pollinators as result of floral morphology and scent (van der Cingel, 1995; Stökl et al., 2008), a specificity that contributes to premating isolating mechanisms between co-occurring orchid species (van der Cingel, 1995; Waser, 2001; Cozzolino et al., 2004; Scopece et al., 2007). In the case of most European orchids, extensive observations over several decades have identified confirmed pollinators, i.e., insects acting efficiently as pollen vectors (van der Cingel, 1995; Schatz, 2006). Although orchids often exhibit strong ecological isolation for pollination (van der Cingel, 1995; Cozzolino et al., 2004), hybrids are frequent (Cozzolino and Widmer, 2005).

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[5] Author for correspondence (e-mail: bertrand.schatz@cefe.cnrs.fr)

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Their frequent occurrence in sympatry with parental species suggests that the latter can share pollen vectors (Schatz, 2006). More generally, barriers preventing cross-pollination in orchids are not completely effective (Dafni, 1987; van der Cingel, 1995; Schatz, 2006), so that prezygotic isolation is not absolute, e.g., in the Mediterranean species from the genus *Orchis* (van der Cingel, 1995; Aceto et al., 1999; Cozzolino and Widmer, 2005; Schatz, 2006).

It is a matter of debate whether postzygotic barriers among orchids are low, especially at stages immediately following fertilization, or high, when considering sterility of F1 hybrids (Moccia et al., 2007). And it is often unknown whether hybrids have reduced fitness because their fruit set is rarely quantified. However, although distribution patterns of orchid hybrids have not been well studied, most of them do not seem to colonize novel ecological niches (van der Cingel, 1995; Schatz, 2006; Moccia et al., 2007), suggesting that they are mainly adapted to the ecological niche of one or both parental species. In this context, if parental species display a higher rate of pollination than hybrids, this difference of fitness should be mainly due to maladaptation of the hybrid in pollinator attraction. As pointed out in a few previous studies (Nilsson, 1983; Salzmann et al., 2007; Stökl et al., 2008), comparisons of the nature and quantity of the emitted scent is one of the main ways of explaining different degrees of pollinator attraction among hybrids and their parents.

Orchids also strongly depend on another biotic interaction: throughout their vegetative life, orchids roots obligatorily associate with soil fungi to form mycorrhizae (Rasmussen, 1995), which provide them with water, minerals, and sometimes even organic compounds (Dearlaney, 2007). This symbiosis is required for germination of orchid seeds, which are devoid of reserves and receive all nutrients from fungi during early development (Rasmussen, 1995). Orchid distribution is likely to be limited by the local absence of their associated fungi (McCormick et al., 2004). Although the benefits (or costs) of this association for the fungus are still unclear, mycorrhizal association may be overlooked in the diversification of orchids (Otero and Flanagan, 2006). Mycorrhizal specificity is often high in orchids, ranging from a few genera to a single fungal species (McCormick et al., 2004; Dearlaney, 2007; Shefferson et al., 2008). Hybrids could display the symbionts of one or both parents or have totally different partners. For hybridizing *Caladenia* spp., Hollick et al. (2005) showed that (1) fungi of one or both parents, depending on the hybrid, can support germination of hybrid seeds, and (2) these hybrids often associate with fungi genetically different from those associated with the parents. Hollick et al., (2005) concluded that these hybrids were thus possibly on pathways to speciation, but their study did not explicit the level of genetic divergence between the fungi involved, i.e., whether they are different species or not. Up to now, no investigation has been performed on hybrids with low fitness (e.g., due to reduced pollination), whereas mycorrhizal symbiosis can be a source of maladaptation if the parents have diverging symbiont preferences. In nonorchids at least, mycorrhizal infection is known to positively affect plant survival and the number of seeds (Bryla and Koide, 1990; Nuortila et al., 2004) and to influence visitation by pollinators by modifying flower number, inflorescence size, or nectar quantity (Gange and Smith, 2005; Cahill et al., 2008). Mycorrhizal status may thus influence the fitness of hybrids.

The relative importance of multispecies mutualisms, and especially the link between aboveground and belowground interactions, has been neglected hitherto (Strauss and Irwin, 2004; Wolfe et al., 2005). Watermann and Bidartondo (2008) recently suggested that combining analyses of both orchids' associations may provide greater insights into speciation. Our knowledge of orchid mycorrhizal fungi and of the role of pollinators in generating orchid diversity has increased greatly in recent years (Cozzolino et al., 2006; Otero and Flanagan, 2006). Although accurate assessments of the nature of the pollinator spectrum and mycorrhizal fungi remain challenging (Watermann and Bidartondo, 2008), some models may now allow integrated investigations. Here, we investigate how hybrids cope with their symbionts, i.e., interact with (1) pollinators required at the reproductive stage and (2) the mycorrhizal symbionts during vegetative life. Hybrid zones, where parental species and hybrids co-occur (Cozzolino et al., 2006), are highly suitable for addressing these questions. We investigate here two food-deceptive (nectarless) species, *Orchis simia* Lam. and *O. anthropophora* L., which hybridize to form *O.* ×*bergonii* (Nanteuil) Camus (Fig. 1). Little is known about the identity of mycorrhizal fungi in *Orchis* spp. (including the species formerly placed in the genus *Aceras*). In vitro cultivation of the fungi identified two usual taxa of orchid symbionts, Ceratobasidiales and/or Tulasnellales (Currah and Sherburne, 1992; Rasmussen, 1995), depending on the species. Recent molecular work has supported the association of Tulasnellales with *O. militaris* (Shefferson et al., 2008) and *O. anthropophora* (Lievens et al., 2010), but no data are yet available for *O. simia* and *O.* ×*bergonii*. Concerning interactions with flower-visiting insects, Schatz (2006) established the suite of pollinators for these taxa and demonstrated that one pollinator (the coleopteran *Cidnopus pilosus* Leske [Elateridae]) shared by the two parental species performs cross-pollination. However, pollination success in hybrids remains very low, for unknown reasons (Schatz, 2006).

Here we compared the two parental species *O. simia*, *O. anthropophora*, and their hybrid *O.* ×*bergonii* at the adult stage. First, we compared among these taxa leaf growth, as proxy for vegetative development and carbon availability, as well as seed viability to determine the existence of a postzygotic barrier in hybrids. Second, we examined the characteristics of the bouquet emitted by flowers, to determine whether floral scent variations can account for the observed differences in pollination rates among the three taxa. Third, we investigated the identity and abundance of mycorrhizal associates in hybrids and their parents to determine whether lack of appropriate fungal symbionts can alter hybrid viability or scent production.

MATERIALS AND METHODS

Species and study site—We investigated plants of *O. simia*, *O. anthropophora*, two deceptive species with nectarless spurs, and their hybrid *O.* ×*bergonii* present in natural populations on the Causse de Blandas plateau (43°54′46N, 03°30′49E), situated 50 km north of Montpellier, France. *Orchis anthropophora* (L.), previously known as *Aceras anthropophorum* (L.) Aiton f., belongs to the genus *Orchis* (Pridgeon et al., 1997; Aceto et al., 1999; Bateman et al., 2003), as does the other parental species *O. simia*. Both species have an Atlantic–Mediterranean distribution (Great Britain to North Africa and Iran), while the hybrid *O.* ×*bergonii* has been reported in Central Europe and in North Africa (Bateman and Farrington, 1987; Menale et al., 1996; Kretzschmar et al., 2007). Sometimes sympatric, the two species *O. simia* and *O. anthropophora* usually occur in patches with 30–200 individuals within a few square meters (Schatz, 2006). *Orchis simia* starts opening flowers from the top to the bottom, whereas *O. anthropophora* and their hybrid adopt the reverse (and more classic) order; this difference does not affect the fruit set for the parental species (Schatz, 2006). Our surveys of populations over 5 years before this study allowed us to identify individuals with clear parental or hybrid phenotypes. These three taxa are easily distinguishable (Fig. 1): first, flowers of

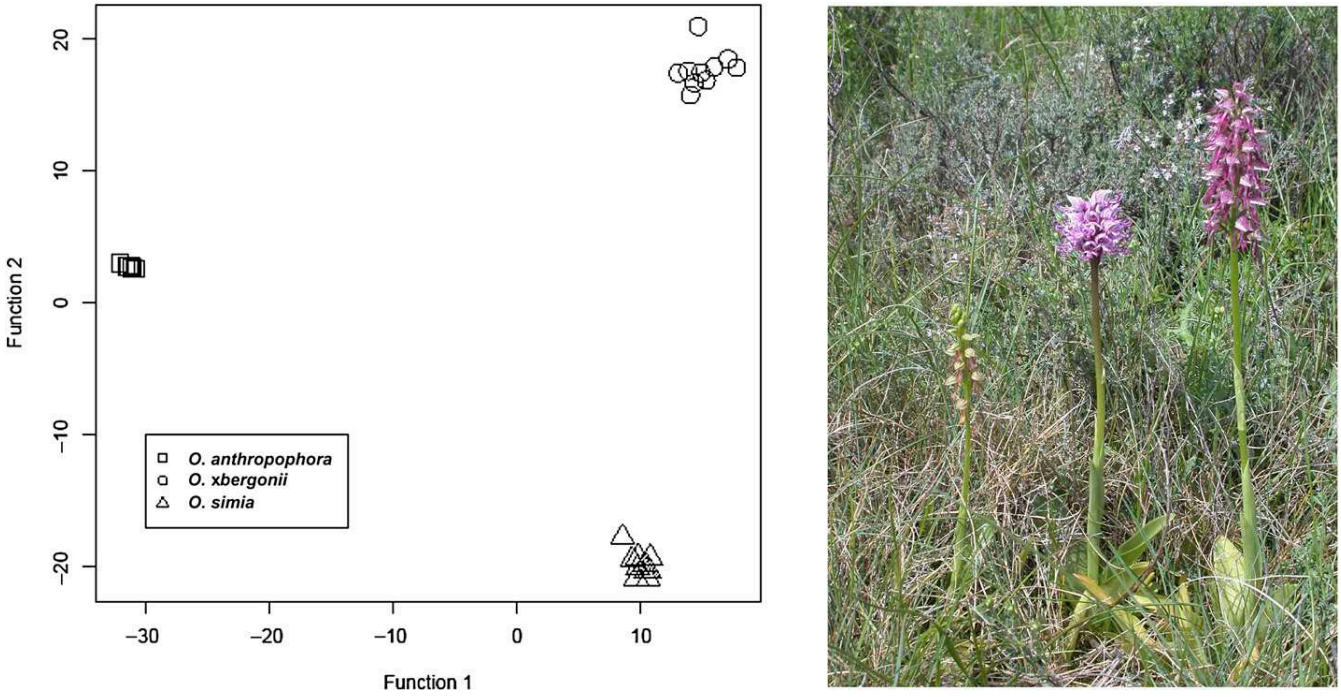

Fig. 1. *Orchis anthropophora*, *O.* ×*bergonii*, and *O. simia* and their emitted scent. (Left) Discriminant function analysis of scent emitted by the three taxa. This analysis was based on the relative proportions of volatile compounds in the 21 most abundant compounds emitted by the three taxa. Both discriminant functions accounted for 99.3% of the total variance. (Right) The three taxa, in a natural situation: *O. anthropophora* on the left, *O. simia* in the center and hybrid on the right.

O. simia are pink, while those of *O. anthropophora* are yellow-orange with a purple border, and those of *O.* ×*bergonii* are intermediate (Bateman and Farrington, 1987; Cozzolino and Aceto, 1994; Bournérias and Prat, 2005; Schatz, 2006; Kretzschmar et al., 2007). Second, the spur, the "tail" of the labellum and dark spots on the flowers are all present in *O. simia*, but absent in *O. anthropophora* and intermediate in *O.* ×*bergonii* (Bateman and Farrington, 1987; Schatz, 2005, 2006). At the study site, *O. simia*, *O. anthropophora*, and *O.* ×*bergonii* displayed distinct morphologies: they were 24.0 ± 5.6 cm, 23.0 ± 3.2 cm and 36.9 ± 8.1 cm in total height; 4.4 ± 1.8 cm, 10.4 ± 2.5 cm and 8.7 ± 3.4 cm in inflorescence height; and had 22.1 ± 8.2, 60.8 ± 21.8 and 53.0 ± 28.7 flowers, respectively (mean ± SD (SD); $N = 20$ individuals for each taxon; Schatz [2005]).

Leaf growth comparison—Length and width of leaves were measured for 30 individuals of each parental species and the hybrid in natural conditions (on five individuals in each of six different populations). For each individual, all leaves were measured (even those around the stem) and added to obtain the individual mean of total leaf area. To estimate the area of each leaf, we used the simple model of a lozenge, i.e., a figure with four equal sides inscribed in the leaf for which the area corresponds to one-half the product of the maximum length by the maximum width. We made use of six flowering individuals (those sampled for the identification of mycorrhizal fungi) to validate this approximation. All leaves of these six individuals (two individuals for each taxon) were used to measure their area using an mk2 area meter (Delta-T Devices, Cambridge, UK) as well as to estimate their area by our method of European lozenge approximation. No significant differences were found among measured and estimated values (Wilcoxon test $W = 23$; $P = 0.49$), indicating that the lozenge model can reliably approximate the leaf area. Log transformation of mean leaf area per individual allowed us to obtain a normal distribution (Shapiro test $P = 0.24$). We then compared the total leaf area among the three taxa (fixed effect) using an ANOVA (R software v.2.6.2; R Development Core Team, 2008) combined with pairwise Tukey tests for comparison of means.

Seed viability comparison—We also estimated the percentage seed viability by viewing a sample of the minute transparent seeds (means of 405, 1365, and 1191 seeds per individual were respectively examined for 22 crosses between two individuals of *O. anthropophora*, 12 crosses within *O.* ×*bergonii* and 33 crosses within *O. simia*), produced by intraspecific hand pollination. The fruit pods were collected 5 wk after hand pollination and dried and the seeds were extracted. Seeds were subsequently examined under an optical microscope with 100× magnification and assigned to two mutually exclusive categories: viable seed with one large embryo or nonviable seed with deficient embryo (i.e., small or aborted embryos or no embryo). We analyzed differences in seed viability between different kinds of crosses using an ANOVA combined with pairwise Tukey tests.

Collection and analysis of volatile compounds—From randomly chosen inflorescences in populations in the field, we sampled the volatile compounds emitted by flowers of eight *O. anthropophora* plants, 10 *O. simia*, and 10 *O.* ×*bergonii* f. From all plants at the peak of flowering, volatile compounds were collected by dynamic headspace adsorption. The entire inflorescence was enclosed in a polyethyleneterephthalate (Nalophan) odor-free bag. Purified air was blown into the bags at 400 mL·min^{-1} and sucked out at 300 mL·min^{-1} through a trap containing 30 mg of Alltech SuperQ absorbant (ARS, Gainesville, Florida, USA). The net influx ensured that the system was continuously purged through the inevitable leaks and that no contaminated outside air would enter the system. Blanks to check for potential air contamination were collected in parallel using an empty bag. After 6 h of collection (1000 to 1600 hours), odor traps were kept at –18°C until analysis. Trapped volatiles were eluted with 150 μL of dichloromethane. Two internal standards (nonane and dodecane, each at 200 ng·L^{-1}) were added to each sample for gas chromatography. Volatile compounds were analyzed by injection in a CP-8741 gas chromatograph (GC) with a flame ionization detector and in a gas chromatograph–mass spectrometer (GC-MS) (Varian CP 5860; Varian, Palo Alto, California, USA). For both analyses an Alltech (Deerfield, Illinois, USA) Heliflex column EC-1 (30 m, ID 0.25 mm, film thickness 0.25 μm; carrier gas: helium) was used. The injector split vent was opened (1/20). Oven temperature was programmed to remain at 50°C for 3 min, then increase by 3°C·min^{-1} to 100°C, by 27°C·min^{-1} to 180°C and by 6°C·min^{-1} to 250°C. Component identification was based on computer matching of the mass spectra with Wiley 138 and NBS 75 K libraries and on retention indices reported in the literature (Adams, 2001) and when possible by injection of reference compounds. We considered here the proportion of individuals in which the compound was detected with a relative abundance greater than 1% in at least one of the three taxa.

We estimated the total quantities of volatiles produced by orchids by using the peak areas of the two internal standards (nonane and dodecane) as a scale. The relative proportions of the 21 most abundant compounds emitted by the three taxa were compared using a discriminant function analysis. The standardized discriminant function coefficients were used to assess the differences among the three taxa (R software v.2.6.2). We then tested the global effect of taxon on the relative proportion of the different volatile compounds using a multivariate analysis of variance (MANOVA, PROC GLM, SAS v9, SAS Institute, Cary, North Carolina, USA) followed by a multiple comparison of means (Tukey–Kramer multiple comparison tests).

Molecular identification of the mycorrhizal fungi—Mycorrhizal samples were obtained by harvesting two individuals of each taxon on three occasions in 2006 to account for phenological variations: 8 February (rosette stage), 3 April (growing shoot), and 10 May (flowering). Summer is a resting period when the plant only has a globose, rootless tuber (Rasmussen, 1995). Root systems of these 18 plants were washed thoroughly and thin root sections were observed under a light microscope to check for mycorrhizal infection. Up to 12.5 mm long root mycorrhizal fragments were harvested per plant (in some cases, small size of the root system or low infection intensity limited the number of samples). The 190 resulting samples were kept at –80°C before molecular analysis. DNA extraction was carried out using the DNeasy Plant Mini Kit (Qiagen S.A., Courtaboeuf, France), according to the manufacturer's advice. The DNA was recovered in 100 μL of distilled water. To identify the fungus, we amplified the fungal intergenic transcribed spacer (ITS, encompassing the ITS1, 5.8S, and ITS2 sequences) using three primer sets: ITS1F+ITS4 and ITS1F+ITS4B (which amplify respectively most fungi and most Basidiomycota [Gardes and Bruns, 1993]), and ITS1-ITS4Tul (ITS4Tul, 5′-CCGCCAGATTCACACATTGA-3′, is specific for some tulasnelloid fungi; Selosse et al. [2004]). PCR conditions were the same as in Selosse et al. (2002). Sequences were obtained from both strands, using the primers used for PCR on an ABI3130xl sequencer (Applied Biosystems, Courtaboeuf, France) using the Big Dye Terminator kit. All sequence stretches that were ambiguous were pruned from the edited sequence. Sequences were edited and aligned using the program Sequencher 4.7 for MacOsX from Gene Codes Corp. (Ann Arbor, Michigan, USA). Data were deposited in GenBank (accession numbers EU583690–EU583721) and searches for similar sequences allowing taxonomic identification were conducted using the BLASTN algorithm available through the NCBI database (http://www.ncbi.nlm.nih.gov/BLAST/index.html).

Estimation of mycorrhizal infection—Thirty randomly chosen root sections (independent of the previous ones) were analyzed for each of the 18 individuals under the light microscope (magnification: 200 or 600). After staining with trypan blue (2% dilution in water), their infection level was estimated as the percentage area of the whole root section showing hyphal pelotons in root cells. Each section was classified in one of the five following classes, depending on the area infected: 0–20%, >20–40%, >40–60%, >60–80%, >80–100% infection. An average infection value was estimated for each individual by considering that a section in the class $x - y$ % had an infection intensity of $(x + y)/2$% (e.g., root sections 0–20% are considered to be 10% infected for this estimation). We used an ANOVA (R software v.2.6.2) to compare the level of infection of each taxon.

RESULTS

Leaf growth comparison between hybrids and parent species—Leaf area was highly significantly different among the three taxa (ANOVA; $F_{2, 87} = 31.1$; $P < 10^{-10}$). The hybrids displayed a significantly higher leaf area than either of the two parental species (Tukey tests: hybrid vs. *O. simia*: $P < 10^{-7}$; hybrid vs .*O. anthropophora*: $P < 4 \cdot 10^{-4}$), whereas the leaf area of *O. simia* was significantly higher than that of *O. anthropophora* (Tukey test: $P < 7 \cdot 10^{-4}$). Leaf area of *O. simia* was 1.5 times higher than that of *O. anthropophora*, and leaf area of the hybrids was 2.5 higher than that of *O. anthropophora*.

Seed viability comparison of hybrids and parent species—The proportions of viable to nonviable seeds in intraspecific crosses did not different among the three taxa (ANOVA; $F_{2, 64} = 0.27$; $P > 0.77$). Mean (±SD) values of this proportion were 75.4 ± 20.6% for *O. anthropophora* ($N = 22$), 75.1 ± 8.5% for *O.* ×*bergonii* ($N = 12$) and 72.2 ± 17.9% for *O. simia* ($N = 33$).

Comparison of the bouquet of floral volatile compounds emitted by the three taxa—The bouquets emitted by inflorescences of *O. simia*, *O. anthropophora*, and their hybrid, *O.* ×*bergonii*, included a total of 23 volatile compounds (Table 1). The compounds, divided into three groups based on their biosynthetic pathways (Knudsen et al., 2006), are presented in Table 1. The number of compounds produced by individuals differed significantly among species (ANOVA, $F_{2, 25} = 17.83$; $P < 10^{-4}$: *O. simia* (mean ± SD): 19.1 ± 2.3, *O. anthropophora*: 14.4 ± 3.8, and *O.* ×*bergonii*: 21.3 ± 0.7). The number of compounds emitted by *O. simia* and by *O.* ×*bergonii* were not significantly different ($P = 0.13$), but *O. anthropophora* emitted significantly fewer compounds than *O. simia* ($P < 2 \cdot 10^{-3}$) and even more significantly fewer than *O.* ×*bergonii* ($P < 10^{-4}$).

All compounds emitted by the hybrids were also produced by at least one of the two parental species. However, the discriminant function analysis of scents emitted showed three distinct clusters corresponding to the three taxa studied (Fig. 1). The first discriminant function, which accounted for 60.1% of the total variance, was mainly important for discriminating between the two parental species (DF1: $\chi^2 = 5539$, df = 50; $P < 2.4\ 10^{-12}$). The second function, which accounted for 39.2% of the total variance, was mainly important for discriminating between the hybrid and the parental species on the basis of the proportions of common compounds (DF2: $\chi^2 = 3574$, df = 24; $P < 7.4\ 10^{-7}$). All of the 28 samples used in the discriminant function analysis were correctly assigned to taxa.

The total quantity of volatile compounds emitted per inflorescence differed significantly among species (ANOVA, $F_{2, 25} = 19.78$, $P < 10^{-3}$) and between pairs of species ($P < 10^{-3}$ for each of the three comparisons). The quantities of volatile compounds emitted by inflorescences were 157.40 ± 76.64 ng·h^{-1} for *O.* ×*bergonii*, 10.57 ± 7.88 ng·h^{-1} for *O. anthropophora*, and 55.13 ± 37.73 ng·h^{-1} for *O. simia*. The total amount of scent emitted by the hybrid was thus ~15 times higher than for *O. anthropophora*, and ~3 times higher than for *O. simia.*

Terpenes were the most abundant compounds in the floral bouquets of all three taxa (69.0% of the compounds for *O. anthropophora*; 78.4% for *O. simia* and 80.2% for *O.* ×*bergonii*). However, for *O. simia* and for the hybrid, the floral scent was essentially composed of monoterpenes, whereas a mixture of monoterpenes and sesquiterpenes in similar relative abundance was observed for *O. anthropophora*. Benzenoid compounds had similar relative abundances in *O. simia* and in *O. anthropophora*, while they were rare in *O.* ×*bergonii*. Relative abundances of fatty acid derivatives decreased from *O. anthropophora* to the hybrids and to *O. simia*. MANOVA analysis of the chemical profiles of the three taxa showed a significant compound × taxon interaction ($F_{44, 550} = 13.33$; $P < 10^{-3}$). Some compounds were dominant, such as α-pinene in *O. simia* and in the hybrid, or β-caryophyllene, followed by caryophylladienol, nonanal, and undecane in *O. anthropophora* (Table 1). Among the 23 volatile compounds identified, the relative proportions of the compounds emitted by the hybrids were intermediate between those in the two parental species in 13 cases, lower than in both parental species in seven cases and higher than in both parental species in three cases. The three most frequent compounds emitted by hybrids (α-pinene, undecane, and sabinene) accounted for 64.2% of the whole bouquet.

TABLE 1. Mean relative proportions of compounds (±SD) in *Orchis simia*, *O. anthropophora* and *O. ×bergonii*. Each compound included represents more than 1% of the whole bouquet emitted by the taxon considered. Different letters after the mean value of each volatile compound indicate a significant difference among the three taxa (Tukey–Kramer multiple comparison tests); *P*: overall probability of the multiple comparison test; CV: coefficient of variation.

		O. anthropophora		*O. ×bergonii*		*O. simia*	
Species	*P*	Mean ± SD	CV	Mean ± SD	CV	Mean ± SD	CV
Fatty acid	*	21.00 ± 6.04a	0.29	17.64 ± 13.35a	0.76	9.14 ± 3.17ab	0.35
Nonanal	*	9.46 ± 5.36a	0.57	0.27 ± 0.26b	0.95	0.10 ± 0.11b	1.10
Undecane	ns	8.72 ± 3.82a	0.44	13.93 ± 14.10a	1.01	1.21 ± 1.99a	1.64
Methyldodecane	*	0.07 ± 0.19a	2.83	1.26 ± 0.76	0.60	2.45 ± 1.25	0.51
Alkanoid	***	2.76 ± 3.84a	1.39	2.18 ± 1.33b	0.61	5.38 ± 2.97c	0.55
Benzenoids	**	10.02 ± 8.51a	0.85	2.17 ± 1.46b	0.67	12.49 ± 6.46a	0.52
Benzene acetaldehyde	***	4.87 ± 4.78a	0.98	0.65 ± 0.91b	1.40	3.65 ± 5.24b	1.44
Methyl salicylate	*	2.41 ± 2.41a	1.00	0.64 ± 0.32	0.50	2.05 ± 1.24	0.61
Ethylacetophenone	***	2.74 ± 4.80a	1.75	0.87 ± 0.45	0.51	6.79 ± 4.68	0.69
Monoterpene	***	36.73 ± 15.69a	0.43	78.81 ± 13.50b	0.17	76.67 ± 6.49b	0.08
α-Pinene	***	5.77 ± 5.96a	1.03	43.21 ± 18.56b	0.43	32.68 ± 10.44b	0.32
β-Pinene	***	2.59 ± 1.35a	0.52	6.10 ± 0.45b	0.07	6.42 ± 1.25b	0.19
Sabinene	***	0.82 ± 0.86a	1.06	7.07 ± 1.55b	0.22	5.23 ± 1.52c	0.29
Myrcene	ns	2.22 ± 1.57a	0.71	4.95 ± 4.09a	0.83	5.45 ± 1.77a	0.33
Limonene	*	5.22 ± 6.27a	1.20	1.22 ± 0.98ab	0.80	1.04 ± 1.43b	1.37
1.8 cineole	**	7.49 ± 6.66a	0.89	0.63 ± 0.41b	0.64	1.46 ± 2.19b	1.50
Eucalyptol	*	1.82 ± 2.26a	1.25	5.15 ± 1.11ab	0.21	7.89 ± 1.90a	0.24
cis-Hydrate sabinene	***	0.16 ± 0.29a	1.86	1.71 ± 0.27b	0.16	2.37 ± 1.03c	0.44
Linalool oxide	ns	0.32 ± 0.62a	1.95	0.65 ± 0.55a	0.84	2.64 ± 1.10a	0.42
Linalool	***	0a		4.99 ± 1.95b	0.39	7.41 ± 2.61b	0.35
α-Campholenal	**	0		1.78 ± 0.35	0.20	2.83 ± 1.27	0.45
m-Myrcene	***	3.24 ± 4.00a	1.23	0.43 ± 0.28b	0.65	0.26 ± 0.39c	1.50
Pinocarvone	***	0.66 ± 0.94a	1.41	0.36 ± 0.13a	0.37	0.29 ± 0.21b	0.73
Terpenoid	*	6.42 ± 8.67a	1.35	0.55 ± 0.21ab	0.39	0.71 ± 0.82a	1.16
Sesquiterpene	***	32.24 ± 20.07a	0.62	1.38 ± 0.85b	0.62	1.70 ± 3.48b	2.05
β-Caryophyllene	***	22.33 ± 17.77a	0.80	1.38 ± 0.85b	0.62	1.70 ± 3.48b	2.05
Caryophylladienol	***	9.91 ± 4.61a	0.47	0b	0	0b	0

Mycorrhizal infection—Infection, showing living or decaying pelotons, was seen on several cell layers up to the vascular bundle (Fig. 2). Root sections from the same individual or from the same taxon had variable infection levels, but the distribution of root sections among the five infection classes differed significantly among the three taxa ($\chi^2 = 221.3$, df = 8, $P < 2.10^{-16}$; Fig. 2). The hybrid always had more than 20% of the section area infected. Infection levels differed significantly among the three taxa ($F_{2, 267} = 216.83$, $P < 2.10^{-16}$), and the average area infected decreased from the hybrid to *O. anthropophora* and *O. simia* (Fig. 2). When the three sampling dates are compared, the two parental species showed increasing infection over time (*O. anthropophora*: $\chi^2 = 39.7$, df = 6, $P < 10^{-6}$; *O. simia*: $\chi^2 = 31.4$, df = 6, $P < 10^{-5}$), while infection level changed much less over time for the hybrid ($\chi^2 = 12.9$, df = 6, $P = 0.044$; data not shown).

Identification of the mycorrhizal fungi—In all, 65% of the PCR amplifications were successful using the primer set ITS1F+ITS4, whereas no PCR product was obtained using the other primer sets. A total of 33 divergent sequences were retrieved (Table 2), and BLAST analysis showed that they were all from Tulasnellales (EU583690–EU583720), with the single exception of a sequence affiliated to *Verticillium*, found on a hybrid individual (EU583721; Table 2). The latter sequence was likely from a parasitic or symptomless endophyte and was therefore ignored in further analysis. Because Tulasnellales were rarely amplified by primer ITS4B and sometimes did not amplify with primer ITS4Tul, our identification agreed well with the PCR results. Because of high divergence among sequences, alignment of ITS sequences for the Tulasnellales was not possible. Nevertheless, a threshold of 70% identity separated these sequences into two groups, A and B (only one sequence from the hybrids, EU583690, did not group in A or B; Table 2), each of which was found in association with all three taxa. There was no evidence that fungi of either group were more common on *O. simia* than on *O. anthropophora* ($\chi^2 = 0.67$, df = 2, $P > 0.05$), *O. simia* than on *O. ×bergonii* ($\chi^2 = 0.60$, df = 2, $P > 0.05$) or *O. ×bergonii* than on *O. anthropophora* ($\chi^2 = 0.08$, df = 2, $P > 0.05$) (χ^2 tests with Yates correction). In addition, several sequences were more than 95% identical (groups *a–d* Table 2): *a* was found on all taxa; *b* and *c* were both found on the two parental species, *O. anthropophora* and *O. simia*; and *d* occurred on *O. anthropophora* only. In all, our data suggested that taxonomically similar symbionts were associated with the two parents, that symbionts of the hybrids mostly belonged to groups infecting the two parents, and that the fungi associated with hybrids had less-diverse sequences than those associated with the parents. Because only one sequence was found twice, we did not exhaust the whole fungal diversity associated with these orchid taxa.

DISCUSSION

This study demonstrates that the hybrid displays marked differences in comparison with its two parental species in

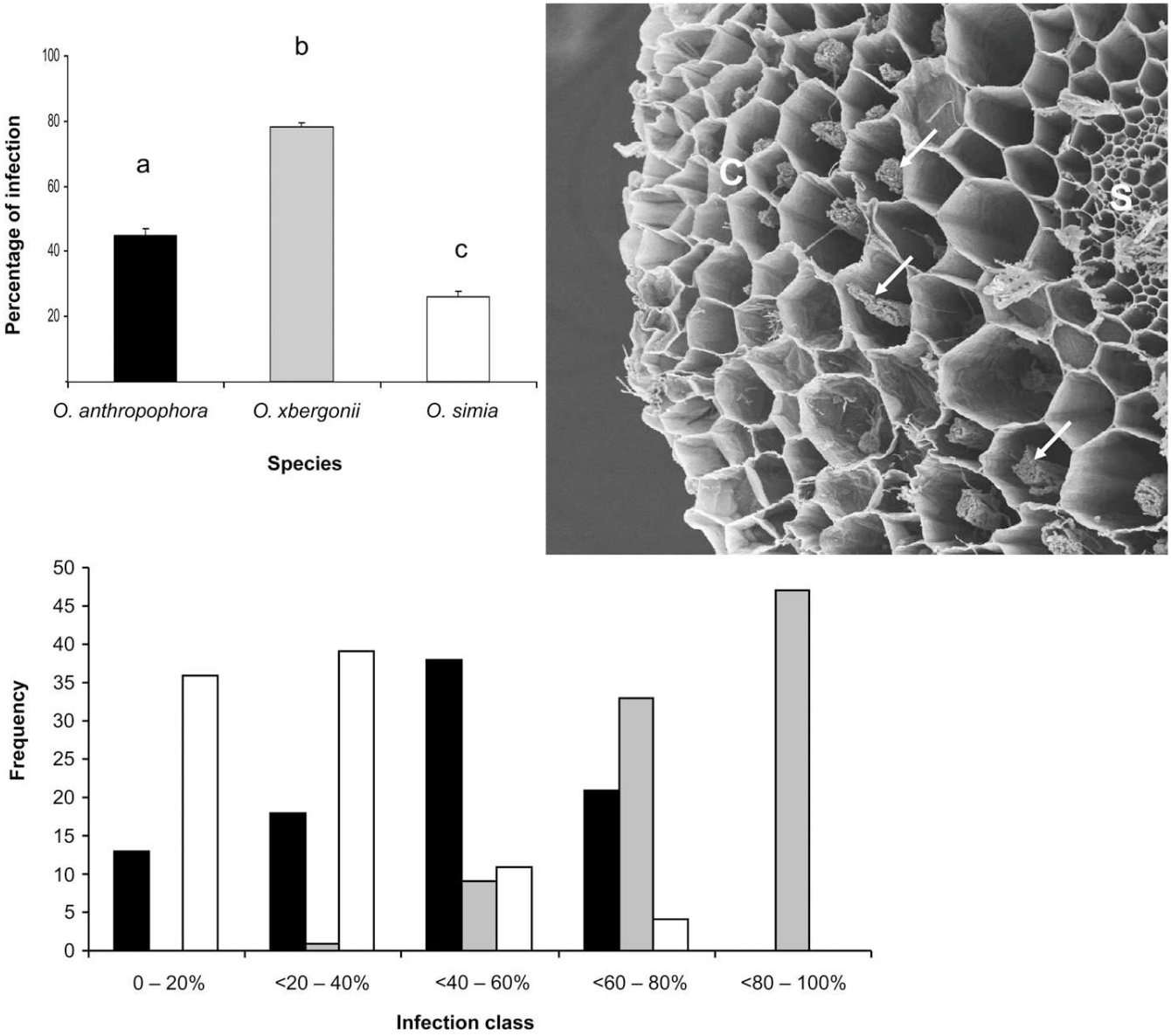

Fig. 2. Mycorrhizal interactions of *Orchis anthropophora*, *O.* ×*bergonii*, and *O. simia*. (Top left) Mean mycorrhizal infections of the three taxa (±SE) (black: *O. anthropophora*; gray: *O.* ×*bergonii*; white: *O. simia*); different letters indicate significant between-species differences according to one-way ANOVA and mean comparison with Tukey–Kramer tests. (Top right) Scanning electron micrograph of *O.* ×*bergonii* mycorrhizal roots with stele (s), cortex (c) and old pelotons at collapsing stage (arrows). (Bottom) Distribution of the analyzed root sections among five infection classes (see Materials and Methods; colors as in top left).

vegetative traits, floral odor traits, and the rate of mycorrhizal infection, but not in the identity of its associated mycorrhizal fungi. Such a multidisciplinary approach has never applied, to our knowledge, to hybrid orchids, although pollination and mycorrhizal symbioses are both likely to strongly shape their biology and evolution (Waterman and Bidartondo, 2008). Examining both associations in the same study allowed us to propose a general model of development and interactions in these hybrids. This study yields new insights into the hybridization process and the factors that allow or limit gene flow (Rieseberg et al., 1999; Waser, 2001; Cozzolino et al., 2004).

Bouquet emitted by flowers and consequences for pollination—In orchids, hybrid morphology (Dafni and Ivri, 1979; Schatz, 2005; Cozzolino et al., 2006) and genetics (Aceto et al., 1999; Chung et al., 2005; Moccia et al., 2007) have received more attention than volatile compounds emitted by flowers of hybrid individuals. This aspect of hybridization is still poorly studied (see Salzmann et al., 2007). Floral volatiles have a major role in attracting insects for deceptive species (Borg-Karlson, 1990; Salzmann et al., 2007). All volatile compounds emitted by *O.* ×*bergonii* hybrids are emitted by at least one of the parental species. Such similarity among floral odors has already been observed in other hybrids among *Orchis* (Salzmann et al., 2007,

TABLE 2. GenBank accessions, taxonomic identity, and frequency of fungal symbionts found on the roots of *Orchis anthropophora*, *O.* ×*bergonii*, and *O. simia*. Tulasnellales were grouped according to ITS sequence identity, at a threshold of 70% (groups A and B) or 95% (groups *a–d*).

	Similarity group		Number of occurrences on		
Accession	70%	95%	*O. anthropophora*	*O.* ×*bergonii*	*O. simia*
Tulasnellales					
EU583699	A	*a*	1	—	—
EU583700	A	*a*	1	—	—
EU583703	A	*a*	1	—	—
EU583697	A	*a*	2	—	—
EU583702	A	*d*	1	—	—
EU583704	A	*d*	1	—	—
EU583705	A	*a*	1	—	—
EU583701	A	*a*	1	—	—
EU583708	B		1	—	—
EU583706	B	*c*	1	—	—
EU909163	B	*b*	1	—	—
EU583707	B	*c*	1	—	—
EU583698	A	*a*	2	—	1
EU583709	A	*a*	—	—	1
EU583710	A	*a*	—	—	1
EU583711	A	*a*	—	—	1
EU583713	A	*a*	—	—	1
EU583712	A	*a*	—	—	1
EU583718	B	*c*	—	—	1
EU583719	B		—	—	1
EU583717	B	*b*	—	—	1
EU583714	B	*c*	—	—	1
EU583716	B	*b*	—	—	1
EU583720	B	*c*	—	—	1
EU583715	B	*c*	—	—	1
EU583690			—	1	—
EU583692	A	*a*	—	1	—
EU583693	A		—	1	—
EU583694	A	*a*	—	1	—
EU583691	A	*a*	—	1	—
EU583696	B		—	1	—
EU583695	B		—	1	—
Verticillium					
EU583721			—	1	—

B. Schatz, unpublished data) or among other European orchids (Nilsson, 1983; Stökl et al., 2008), and even in nonentomophilous species from other families, such as oaks (Schnitzler et al., 2004). Moreover, substantial variation in the relative proportions of the different volatile compounds was observed within each of the three taxa studied, which may limit the ability of pollinators to discriminate unrewarding orchids, as previously observed in other cases of hybridization among *Orchis* spp. (Johnson et al., 2004; Salzmann et al., 2007).

Interestingly, *O.* ×*bergonii* hybrids emit larger quantities of volatiles than parental species. To our knowledge, such a heterosis effect was never observed before. In previous studies, hybrids and parental species have been shown to emit similar quantities of volatile compounds, i.e., in *O. mascula* L., *O. pauciflora* Tenore and their hybrid (Salzmann et al., 2007) or in *Platanthera bifolia* L., *P. chlorantha* Custer, and their hybrid (Nilsson, 1983). Moreover, the relative proportions of volatile compounds emitted by *O.* ×*bergonii* hybrids are greater than those emitted by each of the parents for 10 of 23 compounds, in contrast with other orchids, for which the mean relative proportions for most of the compounds emitted by hybrids are intermediate compared with the parental species (Nilsson, 1983; Salzmann et al., 2007; Stökl et al., 2008). The bouquet of *O.* ×*bergonii* is marked by two particularities, i.e., larger emitted quantities and different relative proportion for several compounds. These two differences offer a potential explanation for the observed absence of effective pollinators in hybrids (Schatz, 2006). Certain volatile compounds reaching higher concentrations in hybrids could even act as repellents for pollinators, a possibility that could be tested in further experiments because the bee-pollinated orchid may repel flies and vice versa. Conversely, benzenoids were significantly lower (about 5-fold) in hybrids, whereas they their levels are intermediate in another orchid hybrid (Salzmann et al., 2007). They are known to be involved in the attraction of pollinators, notably in orchids (Huber et al., 2005; Salzmann et al., 2006). Among benzenoids, methyl salicylate is frequent in plants and often involved in defense responses (Knudsen et al., 2006; Schatz et al., 2009), whereas ethylacetophenone is often present in orchid scent (Knudsen et al., 2006). Such a potential role of benzenoids in pollinator attraction may also constitute an explanation of the observed absence of effective pollinators in hybrids (Schatz, 2006).

Differences in morphology (total height, height of inflorescence, floral color, and morphology, see Materials and Methods) and scent emission allow discrimination of the insects of the three taxa. The respective roles of these two cues should be the subject of future experiments. However, such a distinction certainly explains the differences observed in the suite of effective pollinators (Schatz, 2006). As a result, the fruit set in hybrids is 10 times lower than in the two parental species, over several years (Schatz, 2006; B. Schatz unpublished data). Similarly, the fruit set of another orchid hybrid (*Anacamptis morio* L. × *A. papilionacea* L.) was significantly lower than in its parental species (Moccia et al., 2007). However, in that study the fruit set of the hybrid was almost 30% in some populations, whereas in our case the mean fruit set of *O.* ×*bergonii* never exceeded 8.1% (Schatz, 2006). The low fruit set of *O.* ×*bergonii* was not caused by reduced fertility because hand pollination experiments have shown that this hybrid is potentially as fertile as its parents (Schatz, 2006). Otherwise, estimations of seed viability after controlled intraspecific pollination did not differ among the three taxa and were very similar to the proportion of 72.7% of viable seeds previously observed during a general study on 29 orchid species (Scopece et al., 2007).

The two parental species displayed pollination syndromes that are well differentiated, because effective and confirmed pollinators differ between the two parental species—with the exception of the beetle *Cidnopus pilosus*, which is responsible for the cross-mating that produces hybrids (Schatz, 2006). We conclude that the distinct traits displayed by *O.* ×*bergonii* hybrids, i.e., its general morphology and scent, represent a pollination syndrome inefficient for the attraction of pollinating insects (Schatz, 2006) and especially of the parental pollinators.

Mycorrhizal diversity in hybrids and parents—Beyond the pollinators required for reproduction, a second set of biotic partners, mycorrhizal fungi, is required for germination and survival of orchids. How do hybrids fare in this interaction? Our data indicate two important conclusions. First, all mycorrhizal fungi identified belong to Tulasnellales, a large, common group of orchid mycorrhizal fungi with highly divergent ribosomal DNA sequences (Rasmussen, 1995) that have already been recorded in *O. militaris* L. (Shefferson et al., 2008) and recently in *O. anthropophora* (Lievens et al., 2010). The latter study also found other rarely occurring taxa (Thelephoraceae, Cortinariaceae), perhaps because the authors cloned PCR

products, or as a result of differences in study sites. Similar fungi (and even one identical ITS sequence) occurred in the two parents, perhaps due to the close phylogenetic positions of the two parental species (Aceto et al., 1999). Hybrids had very closely related fungi, suggesting that the mycorrhizal interaction does not constrain hybrid germination and survival.

Tulasnellales taxonomically similar to the symbionts of both parents allow hybrids to germinate and establish mycorrhizae, and these fungi obviously occur at the sites where the parents grow. Similarly, in the genus *Caladenia*, Hollick et al. (2005) showed that fungi of one or both parents can support the germination of hybrid seeds. These authors reported significant genetic divergence among fungi of hybrids and parents, using amplified fragment length polymorphisms (AFLPs). Because the fungal species were not identified, it is unknown whether the variation observed was at the intraspecific or interspecific level; similarly, in our study, most Tulasnellales ITS sequences differed between hybrids and parents (Table 2), but we don't know whether this reflects intraspecific or interspecific differences. The use of different markers in both studies limits further comparison. It would be interesting to check, comparing a larger number of species and identifying the associated fungal species, whether hybrids in orchids are restricted to parental species having similar mycorrhizal fungi, or whether some orchid species pairs associated with unrelated fungal species can produce hybrids that find partners capable of supporting their germination.

A second important result is that infection is higher and more constant over the year in hybrids, as compared with parental species, in which the infection level is lower and increases from spring to early summer. In *Orchis* spp., the rootless, resting stage in summer is followed by growth of roots that undergo inoculation in autumn, mostly relying on the plant's stored reserves. The progressive increase in infection level of individuals of the parental species over the year may be linked to increased availability of photosynthate after leaf expansion. Assuming that the orchid provides carbon to its fungi, as recently demonstrated for *Goodyera repens* L. (Cameron et al., 2006), orchid reserves derived from these photosynthates may allow development of the autumnal inoculum. Compared with the parental species, the hybrid again shows heterosis for fungal colonization. A possible interpretation is that hybrids cannot control the growth of the mycorrhizal fungus: balance of the symbiosis in parental species is a result of a long coevolution that could break down during hybridization (Frenkel et al., 2010), exactly as observed here for pollinators. However, the good development of hybrids (leaf parameters and height, see Materials and Methods and Schatz [2005]) does not support that the fungi behave in a parasitic way or that hybrids lose their adaptations to avoid over-exploitation of carbon by the fungus. Alternatively, the higher level of mycorrhizal infection in hybrids could be explained by greater availability of reserves in spring (discussed later), which may account for the higher infection early in the annual cycle, and/or for greater leaf growth (e.g., greater leaf area), which may also provide a more abundant supply of carbon to mycorrhizal fungi.

The identity of the fungi allowing seed germination in these taxa of *Orchis* remains unknown. Considering in vitro germination experiments conducted with *Orchis* spp. (reviewed in Rasmussen [1995]) and the usual persistence of fungi allowing germination as mycorrhizal associates in adults (McCormick et al., 2004), it can be speculated that Tulasnellales identical to those shown in this study to form mycorrhizal associations with adult plants are involved, but this remain to be rigorously demonstrated.

***Ecological consequences of hybridization in* O. ×bergonii**—The main interest for examining both associations was to build a model of the lifestyle of this hybrid orchid. Here, we offer a tentative model, the most parsimonious one, derived from the correlations observed among data, as a testable hypothesis of interplay between above- and belowground mutualisms for future work on this or other hybrids (Fig. 3). Previous studies showed that mycorrhizal fungi can alter reproductive traits in most higher plant species (Koide and Dickie, 2002): more precisely, a below- to aboveground link was observed, with reduction in mycorrhizal infection resulting in decreased floral attractiveness and pollination (Gange and Smith, 2005; Wolfe et al., 2005; Cahill et al., 2008). Congruently, in *O. ×bergonii*, increased mycorrhizal infection correlated with greater emission of volatile compounds, but, due to unsuccessful pollinator attraction, this did not improve the seed set.

We additionally speculate a second above- to belowground link, where disruption of pollination interactions, by reducing investment in seed production, allows improved mycorrhizal infection in hybrids. *Orchis ×bergonii* displayed significantly higher values for total leaf area and mycorrhizal infection level, and Schatz (2005) reported similar trends for total height, number of leaves, and length of leaf. Interestingly, the three taxa were sorted in the same order (*O. ×bergonii* > *O. simia* > *O. anthropophora*, except for mycorrhizal infection in parental species). We suggest that reducing allocation of resources to fruit production in hybrids may contribute to greater vegetative growth (Fig. 3) and higher accumulation of reserves in tubers, allowing faster vegetative growth in early spring. In particular, higher carbon availability could enhance mycorrhizal infection, at the beginning of the growing season, due to accumulated reserves, as well as later in the season, due to the larger total leaf areas (and so probably higher photosynthesis) and avoidance of fruit cost. Although disruption of coevolution with the fungus may be the reason for increased mycorrhizal infection, the modified carbon budget allows the higher fungal cost in hybrids.

Observed differences among taxa are proportionally similar for the total leaf area and the level of mycorrhizal infection (*O. simia* and *O. anthropophora*, respectively, have 60% and 40% of the total leaf area of *O. ×bergonii* at comparable stages of development, and 57.5% and 33.5% of its level of infection). However, no proportional relationship exists between the total leaf area and the quantity of volatile compounds emitted (values obtained for *O. simia* and *O. anthropophora*, respectively, represent 33.3% and 10% of values obtained for *O. ×bergonii*). Indeed, production of volatile compounds at levels typical of natural populations may not be energetically costly (Borg-Karlson, 1990; Grison-Pigé et al., 2001), perhaps explaining the lack of correlation between quantities of volatile compounds emitted and the leaf area. Thus, in hybrids, reduced fruit costs may allow a larger total leaf area. Both parameters may in turn allow a higher level of mycorrhizal infection; a connection between the higher emission of volatile compounds and the higher carbon supply is possible, but not necessary. Further experiments are required to test this, notably by measuring accumulated reserves and rates of mycorrhizal infection and emission of volatile compounds in hand-pollinated hybrids (pollinated over several years) that would not avoid fruit costs. Moreover, our resulting model (Fig. 3) should be interpreted with great caution, because we did not directly assess (1) photosynthetic activity, (2) carbon flux to roots, or (3) reserves in the tubers at the end of the growing season. The model also should be tested on other hybrids before any generalization can be made.

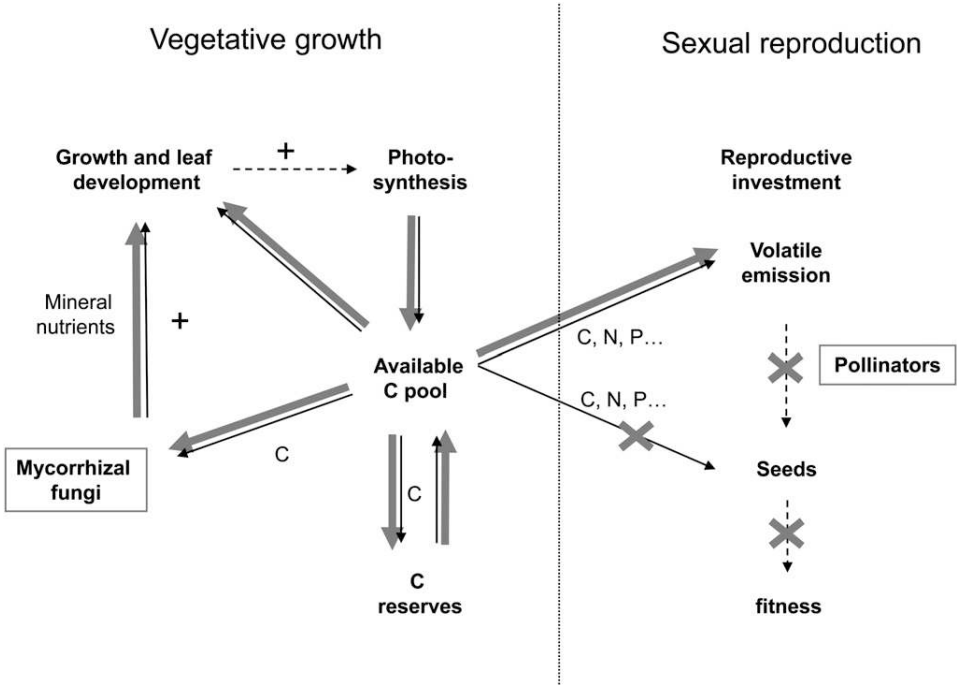

Fig. 3. A model for biological interactions and metabolism in *Orchis ×bergonii*, as compared with the parental species. Black lines: functions expected in mycorrhizal, insect-pollinated *O. anthropophora* and *O. simia*; red crosses: functions disrupted in *O. ×bergonii*; thick gray lines: flux and positive effects expected to be higher in *O. ×bergonii* than in its parents.

Essential conditions for speciation by hybridization (Arnold, 1997) are that the hybrid (1) exploits an ecological niche, either the parental one or a totally new one, and (2) produces a sufficient number of seeds for its ecological maintenance (as much as parental species in the case of sympatry). For *O. ×bergonii*, association with mycorrhizal fungi very similar to the parental ones allows vegetative survival, at least during the first generation. We have no data on the presence of F2 (or later) generations, a point worthy of further analysis using molecular tools (Moccia et al., 2007). However, the mycorrhizal symbiosis imposes no constraints on the fate of hybrids, while the lack of pollinators appears to strongly limit their fitness. The coexistence of *O. ×bergonii* with its parents suggests that this hybrid is a short-term by-product of the hybridizing behavior of a unique and common pollinator (Schatz, 2006).

Conclusion—Food-deceptive strategies in orchids are associated with few pollinator species (Johnson et al., 2004). Thus, selection against hybridization may be important (Dafni, 1987; Johnson et al., 2004; Schatz, 2006), as exemplified by *O. ×bergonii*, which is unattractive to pollinators. In this context, hybrids are a by-product of food deception, as suggested in other cases of hybridization between unrewarding species in *Orchis* (Cozzolino and Widmer, 2005; Cozzolino et al., 2006). Long-term surveys of hybrids and comparative studies in other species (van der Cingel, 1995; Bateman et al., 2003; Schatz, 2006; Moccia et al., 2007) are required to clarify the simultaneous roles of the two obligate orchids' mutualisms in the fate of individual hybrids. In this study, disruption of the pollination interaction probably reallocated resources from reproduction to the mycorrhizal interaction, suggesting that interplay between the two interactions shapes the evolution of hybrids and limits hybridization to vigorous, but unfertile F1 hybrids.

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