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NO EVIDENCE OF REPRODUCTIVE CHARACTER DISPLACEMENT BETWEEN TWO SISTER FUNGAL SPECIES CAUSING ANTHER SMUT DISEASE IN *SILENE*

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Reproductive isolating mechanisms that are stronger for sympatric populations than for allopatric populations of a given species pair are indicative of reproductive character displacement, that is, selection for increased barriers to avoid the costly production of hybrid offspring. Evidence of reproductive character displacement in nature remains equivocal and requires further experimental studies. The genus *Microbotryum* includes species of anther-smut fungi, which castrate pathogens specialized on different plants in the Caryophyllaceae and which serve as excellent models for studying mechanisms of speciation. *Microbotryum lychnidis-dioicae* and *Microbotryum silenes-dioicae* are sister species that show no assortative mating and relatively high hybrid viability and, yet, display a lack of gene flow in natural populations. We wanted to test whether these apparently contradictory results could be explained by reproductive character displacement. We first confirmed the absence of detectable gene flow between the two species in two sympatric populations. Then, using experimental crosses and inoculations of host plants (*Silene latifolia* and *Silene dioica*), we compared intrinsic reproductive barriers between *M. lychnidis-dioicae* and *M. silenes-dioicae* for sympatric versus allopatric populations. We found no evidence for strong reproductive character displacement at any of the following stages: selfing propensity, assortative mating, or hybrid infectivity. Altogether, our results suggest that ecological differences and a tendency for high selfing rates constitute barriers that are strong enough to effectively prevent interspecific gene flow.

Keywords: speciation, prezygotic isolation, postzygotic isolation, specialization, intratetrad mating, kin selection.

Online enhancement: appendix.

Introduction

Geographic separation strongly influences the process of species divergence and the evolution of mating barriers. For example, selection for increased reproductive isolation is expected when species come into secondary contact after having originally diverged in allopatry and when hybrid progeny are costly to produce and suffer from inviability or sterility. Such selection usually acts on premating barriers (Noor 1999; Servedio 2000; Kirkpatrick and Ravigné 2002) and is referred to as "reproductive character displacement" (Brown and Wilson 1956; Grant 1972). Selection to avoid the production of hybrid offspring can occur between already well-established species or before speciation is complete; in the latter case, it is referred to as "reinforcement" (Blair 1955). Investigations of reproductive character displacement between sympatric and allopatric populations of a sister species pair are therefore highly informative about their history of divergence, potentially revealing secondary contact after allopatric speciation and selection for higher reproductive isolation.

Stronger isolating mechanisms between sympatric than allopatric populations have in fact been reported in many natural systems, including insects, mammals, amphibians, fishes, birds, a few plants, and fungi (Levin 1970; Jiggins et al. 2001; Dettman et al. 2003; Coyne and Orr 2004; Lukhtanov et al. 2005; Smadja and Ganem 2005; Urbanelli and Porretta 2008), but other studies have failed to detect such a pattern in organisms covering an equally wide range of taxa (Coyne et al. 2002; Coyne and Orr 2004, pp. 361–362; Moyle et al. 2004; Geyer and Lessios 2009; Widmer et al. 2009). Servedio and Noor (2003) suggested that inconsistent evidence of reproductive character displacement could be due to differences between taxa in the strength of selection, levels of gene flow, or evolutionary constraints on the nature of the isolating mechanism. Indeed, selection for increased reproductive isolation is weak when hybrid fitness is high or the costs of finding a mate or producing offspring are low, selection may be too ineffective if gene flow is high, and mate recognition mechanisms may have few mutational options that would enhance mating efficiency (Servedio and Noor 2003). Alternatively, in-

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consistent observation of reproductive character displacement may result from the failure to identify the trait targeted by selection. While premating reproductive isolation is thought to be primarily affected, postmating stages can also be targeted by a selection for higher isolation under conditions of competition among kin or extended parental care (Levin 1970; Coyne and Orr 2004, pp. 125–178, 360). In fact, a recent study has shown the existence in a fungus of a pattern of reproductive character displacement at a postmating stage that was linked to parental care (Turner et al. 2010). Additional studies have emphasized that mechanisms preventing gene flow may occur at multiple steps in the life cycle (Ramsey et al. 2003; Britton-Davidian et al. 2005; Lai et al. 2005; de Vienne et al. 2009*b*), making the examination of all relevant barriers important for assessing the occurrence of reproductive character displacement in order to avoid missing the trait targeted by this phenomenon.

Fungi are important models for the evolution of reproductive isolation (Burnett 2003; Kohn 2005; Giraud et al. 2008*a*), but they are still poorly represented in studies on speciation. Here we assessed whether a pattern indicative of reproductive character displacement exists between species of the basidiomycete fungus *Microbotryum*, which cause anther-smut disease on different host plants in the Caryophyllaceae (Lutz et al. 2005; Kemler et al. 2006; Le Gac et al. 2007*a*, 2007*b*; de Vienne et al. 2009*a*). This system is well suited for addressing such questions, in particular, when using the species *Microbotryum lychnidis-dioicae* and *Microbotryum silenes-dioicae* parasitizing *Silene latifolia* and *Silene dioica*, respectively. For these closely related species, (1) they are found both in sympatric and allopatric populations (Van Putten et al. 2005; Gladieux et al., forthcoming) and (2) the existence of hybrids has been suggested (Van Putten et al. 2005; Gladieux et al., forthcoming), but (3) the species do not show genome-wide introgressions (Le Gac et al. 2007*a*; Devier et al. 2010; Gladieux et al., forthcoming). Finally, (4) the system is highly amenable to laboratory experiments of in vitro crosses to examine the strength of reproductive barriers at several life-cycle stages (Van Putten et al. 2003; Le Gac et al. 2007*b*; de Vienne et al. 2009*b*), and (5) experimental hybrids show slightly reduced fitness (Le Gac et al. 2007*b*; de Vienne et al. 2009*b*).

Previous studies have investigated the barriers to gene flow between *Microbotryum* species. Partial premating isolation results from ecological differences between the host plants (habitat and pollinator preferences) and from the pathogen's selfing propensity (Giraud et al. 2005, 2008*a*). No assortative mating on the basis of gamete recognition has been detected so far (Van Putten et al. 2005; Le Gac et al. 2007*b*); however, no strains collected at contact sites between different *Microbotryum* species have been tested. Altogether, barriers to hybridization seem to be insufficient to create complete isolation between species pairs, especially between the most closely related species *M. lychnidis-dioicae* and *M. silenes-dioicae* (van Putten et al. 2007; de Vienne et al. 2009*b*). Possible explanations for the lack of introgression between these two species despite weak barriers to gene flow include selection for stronger barriers in sympatric populations (i.e., reproductive character displacement), which we wanted to test here.

We therefore compared reproductive isolation between sympatric and allopatric populations of *M. lychnidis-dioicae* and *M. silenes-dioicae* at several stages of the life cycle and under different mate-choice conditions. We investigated (1) whether hybrids were present in two sympatric populations not previously studied, (2) whether developmental patterns that favor intraspecific mating in the form of selfing differed between sympatric and allopatric populations, (3) whether rates of successful interspecific mating were lower for sympatric populations under conditions of forced hybrid crosses, (4) whether forced hybrid crosses were less likely to result in successful infections when performed between sympatric rather than allopatric strains, and (5) whether hybrids were less likely to be successful at infecting plants under mate-choice conditions when using sympatric strains compared with allopatric strains.

Material and Methods

Biological Model

The life cycle of anther-smut fungi in the genus *Microbotryum* is illustrated in figure A1 in the online edition of the *International Journal of Plant Sciences* (see also Giraud et al. 2008*b*). Diploid teliospores of the pathogen are produced in the anthers of infected plants, replacing the pollen. Teliospores germinate on healthy hosts to produce a septate basidium (i.e., the promycelium), in which meiosis occurs, leading to the production of haploid, yeastlike sporidia of opposite mating types (A1 and A2). These sporidia and their mitotic descendants act as the gametes of the fungus and undergo conjugation to produce the infectious stage. The infectious stage can also result from conjugation between postmeiotic cells of the septate basidium, a developmental process that often precludes the production of sporidia. Such intrapromycelial mating strongly favors selfing over outcrossed mating (Hood and Antonovics 2000). Because each diploid genotype is heterozygous at the mating-type locus, mating among the products of the same meiosis (i.e., automixis) and among the products of separate meioses of the same diploid genotype (i.e., diploid selfing) are frequent (Hood and Antonovics 2000; Granberg et al. 2008).

Microbotryum lychnidis-dioicae and *Microbotryum silenes-dioicae* are sibling species that have recently been given different Latin names (Denchev et al. 2009); they were previously referred to as MvSl and MvSd, according to the hosts they infect, *Silene latifolia* and *Silene dioica*, respectively (Le Gac et al. 2007*a*). Their geographic distributions mirror those of their hosts: both plant species are abundant in Europe but have specific ecological preferences—*S. latifolia* withstanding drier conditions and *S. dioica* occupying more mesic sites—and the two hosts persist in classic metapopulation structures, with frequent local extinction and recolonization (Karrenberg and Favre 2008). The hosts overlap broadly in central Europe, but *S. latifolia* does not grow in northern Scandinavia and *S. dioica* is largely absent from the Iberian Peninsula. *Silene latifolia* has been introduced to North America together with its pathogen, whereas the disease has not been reported in introduced populations of *S. dioica*. In central Europe, it is not uncommon to find the two host species growing in sympatry, that is, intermingled in the same field (Minder and Widmer 2008; Rahme et al. 2009), leading to occasional sympatry of their two pathogen species.

Microbotryum *Strains*

In order to contrast sympatry versus allopatry without ambiguity caused by local metapopulation dynamics, a very strict definition of sympatry was adopted: we considered only sites where individuals of both *M. lychnidis-dioicae* and *M. silenes-dioicae* were found less than 50 m away (see "Results"): Ecault, France (50°39′39″N, 1°34′51″E), and Priddy, Somerset, United Kingdom (51°15′06″N, 2°39′10″W). In the corresponding plant populations, the total disease prevalence was high (more than 50%) and systemically infected plants were common (many diseased branches), suggesting that the two species have coexisted for at least several years. For allopatric strains, we chose those that were collected in regions where, to our knowledge, no individual of the other species has been found, that is, strains located at least 100 km from any individual from the other species (see map in fig. A2 in the online edition of the *International Journal of Plant Sciences*). In the restricted experimental scheme targeting hybrid infectivity, only the most distant strains were retained as allopatric (table 1; see also fig. A2).

Genotyping of Fungal Strains

In order to confirm the *Microbotryum* species identity in relationship to the host from which they were isolated and to thus detect potential cross-species disease transmissions (Antonovics et al. 2002; Refrégier et al. 2008) or to identify

Table 1

***Microbotryum* Strains Used in This Study**

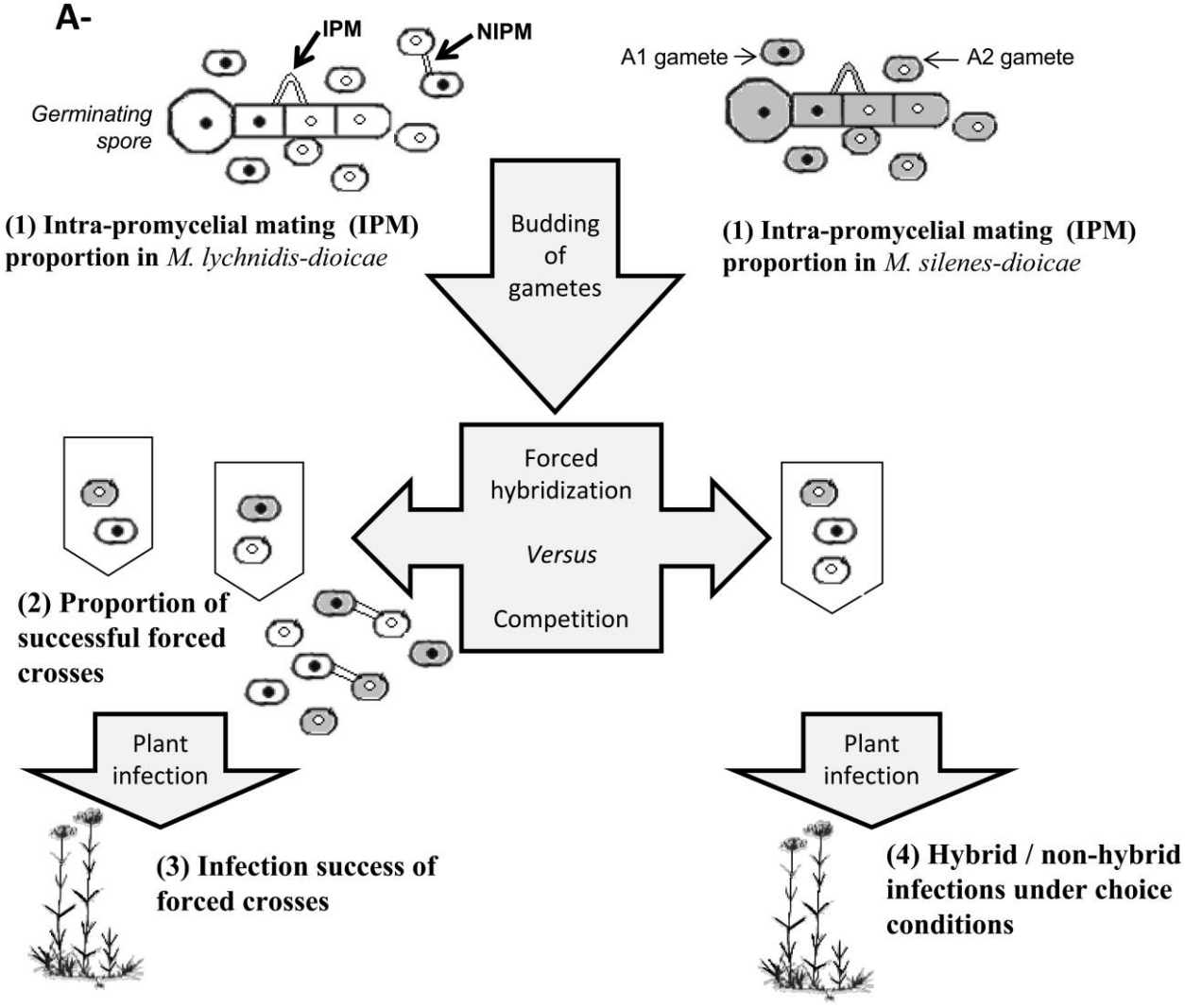
Species, internal code	Collecting site	GPS position	Sympatry with the other species
M. lychnidis-dioicae:			
502.1.1	Centre National de la Recherche Scientifique (CNRS) Gif, Ile-de-France, France	48°42′16″N, 2°7′60″E	No
502.2	CNRS Gif, Ile-de-France, France	48°42′16″N, 2°7′60″E	No
505.1[a,b]	Marais des Bris, Ile d'Oléron, France	45°49′29″N, 1°12′27″W	No
505.2[a]	Marais des Bris, Ile d'Oléron, France	45°49′29″N, 1°12′29″W	No
605.1	Halle, Germany	51°26′07″N, 11°57′27″E	No
605.2	Halle, Germany	51°26′07″N, 11°57′27″E	No
534.1[a]	Montluçon, Centre, France	46°17′58″N, 2°39′11″E	No
506.1[a]	Mendel Garden, Brno, Czech Republic	49°11′28″N, 16°35′40″E	No
583[b,c]	Blue Ridge Park, Virginia, U.S.A	42°11.95′N, 2°11.10′W	No
585[b,c]	Spain	42°11′58″N, 2°11′06″W	No
515.1.1[a]	Ecault, Nord, France	50°39′48″N, 1°34′48″E	Yes
515.2.1	Ecault, Nord, France	50°39′48″N, 1°34′48″E	Yes
515.3[a,b]	Ecault, Nord, France	50°39′48″N, 1°34′48″E	Yes
515.4[a]	Ecault, Nord, France	50°39′48″N, 1°34′48″E	Yes
517.1.1[a]	Ecault, Nord, France	50°39′40″N, 1°35′4″E	Yes
517.1.2	Ecault, Nord, France	50°39′40″N, 1°35′4″E	Yes
460.6[a]	Priddy, Somerset, United Kingdom	51°15′06″N, 2°39′10″W	Yes
460.1[a,b]	Priddy, Somerset, United Kingdom	51°15′06″N, 2°39′10″W	Yes
M. silenes-dioicae:			
526.1[a,b] (A2)	Plougasnou, Finistère Nord, France	48°40′4″N, 3°50′47″W	No
526.2	Plougasnou, Finistère Nord, France	48°40′4″N, 3°50′47″W	No
532.1	Roscoff, Finistère Nord, France	48°43′36″N, 3°59′10″W	No
532.2	Roscoff, Finistère Nord, France	48°43′36″N, 3°59′10″W	No
532.3	Roscoff, Finistère Nord, France	48°43′36″N, 3°59′10″W	No
573	Le Saulcy, Vosges, France	48°24′43″N, 7°2′15″E	No
418.1[a,b] (A2)	St Anthème, Auvergne, France	45°33′38″N, 3°52′12″E	No
336[a] (A1)	Taulé, Finistère Nord, France	48°35′29″N, 3°53′05″W	No
602.1[a,b] (A2)	Alesjaure, Laponia, Sweden	68°8′08″N, 18°26′13″E	No
460.8[a] (A2)	Priddy, Somerset, United Kingdom	51°15′06″N, 2°39′10″W	Yes
460.10[a] (A2)	Priddy, Somerset, United Kingdom	51°15′06″N, 2°39′10″W	Yes
514a5[a,b] (A1)	Ecault, Nord, France	50°39′50″N, 1°34′42″E	Yes
514a1	Ecault, Nord, France	50°39′50″N, 1°34′42″E	Yes
514a2	Ecault, Nord, France	50°39′50″N, 1°34′42″E	Yes
514c5	Ecault, Nord, France	50°39′50″N, 1°34′42″E	Yes
516.1[a,b] (A2)	Ecault, Nord, France	50°39′42″N, 1°34′55″E	Yes

Note. All strains except those indicated were involved in experiments on intratetrad mating propensity. Sympatry was considered to occur when the nearest individual belonging to the alternative species was growing less than 50 m away. Sympatry was considered to be absent when the nearest individual belonging to the alternative species was located at least 100 km away, as experienced from our collecting campaigns.

[a] Strains tested for heterospecific conjugation ability.

[b] Strains also tested for their infectivity when hybridizing with a heterospecific strain.

[c] Strains not involved in experiments on intratetrad mating propensity.



B-

M. lychnidis-dioicae. / *M. silenes-dioicae*		**Sympatry**	**Allopatry**		
		Ecault, North France	Spain	US	Aquitaine, South France
Sympatry	Ecault, North France	X **Sympatric pair**			
Allopatry	North Scandinavia		X **Allopatric pair**	X **Allopatric pair**	X **Allopatric pair**

Fig. 1 Experimental scheme of the different steps of hybridization. *A*, Summary of all experiments designed to investigate reproductive character displacement: experiments targeting premating barriers (*1* and *2*) or taking into account all pre- and postmating barriers (*3* and *4*). All experiments compared combinations of strains coming from sympatric versus allopatric populations. *Microbotryum lychnidis-dioicae* cells are shown in white, while *Microbotryum silenes-dioicae* cells are in gray. The different mating types are represented by open (*A2*) or closed (*A1*) circles. (1) The first experiment compares the proportion of intrapromycelial mating (*IPM*) to the overall number of conjugations when germinating teliospores in water (IPM + non-intrapromycelial mating). Intrapromycelial mating favors selfing and discourages outcrossing, including interspecific hybridization. (2) The second experiment compares the proportion of gametes involved in conjugation (= mating) with the

hybrids, genotyping was performed on teliospores harvested from sympatric populations. DNA extraction from teliospores was performed using the Chelex protocol (Bucheli et al. 2001). Genotypes were analyzed as described in Giraud (2004) using microsatellite markers that have been shown to discriminate by species. Eight markers were used for the Ecault populations: GR15, GR21 (Giraud et al. 2002), SL9, SL16, SVG5, SVG14, SVG15, and SN5 (Giraud et al. 2008*c*); six markers were used for the Priddy populations: SL5, SL9, GR11, GR15, SVG5, and SVG14. Primers for the SL5 microsatellite marker were developed as in Giraud et al. (2008*c*): SL5-F 5′CATCCAAGTCAACCTTCGTG; SL5-R: 5′TGAAGCAAGAAAGCCAGAGAG (GenBank accession number HM002777).

Selfing Rates in the Form of Intrapromycelial Mating

Intrapromycelial mating rates were compared for strains from sympatric versus allopatric populations (all listed in table 1 except those indicated) to assess whether there has been selection for higher rates of selfing (fig. 1*A1*). Intrapromycelial mating often occurs before the budding of haploid, yeastlike cells from the basidium, which ensures intratetrad mating and discourages the mixing of gametes from different individuals (Hood and Antonovics 2000; Giraud et al. 2008*b*). This can easily be quantified under a microscope as conjugation bridges between cells of the promycelium instead of between the ovoid-shaped sporidial cells (Granberg et al. 2008), as is schematically presented in figure 1*A*. Intrapromycelial mating propensity was measured in vitro as the number of intrapromycelial matings divided by the total number of mating events among 100–500 conjugations (intrapromycelia + non-intrapromycelia]. Teliospores from one infected anther were allowed to germinate in 100 μL of sterile tap water for 6 d at 10°C. Two or three independent replicate measures were performed, and the resulting proportions were highly reproducible (Pearson correlation coefficient; $r_{20} = 0.93$; $P < 0.001$).

Interspecific Conjugation Rates

In vitro conjugation rates in sympatric versus allopatric interspecific crosses were measured for strains indicated by footnote c in table 1 (a total of 10 sympatric crosses and nine allopatric crosses) to assess whether there had been selection for assortative mating in sympatry (fig. 1*A2*). Note that strains used in this experiment were randomly chosen from all strains included in the first study, but we ensured that all major regions of collection were represented. Gamete isolation and crosses were performed according to Le Gac et al. (2007*b*). Briefly, for gamete isolation, diploid teliospores were allowed to germinate at 23°C on GMB2-agar-rich medium for 5 d. Diluted sporidial suspensions were spread on GMB2-agar-rich medium and incubated at 23°C for 5 d to obtain colonies derived from single haploid sporidia. Mating types (A1 or A2) were determined for each sporidial isolate (gamete) according to their mating behavior with stock cultures of known mating types. In this set, all *M. silenes-dioicae* strains were found to exhibit a biased mating-type ratio: only one mating type could be recovered (table 1). Biased mating-type ratio is common for *Microbotryum* species, although it is not fixed in *M. silenes-dioicae* (Hood and Antonovics 2000; Thomas et al. 2003). Mating-type bias does not impede conjugation, only the proliferation of sporidia beyond a few mitotic divisions (Oudemans et al. 1998). Conjugations typically occur rapidly on the surface of the plant, without substantial haploid replication, so that the presence of haplolethal alleles should not affect the probability of selection for assortative mating. Gamete concentration was adjusted to $\sim 1.5 \times 10^9$ cells per L tap water after cell counting with a hemocytometer. Thirty μL of gamete suspension from each species was mixed and incubated in microplates at 10°C for 10 d, as in Le Gac et al. (2007*b*). Conjugation rates were measured as the number of conjugating gametes divided by the total number of gametes out of at least 300 cells observed under a microscope. No conjugations were observed in control treatments consisting of gametes all carrying the same mating type.

Infection Success of Hybrid Crosses

Infection successes of hybrids originating from sympatric versus allopatric crosses were compared in order to assess whether there had been selection for early hybrid abortion in sympatry (fig. 1*A3*). This experiment was performed on a restricted scheme, including only two reciprocal sympatric crosses from the Ecault populations and three allopatric crosses (fig. 1*B*) because of space limitations. Seeds were sterilized by 25 min of incubation in a basic solution (1.2% $Ca(ClO)_2$; 0.4% NaOH) followed by three rinses in sterile tap water. Approximately 80 seeds were plated on a petri dish with 0.8% agar. They were stored for 2 d at 4°C to ensure synchronization of germination. They were then put in a growth chamber at 23°C for 1 d before we proceeded to inoculation.

Gamete suspensions were prepared for *M. silenes-dioicae* and *M. lychnidis-dioicae* at cell concentrations of 1.5×10^9 cells per L tap water, as described above. A total of 400 μL (i.e., 6×10^6 cells) of A1 gamete from one species and 400 μL of A2 gamete from the other species were mixed and spread onto petri dishes after 1 d of incubation with the germinating seeds. These steps were performed at room temperature (23°C). To check for uncontrolled infections during the processes of inoculation and growing plants to flower, some plants were grown without inoculation.

total number of gametes when mixing gametes of opposite mating types coming from either the same species (intraspecific crosses) or two different species (interspecific crosses). Low interspecific conjugation success confers isolation between the two species. (3) The third experiment compares the infection success of sympatric versus allopatric interspecific hybrids. Low infection success results from either premating or postmating barriers, and it indicates isolation between the two species. (4) The fourth experiment compares the proportion of hybrid and nonhybrid genotypes after infection with a mixture of gametes from the two species (mate-choice conditions). Low hybrid proportion indicates isolation due to lower efficiency of hybrids at either the premating or the postmating stage. *B*, Restricted experimental scheme used for infection tests (experiments 3 and 4 as defined in *A*).

Plants were allowed to grow for two more weeks in petri dishes at 20°C, and 30 plants per cross and for each plant species were then transferred to 0.25-L plastic pots in the greenhouse. Inoculation treatments were randomized with regard to their position in the greenhouse, and plants were kept until flowering. Approximately 50% of the plants flowered within 6 mo, after which time all plants were discarded. Rates of infection were ~45% on *S. latifolia* and 15% on *S. dioica*. On flowering, the date and infection status for each plant were recorded and the plant was discarded to avoid secondary disease transmission. Flower buds showing symptoms of infection (i.e., teliospores) were collected and genotyped as described above, using one to three microsatellite markers: GR15 (Giraud et al. 2002), SVG14, and SN5 (Giraud et al. 2008*c*). None of the control plants were found to be diseased, and all diseased plants were infected by interspecific hybrids.

Infection Success of the Different Genotypes under Mate-Choice Conditions

Parallel inoculations were performed with gamete mixtures from the two *Microbotryum* species such that both nonhybrids and hybrids could be formed, in order to assess whether there had been selection for increased reproductive isolation between species in sympatry under conditions of mate choice (intraspecific vs. interspecific matings and/or competition during infection; fig. 1*A4*). Again, inoculation treatments were randomized with regard to their position in the greenhouse, and plants were kept until flowering. Rates of infection were also ~45% on *S. latifolia* and 15% on *S. dioica*. The success of intraspecific versus interspecific mating was determined by genotyping the strains that managed to infect plants. Gamete suspensions were prepared as described above. Because of mating-type bias in the single allopatric *M. silenes-dioicae* strain, a "first trial" was performed with only A2 gametes from *M. silenes-dioicae* mixed with A1 and A2 gametes of *M. lychnidis-dioicae*, both for crosses with allopatric populations and for those with sympatric populations from Ecault (the Priddy populations were discarded because of contamination of *M. silenes-dioicae* gametes). Two types of genotypes could therefore be formed with equal probability and possibly compete: pure *M. lychnidis-dioicae* nonhybrids and hybrids between *M. lychnidis-dioicae* and *M. silenes-dioicae*. Mixtures of each gamete were prepared and 400 μL (6×10^6 cells) of each type of gamete (A1 and A2)—that is, 200 μL of each species for A2 competing gametes—were mixed as above to infect the plants. A total of 60 plants per cross and for each plant species were grown to flower in pots, and data were collected as described above. A "second trial" was simultaneously performed for the sympatric Ecault populations by mixing all gametes (again, with 400 μL for each type of gamete and by providing equal probabilities of encounters between hybrid and nonhybrid gamete combinations) and inoculating plants that were randomized with regard to their position in the greenhouse.

Data Analyses

Statistical analyses were performed in R (http://www.r-project.org/). Details about each test can be found in the text and in the legends and notes of the figures and tables. To test for homogeneity among the means of conjugation rates, we used a Student's *t*-test after normalization of the data by square-root transformation. Normality was confirmed using Shapiro-Wilk's test in R. Normalized allelic diversities were computed using FSTAT (http://www2.unil.ch/popgen/softwares/fstat.htm).

To ensure that we did not experience a too-high Type II statistical error (β) by not rejecting the null hypothesis even though the alternative hypothesis was true (i.e., considering that isolation was not significantly higher in sympatry than in allopatry, when in fact it is), we computed power analyses in G*Power (http://www.psycho.uni-duesseldorf.de/aap/projects/gpower/). We calculated the power $1 - \beta$, that is, the probability of having detected a difference of a given magnitude if one existed. Input values include the P value of our test as α, the effect size (reported as d or w) as the normalized difference between the two samples, and the sample size. We followed the recommendations of software and Thomas (1997) regarding the values of intermediate and large effect sizes to account for the genetic isolation. Namely, for χ^2 tests, the effect size w is medium when $w = 0.3$ and large when $w = 0.5$. For the Wilcoxon W test, the index effect size d is considered to be medium when $d = 0.5$ and large when $d = 0.8$. Note that if α is close to 0.05, a high power indicates that the null hypothesis should be accepted. A power was considered to be high when $1 - \beta > 0.8$, as is usually recommended (Thomas 1997).

Results

Strong Isolation between Microbotryum silenes-dioicae *and* Microbotryum lychnidis-dioicae *in Natural Sympatric Populations*

We identified two sites where disease was present on *Silene latifolia* and *Silene dioica* plants (white and red campions, respectively) and/or on potential hybrid plants growing in complete sympatry: one in northern France, called Ecault, and the other in the southern United Kingdom, called Priddy. Plants with an intermediate flower color (light pink) have long been considered to be hybrids, but they may instead belong to one of the two species (Karrenberg and Favre 2008). At both sites, a high proportion (more than 50%) of all plants showed symptoms of anther-smut disease, with no apparent difference according to flower color. For the Ecault populations, 20 *Microbotryum* strains were genotyped using eight microsatellite markers. Principal component analysis revealed two groups of *Microbotryum* genotypes separated along the PC1 axis that explained most of the variance (75.8%; fig. 2*A*). The first group encompassed strains collected on *S. dioica* and plants of intermediate flower color, and the second group contained strains collected on *S. latifolia*. The first group, corresponding to *M. silenes-dioicae*, presented a significantly higher allelic diversity ($\text{mean}_{[\text{normalized}]} = 3.160$ alleles per loci) than the second group, corresponding to *M. lychnidis-dioicae* ($\text{mean}_{[\text{normalized}]} = 1.625$ alleles per loci; Wilcoxon one-sided rank sum test $W = 56$, $P = 0.006$). No strain appeared to be intermediate between the two groups, suggesting that no hybrids were present at the Ecault site. These results support earlier studies indicating that the isolation between the

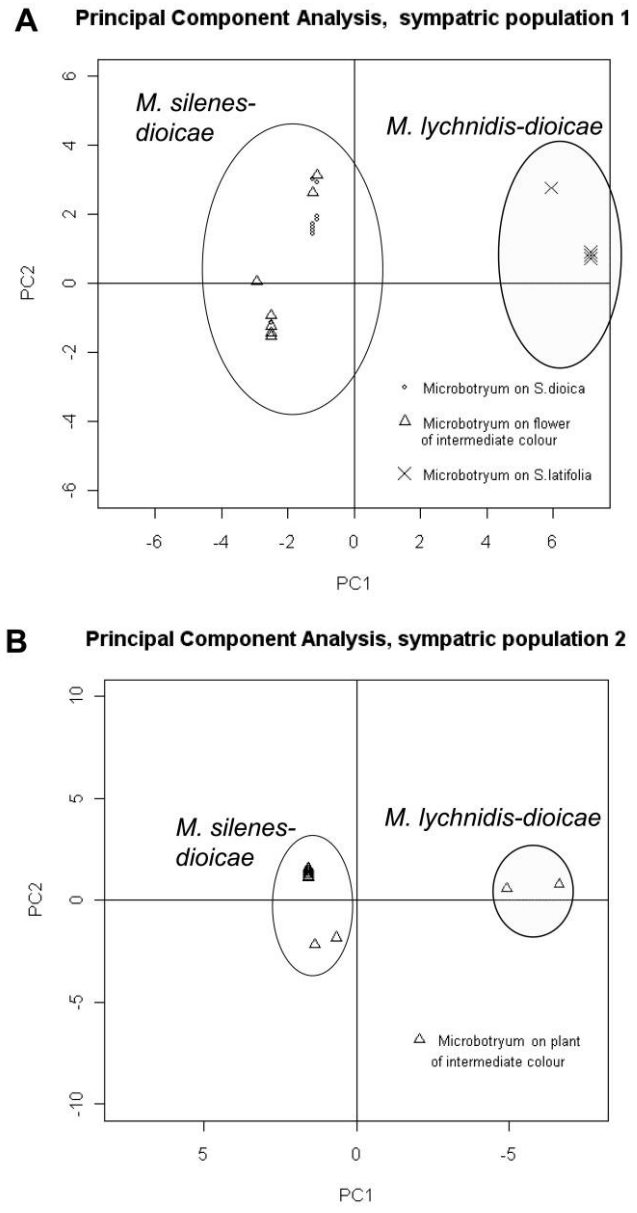


Fig. 2 Genotypic clustering of individuals collected at sympatric sites. *A*, Projection of PC1 (75.8% of the variance) and PC2 (15.2% of the variance) after principal component analysis of 20 *Microbotryum* strains found in Ecault (France) and genotyped using eight microsatellite markers. The plant on which they were found is indicated by symbols (crosses [×] for strains collected on *Silene latifolia*, circles for *Silene dioica*, and triangles for *Silene* plants with intermediate flower colors). The two identified clusters correspond respectively to *Microbotryum lychnidis-dioicae* and *Microbotryum silenes-dioicae*. *B*, Projection of PC1 (78.4% of the variance) and PC2 (12.1% of the variance) after principal component analysis of 10 *Microbotryum* strains found in Priddy (United Kingdom) and genotyped using six microsatellite markers.

two fungal species is strong (Le Gac et al. 2007*a*; Refrégier et al. 2008). At the Priddy site, 10 individuals collected on plants of intermediate flower color were genotyped using six microsatellite markers. Two groups without evidence of hybrids were also well distinguished along PC1, accounting for 78% of variance, although the group corresponding to *M. lychnidis-dioicae* contained only two individuals (fig. 2*B*).

High Variability in Selfing Rates with No Pattern of Reproductive Character Displacement

We investigated whether the propensity for selfing, in the form of intrapromycelial mating (figs. 1*A1*, 3*A*), was higher for *Microbotryum* strains from sympatric than allopatric populations. Mean intrapromycelial mating rates were close to 0.5 (50%) for all strains (fig. 3*B*). Sympatry was found to have no effect on intratetrad mating propensity (ANOVA; table 2). Nor could a sympatry effect be detected in both species taken separately (for *M. lychnidis-dioicae* strains in sympatry with the other species, mean intratetrad mating propensity = 48% vs. 52% for strains in allopatry, Wilcoxon signed-rank test $W_{n_A=8,\, n_S=8} = 29$, $P = 0.64$; for *M. silenes-dioicae*, 49% in sympatry vs. 43% in allopatry, Wilcoxon signed-rank test $W_{n_A=8,\, n_S=7} = 34$, $P = 0.27$). The power achieved for detecting an intermediate difference between the two groups was good ($\text{power}_{d=0.5} = 0.91$ and 0.63, respectively), and it was high for detecting large differences ($\text{power}_{d=0.8}$0.97 and 0.81). Altogether, we can conclude that sympatric strains did not exhibit a strong increase in selfing propensity as compared with allopatric strains. Therefore, no strong reproductive character displacement on selfing rates has occurred that could significantly contribute to genetic isolation between the two sister species.

Interspecific Conjugations as Frequent as Intraspecific Conjugations in Both Sympatric and Allopatric Populations

The degree of assortative mating was measured in vitro under forced hybridization conditions (i.e., no competition between hetero- and conspecifics). Interspecific conjugation rates were not significantly different between sympatric and allopatric crosses (fig. 4; Wilcoxon $W_{n_A=9,\, n_S=10} = 41$, $P = 0.64$, $\text{power}_{d=0.5} = 0.92$; and see ANOVA, table 3). The population from which the strain was collected significantly affected its conjugation efficiency (table 3). Strain identity from the *M. lychnidis-dioicae* parent also significantly affected the conjugation efficiency but not the identity of the *M. silenes-dioicae* parent. Altogether, these data suggest that no strong reproductive character displacement occurred on assortative mating that could explain the lack of gene flow.

Successful Infections by Hybrids from Both Sympatric and Allopatric Crosses under Forced Hybridization

To investigate potential barriers at a postmating stage, we performed in vitro infections. Because the major effect observed in the conjugation experiment for both species was the population origin of strains, we set up a restricted experimental plan in which a single strain per population was included that had selfing propensity and conjugation ability that were close to the means of all strains analyzed from the same population. We also limited the number of populations for practical reasons but kept a high number of plants that were analyzed

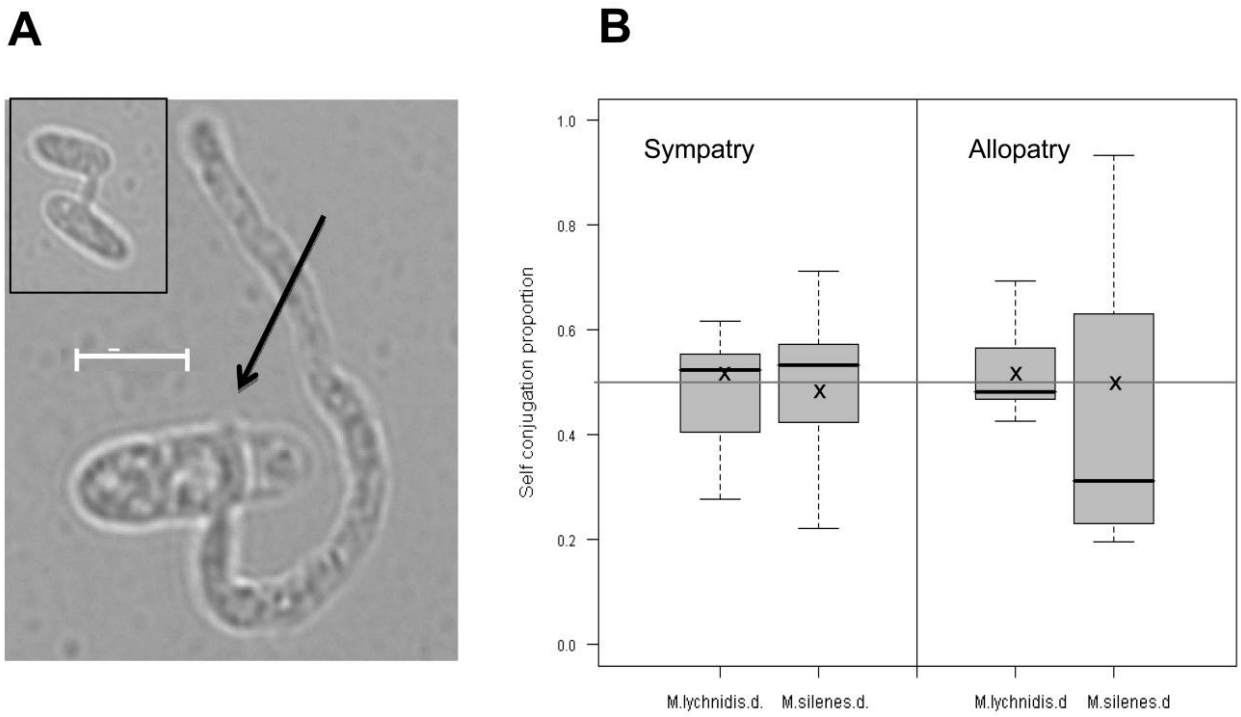


Fig. 3 Selfing in the form of intrapromycelial mating. *A*, Intrapromycelial mating. The arrow indicates the conjugation tube; successful conjugation is confirmed by the growth of an infectious hyphae. Scale = 10 μm. *B*, Box plot of the selfing rate, in the form of intrapromycelial mating proportion, of *Microbotryum lychnidis-dioicae* and *Microbotryum silenes-dioicae*, respectively, and for strains that were collected in either sympatry or allopatry with the other species. Medians are highlighted by a black line. Means are indicated by a cross (×). Boxes extend from 0.25 to 0.75 quartiles, and whiskers extend to the most extreme values. Conjugations other than intrapromycelial ones occur between round-shaped gametes (sporidia).

for each hybrid cross (30 in each species). Using forced interspecific crosses, the proportion of successful infections was similar between sympatric and allopatric crosses, both on *S. latifolia* and *S. dioica*, despite high power to detect a difference between the two groups (fig. 5; table 4; pooled test powers: $\text{power}_{w=0.3;\ N=150} = 0.98$, $\text{power}_{w=0.3;\ N=150} = 0.997$). The mean infection efficiency was even higher for sympatric than for allopatric crosses infecting *S. latifolia* (45% vs. 30%). Overall, hybrids from sympatric crosses performed no worse than hybrids from allopatric crosses, in opposition to expectations under reproductive character displacement.

No Decrease in Hybrid Genotype Proportion in Sympatric Crosses when Allowing for Competition

In order to maximize the probability of identifying reproductive character displacement targeting either a premating or a postmating stage, we set up an experiment that included all steps of the life cycle and allowed the choice between mating with a conspecific or a heterospecific gamete. This experiment included two different trials because of the mating-type bias found in the chosen allopatric *M. silenes-dioicae* population, that is, the inviability of haploid cells carrying one mating type due to linkage of the mating-type locus with deleterious alleles (Hood and Antonovics 2000). The first trial of crosses included sympatric and allopatric strains, such that *M. lychnidis-dioicae* A1 gametes had a choice between intraspecific and interspecific A2 gametes. The second trial, which included both mating types from both species, could be performed only for sympatric strains from Ecault (having no mating-type bias), but the proportion of resulting infections that were hybrid genotypes can be compared with the results of first set of the experiment, including allopatric crosses.

There was no significant effect of sympatry versus allopatry on the proportion of infections caused by hybrid genotypes in the first trial when letting *M. lychnidis-dioicae* A1 gametes have the choice between hybrid and nonhybrid matings on both *S. latifolia* and *S. dioica* (on *S. latifolia*: $\chi^2_1 = 2.05$, $P = 0.15$, $N = 89$, $\text{power}_{w=0.3} = 0.92$; on *S. dioica*: $\chi^2_1 = 0.14$, $P = 0.70$, $N = 28$, $\text{power}_{w=0.3} = 0.91$; pooled data: $\chi^2_1 = 1.6$, $P = 0.20$, $N = 117$, $\text{power}_{w=0.3} = 0.97$; fig. A3 in the online edition of the *International Journal of Plant Sci-*

Table 2

ANOVA on Selfing Propensity

Factor	df	Sum of squares	Mean of squares	*F*	*P*
Species	1	.0114	.0114	.430	.52
Sympatry	2	.0184	.0092	.348	.71
Population	4	.1838	.0459	1.737	.18
Residuals	23	.6083	.0264		

Note. Sympatry effect was nested into species effect, and population effect was nested in both species and sympatry effects.

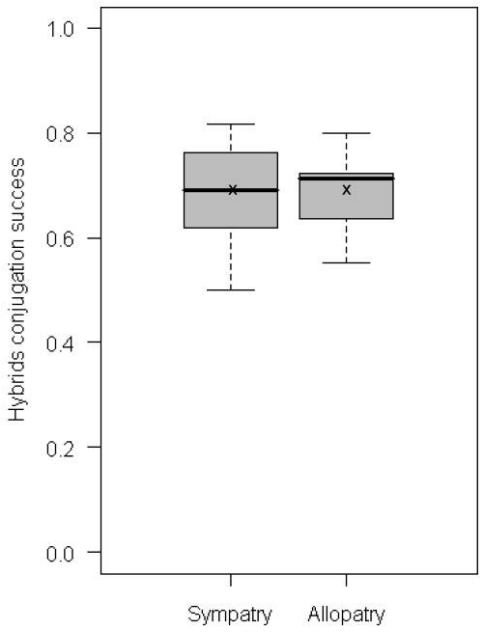


Fig. 4 In vitro conjugation success for interspecific crosses. Box plots of the conjugation success of interspecific crosses between *Microbotryum lychnidis-dioicae* and *Microbotryum silenes-dioicae* gametes of opposite mating types. Medians are highlighted by a black line. Means are indicated by a cross (×). Boxes extend from 0.25 to 0.75 quartiles, and whiskers extend to the most extreme values.

ences, cross "1"). When including all gametes from both species in the second trial (A1 and A2 for both species in Ecault strains), the proportion of hybrid genotypes was not significantly different from that of allopatric populations carrying mating-type bias (on *S. latifolia*: $\chi^2_1 = 1.43$, $P = 0.23$, $N = 105$, power$_{w=0.3} = 0.97$; on *S. dioica*: $\chi^2_1 = 0.02$, $P = 0.89$, $N = 24$, power$_{w=0.3} = 0.96$; pooled data: $\chi^2_1 = 1.71$, $P = 0.19$, $N = 129$, power$_{w=0.3} = 0.98$; fig. A3, cross "2"). Of note, this trial resulted in no pure *M. silenes-dioicae* genotypes on *S. dioica*, although they could have formed, while several *M. lychnidis-dioicae* and hybrid genotypes infected *S. dioica*. Altogether, no significant trend consistent with reproductive character displacement could thus be detected when allowing for competition between hybrids and nonhybrids in greenhouse conditions.

Discussion

No Hybrids in Sympatric Populations, But No Evidence for Strong Reproductive Character Displacement

We detected no hybrids in the two sympatric populations of *Microbotryum lychnidis-dioicae* and *Microbotryum silenes-dioicae*, in agreement with previous reports of lack of introgression between these species (Le Gac et al. 2007*a*). Previous studies showed no evidence of assortative mating between species of *Microbotryum*, but only allopatric populations had been examined (Le Gac et al. 2007*b*). In this study, we wanted to test whether the absence of hybrids and introgression between *M. lychnidis-dioicae* and *M. silenes-dioicae* could be due to the evolution of strong assortative mating in sympatry, that is, whether strong reproductive character displacement had occurred.

We could not detect any significant trend that would be consistent with reproductive character displacement in the production of experimental hybrids, either at the stage of syngamy or at later stages of infectivity on both *Silene dioica* and *Silene latifolia* hosts. Our experimental design included numerous replicates for premating stages but for practical reasons was restricted to a few populations and a single strain for the postmating stages. Thus, for postmating barriers, we could have detected only a strong increase in reproductive isolation in sympatry. In addition, these results could be due to a specific behavior of the strains and populations we studied. However, for reproductive character displacement to explain the complete lack of hybrids in our sympatric populations, it would have to be present and very strong given the high viability and fertility of *M. lychnidis-dioicae* × *M. silenes-dioicae* hybrids (Le Gac et al. 2007*b*; de Vienne et al. 2009*b*) and the complete lack of assortative mating between allopatric strains (Le Gac et al. 2007*b*). Altogether, our results thus indicate that reproductive character displacement probably contributes little or nothing to the lack of hybrids in the sympatric populations examined here.

Possible Causes for Lack of Evidence of Reproductive Character Displacement due to the Experimental Design

A possible explanation for the lack of evidence for strong reproductive character displacement between *M. lychnidis-*

Table 3

ANOVA on Interspecific Conjugation Rates

Factor	df	Sum of squares	Mean of squares	*F*	*P*
Sympatry	1	.0025	.0025	.504	.48
M. l-d. population	5	.1818	.0364	7.352	<.001
M. s-d. population	5	.0612	.0123	2.477	.057
M. l-d. strain	5	.2754	.0551	11.136	<.001
M. s-d. strain	4	.0150	.0038	.760	.56
Residuals	27	.1335	.0049		

Note. *M. l-d.* = *Microbotryum lychnidis-dioicae*; *M. s-d.* = *Microbotryum silenes-dioicae*. Population effects were nested in sympatry effect and strain effect was nested in population and sympatry effects.

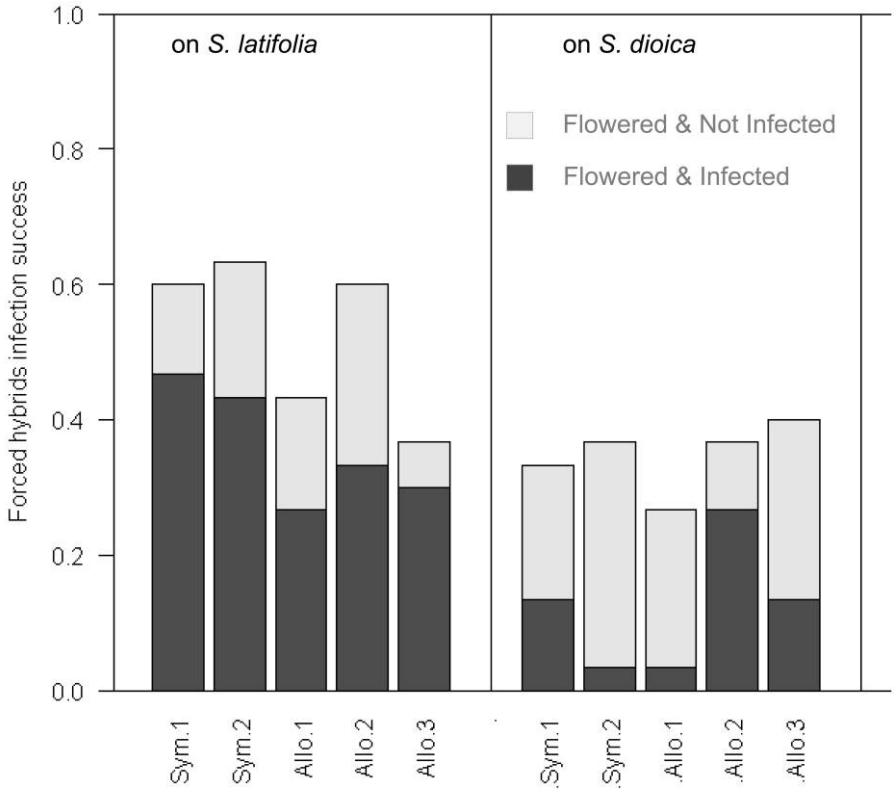


Fig. 5 Floral and infection statuses of plants infected with hybrid crosses. Infections were performed using forced hybrid crosses. The number of actual plants that did not flower, were healthy, or were diseased is indicated for each plant species (*Silene latifolia* and *Silene dioica*).

dioicae and *M. silenes-dioicae* is that we could not detect it with the populations we chose. The first difficulty in assessing reproductive character displacement is to identify what is allopatry for the species under consideration. While the issue of identifying allopatry may be problematic when dealing with fungal species that have spores that are wind dispersed, this should not be the case in *Microbotryum* because its spores are mainly dispersed by pollinators over short distances (Alexander and Antonovics 1995; Delmotte et al. 1999; Giraud 2004). Furthermore, we maximized the possibility of detecting a reproductive character displacement pattern by using (1) sympatric strains collected on plants growing in the same field that were separated by less than 50 m, (2) allopatric strains collected in fields that were located at least 100 km away from any population of the alternative species (known on the basis of a 780-population collection), and (3) (in the restricted scheme dealing with postmating barriers) allopatric strains from the most distant populations available, that is, *M. lychnidis-dioicae* strains from the United States (where *M. silenes-dioicae* does not occur), central Spain, and the Aquitaine region in France and *M. silenes-dioicae* strains from northern Scandinavia, where *S. latifolia* and *M. lychnidis-dioicae* are absent (Prentice et al. 2008; Hathaway et al. 2009).

An alternative explanation for lack of hybridization events with strong reproductive character displacement between *M. lychnidis-dioicae* and *M. silenes-dioicae* is that the two species have come too recently into contact in the two sympatric zones that we identified. Indeed, these species exist in a metapopulation structure in ephemeral patches (Antonovics et al. 1994), so that local sympatry, the product of the probabilities at which each pathogen species occurs, is expected to be rare and transient. That these populations have experienced very recent contact, however, is unlikely, as many plants were infected for both species and on both sites.

Finally, another possibility is that experimental conditions failed to mimic natural conditions under which reproductive displacement occurs. In fact, no pure *M. silenes-dioicae* genotypes successfully infected *S. dioica* under our greenhouse conditions, although they could have formed in the competition experiment. This result, which is consistent with other studies involving experimental hybridization (Van Putten et al. 2003), contrasts with results from natural populations where pure *M. silenes-dioicae* genotypes are present on *S. dioica*, even in populations where both fungal species co-occur. This discrepancy suggests that some important ecological factors are missing in experimental inoculations. These factors could play a role in reproductive isolation between the two *Microbotryum* species, especially when infecting *S. dioica*.

Possible Causes for Lack of Selection for Stronger Reproductive Isolation in Sympatry among Fungal Pathogens

Patterns of reproductive character displacement in the form of isolation that is higher in sympatric than allopatric populations have been successfully identified in *Heterobasidion*

Table 4

Homogeneity Tests of Hybrid Infection Success Depending on Type (Sympatric or Allopatric) of Combination

Inoculated plant species, type of fungal cross	χ^2	df	P
Silene latifolia:			
Sympatry	0	1	1
Allopatry	.3175	2	.85
Sympatry versus allopatry:			
Pooled	2.8947	1	.089
Unpooled	3.8773	4	.42
Silene dioica:			
Sympatry	.8727	1	.35
Allopatry	6.6533	2	.036
Sympatry versus allopatry:			
Pooled	.7602	1	.38
Unpooled	10.4798	4	.033

Note. Infections were performed using forced hybrid crosses. For each cross, 30 plants were available and reported as infected or not infected (unflowered and flowered being pooled). Intragroup and intergroup (sympatry vs. allopatry) homogeneity were assessed. Yates correction was used to correct for continuity. For intergroup homogeneity, either pooled or unpooled sets were tested. Pooled set corresponds to the pooling of all infection successes and all infection failures for sympatric crosses and allopatric crosses, respectively; that is, homogeneity for the unpooled set corresponds to considering each hybrid success individually, testing the overall homogeneity of all the data points.

and *Neurospora* (Dettman et al. 2003; Garbelotto et al. 2007; Turner et al. 2010) but have seldom been searched for in other fungi. To our knowledge, the only report of a lack of reproductive character displacement patterns among fungi concerns Ascomycetes (Le Gac and Giraud 2008). The lack of selection for stronger barriers between sympatric species has been suggested to be due to specialization in some plant pathogens because of host specialization pleiotropically inducing reproductive isolation in fungi that mate within their specific host (Giraud 2006*a*, 2006*b*; Giraud et al. 2010). In contrast, *Microbotryum* species mate before infecting their hosts so that specialization cannot act as premating isolation that would preclude selection for reproductive character displacement.

Another reason for a lack of detectable selection for enhanced isolation in sympatry, and one that is more likely to apply here, is that isolation is already strong enough that hybrids are rarely produced (Marshall et al. 2002). Specific pollinators or pollinator behaviors (van Putten et al. 2007; Dotterl et al. 2009), differences in habitats (Refrégier et al. 2008), and selfing (Giraud et al. 2008*b*) combine to induce strong reproductive isolation among *Microbotryum* species, reducing selective pressure for evolving additional barriers to gene flow in sympatry (van Putten et al. 2007; Giraud et al. 2008*b*). In particular, Gladieux et al. (forthcoming) estimated selfing rates higher than 0.9 in natural populations of both species studied here, which leaves little opportunity for hybridization, especially if we also consider the ecological barriers to gene flow. The strength of premating barriers is supported by the absence of hybrids in the populations we studied, as is evidenced by the strong clustering of the two species using neutral markers and the absence of individuals showing heterozygosity. Note, however, that because of high selfing rates and high numbers of spores deposited on plants, hybrids with reduced fitness should be strongly selected against in *Microbotryum* because they will always compete directly with nonhybrids to infect a given plant (Giraud et al. 2005, 2008*b*). Thus, while selfing can reduce selection for further assortative mating on one hand, it can contribute to selection against interspecific crosses on the other.

In contrast to what occurs in natural populations, we obtained many experimental hybrids, as has been observed in previous studies (Biere and Antonovics 1996; Van Putten et al. 2003; Le Gac et al. 2007*b*). This is probably because we inoculated plants using sporidia—that is, multiplied gametes—while teliospores are the dispersal stage that produces gametes upon germination. When mixtures of teliospores from *M. lychnidis-dioicae* and *M. silenes-dioicae* are deposited on a plant in natural populations, many fewer hybrids may be produced because germinating teliospores undergo intrapromycelial selfing at a very high rate (Hood and Antonovics 2000; Granberg et al. 2008) and, most often, because spores of a single diploid individual may be deposited on a given plant. The situation parallels that of their host plants, *S. latifolia* and *S. dioica*, in that they are able to produce hybrids in the greenhouse, where they have partial ecological isolation, but in natural populations hybrids are extremely rare (Minder et al. 2007; Karrenberg and Favre 2008; Minder and Widmer 2008).

Conclusions

Despite our efforts to investigate multiple premating and postmating steps in the life cycle, our study adds to a number of works reporting a lack of evidence for reproductive character displacement (Coyne and Orr 2004, pp. 361–362; Moyle et al. 2004; Le Gac and Giraud 2008; Geyer and Lessios 2009; Widmer et al. 2009). Our experimental design would have allowed for the detection of a medium increase in reproductive isolation for most traits in sympatric strains as compared with allopatric ones, so we can conclude that a lack of hybrids in *Microbotryum* can be achieved without evolution toward strong assortative mating in sympatry. The lack of detectable reproductive character displacement between *Microbotryum silenes-dioicae* and *Microbotryum lychnidis-dioicae* is likely due to insufficient selective pressure to increase intrinsic barriers to gene flow in sympatry because reproductive isolation is already strong, thanks to multiple barriers in the life cycle such as pollinator specificities and developmental processes that favor selfing.

The lack of complete premating barriers between sympatric strains of *M. lychnidis-dioicae* and *M. silenes-dioicae*, however, makes it possible for hybridization to occur in nature. A wider survey of natural sympatric populations of *M. lychnidis-dioicae* and *M. silenes-dioicae* is required to assess whether hybrids are present in some natural populations and from what generation and how frequently. Analyses using coalescent theory should also be useful in elucidating the history of speciation between *M. lychnidis-dioicae* and *M. silenes-dioicae* and assessing whether it occurred with continuous gene flow or whether gene flow recently increased because of secondary contact.

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