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Maximized virulence in a sterilizing pathogen: the anther-smut fungus and its co-evolved hosts

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Abstract

Host sterilization is a common feature of sexually transmitted diseases (STDs). Because host reproductive failure may free up resources for pathogen reproduction and transmission, theory predicts that selection on sterilizing pathogens will favour maximum virulence (i.e. complete sterilization). We examined patterns of infection in sexually transmitted anther-smut fungi (*Microbotryum*) on four of their host species in the Caryophyllaceae. Using controlled fungal matings and experimental inoculations, we compared disease expression in inoculations ranging from host-specific pathogens to hybrids and cross-species treatments. Our data support the existence of host-specific sibling species within the genus *Microbotryum* based on a low infection rate from cross-inoculations and reduced fitness for hybrid pathogens. These patterns of host specificity and reproductive isolation, however, were not absolute. We did observe some successful cross-species and hybrid infections, but the expression of disease was frequently incomplete, including only partial host sterilization and the failed dehiscence of pathogen spores. The prevalence of these maladapted disease phenotypes may greatly inhibit the emergence of novel host pathogen combinations. Infections by hybrid pathogen genotypes were intermediate, in terms of both infection rate and the normality of disease symptoms, between host-specific and cross-inoculated pathogens. In addition, the frequency with which hybrid and cross-inoculated anther-smut pathogens were able to infect but not sterilize new hosts supports the prediction that sterilizing STDs are under selection to maximize virulence in natural populations.

Introduction

Expectations for the evolution of pathogen virulence (i.e. the reduction in host fitness resulting from infection) depend greatly on whether the pathogen causes mortality or sterility in the host. A number of theoretical studies (Baudoin, 1975; Obrebski, 1975; Jaenike, 1996; O'Keefe & Antonovics, 2002) concluded that sterilizing pathogens should be under strong selection to completely sterilize their hosts in contrast to other parasitic relationships in which intermediate levels of virulence are often predicted (Anderson & May, 1982). The general assumption underlying these models is that hosts face a trade-off between reproduction and growth/survival, and therefore, sterilization can increase the host's longevity and thus prolong the pathogen's opportunity for transmission. Any investment by the host in its own reproduction would be resources lost for the reproduction of the pathogen. Sexually transmitted diseases (STDs) are particularly likely to cause host sterilization, which may benefit the pathogen by diverting resources away from its host without diminishing the chances that the host will continue to engage in mating activity (Lockhart *et al.*, 1996; Knell & Webberley, 2004).

Anther-smut fungi are a group of related pathogens belonging to the genus *Microbotryum* and specializing on flowering plants predominantly within

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the Caryophyllaceae (Thrall *et al.*, 1993). The life history of *Microbotryum* on the Caryophyllaceae recently was reviewed (Giraud *et al.*, 2008). Anther-smut disease has relatively little effect on host survival (Alexander & Antonovics, 1995), but infection results in the elimination of pollen production in the host's anthers. In place of pollen, fungal teliospores proliferate in these sexual organs and are subsequently transmitted to new host individuals by pollinators. Thus, because the same act that mediates sexual reproduction (pollination), also facilitates disease transmission, anther-smut has been characterized as a plant STD (Antonovics, 2005). In addition to the elimination of pollen production, *Microbotryum* infection also suppresses development of female sexual organs (styles and ovaries), resulting in complete host sterilization (Thrall *et al.*, 1993). For a sexually transmitted pathogen in plants, the potential benefits of host sterilization are particularly clear. After successful fertilization, many plants curtail production of additional flowers in favor of devoting resources to seed and fruit maturation (Hensel *et al.*, 1994). Therefore, sterilization by *Microbotryum* may allow for increased production of infected flowers and more opportunities for pathogen transmission.

Collectively, anther-smut fungi infect hundreds of host species (Thrall *et al.*, 1993). The existence of multiple potential hosts can have important implications for the evolution of virulence because specialization on one host can come at the expense of poor performance on others (Woolhouse *et al.*, 2001). For decades, anther-smut fungi have been taxonomically grouped under a single species designation (*Ustilago violacea* and more recently *Microbotryum violaceum*). However, cross-inoculation experiments dating back to the early 20th century (Zillig, 1921; Goldschmidt, 1928) and recent molecular phylogenetic studies (Garr *et al.*, 1997; Perlin *et al.*, 1997; Bucheli *et al.*, 2000; Freeman *et al.*, 2002; Lutz *et al.*, 2005; Van Putten *et al.*, 2005; Le Gac *et al.*, 2007b; Refrégier *et al.*, 2008) have made it increasingly clear that this group comprises multiple host-specific sibling species, some of which have been described recently (Lutz *et al.*, 2005).

The existence of sibling species raises important evolutionary and epidemiological questions because related host species often exist in sympatry. The co-occurrence of multiple related hosts presents the opportunity for pathogens to 'jump' to new hosts. The end result of such host shifts may vary greatly, ranging from a transitory nonsustainable phenomenon to a permanent expansion of the pathogen's host range (Fenton & Pedersen, 2005). In addition, if isolating mechanisms are incomplete, the co-occurrence of related pathogens may allow for hybridization and gene flow which can affect host–pathogen co-evolution (Robertson *et al.*, 1995; Ochman *et al.*, 2000; Brasier, 2001; Li *et al.*, 2004; Stavrinides & Guttman, 2004).

There is evidence that both host shifts and hybridization have been important forces in the evolutionary history of *Microbotryum*. Multiple studies of natural populations have presented evidence for transmission of *Microbotryum* between host species (Shykoff *et al.*, 1999; Antonovics *et al.*, 2002; López-Villavicencio *et al.*, 2005; Van Putten *et al.*, 2005; Carlsson-Graner, 2006). On a broader level, the lack of congruence in phylogenies between host and pathogen clearly indicates that host shifts have occurred (Jackson, 2004; Refrégier *et al.*, 2008). Topological differences among separate gene phylogenies of *Microbotryum* are also consistent with episodes of hybridization and gene flow among species (Le Gac *et al.*, 2007b).

To further characterize the physiological potential for host shifts and hybridization in *Microbotryum* and to better understand the consequences of host specificity on the evolution of virulence in this system, we performed a combination of experimental cross-inoculations and controlled fungal matings. We found strong evidence for host-specific sibling species, where *Microbotryum* isolates performed poorly on species other than their hosts-of-origin. Moreover, hybrid pathogen genotypes exhibited partial hybrid sterility and infection rates lower than nonhybrids on their host-of-origin. When these hybrid and cross-inoculated pathogens (which we collectively refer to as 'non-co-evolved') did produce infections, the host often exhibited abnormal disease symptoms that would further limit pathogen transmission. Notably, incomplete suppression of female reproductive organs appeared to be commonplace in infections with non-co-evolved *Microbotryum* genotypes. Here, we discuss the implications of these findings for the evolution of virulence in a sterilizing pathogen.

Materials and methods

Plant material

We used four host species from the Caryophyllaceae – *Silene vulgaris*, *Silene latifolia*, *Silene paradoxa*, and *Dianthus carthusianorum*. All the four are native to Europe where they share overlapping distributions (Jalas and Suominen, 1987). In the last 250 years, *S. latifolia* and *S. vulgaris* have been introduced into North America where they are now considered invasive (Taylor & Keller, 2007). *Silene latifolia* is dioecious, whereas the other three species are all hermaphroditic with at least some incidence of male sterility (gynodioecy). Seeds for each of the four host species (Table 1) were surface sterilized and germinated at 25 °C on 0.8% agar with 0.1x MS salts (Murashige & Skoog, 1962).

Microbotryum cultures and plant inoculation

Microbotryum teliospore samples were field collected from diseased populations of each of the four host species (Table 1). We did not use seeds and fungus from the same population so that an immediate history of

Table 1 Source information for seed and spore collections.

Host species	Seed source	*Microbotryum* code	*Microbotryum* source
Silene vulgaris	Broadway (Virginia), USA	SvA	Chambery, France
		SvB	Bugnei, Switzerland
Silene latifolia	Newport (Virginia), USA	Sl	Lamole, Italy
Silene paradoxa	Lamole, Italy	Sp	Gran Sasso, Italy
Dianthus carthusianorum	Sestriere, Italy	Dc	San Baronto, Italy

co-evolution would not affect our assessment of host specificity. The meiotic products of a single teliospore include two haploid sporidia of each mating type (a1 and a2). These individual cells were isolated to produce yeast-like cultures of single cell descent (Hood & Antonovics, 2000). For each *Microbotryum* species, we used two such cultures of opposite mating type (derived from the same meiotic tetrad). For *Microbotryum* from *S. vulgaris*, we isolated two separate pairs of sporidial cultures, each from a different population (Table 1). Each isolate was maintained in pure culture on potato dextrose agar (PDA) and transferred regularly to maintain active growth. The taxonomic classification of the anther-smut fungi is still in flux, so we refer to each *Microbotryum* isolate using abbreviations based on its host-of-origin (Table 1). To confirm their genetic identity and phylogenetic placement, we sequenced the ribosomal DNA internal transcribed spacer (ITS) region for each *Microbotryum* isolate (GenBank accession numbers: EU687463–EU687466). These sequences were consistent with more extensive phylogenetic sampling, showing characteristic genetic divergence based on host species (M. Hood, unpublished data; Kemler *et al.*, 2006). Our *Microbotryum* isolates for *S. vulgaris* correspond to the clade previously referred to as MvSv1 (Le Gac *et al.*, 2007b) and recently characterized as *Microbotryum lagerheimii* (although it was described on *Lychnis viscaria*; Denchev, 2007). The *Microbotryum* from *D. carthusianorum* corresponds to the clade previously referred to as MvDsp (Le Gac *et al.*, 2007b).

We determined the mating type of each culture by growing it on water agar plates coated with 200 μL of a 0.01% α-tocopherol (Sigma T-1539; Sigma-Aldrich Corp., St. Louis, MO, USA) solution (v/v) in the presence of tester strains of known mating type (Castle & Day, 1984). Successful mating was confirmed by observing conjugation between sporidia and production of hyphae after 24–72 h of incubation at 15 °C. We subsequently performed all possible pairwise mating crosses among our five *Microbotryum* samples, assaying mating success in the same fashion. We also performed negative controls by plating each culture with a tester strain of the same mating type and by itself. Mating was never observed in any of the negative controls.

Seven days after seeds were sown, we inoculated the resulting seedlings with *Microbotryum*. We prepared the inoculations by suspending sporidial cultures in sterile distilled water at a concentration of 200 μg/mL (or approximately two million sporidia/mL). To generate each cross, we mixed equal parts of two sporidial suspensions of opposite mating type and pipetted 2 μL of the resulting mixture directly onto the shoot apical meristem of each plant. Three days after *Microbotryum* inoculation, we transplanted all seedlings to soil and grew them in the greenhouse at 21 °C with a 16 h–8 h light–dark cycle. Plants were kept in randomized positions with respect to inoculation treatment, and they were sequestered prior to the maturation of flowers to prevent cross-infection within the study. We verified that observed infections matched the expected inoculation treatment by analysing a sample of fungal teliospores from each treatment with species-specific microsatellite markers using the primers and methods of Giraud *et al.* (2007). The microsatellite genotypes matched expectations in every case.

Experimental design and data collection

The experimental design (Fig. 1) consisted of two major components. First, we performed all possible cross-inoculations such that each host species was inoculated with *Microbotryum* from all four hosts. Second, we utilized the subset of *Microbotryum* hybrids involving sporidial cultures derived from a *S. vulgaris* host crossed with any of the other cultures. Hybrids were inoculated onto the two hosts of their parental species. In total, this design included 46 plant–fungus combinations. For each combination, we performed 49 replicate inoculations, resulting in a sample size of 2254 plants.

a1 Sporidia source \ *a2 Sporidia source*	**SvA**	**SvB**	**Sl**	**Sp**	**Dc**
SvA	Sv, Sl, Sp, Dc	Sv	Sv, Sl	Sv, Sp	Sv, Dc
SvB	Sv	Sv, Sl, Sp, Dc	Sv, Sl	Sv, Sp	Sv, Dc
Sl	Sv, Sl	Sv, Sl	Sv, Sl, Sp, Dc	--	--
Sp	Sv, Sp	Sv, Sp	--	Sv, Sl, Sp, Dc	--
Dc	Sv, Dc	Sv, Dc	--	--	Sv, Sl, Sp, Dc

Fig. 1 The matrix of all possible crosses of the five *Microbotryum* isolates. The abbreviations in each cell correspond to the host species that were inoculated with that particular pathogen treatment. SvA and SvB refer to two distinct isolates both collected from *Silene vulgaris* (see Table 1).

We monitored the plants on a daily basis for one full year after inoculation and examined each plant for signs of infection upon first flowering. We scored each individual as healthy or diseased and further distinguished between 'completely' and 'incompletely' diseased plants; we defined completely the disease based on the phenotypic characteristics required for transmission in the field (i.e. an open flower with mature, dehiscent *Microbotryum* teliospores), whereas plants that failed to meet this definition but had clear disease symptoms were scored as incompletely diseased. The most common symptoms seen in such incomplete infections were malformed anthers with underdeveloped, nondehiscent teliospores or completely aborted flowers.

From each diseased individual, we sampled a single teliospore-containing flower and stored it under desiccation at room temperature. We plated a sample of 57 teliospore collections on PDA and incubated them at room temperature for 24–28 h before observing the teliospores under 400 times magnification to assess germination and meiosis.

Incomplete female sterilization

After observing an abnormal flowering phenotype in one of the *S. vulgaris* individuals (see Results), we systematically monitored the other three host species for indications of incomplete female sterilization. Flowers that had disease symptoms as well as incompletely suppressed styles and ovaries were hand pollinated along with a sample of control individuals with no detectable symptoms. The resulting fruits were collected at maturity and inspected under a dissecting microscope. We pollinated a total of 20, 15 and 12 diseased individuals of *S. latifolia*, *S. paradoxa* and *D. carthusianorum* respectively. In addition, we pollinated 10 healthy individuals of each species. Pollen donors were taken from the same seed stock used for this experiment.

Data analysis

To test for an effect of *Microbotryum* treatment on infection rate, we constructed a two-way (treatment by disease status) contingency table for each host species. For all three *Silene* species, we assessed significance with log-likelihood chi-squared tests (*G*-tests) using the FREQ procedure in SAS software v9.1.3 (SAS Institute, Cary, NC, USA). Because of the smaller sample size resulting from a low flowering rate in *D. carthusianorum*, we used Fisher's exact test. We performed additional comparisons between specific treatments with the same contingency table approach, employing a Bonferroni correction for multiple comparisons.

Results

After 1 year, flowering rate was high for all species except *D. carthusianorum* (Supporting Information Table S1). We observed infections in all the four host species, and inoculation treatment had a significant effect on infection rate ($P < 0.0001$ for all species; *G*-test or Fisher's exact test).

Host specificity

In all cases, *Microbotryum* isolates produced the highest infection rates on their host-of-origin (Fig. 2). By contrast, cross-inoculation generally failed to yield complete infections. Only *Microbotryum* isolated from *S. latifolia* was able to successfully infect cross-inoculated hosts to the extent of complete disease expression, but its infection rates were still low (approximately 20% on both *S. vulgaris* and *S. paradoxa* and no successful infections on *D. carthusianorum*).

Reproductive isolation

All parings between *Microbotryum* from the four hosts (representing interspecific hybridizations of the pathogen;

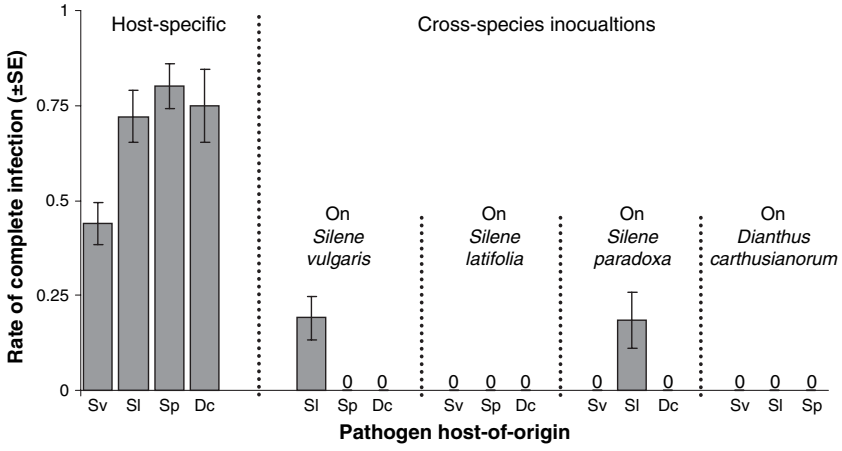

Fig. 2 Rates of complete infection (as defined in the Materials and methods section) for both host-specific inoculations and cross-inoculations. Error bars represent the Standard error of the proportion.

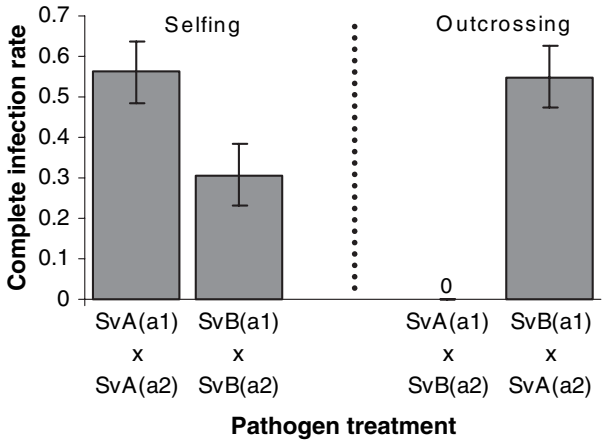

Fig. 3 Rates of complete infection (as defined in the Materials and methods section) for selfed and outcrossed genotypes derived from two separate samples of *Microbotryum* collected from *Silene vulgaris* hosts. Error bars represent the Standard error of the proportion.

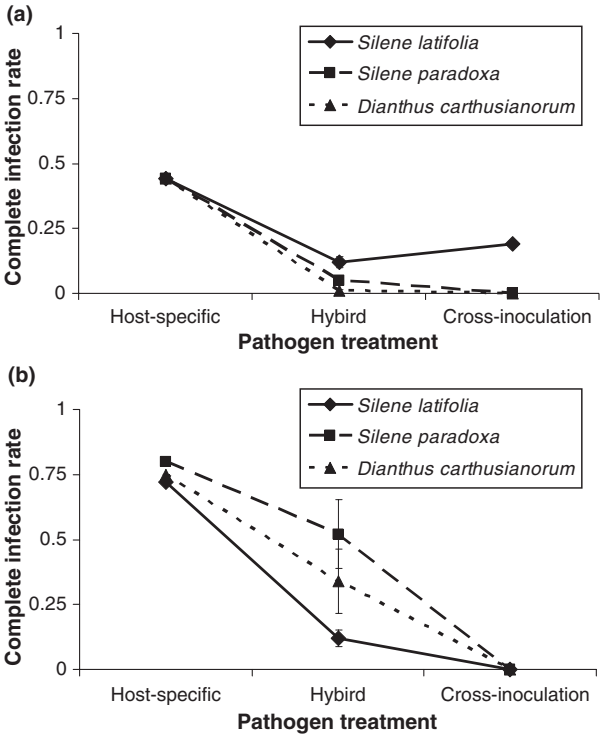

Fig. 4 Rates of complete infection (as defined in the Materials and methods section) for hybrid *Microbotryum* genotypes in comparison with nonhybrid genotypes. Infections rates are shown for (a) inoculations onto *Silene vulgaris* or (b) onto the other three host species. For each series, the host name indicates the source of *Microbotryum* genotypes crossed with *Microbotryum* from *Silene vulgaris*. Error bars represent Standard error when multiple hybrid genotypes were averaged.

Le Gac *et al.*, 2007a) were able to successfully conjugate and initiate hyphal growth *in vitro*. Surprisingly, the only combination that failed to successfully mate (even after repeated attempts) was an intraspecific cross between two isolates from *S. vulgaris* ($SvA_{a1} \times SvB_{a2}$). As expected, given the lack of successful mating, this cross failed to produce any infection whatsoever (Fig. 3). By contrast, the reciprocal cross ($SvB_{a1} \times SvA_{a2}$) demonstrated normal mating behaviour and produced high infection rates on *S. vulgaris*. Moreover, the combinations of SvA_{a1} or SvB_{a2} with mating partners derived from other host species were able to conjugate and cause infections.

Interspecific *Microbotryum* hybrids were consistently less successful in producing complete infections than their nonhybrid parental genotypes on their hosts-of-origin (Fig. 4), confirming that hybrid inviability represents a substantial post-zygotic barrier in *Microbotryum* (Le Gac *et al.*, 2007a). The hybrid genotypes that were able to produce teliospores exhibited further isolating barriers in the form of hybrid sterility. We observed the process of meiosis in a sample of teliospores collected from both interspecific ($n = 37$) and intraspecific ($n = 20$) crosses, representing all plant–pathogen combinations that produced infection. In every case, teliospores from intraspecific crosses underwent normal meiosis, producing sporidial colonies within 24 h regardless of whether they had been isolated from infections on their host-of-origin or a novel host. By contrast, every hybrid genotype exhibited abnormal patterns of sporidial production. Hybrid teliospores produced morphologically normal promycelia, but budding of sporidia was extremely slow or absent altogether.

Abnormal disease phenotypes

In addition to the low rate of complete infection, we also found that hybridization and cross-inoculation frequently resulted in abnormal and incomplete expression of disease symptoms. These infections generally resulted in small, aborted flowers containing partially smutted anthers with immature teliospores. In rare instances, we found individuals with anthers containing both pollen and teliospores (Fig. 5). For all four host species, the fraction of diseased individuals with partial or abnormal symptoms was lower for host-specific inoculations than for the other non-co-evolved pathogen genotypes (Table 2). These differences, however, were only significant for *S. latifolia* and *S. paradoxa*.

Infection is normally associated with suppression of female sexual organs (ovaries and styles; Thrall *et al.*, 1993), but we found that inoculation with non-co-evolved pathogen genotypes often resulted in incomplete female sterilization. We first observed incomplete sterilization in a single *S. vulgaris* plant, representing the only infection with mature teliospores and an open

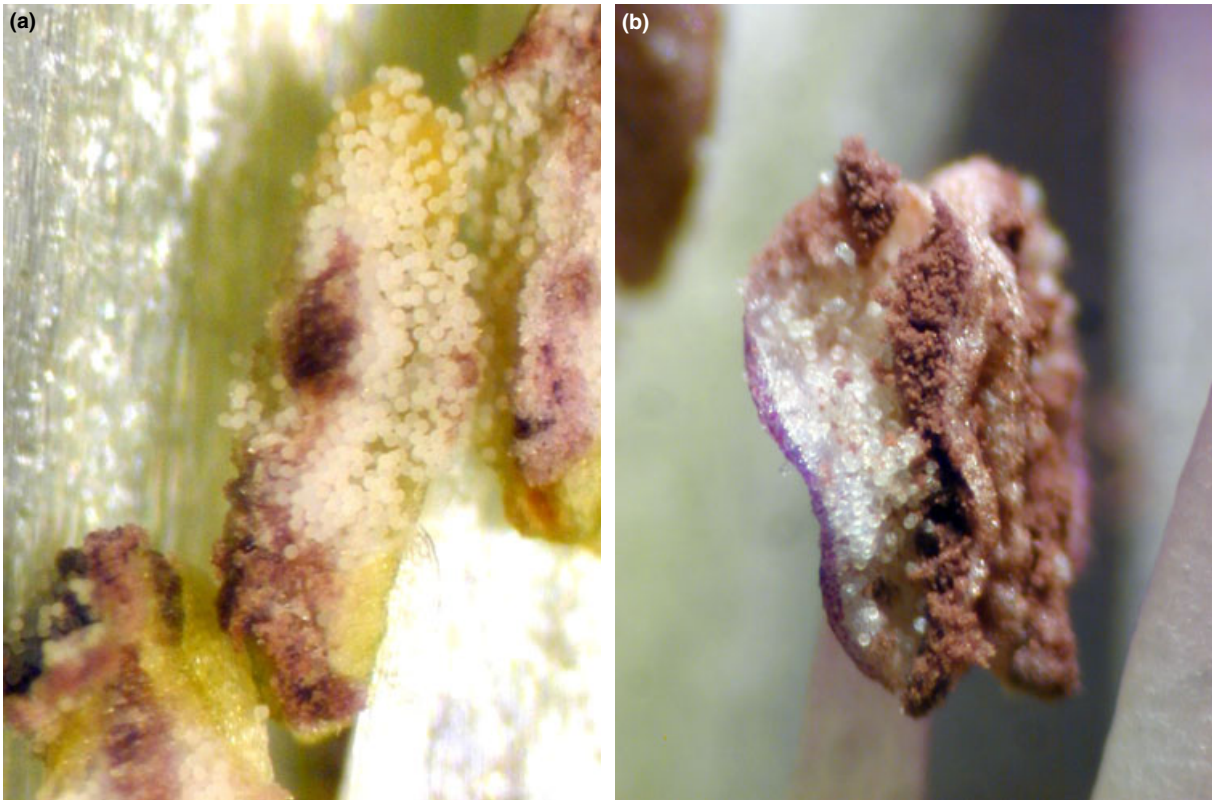

Fig. 5 Anthers from (a) *Silene latifolia* and (b) *Dianthus carthusianorum*, containing a mixture of mature pollen grains and *Microbotryum* teliospores. The *Silene latifolia* individual was inoculated with the hybrid SvB × Sl *Microbotryum* treatment, wheras the *D. carthusianorum* individual was inoculated with nonhybrid SvA × SvA. We did not observe this phenotype in any cases of nonhybrid smut inoculated onto their host-of-origin.

flower by a Sv × Dc hybrid pathogen in that host. The infected flower produced noticeably extended styles, which are not typical of disease in *S. vulgaris*. Thereafter, we systematically monitored the three remaining host species for evidence of incomplete female sterilization (see Materials and methods).

For the dioecious *S. latifolia*, successful *Microbotryum* infection in females necessitates the induction of male sexual organs (Uchida *et al.*, 2003). We found that the complete induction of diseased stamens was always associated with the complete functional suppression of female reproductive organs ($n = 21$). However, we did observe incompletely diseased flowers in which only small rudimentary stamens were induced and functional female sex organs were retained. This latter phenotype was observed in 19% of females inoculated with non-co-evolved pathogen genotypes ($n = 165$), but did not occur at all with host-specific inoculations ($n = 20$).

Hand pollination of flowers from 20 incompletely diseased *S. latifolia* individuals produced varying results, yielding either a seemingly normal capsule with mature seeds ($n = 7$; Fig. 6a) or a capsule filled with fungal teliospores and aborted ovules ($n = 11$; Fig. 6c). The latter phenotype appeared to fit Baker's (1947) description of a diseased *S. latifolia* plant found in the field. The two remaining capsules contained both seeds and teliospores (Fig. 6b). Germination of these seeds in soil resulted in healthy plants with no symptoms of disease upon flowering.

In contrast to *S. latifolia* in which female fertility was observed only in incompletely diseased individuals, *S. paradoxa* and *D. carthusianorum* (both of which are

Table 2 Rates of incomplete infection.

	Proportion of diseased individuals with incomplete symptoms		
Host species	Host-specific inoculations	Hybrid/cross-inoculations	*G*-test
Silene vulgaris	0.35	0.39	$P > 0.5$
Silene latifolia	0.09	0.74	$P < 0.0001$
Silene paradoxa	0.00	0.40	$P < 0.0001$
Dianthus carthusianorum	0.25	0.33	$P > 0.5$

Rates of partial infection represent the proportion of all diseased individuals that show incomplete symptoms.

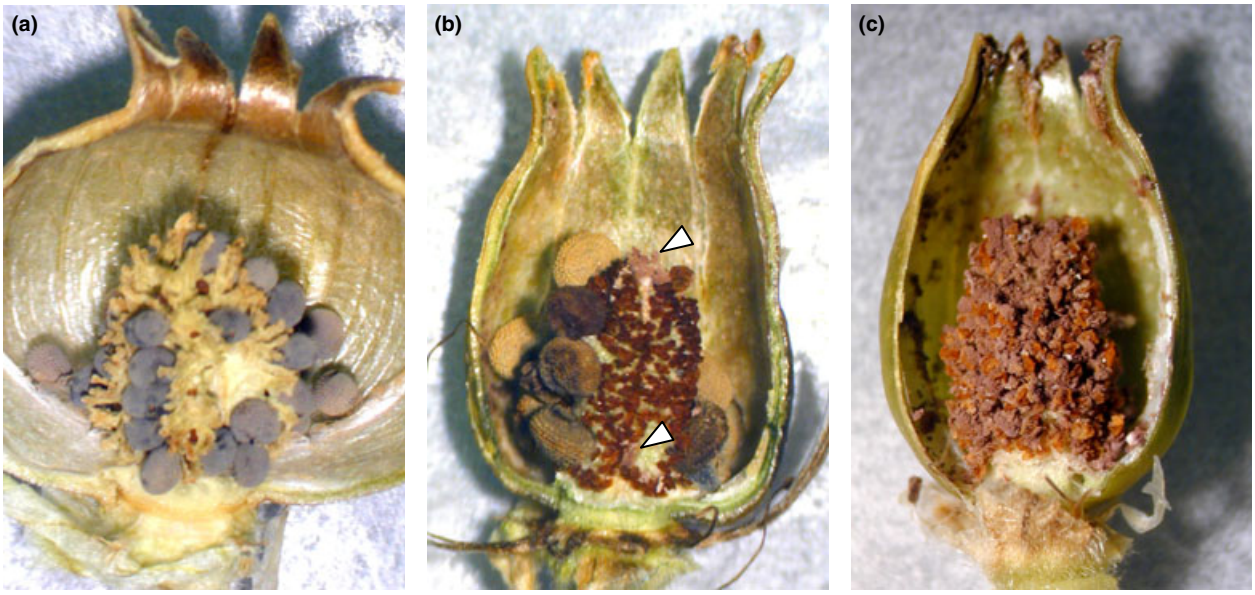

Fig. 6 Mature *Silene latifolia* capsules containing (a) healthy seeds only, (b) a mixture of healthy seeds, aborted ovules and *Microbotryum* teliospores (arrow heads) or (c) aborted ovules and *Microbotryum* teliospores only.

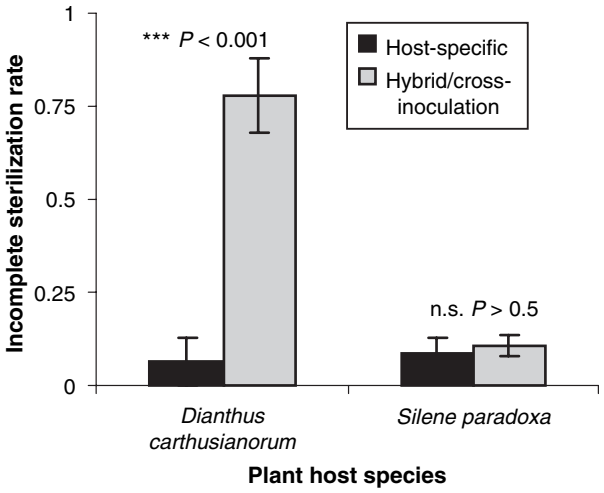

Fig. 7 Rates of incomplete sterilization (i.e. female fertility) in completely diseased *Dianthus carthusianorum* and *Silene paradoxa* resulting from inoculation with either host-specific or non-co-evolved pathogens. *Silene latifolia* data are not included in this figure because we found no individuals with both fully diseased anthers and functional female organs. Error bars represent the Standard error of the proportion, and *P*-values are based on Fisher's exact test.

hermaphroditic/gynodioecious) exhibited incomplete female sterilization in individuals that were otherwise fully symptomatic with regard to anther morphology. In *S. paradoxa*, this phenotype was rare and did not depend on whether the pathogen was host specific (Fig. 7). On the other hand, the retention of some female fertility in diseased individuals was very common in *D. carthusianorum* but only with non-co-evolved pathogen genotypes (Fig. 7). For both *S. paradoxa* and *D. carthusianorum*, these diseased flowers responded to hand pollination, producing swollen capsules with mature seeds. All diseased *D. carthusianorum* fruits ($n = 12$) also contained *Microbotryum* teliospores along with seeds. Only one of 15 fruits from diseased *S. paradoxa* individuals contained teliospores in addition to seeds. This individual had been inoculated with the nonhybrid and host-specific Sp × Sp *Microbotryum* treatment. For all three species, healthy hand pollinated flowers produced capsules with mature seeds ($n = 10$ per species). Interestingly, we found teliospores among the seeds in a single 'healthy' *D. carthusianorum* fruit (Dc × SvA treatment), indicating that *Microbotryum* infection may be present even in individuals that exhibited no detectable symptoms with regard to anther morphology.

Discussion

Evolution of virulence in sterilizing STDs

We found that non-co-evolved *Microbotryum* genotypes frequently induced abnormal disease symptoms including malformed or aborted flowers, partial infection of anthers and nondehiscent teliospores. Furthermore, the prevalence of incomplete sterilization associated with non-co-evolved pathogens was particularly interesting in the light of expectations for the evolution of virulence in sterilizing STDs. Theory predicts that selection will favour a highly virulent pathogen that completely sterilizes its host (Baudoin, 1975; Obrebski, 1975; Jaenike, 1996;

O'Keefe & Antonovics, 2002). Accordingly, anther-smut disease generally results in the complete suppression of both male and female sexual organs in natural populations. An earlier study (López-Villavicencio *et al.*, 2005), however, documented an exception to this pattern for the host *Gypsophila repens* in which smutted individuals maintain partial female fertility. The authors attributed the apparent suboptimal behaviour on the part of the pathogen to the fact that it had only recently undergone a host shift onto *G. repens* and, therefore, was poorly adapted to its new host.

Our greenhouse study showed that incomplete sterilization may be a more general phenomenon for novel host–pathogen combinations in this system, as it was documented on four different host species. Moreover, our data support the view that incomplete sterilization is an indication of suboptimal performance by pathogens that are poorly adapted to their hosts. In both *D. carthusianorum* and *S. latifolia*, incomplete suppression of female reproductive function was significantly associated with non-co-evolved hybrid and cross-inoculated infections. Moreover, the only observed instance of incomplete sterilization in *S. vulgaris* occurred in response to inoculation with a hybrid pathogen cross that demonstrated very low fitness (i.e. low infection rate). It should be noted, however, that this bias was not observed in the case of *S. paradoxa*.

Overall, these observations provide empirical support for an extensive body of theory that predicts that sterilizing STDs are under selection to maximize virulence. By suppressing female sexual organs, *Microbotryum* may be able to prevent the allocation of host resources to seed production and promote continued flowering, providing more extensive opportunities for pathogen transmission. Further experiments are needed to quantify the fitness consequences of female sterilization in natural ecological conditions by comparing differences in flower production between hosts with sterilizing and nonsterilizing infections. In addition, further studies should address the consequences of co-evolution at the intraspecific level by comparing host–pathogen combinations from sympatric and allopatric sources. Our study utilized seeds and spores collected from distinct populations so that a short-term history of co-evolution would not be confused for host-specificity at the species level. Cross-inoculations among populations within a single host species may reveal more subtle co-evolutionary patterns which were not addressed in our experiment. For example, a comparison of sympatric and allopatric inoculations of *S. latifolia* has revealed different infection rates, suggesting a pattern of local co-evolution (Kaltz *et al.*, 1999), but such studies have not specifically investigated the effects on host sterility.

Previous studies have found that STDs are significantly more likely to sterilize their hosts than other infectious diseases, but the cause of this association has remained uncertain (Lockhart *et al.*, 1996; Knell & Webberley, 2004). One potential explanation is that host sterilization is merely a by-product of the fact that STDs generally infect sexual organs (Knell & Webberley, 2004). Our findings are not consistent with this argument. We observed that some maladapted hybrid pathogens failed to sterilize the ovary of their host despite producing otherwise full and vigorous infections in the anthers, suggesting that the suppression of female sexual organs is actually an adaptation on the part of the pathogens to maximize their fitness.

Host specificity, reproductive isolation and transmission mode

Our results confirm the existence of host-specific sibling species within the *Microbotryum*, supporting earlier studies based on cross-inoculation (Zillig, 1921; Goldschmidt, 1928), electrophoretic karyotypes (Perlin *et al.*, 1997) and molecular phylogenetics (Lutz *et al.*, 2005; Kemler *et al.*, 2006; Le Gac *et al.*, 2007b; Refrégier *et al.*, 2008). Consistent with previous work (Le Gac *et al.*, 2007a), we found no evidence for prezygotic isolation among *Microbotryum* species but substantial support for post-zygotic barriers. We found that cross-inoculations and hybrid genotypes were generally maladapted based on: (1) low rates of successful infection; (2) a high frequency of abnormal and incomplete disease symptoms; and (3) partial hybrid sterility (in the case of interspecific crosses).

Nevertheless, there were cases of successful hybrid infections and host shifts. All interspecific *Microbotryum* crosses resulted in successful mating and infection in at least some individuals of both parental hosts. Although gamete production by the hybrid genotypes was clearly reduced, these teliospores did generate some viable sporidia, suggesting that hybridization may still be a relevant mechanism in the evolution of these pathogens. In addition, *Microbotryum* isolated from *S. latifolia* was able to successfully infect both *S. vulgaris* and *S. paradoxa*. Interestingly, host shifts from *S. latifolia* onto *S. vulgaris* have been observed in the field (Antonovics *et al.*, 2002), but a shift onto *S. paradoxa* has not, even though both species can be found in sympatry (Jalas and Suominen, 1987). Further investigation may find that transmission between *S. latifolia* and *S. paradoxa* is actively occurring in natural populations. Alternatively, there may be additional ecological factors such as pollinator activity or phenology that are preventing this potential host shift.

When hybridization brings together the genomes of divergent host-specific pathogens, two distinct classes of genetic effects may reduce hybrid fitness (Le Gac *et al.*, 2007a). First, hybrids may experience *intrinsic* genetic incompatibilities. Genomes that have evolved under reproductive isolation will generally accumulate mutations that lead to negative epistatic interactions in hybrids (Coyne & Orr, 1998; Lynch & Force, 2000). Second, hybrids may experience *extrinsic* incompatibilities with

their environment. Because half of the hybrid's genome will always be expressed in an environment (i.e. a host species) to which it is not adapted, these genotypes may often perform poorly in both parental environments (Grant & Grant, 1992; Schluter, 1996).

We found that hybrids were generally intermediate in their infection frequencies and their ability to manifest normal disease symptoms relative to their host-specific and cross-inoculated parental species (Fig. 4), suggesting that infection ability may be determined by extrinsic genetic factors of largely additive effect. By contrast, we found that only hybrids exhibited abnormal patterns of meiosis and sporidial production (i.e. hybrid sterility). Nonhybrid teliospores underwent normal meiosis regardless of whether they resulted from a host-specific infection or cross-inoculation. Therefore, hybrid sterility in *Microbotryum* appears to be the product of intrinsic genetic incompatibilities, most likely resulting from karyotypic differences that prevent proper chromosome pairing in meiosis (Perlin *et al.*, 1997). Interestingly, the complete failure of the intra-specific $SvA_{a1} \times SvB_{a2}$ cross to even mate also appears to be the result of intrinsic genetic incompatibilities, because the reciprocal cross and both parental crosses successfully mated and produced high infection rates (Fig. 3). This finding raises intriguing questions about the segregation of genetic variation and the role of mating system in maintaining that variation (Hood & Antonovics, 2004).

The expression of fungal spores in the ovaries of plants inoculated with non-co-evolved pathogens also suggests a mechanism by which *Microbotryum* may evolve alternate modes of disease transmission. The potential for novel host–pathogen combinations to exhibit substantial changes in development and the disease cycle has described in diverse systems (Shirai & Morimoto, 1997; Nikoh & Fukatsu, 2000; Vanbergen *et al.*, 2003). The co-occurrence of seeds and fungal teliospores in the same fruits raises the possibility of vertical transmission, although we found no symptoms of disease in the plants grown from these seeds. In addition, floral smuts of the Asteraceae and Dipsacaceae caused by *Microbotryum* species produce spores in the ovary tissue which allows for environmental contamination and nonvectored transmission (Berner *et al.*, 2006; Kemler *et al.*, 2006). Our observation of teliospore production in ovaries by an anther-smut pathogen supports the phylogenetic relationship with other ovary smuts and suggests that the physiological and developmental barriers that divide these parasitic strategies may be relatively minor.

A number of species in the Caryophyllaceae including some in this study are classified as invasive (McCauley *et al.*, 2003; Wolfe et al., 2004). As the distributions of these host species expand under the influence of human activity, new combinations of sibling species within *Microbotryum* are likely to occur in sympatry, creating novel opportunities for host shifts and pathogen hybridization. The barriers of host specificity and reproductive isolation will determine the boundaries for the emergence of novel pathogen lineages. Investigating the epidemiological dynamics of this multi-host/multi-pathogen system should provide useful insights into anthropogenic effects on the evolution of infectious diseases.

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Supporting information

Additional supporting information may be found in the online version of this article.

Table S1 Survival, flowering and infection data for each treatment

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