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PERFORMANCE OF A HYBRID FUNGAL PATHOGEN ON PURE-SPECIES AND HYBRID HOST PLANTS

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Premise of research. Recent hybridization events in fungi have produced emerging pathogens characterized by novel host specificities, increased infectivity, and/or elevated severity. We investigated the potential for host shifts and increased infectivity following hybridization of fungal pathogens in the genus *Microbotryum*, which causes anther-smut disease on Caryophyllaceae hosts. Hybrids of the closely related species *Microbotryum lychnidis-dioicae* (MvSl) and *Microbotryum silenes-dioicae* (MvSd) are viable and fertile. Although historical genetic exchange between MvSl and MvSd is rare, there is evidence of recent hybridization of these fungal species, as well as of their plant hosts, *Silene latifolia* and *Silene dioica*.

Methodology. We examined the fitness of hybrid pathogens and hosts by using F₁ hybrids of MvSl × MvSd to inoculate *S. latifolia* × *S. dioica* hybrids. Experimental inoculations of *S. latifolia* and hybrid hosts with pure-species and hybrid pathogens allowed assessment of the likelihood of hybrid emergence on a novel host (the hybrid plant) and of increased infectiousness of the hybrid pathogen on the pure-species host.

Pivotal results. We found no evidence for pathogen hybrid inferiority, arguing against interspecific incompatibilities at small genetic distances. Instead, we found that hybrid pathogens are more infectious on pure-species hosts, while pure-species pathogens are more infectious on hybrid hosts, indicating an interaction of host and parasite genotypes.

Conclusions. This finding argues against emergence of hybrid pathogens on a novel hybrid host. However, our study suggests that hybridization of pathogens and hosts in natural populations may lead to elevated disease prevalence overall, thus furthering the impact of anther-smut disease in these *Silene* species.

Keywords: hybridization, genotype-by-genotype interactions, virulence, aggressiveness, infectivity, ecologically based inviability.

Online enhancements: appendix tables.

Introduction

The merging of genomes through interspecific hybridization can generate phenotypes capable of exploiting new ecological and evolutionary opportunities (Lewontin and Birch 1966; Rieseberg 2003; Gross and Rieseberg 2005; Mallet 2007). There is great concern, therefore, that hybridization influences the emergence of novel infectious diseases, with hybrid pathogens able to colonize new hosts or to be more infectious and severe than their pure-species parents (Brasier 2001; Arnold 2004; Farrer et al. 2011). Indeed, the reassortment of influenza A lineages poses one of the greatest threats to human health today (Brown et al. 1998; Holmes et al. 2005; Nelson et al. 2008; Lam et al. 2013). Fungal and fungal-like diseases ex-

emplify this phenomenon: in the past 15 yr, emerging fungal and oomycete pathogens have been repeatedly linked to hybridization events. For example, the global expansion of Dutch elm disease and its destructiveness in the second half of the twentieth century are attributed to genetic exchange between two *Ophiostoma* species, the causative agents of this wilt disease (Brasier et al. 1998; Brasier 2001). This case and many others (Brasier et al. 1999; Newcombe et al. 2000; Brasier 2001; Kamoun 2001; Olson and Stenlid 2002; Giraud et al. 2008; Stukenbrock and McDonald 2008; Farrer et al. 2011; Stukenbrock et al. 2012) attest to the importance of hybridization and recombination in shaping the distribution of infectious disease, as well as the infectiousness and harm inflicted by pathogens.

The fungal genus *Microbotryum* (Basidiomycota: Microbotryales) provides an interesting system in which to investigate hybrid pathogens. It has a documented history of hybridization, potentially coupled with shifts to novel hosts

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(Refrégier et al. 2008; Devier et al. 2010), and indications of an ongoing increase in hybridization between certain species (Gladieux et al. 2011). Many *Microbotryum* pathogens infect plants in the Caryophyllaceae family, causing the sterilizing disease anther-smut, with different *Microbotryum* species specialized to infect different host species (Kemler et al. 2006; Le Gac et al. 2007a; Lutz et al. 2008; Denchev et al. 2009). The primary symptom of anther-smut disease is the replacement of host pollen by dark fungal spores. Pollinators vector spores from the anthers of infected hosts to floral and vegetative surfaces of healthy plants, where the diploid spores germinate, undergo meiosis, and conjugate to form dikaryotic hyphae (Schäfer et al. 2010). These hyphae invade host tissue and colonize meristems of the host, which is sterilized by pollen replacement and abortion of ovaries. There is clearly a genetic basis to variation in host resistance, both within (Alexander and Maltby 1990; Thrall and Jarosz 1994; Alexander and Antonovics 1995; Biere and Antonovics 1996; Carlsson-Granér 1997; Giles et al. 2006; Cafuir et al. 2007) and between (Antonovics et al. 2002; Le Gac et al. 2007b; Giraud and Gourbière 2012) closely related host species, as well as to variation between species of *Microbotryum* in their ability to cause disease (Antonovics et al. 2002; Le Gac et al. 2007b; Giraud and Gourbière 2012).

Hybrids of closely related *Microbotryum* species infecting closely related plants are viable and fertile (Le Gac et al. 2007b; Sloan et al. 2008; de Vienne et al. 2009), as seen for *Microbotryum lychnidis-dioicae* (MvSl) and *Microbotryum silenensis-dioicae* (MvSd), parasitizing *Silene latifolia* and *Silene dioica*, respectively. In natural populations, ample opportunity seems to exist for hybridization of MvSl and MvSd: sympatry of the host and pathogen species is common, and pollinators that disperse the fungal spores from smutted anthers are frequently generalist (Goulson and Jerrim 1997; Van Putten et al. 2005, 2007; Minder et al. 2007; Karrenberg and Favre 2008). Though evidence of hybridization is rare (Refrégier et al. 2010; Gladieux et al. 2011), gene flow appears to be increasing, possibly due to secondary contact following a period of allopatry (Gladieux et al. 2011).

Hybrids between the host plants *S. latifolia* and *S. dioica* are also fertile, and the two species are frequently sympatric or parapatric (Baker 1948). They are, however, clearly delineated as distinct species by ecological habit, morphology, and molecular data (Delmotte et al. 1999; Karrenberg and Favre 2008; Minder and Widmer 2008; Goulson 2009). Rare hybrids do occur in nature (Minder et al. 2007; Karrenberg et al. 2008), especially at contact zones between plant species (Goulson 2009). Human disturbances (e.g., farming, roadsides) are hypothesized to create habitat suitable for both plant species (Karrenberg and Favre 2008; Goulson 2009). Contact between *S. latifolia* and *S. dioica* can create opportunities for both host hybridization and pathogen hybridization, via pollinator movement of pollen and fungal spores, respectively, between host species. The dynamics of such multihost, multipathogen interactions between closely related species are poorly understood, and studies are required to illuminate the dynamics expected in cases of secondary contact.

Accordingly, we experimentally model this natural scenario in which contact between closely related hosts promotes formation of pathogen hybrids, which may then interact with

pure-species and hybrid hosts. Specifically, we compare fitness, measured as the rate of disease, of hybrid and pure-species pathogens on hybrid and pure-species plants. We perform reciprocal artificial inoculations using lab-bred F₁ hybrids between *S. latifolia* and *S. dioica* and their pathogens, MvSl and MvSd. While the general alarm over the emergence of hybrid pathogens suggests the hypothesis that interspecific crosses will result in increased disease, there are several possible outcomes. Hybrid pathogens might have consistently lower or higher fitness, due to hybrid incompatibilities or vigor, respectively. Relatedly, hybrid hosts could be more susceptible or more resistant regardless of pathogen genotype. In contrast, more complex patterns might emerge based on the interaction of host and pathogen alleles. For example, hybrid hosts could constitute an intermediate habitat most suitable for hybrid pathogens. We thus addressed the following questions: (1) Do *Microbotryum* hybrids show altered ability to cause disease on all hosts, suggestive of either genetic incompatibilities or hybrid advantage? (2) Is the ability to cause disease alternately mediated by an interaction of pathogen and host genotypes, such that pure-species and hybrid pathogens differ in disease rates on pure-species and hybrid hosts? If hybrid pathogens cause more disease on hybrid hosts, then a hybrid pathogen has greater potential to establish on emerging hybrid hosts. If hybrid pathogens instead cause more disease on pure-species hosts, pathogen hybridization could elevate the disease burden of pure-species-host populations.

Methods

Preparation of Fungal Strains

All *Microbotryum* strains used as inocula are detailed in table A1 (tables A1–A4 available online). Three to four field-collected samples were chosen to represent each of two fungal species: MvSl, collected from natural populations of the host *Silene latifolia* and MvSd, collected from the host *Silene dioica*. There is no known variation in the propensity of different strains of MvSl and MvSd to hybridize (Refrégier et al. 2010).

Inoculum preparation was conducted as described in Gibson et al. (2012). Briefly, for each *Microbotryum* sample, a single anther was extracted from preserved diseased flowers and serially diluted prior to growth on GMB2 medium (Thomas et al. 2003). Following serial dilution and further growth, colonies derived by mitotic replication of a single haploid sporidium were isolated and tested for mating type under a conjugation assay (Le Gac et al. 2007b). As often occurs, some MvSl isolates showed mating-type bias (Oudemans et al. 1998; Thomas et al. 2003): only the a₁ mating type was available for the MvSl strain 703.2, so the a₂ mating type from an additional strain, MvSl 729.2, was included (table A1).

Preparation of Host Populations

Seeds of *S. latifolia* and *S. dioica* were derived from populations in Orsay and Brittany, France, respectively (table A2). Seeds of F₁ hybrids from these host species were derived from artificial greenhouse crosses conducted in Orsay by reciprocally pollinating individuals of the two plant species. F₁ seeds from *S. latifolia* and *S. dioica* mothers were then pooled. Pure-species host plants of *S. dioica* were initially included in this

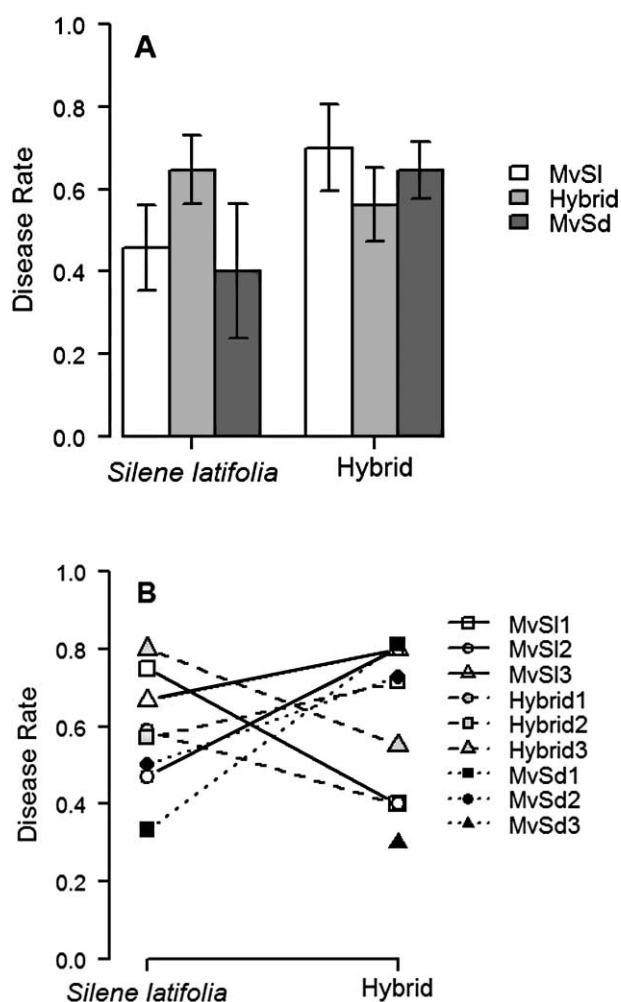


Fig. 1 Disease rate by host and pathogen combination. *A*, The proportion of inoculated plants that became infected is shown for *Silene latifolia* and the hybrid host inoculated with *Microbotryum lychnidis-dioicae* (MvSl), *Microbotryum silenes-dioicae* (MvSd), and the hybrid pathogen (MvSl × MvSd), averaged across all strains. Error bars show standard error of the proportion. *B*, The proportion of inoculated plants that became infected is shown according to the pathogen strain used. Strains included three strains for each of MvSl, MvSd, and the hybrid pathogen. Hybrid strains are numbered according to the MvSl and MvSd strains crossed to generate the hybrid (e.g., hybrid 1: MvSl1 × MvSd1). None of the *S. latifolia* plants that were inoculated with MvSd3 flowered, so disease rate could not be assessed for this treatment group.

experiment as well. However, very few *S. dioica* plants successfully flowered under greenhouse conditions, so this host was excluded from the following analyses. Seed sterilization and germination in preparation for inoculation were conducted as described in Gibson et al. (2012).

Inoculation and Treatment Design

The inoculum was prepared by combining equivalent amounts of a_1 and a_2 sporidial cultures in 400 μ L of water. Seedlings were treated with 4 μ L of inoculum on first emer-

gence of their cotyledons. *Microbotryum* crosses were either intraspecific, between haploid genotypes of MvSl or haploid genotypes of MvSd, or interspecific, between haploid genotypes of MvSl and MvSd. A total of six intraspecific crosses (three MvSl and three MvSd crosses) and three interspecific (hybrid) crosses were conducted. The particular combinations of sporidia for each cross are detailed in table A3. Each of these nine crosses was inoculated on both pure-species *S. latifolia* and F₁ hybrid *S. latifolia* × *S. dioica* host plant populations, resulting in 18 treatment groups. Twenty-five plants were inoculated per treatment group and maintained at 15°C for 2–4 d of incubation. All treatments are presented in detail in table A4.

Data Collection and Genotyping

Following incubation, seedlings were transplanted in a randomized fashion to soil in the greenhouse. Upon flowering, plants were visually assessed for symptoms of anther-smut disease. All flowering plants were removed from the flower beds as soon as the first flower appeared in order to avoid secondary contamination. The identity of healthy and diseased plants was noted. Disease rates were calculated as the percentages of flowering plants that were visibly infected within each treatment.

Statistical Analysis

To analyze the factors affecting the probability of infection, a logistic regression was performed with JMP 3 (SAS Institute, Cary, NC). For each inoculated plant, presence or absence of infection was treated as the response variable. Host (*S. latifolia* or hybrid), pathogen (MvSl, MvSd, or hybrid), and the interaction of host and pathogen were treated as predictor variables. An additional larger model was also performed in order to assess pathogen strain, nested within pathogen, as a predictor of disease rate.

Results

An average of 37.3% of plants ($N = 168$ of 450) flowered in the 18 treatment groups, ranging from 0% to 88% (table A4). Excluding all plants that failed to flower, artificial inoculations resulted in high disease rates ($\geq 40\%$) on *Silene latifolia* and hybrid plants for all inoculum treatments (fig. 1), allowing analysis of the explanatory variables. Host (*S. latifolia* or hybrid) and pathogen (MvSl, MvSd, or hybrid) were not significant predictors of disease rate (table 1). This result addresses our first question: we conclude that there is no evi-

Table 1

Results of Logistic Regression for Disease Rate			
Source	χ^2	df	<i>P</i>
Host	.244	1	.6213
Pathogen	1.053	2	.5908
Host × pathogen	10.185	2	.0061

Note. Host (*Silene latifolia*, hybrid), pathogen (*Microbotryum lychnidis-dioicae*, *Microbotryum silenes-dioicae*, and hybrid), and the interaction of host and pathogen are examined as predictor variables (whole model: $P = 0.0286$, $r^2 = 0.0394$).

dence for either universal depression of hybrid fitness in the host or pathogen, consistent with hybrid inferiority, or universal elevation of hybrid fitness, consistent with hybrid superiority.

The interaction of host and pathogen type was a significant predictor of disease rate (table 1). This result addresses our second question: disease rate is mediated by an interaction of host and parasite genotype. Specifically, we found that hybrid pathogens performed worse on hybrid hosts and better on pure-species hosts relative to pure-species pathogens (fig. 1A). Disease rates of hybrid pathogens are higher on pure-species *S. latifolia* plants than on hybrid plants, while the disease rates of pure-species pathogens are higher on hybrid plants than on pure-species plants. We note that the variation of strains within a pathogen type is substantial (fig. 1B). A larger model including the effect of pathogen strain nested within pathogen nevertheless found the strain effect to be insignificant and the interaction of host and pathogen to be marginally significant in predicting disease rate (table 2).

Discussion

Our results show that hybrid pathogens between MvSl and MvSd do not have uniformly higher or lower fitness than their parents. Rather the relative fitness of hybrid pathogens is contingent on host genotype. Hybrid pathogens are more infectious on pure-species hosts than are pure-species pathogens, indicating a potential link between pathogen hybridization and increased virulence.

The insignificance of pathogen genotype as a predictor of disease rate overall indicates that hybrid pathogens are not uniformly less fit than their parents, as might be expected if genetic incompatibilities on hybrid viability were present (Dobzhansky 1936; Muller and Pontecorvo 1940; Muller 1942; Burke and Arnold 2001). This is consistent with previous work in *Microbotryum*. Although distantly related species show reduced viability and fertility (Le Gac et al. 2007b; Sloan et al. 2008; de Vienne et al. 2009), hybrids of closely related MvSl and MvSd are consistently viable and fertile (Le Gac et al. 2007b; de Vienne et al. 2009). Previous results on the post-mating performance of backcrossed hybrids of MvSl and MvSd on pure-species host indicate that isolation results mainly from ecological incompatibility of hybrids: backcross progenies with a higher proportion of the pathogen genome native to the particular host environment were most fit (Büker et al. 2013). Even at greater genetic distances, Giraud and Gourbière (2012) found no evidence that negative interactions between loci in divergent lineages contribute to reproductive isolation of fungal species. They again support an ecological basis for hybrid inviability, in which the fitness of hybrid genotypes depends on their ability to cause disease on available hosts (Giraud et al. 2010).

The insignificance of pathogen genotype as a predictor of disease rate also signifies that there is no uniform fitness advantage of hybridization. Hybrid progenies may be predicted to have elevated fitness for multiple reasons, including heterosis and positive epistasis of newly combined loci (as reviewed in Burke and Arnold 2001). No fitness advantage of hybridization was apparent here, however. Importantly, this study examined the fitness of three unique F₁ hybrids, derived from

Table 2
Results of Logistic Regression for Disease Rate including Strain Effect

Source	χ^2	df	P
Host	1.268	1	.2601
Pathogen	3.097	2	.2126
Strain [pathogen]	8.141	6	.2258
Host \times pathogen	4.961	2	.0837

Note. Host (*Silene latifolia*, hybrid), pathogen (*Microbotryum lychnidis-dioicae*, *Microbotryum silenens-dioicae*, and hybrid), strain nested within pathogen (1, 2, 3), and the interaction of host and pathogen are examined as predictor variables (whole model: $P = 0.3180$, $r^2 = 0.0561$).

genetically divergent parents (Delmotte et al. 1999; Bucheli et al. 2001; Giraud 2004). We might then expect the fitness of hybrid genotypes to vary widely. If hybrid genotypes with elevated fitness arise because hybridization increases the variance of a trait (Arnold and Hodges 1995), then examining more hybrid genotypes might reveal a subset with elevated fitness. Variation in infectivity between strains was substantial but no higher between hybrid strains than between pure-species strains (fig. 1B). Nevertheless, with only three hybrid genotypes, we cannot exclude low sample size as an explanation for the failure to observe hybrid vigor.

While no uniform difference in hybrid- and pure-species-pathogen fitness was observed, the interaction of host and pathogen genotype was a significant predictor of disease rate. Thus, novel gene combinations may be fitter on certain hosts, indicating a role for genotype-by-genotype interactions in determining infection success (Carius et al. 2001; Laine 2004; Lively et al. 2004; Little et al. 2006; Salvaudon et al. 2007). This interaction effect in and of itself is an important result given that little is currently known regarding the genetics underlying the interaction between *Silene* and *Microbotryum*. The hybrid pathogen had a lower disease rate on the hybrid host and a higher disease rate on the pure-species host than did pure-species pathogens. The F₁ hybrid pathogens combine infection loci from both MvSd and MvSl, which have co-evolved with *Silene dioica* and *Silene latifolia*, respectively. The advantage of the hybrid pathogen on the pure-species host may arise from the combination of proteins actively involved in host infection (i.e., effectors) from both MvSl and MvSd, with which the nonhost pure-species plants have not coevolved. In contrast, the hybrid host combines resistance loci of both *S. latifolia* and *S. dioica*, which have evolved to recognize MvSl or MvSd effectors, respectively. This combination of resistance loci from both parent hosts may put the hybrid pathogen at a disadvantage on the hybrid host. We were unable to obtain data from artificial inoculations of pure-species *S. dioica* hosts in this study, but Le Gac et al. (2007b) found that the disease rate of MvSl \times MvSd hybrids on *S. dioica* was at least as high as that of MvSd on *S. dioica*. This is consistent with our finding that hybrid pathogens are successful on pure-species hosts, perhaps more so than are pure-species pathogens.

Our findings have important implications for natural populations undergoing secondary contact of closely related hosts and their pathogens. In mixtures of pure-species and hybrid hosts and pathogens, the hybrid host may negatively influence

the potential for hybrid-mediated gene flow in the pathogen, as hybrid pathogens have low fitness in the presence of hybrid hosts. The pathogen may likewise negatively influence the potential for hybrid-mediated gene flow between host species as hybrid hosts have low fitness in the presence of pure-species pathogens. Our results thus suggest that increased disease susceptibility could be an additional ecological factor explaining the rarity of hybrids between *S. dioica* and *S. latifolia* (Goulson and Jerrim 1997; Fritz et al. 1999; Minder et al. 2007; Karrenberg and Favre 2008; Goulson 2009). This finding may prove to be quite general: in many systems, including in the *Silene-Microbotryum* system (Refrégier et al. 2008), closely related hosts are infected by closely related pathogens (as reviewed in de Vienne et al. 2013; see also Hafner et al. 1994; Jackson and Charleston 2004; Sorenson et al. 2004; Huysse and Volckaert 2005), making hybridization of both hosts and pathogens a possibility.

In several other fungal pathogen systems, hybridization and recombination have been associated with increased infectivity and/or elevated disease severity (e.g., *Batrachochytrium dendrobatitis* of amphibian chytridiomycosis, *Ophiostoma* species of Dutch elm disease; Brasier et al. 1999; Newcombe et al. 2000; Fraser et al. 2005; Brasier and Kirk 2010; Farrer et al. 2011; Goss et al. 2011; McKenzie and Peterson 2012; Schloegel et al. 2012; Stukenbrock et al. 2012). We see potential for a similar association of hybridization and infectivity in *Microbotryum*. If the frequency of sympatry and hybridization between MvSl and MvSd is indeed on the rise (Gladieux et al. 2011), increased infectivity of hybrid pathogens on pure-spe-

cies hosts may limit host population growth, via sterilization, and even drive populations to greater local extinction rates (Antonovics 2004). In turn, increased local extinction of hosts with hybrid pathogens may contribute to the rarity of hybrid pathogens observed in nature (Gladieux et al. 2011). Finally, the elevated success of F₁ hybrid pathogens on pure-species hosts could facilitate gene flow between pure-species pathogens. Such introgression might increase the infectivity of pure-species pathogens and elevate the burden of disease, as has been seen in other systems (Stukenbrock and McDonald 2008). The results of our study and contemporary increases in hybridization between host and pathogen species suggest the *Silene-Microbotryum* system as a tractable, natural model in which to further investigate potential increases in pathogen infectivity and severity as a consequence of hybridization.

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