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SIBLING COMPETITION ARENA: SELFING AND A COMPETITION ARENA CAN COMBINE TO CONSTITUTE A BARRIER TO GENE FLOW IN SYMPATRY

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Selfing and sibling competition prevent gene flow

ABSTRACT

Closely-related species coexisting in sympatry provide critical insight into the mechanisms underlying speciation and the maintenance of genetic divergence. Selfing may promote reproductive isolation by facilitating local adaptation, causing reduced hybrid fitness in parental environments. Here, we propose a novel mechanism by which selfing can further impair interspecific gene flow: selfing may act to ensure that non-hybrid progeny systematically co-occur whenever hybrid genotypes are produced. Under a competition arena, the fitness differentials between non-hybrid and hybrid progeny are then magnified, preventing development of interspecific hybrids. We investigate whether this "sibling competition arena" can explain the coexistence in sympatry of closely-related species of the plant fungal pathogens (*Microbotryum*) causing anther-smut disease. The probabilities of intratetrad mating (automixis), outcrossing, and sibling competition were manipulated in artificial inoculations to evaluate their contribution to reproductive isolation. We report that both intratetrad mating and sibling competition significantly reduced rates of hybrid infection, beyond that expected based solely upon selfing rates and non-competitive fitness differentials between hybrid and non-hybrid progeny. Our results thus suggest that selfing and a sibling competition arena can combine to constitute a barrier to gene flow and diminish selection for additional barriers to gene flow in sympatry.

KEYWORDS

Hybridization, *Silene*, selection arena, speciation, fungi, pre-zygotic isolating mechanism, assortative mating, post-zygotic reproductive isolation

INTRODUCTION

The emergence of reproductive barriers that preserve locally adapted gene complexes is critical to the genetic divergence or coexistence of sympatric populations and, more broadly, to the process of speciation. Understanding the origins of reproductive isolation in sympatry, however, has proven difficult. Frequently, multiple isolating mechanisms are observed between well-established species, obscuring the primary barriers to gene flow (Ramsey et al. 2003; de Vienne et al. 2009). Some insights have been gained from studying closely related species occurring in sympatry. Studies of sympatric sister taxa, for example, have consistently shown the importance of pre-mating mechanisms in barring gene flow (Husband and Sabara 2004; Kay 2006; Martin and Willis 2007; Sánchez-Guillén et al. 2011).

Though tremendous variation in mating systems exists across all major groups of sexual eukaryotes, selfing as a pre-mating barrier to gene flow is studied infrequently and almost exclusively in plant systems (Levin 2010). Frequent cleistogamy (i.e. non-opening, self-pollinating flowers), for example, has been found to significantly reduce hybridization between sympatric *Mimulus* species (Martin and Willis 2007). The reduction in outcrossing accompanying selfing can protect reproductive investment by preventing the formation of hybrid progeny with maladaptive genetic combinations (Antonovics 1968; Allard 1975).

Selfing is considered to be an unusual isolating barrier, however. Because it usually isolates intra- and interspecific individuals equivalently, it has been argued that selfing cannot be regarded as directly promoting speciation (Coyne and Orr 2004 pg. 212). Nonetheless, selfing commonly facilitates speciation indirectly by generating other isolating barriers; for example, the reduction in gene flow from maladapted populations promotes local adaptation, thereby accelerating genetic divergence (Coyne and Orr 2004 pg. 212).

Here, we propose a novel mechanism by which selfing functions as a genuine isolating barrier that limits the success of interspecific hybrids to a greater extent than the progeny of intraspecific crosses. When reproduction is associated with early, intense competition between numerous sibling progeny for a limited resource, hybrids systematically compete with non-hybrids for establishment. If hybrids suffer any degree of fitness reduction, they will be unable to develop when the available resources restrict establishment to only a subset of the competing progeny. Interspecific gene flow is thus directly reduced by the combination of selfing and competition. We will henceforth refer to this mechanism as the "sibling competition arena." The use of the term "arena" reflects the requirement for systematic and intense local competition for successful establishment prior to further development of the zygote. It thus resembles Stearns' selection arena (1987), which proposes early selection of high fitness progeny by maternal choice and resource limitation among abundant offspring. However, several key assumptions are unique to our proposed model, as detailed in Table 1 and below.

Under this model, the systematic presence of numerous non-hybrid progeny promotes intense competition, magnifying the fitness handicap of hybrids. This strong

selective sieve reduces the rate of hybrid production in the population well below that predicted solely by the selfing rate and the non-competitive fitness differentials between hybrids and non-hybrids. Selfing and the production of numerous progeny strongly promote this process and may in fact be essential (Table 1, points 2.1 and 3.1). First, these factors generate intense competitive pressure. Secondly, they ensure that hybrid progeny are always accompanied by non-hybrid (selfed) progeny, even when the density of conspecifics is locally reduced. Some degree of divergence between hybridizing genotypes is required, such that hybrid progeny face reduced competitive ability relative to non-hybrid progeny (Table 1, point 1.4). These factors in combination may then function as a true isolating barrier, with gene flow reduced in association with interspecific hybridization but not intraspecific outcrossing, provided that outcrossed progeny do not suffer reduced viability relative to selfed progeny.

The reproductive traits of many taxa, most notably plant and fungal taxa, suggest that the sibling competition arena may constitute an isolating barrier across many systems. Importantly, such a barrier does not require sibling competition: competition between any hybrid and non-hybrid individuals would suffice. However, mixed broods of hybrid and non-hybrid siblings automatically yield the early competitive arena, and thus siblings are likely to be the most relevant competitors in many systems, including this study's focal species (Table 1, 3.1). Moreover, the mechanism of isolation need not be adaptive (Table 1, 4.1), a key distinction between our model and Stearns' selection arena (1987). In fact, sibling competition and any resulting reproductive isolation may merely be byproducts of the mating system. For example, fungal pathogens commonly produce large quantities of spores and predominantly perform haploid or diploid selfing (Giraud et al. 2008a). Both of these strategies facilitate reproduction and dispersal to new hosts. We hypothesize that, in the context of selfing and the sibling competition arena, these isolating mechanisms may additionally help explain, in an adaptive or non-adaptive manner, the abundance of cryptic, host-specific species within fungal pathogen taxa. The same hypothesis might be applied to plant taxa in which selfing and the over-production of seeds are common strategies (Vogler and Kalisz 2001).

We tested this model using *Microbotryum violaceum sensu lato,* a complex of basidiomycete fungi causing anther-smut disease on plants of the Caryophyllaceae family (Le Gac et al. 2007a). Species of *Microbotryum* are highly host-specific and apparently evolve without significant gene flow between closely related taxa (Le Gac et al. 2007b; Gladieux et al. 2011). Yet the barriers to hybridization that have been described for this system thus far are insufficient to account for such extensive reproductive isolation. Host and pathogen ranges overlap significantly, with frequent sympatry of diseased hosts (Van Putten et al. 2005; Le Gac et al. 2007b; Refrégier et al. 2010). These fungi are spread by pollinators showing only a partial host specificity that fails to explain the observed species integrity in sympatry (Goulson and Jerrim 1997; Minder et al. 2007; Van Putten et al. 2007; Karrenberg and Favre 2008; Refrégier et al. 2010; Gladieux et al. 2011). Moreover, mating occurs prior to plant infection, so host specialization alone cannot act as a barrier to gene flow, as is suggested for ascomycete fungi (Le Gac and Giraud 2008; Giraud et al. 2010). In addition, there is little evidence of assortative mating in the form of diminished conjugation rates between species (Le Gac et al. 2007b), even in sympatry (Refrégier et al. 2010). Post-mating reproductive isolation has been detected, with hybrids showing reduced infection ability, but closely related species can produce viable

hybrids (Sloan et al. 2008; de Vienne et al. 2009). Overall, the high viability of experimental crosses between closely related pathogen species and the weak ecological barriers to hybridization in the field are not consistent with the near total absence of gene flow in natural populations, which calls for investigation of additional mechanisms underlying this reproductive isolation (Gladieux et al. 2011).

Microbotryum violaceum exhibits a high rate of selfing, which may serve to explain the observed rarity of hybrids in the field (Giraud et al. 2008b; Refrégier et al. 2010; Gladieux et al. 2011). Conjugation occurs preferentially within the meiotic tetrad (automixis) (Hood and Antonovics 2000; Giraud et al. 2008b; Granberg et al. 2008). This form of selfing is facilitated by the development of the meiotic products in a multicellular basidium (e.g. promycelium), where neighboring cells readily conjugate. The fungus may also undergo autogamy or outcrossing, primarily through the production and mating of haploid yeast-like cells (sporidia) (Hood and Antonovics 2000; Giraud 2004; Hood and Antonovics 2004; Giraud et al. 2005; Giraud et al. 2008b; Schäfer et al. 2010; Gladieux et al. 2011). Hybridization with other fungal species occurs through this sporidial mating as well (Le Gac et al. 2007b; Gladieux et al. 2011). Meiosis and syngamy, through either intra-promycelial mating and/or sporidial mating, occur following deposition of hundreds of diploid teliospores of *Microbotryum* upon the surface of a new host plant (Schäfer et al. 2010). Some combination of automixis, autogamy, outcrossing, and hybridization results in the formation of infectious dikayrons on the plant surface. The numerous sibling progeny then compete to occupy the host meristem, the seat of *Microbotryum* infection which is limited to colonization by a single diploid individual (Audran and Batcho 1982; López-Villavicencio et al. 2007; Schäfer et al. 2010). These life history traits raise the question of the degree to which developmentally-promoted selfing and the production of multiple progeny may serve as a barrier to interspecific gene flow between sympatric *Microbotryum* species.

In this study, we assessed the significance of the combination of selfing and the sibling competition arena as a mechanism of reproductive isolation between closely related species of *Microbotryum*. Artificial inoculations of host plants were designed to compare rates of overall infection and of hybrid infection in the presence and absence of selfing or the sibling competition arena. First, we tested the effect of developmentally-promoted selfing by intra-promycelial mating on gene flow: we asked if fewer hybrids are formed under inoculation with diploid teliospores, undergoing mostly automixis, as opposed to inoculation with cultures of haploid sporidia. Secondly, we tested the effect of the sibling competition arena by comparing hybrid infection rates when hybridization was forced to situations where competition with non-hybrids was allowed. Under competition, the rate of hybrid infection is expected to be lower than that based solely upon the non-competitive fitness differentials of hybrids and non-hybrids.

MATERIALS AND METHODS

Model System

Fungi of the genus *Microbotryum* (Basidiomycetes: Microbotryales) cause anther-smut disease on plant hosts of the Caryophyllaceae. Host-specific lineages have recently been delineated into species, where the criterion of concordance between multiple gene genealogies demonstrated a lack of gene flow (Kemler et al. 2006; Le Gac et al. 2007a;

Lutz et al. 2008; Denchev et al. 2009). The fungus replaces host pollen with dark fungal spores and is transmitted between hosts via pollinators. As previously described, intra-promycelial or intratetrad mating (automixis) is the dominant life history strategy for *Microbotryum* species (Hood and Antonovics 2000, 2004; Schäfer et al. 2010), though rates of automixis vary between species and populations (Granberg et al. 2008). Following mating, dikaryotic hyphae form and invade host tissues, with infecting strains ultimately establishing in a limited region of the host meristems (Audran and Batcho 1982; Schäfer et al. 2010). Upon flowering, diploid teliospores are formed and expressed in the anthers, for disease transmission via pollinators.

Natural hybrids have rarely been observed (Gladieux et al. 2011), though Devier et al. (2010) proposed that historic hybridization events between moderately distant species were significant in generating several *Microbotryum* species. Hybridization between the closely related species *M. lychnidis-dioicae* and *M. silenes-dioicae* has been most commonly studied. Their respective hosts, *Silene latifolia* and *S. dioica*, are frequently sympatric (Van Putten et al. 2005; Refrégier et al. 2010), and hybrids of the two fungal species are viable, fertile, and infectious in a laboratory setting (Van Putten et al. 2003; Le Gac et al. 2007b; de Vienne et al. 2009). In natural populations, however, evidence for hybridization is limited. Gladieux et al. (2011) reported only 15 hybrids out of 1028 pathogen individuals based upon microsatellite characterization, suggesting strong reproductive isolation in the field between these closely-related, host-specific pathogen species. Ecological isolation, through specialization of habitat or pollinator, may play some role, but it is far from complete (Goulson and Jerrim 1997; Van Putten et al. 2007). Assortative mating in the form of preference for conspecific gametes could not be detected, even between different species occurring in sympatry (Le Gac et al. 2007b; Refrégier et al. 2010). Selfing has therefore been proposed as the primary barrier to interspecific gene flow (Giraud et al. 2008b).

Preparation of Fungal Strains

The *Microbotryum* collections used as inoculum, as well as the original location of their collection, are identified in Table 2. Collections were sampled from natural populations of six host species, *Silene latifolia*, *S. dioica, S. nutans, S. vulgaris, S. paradoxa,* and *Lychnis flos-cuculi*. These fungal species are known respectively as *Microbotryum lychnidis-dioicae* (MvSl), *M. silenes-dioicae* (MvSd), *M. violaceum sensu stricto* (MvSn), *M. lagerheimii* (MvSv1), *M. violaceum sensu lato* (MvSp), and *M. violaceum sensu lato* (MvLfc). Abbreviated names indicate the host plant species. These species of *Microbotryum* were chosen for their demonstrated ability to hybridize in a laboratory setting with *Microbotryum lychnidis-dioicae* (MvSl) (Le Gac et al. 2007a). Three field-collected samples were chosen from each pathogen species and were genotyped with microsatellites to verify species classification.

For each *Microbotryum* sample, a single anther was taken from archived infections and suspended in 200 µl of distilled water. Dilution series of teliospores were spread on GMB2 medium (Thomas et al. 2003) and incubated at 25°C until sporidial colonies derived from single teliospores could be visualized. Sporidial colonies derived from single teliospores were separately subjected to a dilution series and grown on GMB2 medium until colonies derived from single haploid sporidia could be visualized. For each *Microbotryum* sample, such cultures were isolated and tested for mating type by

a conjugation assay to obtain sporidia of both mating types, a_1 and a_2, derived from a common meiosis (Le Gac et al. 2007b). For *M. silenes-dioicae* (MvSd), *M. violaceum sensu stricto* (MvSn), *M. lagerheimii* (MvSv1), *M. violaceum sensu lato* (MvSp), and *M. violaceum sensu lato* (MvLfc), the mating-type ratios were heavily biased (Oudemans et al. 1998; Thomas et al. 2003), such that samples with only a single mating type were used in certain crosses (Supplementary Table 1). Importantly, for *M. lychnidis-dioicae* (MvSl), both mating types were available for all three strains.

Inoculation and Treatment Design

Seeds from a population of *S. latifolia* in Amherst, Massachusetts, were chosen for inoculation. This population of *S. latifolia* is known to be highly susceptible to infection. Seeds were sterilized in a solution of deionized water, calcium hypochlorite (12 g per liter), and sodium hydroxide (4 g per liter) for 20 minutes, rinsed in a 1:10 dilution of the same solution, and left to dry. For germination, seeds were grown on 1% agar-filled Petri dishes, with 15 seeds per plate, under fluorescent lights at 22°C.

Seedlings were inoculated upon first emergence of their cotyledons by application of *Microbotryum* suspensions to the apical meristem. Teliospore inocula were created by suspending teliospores from two anthers, one from each of the two fungal species to be crossed, in 1000 µL of distilled water. For sporidial inocula, one inoculating loop (10 µL) of each a_1 and a_2 mating type sporidial culture that had been maintained independently on GMB2 medium was individually suspended in 300 µL of distilled water. Equal volumes of various individual cultures were then combined to prepare inocula. *Microbotryum* crosses were either intraspecific between haploid genotypes of MvSl or interspecific between MvSl and one of five other *Microbotryum* species. For each species pair, six mating combinations (i.e. a_1 and a_2 sporidia) were randomly chosen from among the samples available per *Microbotryum* species. The six inoculum combinations were then applied under four different treatments (S-pair, S-mix, T-high, T-low, see below). A total of 144 individual crosses were performed (6 pairs of haploid genotypes for each of 6 species pairs x 4 treatments). These 144 crosses are detailed in Supplementary Table 1. Between 15 and 25 plants were inoculated for each cross.

The four treatments were designed to contrast the rates of selfing with intraspecific outcrossing or interspecific hybridization under conditions of either i) forced hybridization or intraspecific outcrossing, ii) hybridization or selfing possible via sporidia (autogamy), or iii) hybridization or selfing possible via sporidia and intra-promycelial mating (automixis) (Fig. 1). The treatment S-pair (for Sporidial pair) comprised inoculation with a_1 sporidia from one haploid genotype and a_2 sporidia from a second haploid genotype, such that hybridization or intraspecific outcrossing could be forced. The treatment S-mix consisted of inoculation with equal quantities of 4 distinct sporidial types, the a_1 and the a_2 from each of two fungal individuals (except when a second mating type was unavailable for the species to be crossed with MvSl; see Supplementary Table 1). Under the S-mix treatment, competition between outcrossed/hybrid and non-hybrid (selfed) progeny could occur because selfing was possible; however, the developmentally-promoted selfing via intra-promycelial mating was absent. In the T-high (for Teliospore, high concentration) and T-low treatments, the inoculum consisted of a suspension of teliospores from two *Microbotryum* diploids; the use of teliospores allowed intra-promycelial mating, the more common form of mating in nature (Hood and

Antonovics 2000, 2004; Schäfer et al. 2010). With teliospores, outcrossing or hybridization were not forced, and competition between outcrossed/hybrid and non-hybrid progeny could occur. The T-high and T-low treatments differed in that the T-low inoculum was diluted 100-fold relative to the T-high inoculum in order to assess the role of teliospore density in the balance of selfing and outcrossing/hybridization. For each treatment, three intraspecific crosses, consisting of selfing MvSl genotypes from three populations, were conducted. These crosses were designed to obtain a baseline infection rate by selfed progeny for estimations of the reduction in fitness of hybrid progeny. In order to simplify the description of the results, the term "hybrid" will henceforth be used as an umbrella term for both interspecific hybrid and intraspecific outcrossed progeny.

Under the hypothesis that selfing, and in particular the developmental propensity for intra-promycelial mating, plays a role in reproductive isolation, the rate of hybrid infection was predicted to be highest in the absence of competition (S-pair) and moderate under competition with non-hybrids in the absence of developmentally-promoted selfing (S-mix). Developmentally-promoted selfing was predicted to reduce rates of hybrid infection to their lowest level, even below that projected by intrinsic hybrid fitness and selfing rates. The rate of hybrid infection was therefore expected to be lower in the presence of developmentally-promoted selfing by intra-promycelial mating (T-high), and lower still under reduced teliospore concentrations (T-low), due to less frequent contact between teliospores and a decreased density of hybrids relative to non-hybrids, yielding a stronger sibling competition arena.

Data Collection and Genotyping

After 2-4 days of incubation, seedlings were transplanted to soil in the greenhouse. Upon flowering, plants were visually assessed for symptoms of anther-smut disease. All flowering plants were removed from the flowerbeds as soon as the first flower appeared in order to avoid secondary contamination. The number of plants that flowered for each cross is reported in Supplementary Table 1. The identity of healthy and diseased plants was noted, and 1-2 flowers of diseased plants were retained for genetic analysis. Anthers were desiccated on silica gel (Silica gel blue 2-5mm Prolabo) and stored at 4°C.

DNA was extracted, using the Chelex (Bio-Rad) method (Bucheli et al. 2001), from 1-2 anthers from a single flower derived from each diseased sample. Artificial inoculation at the single meristem stage of seedlings largely prevents coexistence of multiple infections, resulting in systemic infection by the single pathogen genotype that persists in subsequently derived meristems (Hood 2003; Gold et al. 2009). Anthers from a single flower were therefore considered accurate for genetic typing of an infection under our inoculation protocol. Even in the unlikely event of multiple infections, they would segregate in different stems (Hood 2003; Gold et al. 2009; López-Villavicencio et al. 2011), and our genotyped strains would represent an unbiased sample of the infecting strains.

Microsatellite genotyping was conducted as described in Giraud (2004). The microsatellite markers SVG8 and SL16 (Giraud et al. 2008c) were used to identify interspecific and intraspecific hybrids, respectively. The majority of strains used in this study were homozygous at the SVG8 microsatellite marker, and different species carried discriminating alleles. Therefore, heterozygotes at this marker indicated infection by a

hybrid pathogen. The marker SL16 was additionally used to distinguish two *M. lychnidis-dioicae* (MvSl) strains (728.6, 729.2) which were not distinguishable using the marker SVG8.

Data Analysis

To assess variation in the overall infection rate, logistic regressions were performed with JMP 3 (SAS Institute Inc., Cary, NC). For each inoculated plant, presence or absence of infection was treated as the dependent variable, and treatment (S-pair, S-mix, T-high, T-low) and genetic distance between the fungal parents of the cross were treated as predictor values. A similar logistic regression was then performed using presence or absence of a hybrid genotype for each infected plant as the dependent variable.

The intrinsic reduction in hybrid fitness relative to non-hybrids (i.e. without any competition effect) was determined in the treatment S-pair by the percent reduction in hybrid infection rate relative to the mean infection rate of the three selfed crosses (i.e. between a_1 and a_2 sporidia of the same MvSl sample; Supplementary Table 1). To test whether the sibling competition arena further reduced the rate of hybrid infection below what was expected based upon intrinsic fitness of hybrids and the observed rate of intraspecific outcrossing, expected hybrid infection rates in the S-mix treatment were computed as the rate of intraspecific outcrossing in S-mix (MvSlxMvSl) corrected by a factor of [1- reduction in intrinsic hybrid fitness]. The expected and observed rates of hybrid infection in the S-mix treatment were compared using Chi-square tests. The same analysis was also performed using a lower rate of intraspecific outcrossing, to determine if the results were robust to the variation in outcrossing rate observed across different pairings of *Microbotryum* strains (Giraud et al. 2005).

RESULTS

Overall Infection Rates

The four treatments (S-pair, S-mix, T-high, T-low) differed significantly in overall infection rates (Fig. 2; Table 3). Broadly, the S-pair and T-low treatments resulted in lower infection rates than the S-mix and T-high treatments (Fig. 2). This is in agreement with our expectations that i) the forced hybridizations in S-pair would yield lower infection rates than treatments with similar inoculum concentration but selfing allowed (S-mix and T-high), and that ii) lower teliospore concentrations (T-low) would yield lower infection rates than higher teliospore concentrations (T-high). Genetic distance between the a_1 and a_2 parents and the interaction of genetic distance with treatment also significantly affected infection rates (Table 3). As shown in Figure 2, the significant interaction term between treatment and genetic distance likely results from the impact of genetic distance being limited to the treatment S-pair, where hybridization was forced. Consistent with previous studies, the fitness of hybrids is shown to decrease with genetic distance between hybridizing species (Le Gac et al. 2007b). Accordingly, the infection rate was influenced little by genetic distance when selfing was allowed, because infections were dominated by selfed progeny (see below). This is consistent with the low hybrid infection rates reported below.

Hybrid Infection Rates

The four treatments also differed significantly in hybrid infection rates, i.e. the proportion of all inoculated plants that became infected with hybrid pathogens (Fig. 3; Table 4). As expected, the highest hybrid infection rates were observed under forced hybridization (S-pair) (Fig. 3). Sporidial mixtures (S-mix), where selfing was allowed but there was no possibility of intra-promycelial mating, showed some interspecific hybrid infection, while the lowest interspecific hybrid infection rates were seen in teliospore treatments (T-high, T-low), where rapid developmental intra-promycelial mating promoted selfing. Additionally, the genetic distance between the two fungal species being crossed was a significant predictor of hybrid infection rate (Table 4): hybrid fitness decreased with increasing genetic distance between hybridizing species. Interestingly, there was no reduction in fitness under MvSlxMvSl outcrossing in comparison to selfing. Under the MvSlxMvSl S-mix treatment, 67% of infections were attributed to outcrossed pathogens and only 33% to selfed pathogen genotypes. This ratio did not differ significantly from a 50:50 null hypothesis for the comparison of selfing and outcrossing rates (χ^2=3.667, DF=1, p=0.0555).

Barriers to Hybridization: selfing and intra-promycelial mating

The significant differences in hybrid infection rates between treatments indicate that the potential for selfing and intra-promycelial mating influences the probability of hybridization between *Microbotryum* species (Table 4, Fig. 3). In comparing only treatments with forced hybridization (S-pair) to those with the possibility of sporidial selfing (S-mix), the potential for selfing reduced the rate of infection by 39% for intraspecific crosses (MvSlxMvSl) and by 75% for interspecific hybrid crosses with the closest species (MvSlxMvSd). The possibility of selfing reduced the rate of hybrid infection by 77% for the next most distant cross (MvSlxMvSn) and by 100% for the three most distant interspecific crosses (Fig. 3). When comparing treatments in which selfing was possible between sporidia (S-mix) to those in which developmentally-promoted intra-promycelial mating was also possible (T-high), the potential of intra-promycelial mating reduced the rate of hybrid infection by 76-78% for both intraspecific and the closest interspecific (MvSlxMvSd) crosses (Fig. 3). This comparison was not informative for the four more distant interspecific crosses (MvSlxMvSn, MvSlxMvSp, MvSlxMvSv1, MvSlxMvLfc) because hybrid infection rates were 5% or less under forced hybridization (S-pair) and were already dramatically reduced to 0-1% in the S-mix treatment (Fig. 3).

The concentration of teliospores used in inoculation affected hybrid infection rates (Fig. 3, Table 4). As predicted, fewer hybrids resulted at low teliospore densities (T-low) relative to high teliospore densities (T-high) for intraspecific crosses (MvSlxMvSl) and the closest interspecific crosses (MvSlxMvSd). This comparison was not possible for more distant interspecific crosses due to the absence of hybrid infections in both treatments.

Barriers to Hybridization: addition of the sibling competition arena

The intrinsic fitness of hybrid progeny relative to selfed progeny, without accounting for competition, was estimated based upon infection rates in the forced mating treatment (S-pair). Using this estimate, a significant negative correlation between intrinsic hybrid fitness and genetic distance between MvSl and the hybridizing species

was found (r=0.85, p=0.031) (S-pair in Fig. 3). The hybrid infection rate in the S-mix treatment for MvSlxMvSd was significantly lower than expected based upon the outcrossing rate measured in intraspecific, S-mix crosses and the intrinsic reduction in MvSlxMvSd hybrid fitness as measured by the S-pair hybrid infection rate (χ^2=13.333, DF=1, p=0.0003) (Fig 4). Thus, competition generated by the presence of selfed progeny further impeded hybrids from successfully infecting, beyond their intrinsic fitness reduction and the selfing rate. This difference remained marginally significant when the intraspecific outcrossing rate was reduced from 0.478 to 0.30 to test for robustness to observed variation in outcrossing rates (χ^2=3.411, DF=1, p=0.0647). The most relevant outcrossing rate here, however, remains the prior value of 0.478, as it was obtained directly from the experimental conditions applied across all treatments. For the four more distant interspecific crosses, observed hybrid infection rates were not significantly lower than expected rates (MvSlxMvSn: χ^2=1.025, DF=1, p=0.3113; MvSlxMvSv1: χ^2=2.026, DF=1, p=0.1546; MvSlxMvLfc: χ^2=3.051, DF=1, p=0.0807; and MvSlxMvSp: χ^2=3.053, DF=1, p=0.0806), though this is likely attributable to the already very low hybrid fitness of these crosses seen in the S-pair treatment.

DISCUSSION

Selfing in combination with the competition arena is shown here to be an important barrier to gene flow between closely-related, sympatric species of the phytopathogenic fungi *Microbotryum*. Intra-promycelial mating, a developmentally-promoted form of automixis, in combination with early, intense competition between selfed and hybrid progeny, yields nearly complete reproductive isolation between species of *Microbotryum.* Results of artificial inoculations confirm our prediction that fewer hybrids successfully establish infection when selfing and intra-promycelial mating are possible, in comparison to the forced hybridization treatment. Furthermore, our results confirm the prediction that fewer hybrids successfully establish infection under competition with non-hybrid siblings than expected based upon selfing rates and intrinsic fitness reductions. Moreover, we demonstrate that low teliospore density further reduces rates of hybrid infection, potentially by reducing the frequency with which hybrids are generated. Reproductive isolation in this fungal system thus appears to be strongly influenced by selfing and the associated competition between non-hybrid and hybrid genotypes, which we refer to as the sibling competition arena.

Treatments differing in the potential for selfing and intra-promycelial mating yielded significantly different rates of hybrid infection. The potential for intra-promycelial selfing alone reduced hybrid infection rates by 76-78% across both the MvSlxMvSl and MvSlxMvSd species pairs, most likely indicating a constant average rate of intra-promycelial mating within the MvSl pathogen species. In natural populations, rates of selfing vary but have been estimated to be as high as 88-94% (Gladieux et al. 2011), which is higher than the rate observed in our experimental crosses. In nature, teliospore concentrations and/or the frequency of occurrence of different mating partners on a given plant may be lower or more variable than they are under artificial inoculation, influencing estimations of selfing rates. Moreover, Oudemans et al. (1998) and Thomas et al. (2003) reported that haplo-lethal alleles, which limit outcrossing for the sporidia of

one mating type, may be as frequent as 100% in some field populations. This was not the case for the three MvSl strains used in this study.

It is possible that mechanisms promoting high frequencies of selfing represent adaptations to limit gene flow, but identifying such adaptations has proven notoriously problematic (Ramsey et al. 2003; Martin and Willis 2007; Matsubayashi and Katakura 2009). Refrégier et al. (2010) found no evidence for a mating preference for conspecifics in sympatric versus allopatric populations of MvSl and MvSd. They attributed this to the presence of a powerful pre-zygotic isolating mechanism (i.e. selfing) that limits selection for additional reproductive barriers, such as assortative mating by mate choice. That study, however, also found no evidence for adaptive reinforcement in the form of higher rates of selfing in sympatric populations. Likely alternative hypotheses for the observed frequencies of selfing are facilitation of mating (e.g. reproductive assurance) or acceleration of hyphal formation (Baker 1955; Lloyd 1992; Granberg et al. 2008); the resulting reproductive isolation may therefore have arisen from selective pressures unrelated to assortative mating.

Though its adaptive significance is difficult to clarify, selfing is shown here to be a promising explanation for the striking absence of interspecific gene flow in natural populations of *Microbotryum*. While hybrid pathogens readily form under experimental conditions (Le Gac et al. 2007b) and phylogenetic analysis supports ancient hybridization events (Le Gac et al. 2007a; Devier et al. 2010), there is negligible evidence of hybridization events occurring in the field, even between closely-related, sympatric species (Refrégier et al. 2010; Gladieux et al. 2011). The prominent role of selfing as a barrier to gene flow is consistent with studies in plants, where selfing is commonly found to facilitate reproductive isolation under sympatric or parapatric conditions. Mattalana et al. (2010) recently reported that self-compatible species of the Bromeliaceae family are more likely to overlap with close relatives in their geographic range or blooming period than are self-incompatible species. Likewise, populations in parapatry and sympatry with close relatives have been shown to have significantly higher rates of selfing than allopatric populations. This has been interpreted as defending against "gametic wastage" on unfit hybrid progeny and promoting local adaptation (Antonovics 1968; Petit et al. 1997; Fishman and Wyatt 1999; Grossenbacher and Whittall 2011). Though selfing has been studied almost exclusively in plants, it offers a potentially important mechanism of reproductive isolation in fungi, where speciation processes are largely undefined. Mating systems of plant and human fungal pathogens are increasingly found to incorporate versions of sexual reproduction that favor inbreeding over outcrossing in a manner proposed to facilitate host adaptation (Giraud et al. 2010; Heitman 2010).

Selfing as a barrier to gene flow has been objected to on the grounds that mating systems with high levels of selfing reduce gene flow within the same species to the same degree that they reduce gene flow between species (Coyne and Orr 2004 pg. 212). Selfing has, nonetheless, been acknowledged to be a major, indirect contributor to the speciation process: small effective population sizes, facilitation of the founder effect, and chromosomal rearrangements are all correlates of selfing that may promote speciation (Lewis 1966; Coyne and Orr 2004 pg. 212). Here, we propose another consequence of selfing: it can function to strongly reduce the likelihood of propagation of hybrid lineages when selfing is associated with production of multiple progeny in a competition arena,

such that non-hybrid and hybrid genotypes always compete prior to establishment and early development (Table 1).

The sibling competition arena hypothesizes that early, intense competition between hybrids and non-hybrids will prevent development of hybrid individuals. This requires systematic competition between numerous progeny for a limited resource in which only a small subset of zygotes can ultimately establish (Table 1). In the fungus *Microbotryum*, this hypothesis takes the shape of hundreds of sibling, dikaryotic hyphae competing for invasion and infection of a host plant meristem, in which only a single individual can persist. In the event that spores of two different pathogen species are deposited on the surface of a single plant, a likely event in sympatric populations, they will self, via intra-promycelial mating, and hybridize, via sporidial mating. Our results here indicate that the hybrid progeny will then fail to survive the sibling competition arena due to direct competition with their superior, non-hybrid siblings. This finding strongly suggests a role for early, post-zygotic competition between selfed and hybrid progeny in depressing gene flow between sympatric pathogen species well below that predicted solely by the intrinsic fitness reductions of hybrids. Importantly, this mechanism of reproductive isolation is a genuine isolating barrier in that it limits gene flow in the event of hybridization but not intraspecific outcrossing, assuming that outcrossed progeny face no reduction in fitness relative to selfed progeny. This assumption is supported by our finding that, within a single pathogen species (MvSl), outcrossed progeny were not less fit than selfed progeny under competition: outcrossed progeny in fact represented a larger proportion of infections in the S-mix treatment than did selfed progeny.

The sibling competition arena bears much similarity to other mechanisms of early post-zygotic reproductive isolation and offspring selection in plants and animals, such as Stearn's "selection arena" (1987). Hauser et al. (2000) hypothesized that, in the plant species *Silene nutans*, strong discrimination against inbred progeny could result from the decreased survival of selfed seed when developing in competition with outcrossed seeds in the same fruit. Lively and Johnson (1994) proposed that brooding organisms are more susceptible to the invasion of parthenogenetic mutants due to a mother's ability to establish a "selection arena" to weed low fitness progeny from her brood, either actively or through sibling competition. The perspectives of these previous works differ from that of the model proposed here, which is tailored for the unique biology of fungal pathogens and some plants and bears directly upon reproductive isolation. They do, however, propose mechanisms of early zygotic selection, through competition or parental control, that may similarly facilitate reproductive isolation and eliminate hybrid fitness. Such studies have been predominantly conducted in animal and plant systems. By extension to the realm of fungi, developmental competition may be found to be a broadly generalizable mechanism for ensuring the early elimination of low fitness offspring (Bruggeman et al. 2004). Likewise, the sibling competition arena should be broadly generalizable to plants, where selfing and early sibling competition for establishment in a saturated environment are frequent (Table 1).

We demonstrate here that selfing in the plant fungal pathogen *Microbotryum* dramatically reduces hybridization between closely-related species by two mechanisms: directly by intra-promycelial mating and indirectly by the sibling competition arena's influence on competitive exclusion. The magnitude of the reduction in gene flow

attributable to selfing appears to strongly diminish natural selection for other pre-zygotic isolating barriers between sympatric species of *Microbotryum*, consistent with observation of natural populations (Refrégier et al. 2010). These results, and the predominance of mating systems that facilitate inbreeding in fungal pathogen and plant species (Billiard et al. 2011), support the inclusion of mating system and selfing rate as critical components of reproductive isolation in the study of speciation.

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FIGURE LEGENDS

Figure 1: Illustration of the four treatments conducted to compare rates of hybrid infection in order to assess the combined effect of selfing and sibling competition as a barrier to hybridization. S-pair: inoculation with the a_1 sporidia from one strain and the a_2 sporidia from a second strain. Hybridization or outcrossing is forced. S-mix: inoculation with the a_1 and the a_2 sporidia from both strains of the cross. Selfing via sporidial mating and hybridization are both possible. T-high and T-low: inoculation with diploid teliospores from both strains at high (T-high) or low (T-low) concentrations. Selfing via sporidial and intra-promycelial mating and hybridization are all possible. Each treatment is defined in terms of hybridization (forced or by choice) and the possibility of selfing, intra-promycelial mating, and competition. The processes of sporidial selfing, intra-promycelial mating, and hybridization are diagrammed.

Figure 2: Overall infection rate according to treatment, cross, and genetic distance. The proportion of inoculated plants that became infected is shown for each cross over the four treatments; see Figure 1 and the text for treatment definitions. The genetic distance between MvSl and the hybridizing pathogen species increases from left to right within each treatment. Error bars show the standard error of the proportion.

Figure 3: Hybrid infection rate according to treatment, cross, and genetic distance. The hybrid infection rate is shown for each cross across all four treatments; see Figure 1 and the text for treatment definitions. Hybrid infection rate indicates the proportion of all inoculated plants that became infected with hybrid pathogens. The genetic distance between MvSl and the hybridizing pathogen species increases from left to right within each treatment. Note that MvSlxMvSl crosses represent outcrossing rather than hybridization and are used for comparison to hybrid crosses. Error bars show the standard error of the proportion.

Figure 4: Reduction of hybrid infection rate resulting from selfing and the sibling competition arena. For the forced mating treatment (S-pair), infection rates are shown for MvSlxMvSl (outcrossed) and MvSlxMvSd (hybrid) crosses. For the hybridization choice treatment (S-mix), the MvSlxMvSd hybrid infection rate is shown relative to the expected infection rate based on the results in the forced mating treatment (S-pair). Assuming no reduction in hybrid success due to the sibling competition arena, the expected hybrid infection rate of MvSlxMvSd in sporidial mixtures (S-mix) is estimated as the rate of intraspecific outcrossing (MvSlxMvSl) in S-mix corrected by the reduction in MvSlxMvSd hybrid fitness observed under forced hybridization (S-pair). The observed rate of hybrid infection is significantly less than that predicted based upon hybrid fitness and selfing rate alone (χ^2=14.070, DF=1, p=0.0002), indicating a role for competition between selfed and hybrid progeny (i.e. sibling competition arena). Error bars show the standard error of the proportion for observed rates.

TABLES

Table 1: Outline modeling the proposed sibling competition arena. Includes the assumptions and conditions of the sibling competition arena, their role in reproductive isolation, and their relevance to *Microbotryum* and other fungal and plant systems.

Sibling competition arena: components that bar gene flow in combination with selfing		**Application to *Microbotryum***	**Application to other systems**
Required 1. Competition	1.1 Limited resources: space/nutrients restrict establishment to a limited number of zygotes at an early stage, prior to detectable development	Occupation of a meristem limited to a single individual, the unsuccessful zygotes being unable to persist in the host	*Fungi* associated with systemic infection: limited number of genotypes occupy host *Plants*: limited space, above and below ground, for early establishment/germination
	1.2 Intense competition for establishment in the environment (e.g. host) through the production of multiple progeny	The number of diploid teliospores deposited on a host far exceeds the number that can ultimately colonize the host plant	*Fungi*: thousands of spores dispersed to each host individual *Plants*: number of seeds dispersed locally exceeds that which the environment can support
	1.3 Mixed population on the required resource: hybrids and non-hybrids always compete for establishment	Hybrid hyphae always compete with non-hybrid hyphae prior to infection (due to selfing, 2.1, and to the presence of numerous siblings, 3.1)	*Fungi and plants*: deposition of mixed broods of hybrid and non-hybrid individuals on/in the same host/environment
	1.4 Reduced competitive ability of hybrids	Infection success of hybrids on parental hosts decreases with genetic distance of species, even measured in the absence of competition	*Fungi*: reduced infection ability of hybrids *Plants*: reduced establishment ability of hybrid seedlings
Contributes to meeting point 1.3 2. High selfing rates	2.1 Selfing: ensures the systematic presence of non-hybrids, even when conspecific density is locally reduced	High intratetrad mating (automixis) rates	*Fungi*: selfing (diploid or haploid) frequent *Plants*: self-compatibility widespread in many plant taxa
Contributes to meeting point 1.2 3. Sibling competition	3.1 Numerous progeny commonly compete intensively, enhancing competition between siblings, hybrid and non-hybrid alike	Many diploid teliospores of a single individual deposited on a host plant: non-hybrid and hybrid siblings, produced by intra-tetrad selfing and sporidial mating, compete for infection of the meristem	*Fungi*: thousands of spores produced by a single infection *Plants*: numerous seeds dispersed locally
Not required 4. Selection to avoid hybridization	4.1 Selfing and the co-occurrence of numerous progeny are not necessarily adaptations *for* avoiding hybridization	The sibling competition arena is likely a byproduct of life-history and reproductive strategies derived from ancestry or other selective pressures (e.g. numerous spores and high selfing rates may function as assurance in host and mate seeking)	*Fungi and plants*: selfing and over-production of seeds or spores may serve as adaptations to facilitate reproduction and dispersal

Table 2: Identification of the 18 strains of *Microbotryum* used as inoculum. Presented are the identifying codes, original host plant from which the strain was isolated, location of the population from which the strain was collected, the date of collection, and the name of the collector.

Microbotryum Species	Strain	Plant Host Species	Original Location	Collector
M. lychnidis-dioicae (MvSl)	729.2	*Silene latifolia*	Marcillat, France	T Giraud
M. lychnidis-dioicae (MvSl)	728.6	*Silene latifolia*	Foxley Corner, UK	H Prentice
M. lychnidis-dioicae (MvSl)	665.2	*Silene latifolia*	Mt. Biokoyo, Croatia	S Yakovlev
M. silenes-dioicae (MvSd)	831.3	*Silene dioica*	Swiss Alps	S Karrenberg
M. silenes-dioicae (MvSd)	700.3	*Silene dioica*	Tomtebo, Sweden	B Giles
M. silenes-dioicae (MvSd)	701.3	*Silene dioica*	Sävartärnögern, Sweden	B Giles
M. violaceum sensu stricto (MvSn)	706.1	*Silene nutans*	Dunes of l'Escault, France	M Fontaine, P Reignault, G Refrégier
M. violaceum sensu stricto (MvSn)	705.3	*Silene nutans*	Dunes of l'Escault, France	M Fontaine, P Reignault, G Refrégier
M. violaceum sensu stricto (MvSn)	719.2	*Silene nutans*	Dunes of l'Escault, France	M Fontaine, P Reignault, G Refrégier
M. lagerheimii (MvSv1)	432.87	*Silene vulgaris*	Bugnei, Switzerland	B Devier, DM de Vienne, J Shykoff, L Salvaudon
M. lagerheimii (MvSv1)	C11.1	*Silene vulgaris*	Swiss Alps	AK Gibson, ME Hood
M. lagerheimii (MvSv1)	C4.1	*Silene vulgaris*	Swiss Alps	AK Gibson, ME Hood
M. violaceum sensu lato (MvSp)	8A.2	*Silene paradoxa*	Swiss Alps	AK Gibson, ME Hood
M. violaceum sensu lato (MvSp)	4B.1	*Silene paradoxa*	Swiss Alps	AK Gibson, ME Hood
M. violaceum sensu lato (MvSp)	Sp1	*Silene paradoxa*	Swiss Alps	AK Gibson, ME Hood
M. violaceum sensu lato (MvLfc)	6-8B	*Lychnis flos-cuculi*	Swiss Alps	AK Gibson, ME Hood
M. violaceum sensu lato (MvLfc)	6-8E	*Lychnis flos-cuculi*	Swiss Alps	AK Gibson, ME Hood
M. violaceum sensu lato (MvLfc)	LF1	*Lychnis flos-cuculi*	Swiss Alps	AK Gibson, ME Hood

Table 3: Results of logistic regression for overall infection rate. Treatment (S-pair, S-mix, T-high, T-low) and genetic distance between crossed species are examined as predictor variables (Whole model: $p<0.0001$, $r^2=0.2652$).

Source	D.F.	χ^2	P-value
Treatment	6	98.624	<0.0001
Genetic distance	2	69.258	<0.0001
Treatment x genetic distance	6	84.237	<0.0001

Table 4: Results of logistic regression for hybrid infection rate. Treatment (S-pair, S-mix, T-high, T-low) and genetic distance between crossed species are examined as predictor variables (Whole model: $p<0.0001$, $r^2=0.4256$).

Source	D.F.	χ^2	P-value
Treatment	6	62.748	<0.0001
Genetic distance	2	129.777	<0.0001

SPORIDIA CROSSES

TELIOSPORE CROSSES

Sporidia of strain 1

Sporidia of strain 2

Teliospore of strain 1

Teliospore of strain 2

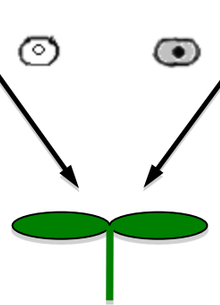
Nucleus of a_1 mating type

Nucleus of a_2 mating type

Diploid nucleus

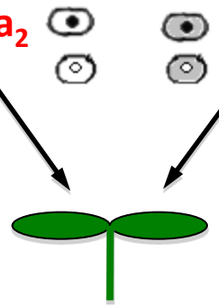
S-pair

Strain 1 (a_1) Strain 2 (a_2)



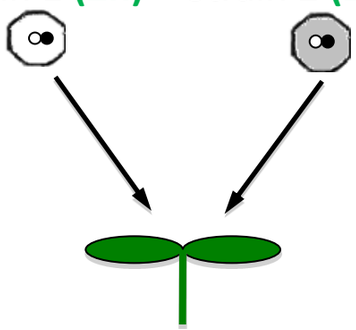
S-mix

Strain 1 (a_1, a_2) Strain 2 (a_1, a_2)



T-high

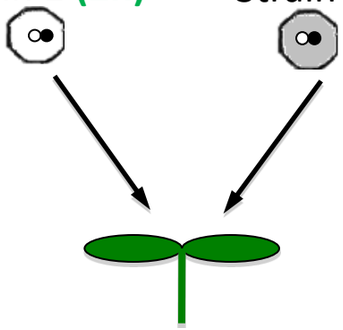
Strain 1 (2n) Strain 2 (2n)



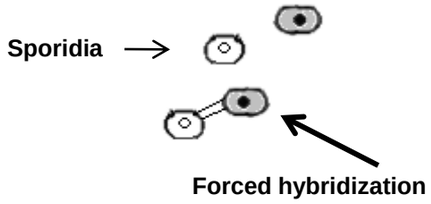
DILUTION

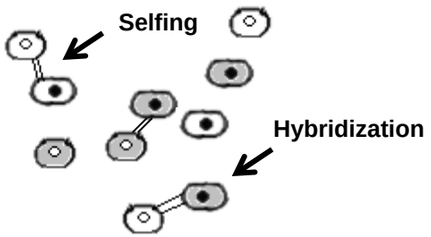
T-low

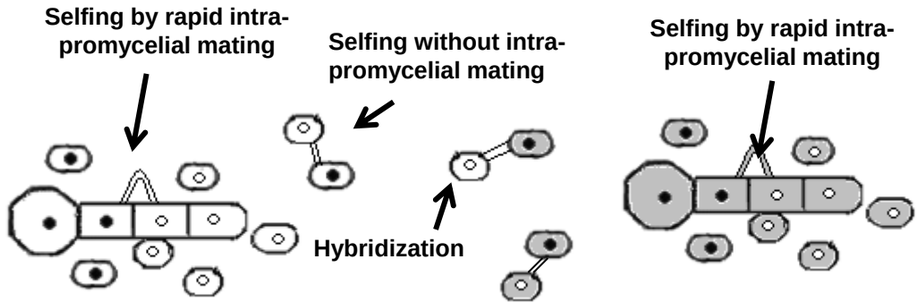
Strain 1 (2n) Strain (2n)

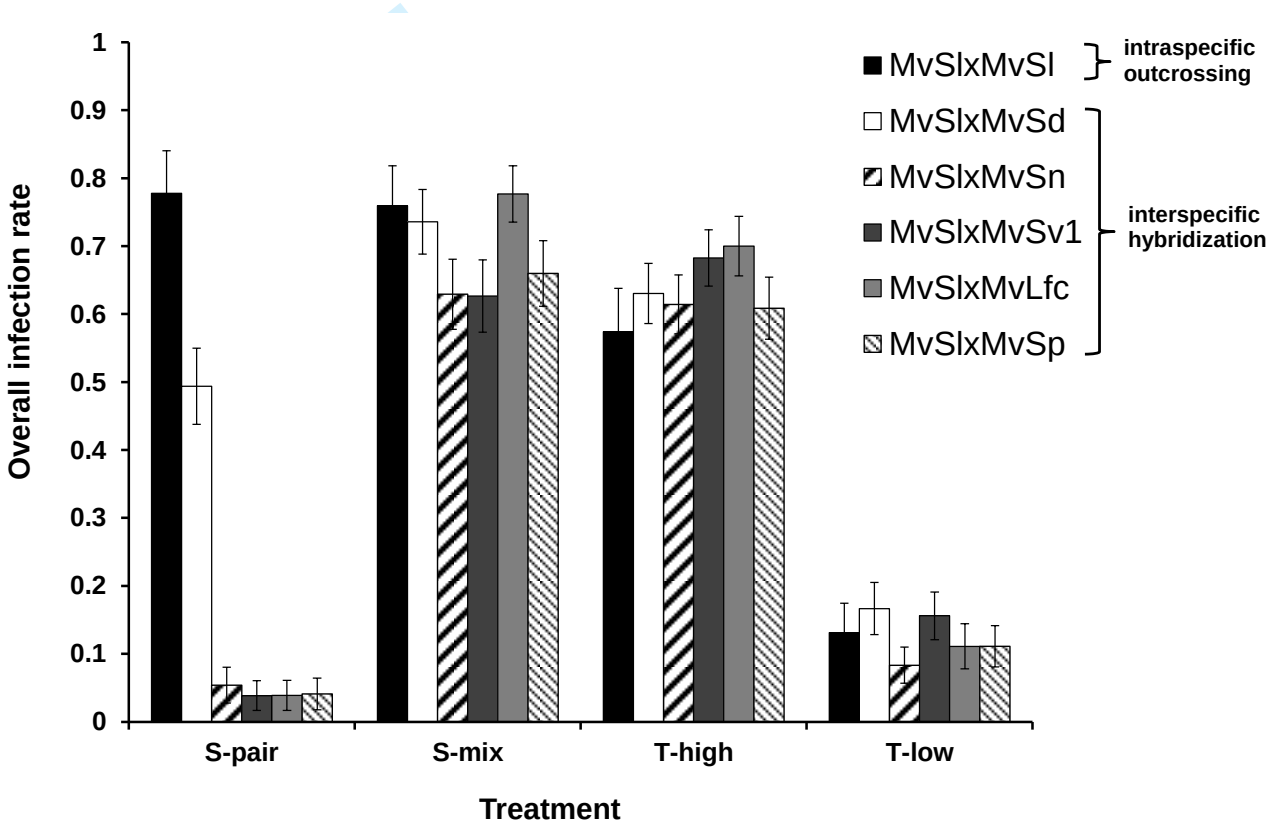


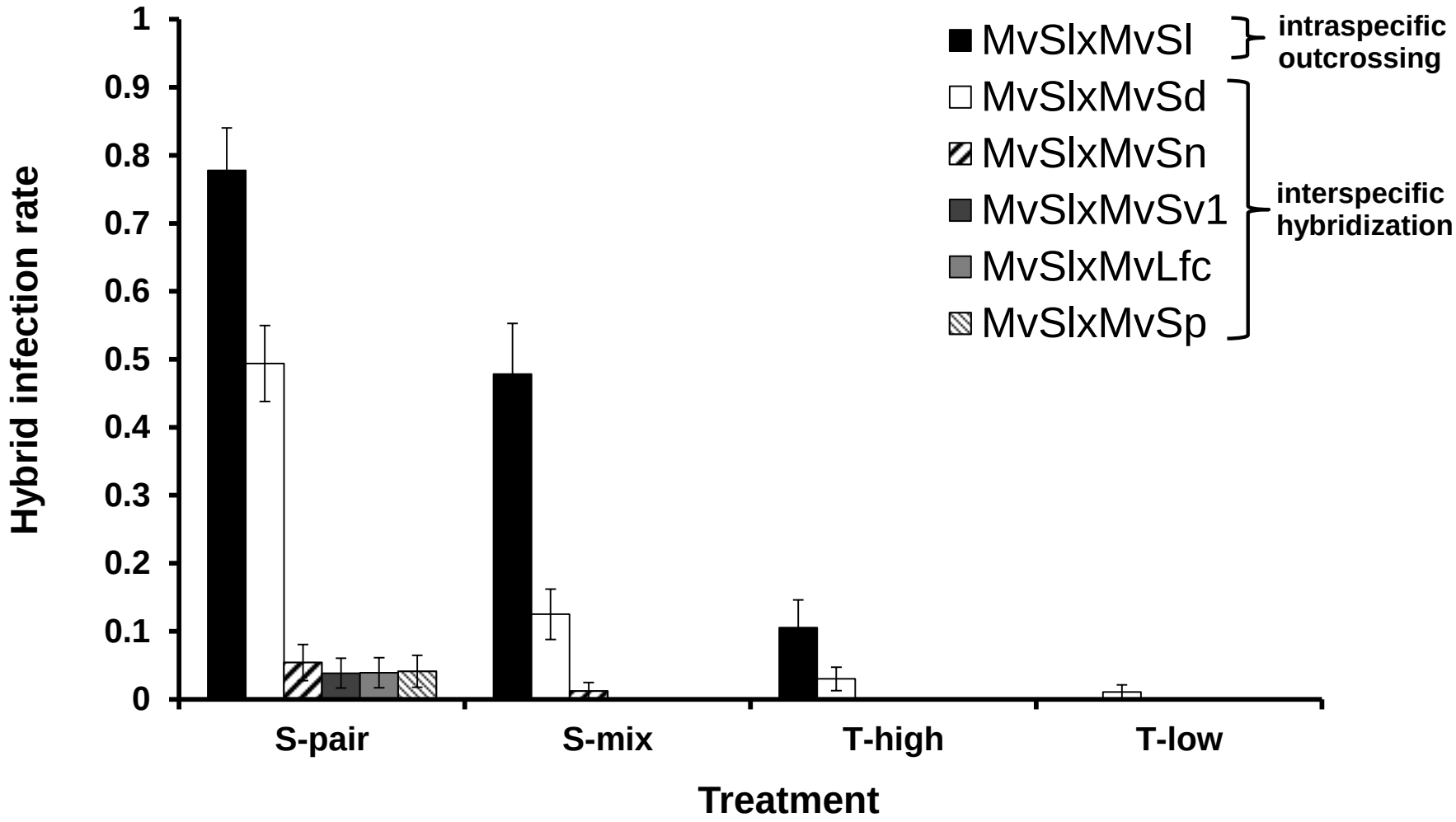
	S-pair	S-mix	T-high	T-low
Hybridization choice?	No	Yes	Yes	Yes
Selfing possible?	No	Yes	Yes	Yes
Intrapromycelial mating?	No	No	Yes	Yes
Competition?	No	Yes	Yes	Yes
Expected Hybrid Fitness	High	Moderate	Low	Lowest

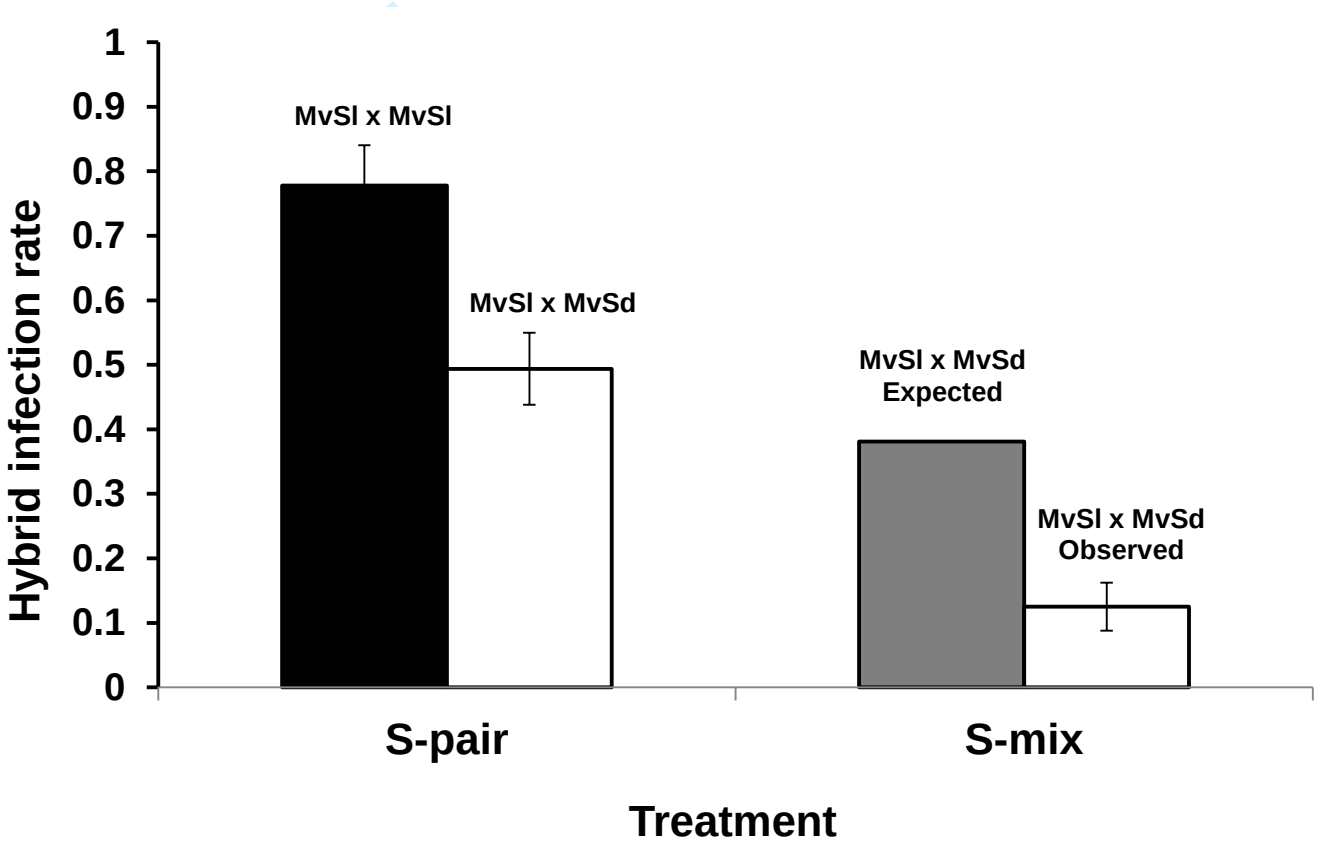







Overall infection rate
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
S-pair
S-mix
T-high
T-low
Treatment
MvSlxMvSl
MvSlxMvSd
MvSlxMvSn
MvSlxMvSv1
MvSlxMvLfc
MvSlxMvSp
intraspecific outcrossing
interspecific hybridization


Hybrid infection rate
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
S-pair
S-mix
T-high
T-low
Treatment
MvSlxMvSl
MvSlxMvSd
MvSlxMvSn
MvSlxMvSv1
MvSlxMvLfc
MvSlxMvSp
intraspecific outcrossing
interspecific hybridization


Hybrid infection rate
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
MvSl x MvSl
MvSl x MvSd
MvSl x MvSd Expected
MvSl x MvSd Observed
S-pair
S-mix
Treatment

Supplementary Table 1: **Identification of the 144 crosses performed.** Presented are the treatment (S-pair, S-mix, T-high, T-low), the crossed species, whether the cross is intra- or interspecific, whether the cross was conducted with teliospores or sporidia, the identity of the a_1 and a_2 sporidia for the S-pair treatment, the concentration of the inoculum (1x or 2x for sporidia, high or low for teliospores), and the total number of plants flowered for each cross. An X in either the a_1 or a_2 column indicates that sporidia of that mating type were not available for the species to be crossed with MvSl.

Treatment	Cross	Type	Teliospore/Sporidia	a_1	a_2	Concentration	No. flowered
	MvSl x MvSl						
S-pair	729.2 x 665.2	Intra	Sporidia	729.2	665.2	1x	18
S-pair	729.2 x 728.6	Intra	Sporidia	728.6	729.2	1x	11
S-pair	665.2 x 728.6	Intra	Sporidia	665.2	728.6	1x	16
S-pair	729.2 x 729.2	Intra	Sporidia	729.2	729.2	1x	22
S-pair	728.6 x 728.6	Intra	Sporidia	728.6	728.6	1x	16
S-pair	665.2 x 665.2	Intra	Sporidia	665.2	665.2	1x	12
S-mix	729.2 x 665.2	Intra	Sporidia			2x	21
S-mix	729.2 x 728.6	Intra	Sporidia			2x	20
S-mix	665.2 x 728.6	Intra	Sporidia			2x	13
S-mix	729.2 x 729.2	Intra	Sporidia			2x	22
S-mix	728.6 x 728.6	Intra	Sporidia			2x	16
S-mix	665.2 x 665.2	Intra	Sporidia			2x	12
T-high	729.2 x 665.2	Intra	Teliospore			high	23
T-high	729.2 x 728.6	Intra	Teliospore			high	20
T-high	665.2 x 728.6	Intra	Teliospore			high	18
T-high	729.2 x 729.2	Intra	Teliospore			high	18
T-high	728.6 x 728.6	Intra	Teliospore			high	17
T-high	665.2 x 665.2	Intra	Teliospore			high	22
T-low	729.2 x 665.2	Intra	Teliospore			low	18
T-low	729.2 x 728.6	Intra	Teliospore			low	18
T-low	665.2 x 728.6	Intra	Teliospore			low	25
T-low	729.2 x 729.2	Intra	Teliospore			low	18
T-low	728.6 x 728.6	Intra	Teliospore			low	19
T-low	665.2 x 665.2	Intra	Teliospore			low	23
	MvSl x MvSd						
S-pair	729.2 x 700.3	Inter	Sporidia	700.3	729.2	1x	15
S-pair	729.2 x 701.3	Inter	Sporidia	729.2	701.3	1x	12
S-pair	665.2 x 831.3	Inter	Sporidia	831.3	665.2	1x	16
S-pair	665.2 x 700.3	Inter	Sporidia	700.3	665.2	1x	14
S-pair	728.6 x 701.3	Inter	Sporidia	728.6	701.3	1x	10
S-pair	728.6 x 831.3	Inter	Sporidia	831.3	728.6	1x	14
S-mix	729.2 x 700.3	Inter	Sporidia		X	2x	17

S-mix	729.2 x 701.3	Inter	Sporidia	X		2x	13
S-mix	665.2 x 831.3	Inter	Sporidia			2x	18
S-mix	665.2 x 700.3	Inter	Sporidia		X	2x	12
S-mix	728.6 x 701.3	Inter	Sporidia	X		2x	13
S-mix	728.6 x 831.3	Inter	Sporidia			2x	14
T-high	729.2 x 700.3	Inter	Teliospore			high	22
T-high	729.2 x 701.3	Inter	Teliospore			high	20
T-high	665.2 x 831.3	Inter	Teliospore			high	23
T-high	665.2 x 700.3	Inter	Teliospore			high	17
T-high	728.6 x 701.3	Inter	Teliospore			high	18
T-high	728.6 x 831.3	Inter	Teliospore			high	19
T-low	729.2 x 700.3	Inter	Teliospore			low	18
T-low	729.2 x 701.3	Inter	Teliospore			low	17
T-low	665.2 x 831.3	Inter	Teliospore			low	12
T-low	665.2 x 700.3	Inter	Teliospore			low	18
T-low	728.6 x 701.3	Inter	Teliospore			low	16
T-low	728.6 x 831.3	Inter	Teliospore			low	15
	MvSl x MvSn						
S-pair	729.2 x 719.2	Inter	Sporidia	719.2	729.2	1x	11
S-pair	729.2 x 705.3	Inter	Sporidia	705.3	729.2	1x	11
S-pair	665.2 x 706.1	Inter	Sporidia	706.1	665.2	1x	18
S-pair	665.2 x 719.2	Inter	Sporidia	719.2	665.2	1x	11
S-pair	728.6 x 705.3	Inter	Sporidia	705.3	728.6	1x	13
S-pair	728.6 x 706.1	Inter	Sporidia	706.1	728.6	1x	10
S-mix	729.2 x 719.2	Inter	Sporidia		X	2x	6
S-mix	729.2 x 705.3	Inter	Sporidia		X	2x	16
S-mix	665.2 x 706.1	Inter	Sporidia		X	2x	18
S-mix	665.2 x 719.2	Inter	Sporidia		X	2x	15
S-mix	728.6 x 705.3	Inter	Sporidia		X	2x	15
S-mix	728.6 x 706.1	Inter	Sporidia		X	2x	19
T-high	729.2 x 719.2	Inter	Teliospore			high	23
T-high	729.2 x 705.3	Inter	Teliospore			high	22
T-high	665.2 x 706.1	Inter	Teliospore			high	20
T-high	665.2 x 719.2	Inter	Teliospore			high	21
T-high	728.6 x 705.3	Inter	Teliospore			high	17
T-high	728.6 x 706.1	Inter	Teliospore			high	24
T-low	729.2 x 719.2	Inter	Teliospore			low	18
T-low	729.2 x 705.3	Inter	Teliospore			low	19
T-low	665.2 x 706.1	Inter	Teliospore			low	17
T-low	665.2 x 719.2	Inter	Teliospore			low	17
T-low	728.6 x 705.3	Inter	Teliospore			low	17

T-low	728.6 x 706.1	Inter	Teliospore			low	20
	MvSl x MvSv1						
S-pair	729.2 x 432.87	Inter	Sporidia	729.2	432.87	1x	8
S-pair	729.2 x C4-1	Inter	Sporidia	C4-1	729.2	1x	13
S-pair	665.2 x C11-1	Inter	Sporidia	C11-1	665.2	1x	16
S-pair	665.2 x 432.87	Inter	Sporidia	665.2	432.87	1x	12
S-pair	728.6 x C4-1	Inter	Sporidia	C4-1	728.6	1x	12
S-pair	728.6 x C11-1	Inter	Sporidia	C11-1	728.6	1x	17
S-mix	729.2 x 432.87	Inter	Sporidia	X		2x	16
S-mix	729.2 x C4-1	Inter	Sporidia			2x	17
S-mix	665.2 x C11-1	Inter	Sporidia			2x	14
S-mix	665.2 x 432.87	Inter	Sporidia	X		2x	11
S-mix	728.6 x C4-1	Inter	Sporidia			2x	8
S-mix	728.6 x C11-1	Inter	Sporidia			2x	17
T-high	729.2 x 432.87	Inter	Teliospore			high	24
T-high	729.2 x C4-1	Inter	Teliospore			high	22
T-high	665.2 x C11-1	Inter	Teliospore			high	24
T-high	665.2 x 432.87	Inter	Teliospore			high	23
T-high	728.6 x C4-1	Inter	Teliospore			high	15
T-high	728.6 x C11-1	Inter	Teliospore			high	18
T-low	729.2 x 432.87	Inter	Teliospore			low	20
T-low	729.2 x C4-1	Inter	Teliospore			low	17
T-low	665.2 x C11-1	Inter	Teliospore			low	17
T-low	665.2 x 432.87	Inter	Teliospore			low	22
T-low	728.6 x C4-1	Inter	Teliospore			low	19
T-low	728.6 x C11-1	Inter	Teliospore			low	14
	MvSl x MvSp						
S-pair	729.2 x Sp1	Inter	Sporidia	729.2	Sp1	1x	12
S-pair	729.2 x 4B-1	Inter	Sporidia	4B-1	729.2	1x	14
S-pair	665.2 x 8A-2	Inter	Sporidia	665.2	8A-2	1x	10
S-pair	665.2 x Sp1	Inter	Sporidia	665.2	Sp1	1x	17
S-pair	728.6 x 4B-1	Inter	Sporidia	4B-1	728.6	1x	2
S-pair	728.6 x 8A-2	Inter	Sporidia	728.6	8A-2	1x	18
S-mix	729.2 x Sp1	Inter	Sporidia	X		2x	16
S-mix	729.2 x 4B-1	Inter	Sporidia		X	2x	16
S-mix	665.2 x 8A-2	Inter	Sporidia	X		2x	16
S-mix	665.2 x Sp1	Inter	Sporidia	X		2x	20
S-mix	728.6 x 4B-1	Inter	Sporidia		X	2x	12
S-mix	728.6 x 8A-2	Inter	Sporidia	X		2x	17
T-high	729.2 x Sp1	Inter	Teliospore			high	22
T-high	729.2 x 4B-1	Inter	Teliospore			high	16

T-high	665.2 x 8A-2	Inter	Teliospore			high	21
T-high	665.2 x Sp1	Inter	Teliospore			high	14
T-high	728.6 x 4B-1	Inter	Teliospore			high	22
T-high	728.6 x 8A-2	Inter	Teliospore			high	20
T-low	729.2 x Sp1	Inter	Teliospore			low	24
T-low	729.2 x 4B-1	Inter	Teliospore			low	14
T-low	665.2 x 8A-2	Inter	Teliospore			low	15
T-low	665.2 x Sp1	Inter	Teliospore			low	18
T-low	728.6 x 4B-1	Inter	Teliospore			low	16
T-low	728.6 x 8A-2	Inter	Teliospore			low	21
	MvSl x MvLfc						
S-pair	729.2 x 6-8B	Inter	Sporidia	729.2	6-8B	1x	12
S-pair	729.2 x 6-8E	Inter	Sporidia	6-8E	729.2	1x	6
S-pair	665.2 x LF1	Inter	Sporidia	665.2	LF1	1x	16
S-pair	665.2 x 6-8B	Inter	Sporidia	665.2	6-8B	1x	14
S-pair	728.6 x 6-8E	Inter	Sporidia	6-8E	728.6	1x	14
S-pair	728.6 x LF1	Inter	Sporidia	728.6	LF1	1x	15
S-mix	729.2 x 6-8B	Inter	Sporidia			2x	20
S-mix	729.2 x 6-8E	Inter	Sporidia		X	2x	23
S-mix	665.2 x LF1	Inter	Sporidia	X		2x	17
S-mix	665.2 x 6-8B	Inter	Sporidia			2x	15
S-mix	728.6 x 6-8E	Inter	Sporidia		X	2x	13
S-mix	728.6 x LF1	Inter	Sporidia	X		2x	15
T-high	729.2 x 6-8B	Inter	Teliospore			high	19
T-high	729.2 x 6-8E	Inter	Teliospore			high	16
T-high	665.2 x LF1	Inter	Teliospore			high	23
T-high	665.2 x 6-8B	Inter	Teliospore			high	19
T-high	728.6 x 6-8E	Inter	Teliospore			high	14
T-high	728.6 x LF1	Inter	Teliospore			high	20
T-low	729.2 x 6-8B	Inter	Teliospore			low	12
T-low	729.2 x 6-8E	Inter	Teliospore			low	17
T-low	665.2 x LF1	Inter	Teliospore			low	13
T-low	665.2 x 6-8B	Inter	Teliospore			low	15
T-low	728.6 x 6-8E	Inter	Teliospore			low	18
T-low	728.6 x LF1	Inter	Teliospore			low	15