From generalist to specialists: variation in the host range and performance of anther-smut pathogens on *Dianthus.*

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Abstract

Determining the processes that drive the evolution of pathogen host range can inform our understanding of disease dynamics and the potential for host-shifts. In natural populations, patterns of host range could be driven by genetically based differences in pathogen infectivity or ecological differences in host availability. In northwestern Italy, four reproductively isolated lineages of the fungal plant-pathogen *Microbotryum* have been shown to co-occur on several species in the genus *Dianthus.* We carried out cross-inoculation experiments to determine whether patterns of realized host range in these four lineages were driven by differences in infectivity and to test whether there was evidence of a trade-off between host range and within-host reproduction. We found strong concordance between field patterns of host range and pathogen infectivity on different *Dianthus* species using experimental inoculation, indicating that infection ability is a major driving force of host range. However, we found no evidence of a trade-off between the ability to infect a wider range of host species and spore production on a shared host.

INTRODUCTION

The host range of a pathogen, defined broadly here by the number of host species that a given pathogen species infects, is a critically important pathogen trait that can impact epidemiology (Cleaveland et al. 2001; Poulin and Keeney 2008), host community composition (Power and Mitchell 2004; Maloney et al. 2005; Alexander 2010; Mordecai 2011) and the risk of pathogen spillover into crop, livestock and human populations (Cleaveland et al. 2001; Woolhouse et al. 2005; Gilbert and Webb 2007). A distinction can be made between the 'realized host range' defined as the range of host species the

pathogen is observed to infect in nature, and the 'potential host range' defined as the range of host species that a given pathogen can infect under experimental conditions. The latter is particularly critical for assessing risks of host shifts and novel disease emergence (Woolhouse et al. 2005; Gilbert and Webb 2007, Antonovics et al. 2013). Determining the processes that drive the evolution of pathogen host range in natural populations has the potential to inform predictions of disease dynamics and the development of control measures.

Modern molecular phylogenetics has reshaped our understanding of the realized host range of many pathogens. Many pathogens with a similar morphology and which were once thought to be generalists on a wide range of host species, have been shown to be species complexes, composed of multiple cryptic species each specialized to a small number of hosts (Szabo 2006; Le Gac et al. 2007; Malenke et al. 2009; Rouxel et al. 2013; Vialle et al. 2013). However, phylogenetic methods alone may be insufficient for determining a pathogen's potential host range if sampling of host diversity is limited. For example, a pathogen may appear to be specialized if only one host species is well sampled. Experimental cross-inoculation studies can help bridge the gap between pattern and process by teasing apart infection ability from host availability (Poulin and Keeney 2008). Indeed, in most cases, inoculation studies have found that the potential host range of pathogens is greater than that observed in nature (Ebert 1998; King and Cable 2007; de Vienne et al. 2009; Parker et al. 2015). However, realized host ranges can be constrained if contact between host species is limited at fine spatial scales (Pedersen and Davies 2009, Stephens et al. 2019).

A key issue in the evolution of host range has been determining the factors that favor the evolution of specialists vs. generalists. One widely hypothesized mechanism is that a trade-off occurs in which pathogen traits that increase transmission within one species come at cost of reduced transmission on the other species (Fry 1990; Jaenike 1990; Kawecki 1994; Joshi and Thompson 1995; Ebert 1998). Evidence of such costs are exemplified by experimental evolution studies, that show a reduction in parasite fitness following several generations of selection on a novel host (Ebert et al. 1998, Bono et al. 2016). In natural populations, support for the trade-off hypothesis comes from observations of higher abundance of specialists compared to generalists on their shared hosts (Straub et al. 2011; Mederios et al. 2014). However, trade-offs are not always evident (Hellgren et al. 2009, Bedhomme et al. 2012). Moreover, the magnitude of trade-offs required to facilitate the evolution of host specialization will also depend on host community composition (Poisot et al. 2011; Asplen et al. 2012) and environmental heterogeneity (Bono et al. 2016). Thus, the trade-off approach to host breadth is best utilized when measures of trade-offs can be combined with knowledge of relative host abundance.

Anther-smut (caused by Basidiomycete fungi in the genus *Microbotryum*) is a pollinator-transmitted sterilizing disease that has become a model system for disease ecology (Bernasconi et al. 2009) and pathogen speciation (Giraud et al. 2008, 2010; Refrégier et al. 2008; Devier et al. 2010; Büker et al. 2013). Hosts infected with anther-smut produce fungal spores in place of pollen, and the ovary is also sterilized. The infection is systemic and can only be sustained on perennial hosts (Thrall et al. 1993; Hood et al. 2010). The

disease is found on a wide range of plant species, most commonly in the Caryophyllaceae (Hood et al. 2010; Kido and Hood 2019). While the disease was initially described as being caused by a single widespread species, *Microbotryum violaceum* (=*Ustilago violacea*), this is now known to be a species complex with multiple host-specific pathogen lineages (Lutz et al. 2005; Le Gac et al. 2007; Kemler et al. 2013). *Microbotryum* species infecting hosts in the genus *Silene* are highly specialized; each pathogen species is generally found on just a single host species (Le Gac et al. 2007). Moreover, this specificity is generally maintained even in sympatry with other closely related host species (Hood et al. 2019; Tang et al. 2019), although occasional host shifts have been identified (Antonovics et al. 2002; Refregier et al. 2008; Tyson et al. 2018; Hood et al. 2019).

Here, we measured the infectivity and disease-related traits associated with pathogen fecundity (i.e., number of diseased flowers and spore production) for anther-smut pathogens on *Dianthus* species that vary in their realized host range in nature. The anther-smuts found on *Dianthus* are an exception to the general rule of strict specificity of *Microbotryum*; we studied the infection potential of four distinct putative species (hereafter referred to as 'lineages') of the anther-smut pathogen associated with *Dianthus* host species in the Mediterranean Alps (Petit et al. 2017). These four lineages have highly overlapping geographic and realized host ranges but are maintained through substantial post-zygotic isolation mechanisms in the form of post-meiotic F1 sterility (Petit et al. 2017). Three of the four lineages were found on two or more host species, indicative of some generalism, while one lineage occurred only on a single host species, *D. pavonius,* indicative of specialization. However, such patterns of host range could also be affected by host geography and variation in ecological opportunity for cross-species transmission, as there is large variation in the degree of overlap of host species (Bruns et al. 2018).

In this study, we investigate three research questions about the realized and potential host range of *Microbotryum* lineages on *Dianthus*:

1. Is there concordance between the experimental infectivity of each pathogen lineage and its observed distribution on *Dianthus* host species in nature?

2. Do generalist pathogen lineages have lower infectivity and spore production than specialist lineages on their shared host species?

3. Does the infection ability of generalist and specialist pathogen lineages vary across host populations within a host species?

To answer these questions, we carried out two greenhouse cross-inoculation experiments. The first experiment tested the infection ability and sporulation capacity of the four fungal lineages described by Petit et al. (2017) on three different *Dianthus* host species, to determine the potential host range and fitness of each pathogen (Questions 1 and 2). The second experiment tested the infection ability of the most specialized and most generalized lineage on multiple different populations of a single host species, *Dianthus seguieri*, to determine the ability of generalists and specialist to infect variable populations *within* a single host species (Question 3).

METHODS

Study system

The Western Mediterranean Alps are a hot spot of diversity for *Dianthus* (Valente et al. 2010). We recently surveyed host and pathogen abundance across 59 elevational transects in this region of the Alps (Bruns et al. 2018) and found anther-smut disease on seven perennial species: *D. pavonius, D. furcatus, D. seguieri, D. sylvestris, D. superbus, D. carthusianorum, D. deltoides.* Here we focus on the three most abundant species, *D. pavonius, D. furcatus,* and *D. seguieri.*

Dianthus pavonius is common in alpine meadow habitats from 1500m-2300m. In this region, it is the most abundant of the three species: it was present in 80% of transects surveyed in the large-scale study of the distribution of hosts and anther-smut disease (Fig. 1A; Bruns et al. 2018). *Dianthus furcatus*, has an elevational range (1400m-2100m) that overlaps with *D. pavonius*, and these species are frequently found in sympatry, in some cases, only a few meters apart (e.g., see the Alberghi population in Fig. 1A). However, *D. furcatus* has a more limited range than *D. pavonius* and was present in just 20% of surveyed transects (Bruns et al. 2018). *Dianthus furcatus* is largely absent from the easternmost populations including the Valle Pesio population.

The third host species, *D. seguieri* is found at lower elevations between 500–1200m. We have only encountered close sympatry between *D. seguieri* and *D. pavonius* once, but they are frequently found in the same valley systems, separated by ca. 3–5km. We have never found sympatry between *D. seguieri* and *D. furcatus* (closest known locations 5km apart).

Petit et al. (2017) identified four unique genetic lineages of *Microbotryum* infecting these focal *Dianthus* species in this same region, based on 11 microsatellite markers and sequence data from 2 nuclear genes. One of these ('Lineage 1') was only ever found on *D. pavonius* plants in the field and thus appeared to be highly specialized. The other pathogen lineages showed a wider host range: Lineage 2 was found on all three *Dianthus* species, Lineage 3 was found on *D. pavonius* and *D. furcatus*, and Lineage 4 was found on *D. pavonius* and *D. seguieri.* Petit et al. (2017) also found that the distributions of the lineages varied in space: with Lineage 2 was more common in the northwest, while Lineage 4 was only found in the southeast (Fig. 1B).

Overview of experiments:

We carried out two cross-inoculation experiments. In **Experiment 1** (Table 1A), we tested the infection ability and sporulation capacity of the four different pathogen lineages (with 2–7 pathogen isolates per lineage) against three different *Dianthus* host species (with 2 populations per host species). Due to a low sample size in the host *D. seguieri*, this experiment included a second round of inoculations on this host in a follow up inoculation trial (Table S1), using a subset of isolates from each of the four lineages, to test repeatability (We did not have enough *D. furcatus* seeds for a repeat inoculation). In **Experiment 2** (Table 1B) we aimed to determine whether the specificity of the tested pathogen lineages was consistent across host populations within a species. We inoculated an additional eight

populations of a single host species, *D. seguieri,* with a single isolate from pathogen Lineage 1 and a single isolate from Lineage 2.

Experiment 1: Host species by pathogen lineage

Pathogen isolates—It would have been ideal to obtain all 12 combinations of host-of-origin and pathogen lineage, but a number of these were unavailable due to field specificities; for example, Lineage 1 was only found on *D. pavonius* (Petit et al. 2017). We eventually selected 19 pathogen isolates (Table 2; Fig. 1B) collected in July 2011 from the three focal host species in the Mediterranean Alps (10 from *D. pavonius,* 1 from *D. furcatus,* and 8 from *D. seguieri*). Microsatellite cluster analysis assigned these isolates to four distinct fungal lineages (Petit at al. 2017), all except two of these isolates (12 and 15) had <80% cluster assignment (Table 2). We therefore ran analyses with and without the inclusion of these two isolates (Supplemental Material S2). In total, there were 7 combinations of host-of-origin by fungal lineage out of a possible 12.

Host species—Seeds were collected in late August 2011, from six different geographic locations including two locations ("populations") per host species (Table 2; Fig. 1A). Where possible, we tried to collect seed from the same populations where spores were collected earlier in the season, however, this was not always possible since some populations had already dispersed their seeds. Within each population seeds were collected from 4 to 20 plants and mixed together to form a bulk population. The number of available seeds varied considerably, due to a combination of host density, temporal variation in seed set, and seed predation.

To ensure even germination, seeds were started in 150mm culture dishes filled with 0.75% Agar media, amended with a 10% Murishage and Skoog plant nutrient medium. Prior to starting, seeds were surface sterilized for two 3-minute washes in 20% commercial bleach, 10% ethanol solution and rinsed 5 times in sterile distilled water. Each seed was then nicked with a sterile razor blade to break the seed coat. Plates were incubated in a growth chamber with 12 h days at 20°C day and 18°C night temperatures.

Inoculations—To standardize inoculation, teliospores from each pathogen isolate were placed on potato dextrose agar media and germination rate was scored after incubation at room temperature for 24 hours. We used these germination rates to make spore suspensions that contained the equivalent of 500 viable spores/uL for each of the 19 isolates. Solutions were made with sterile water and a drop of surfactant (Triton X-100) to break up spore clusters. Seedlings were inoculated 7 days after planting by placing a 2uL drop of spore suspension solution onto the apical meristem of the seedling. Only seedlings that had fully expanded cotyledons were inoculated. In total, we inoculated 140 *D. furcatus*, 431 *D. pavonius,* and 164 *D. seguieri* seedlings. Several seedlings germinated but were not ready for the inoculation and were left as uninoculated controls. (See Supplemental Material).

Following inoculations, seedlings were incubated in the growth chamber at 20°C for an additional 7 days. Each seedling was then transferred to a 4 × 13.5cm 'conetainer' (Stuewe and Sons Inc, Tangent, Oregon) filled with PromixBX, and moved to a greenhouse with 12 hours light/dark cycle, 20° days and 18° nights. Treatments were completely randomized.

Plants were transferred outside in November for vernalization, and were moved back inside the greenhouse in April, prior to flowering.

Data collection—We recorded the date of first flowering, disease status, and number of inflorescences for each plant. To quantify spore production, we collected a single bud from each diseased plant. Each bud was dried and weighed and then cut open and put into vials with 1mL of 70% ethanol. Vials were vortexed and spores counted with an automatic cell counter (Cellometer Mini, Nexcelom Bioscience, Lawrence, Massachusetts). Inoculated plants that did not flower the first year were maintained and treated similarly for a second winter exposure and scored for disease the following spring.

***Repeat inoculation trial with* D. seguieri**—Final samples sizes for the *D. seguieri* and *D. furcatus* host treatments were much smaller than anticipated due to lower starting seed numbers, reduced survival, and low flowering rates. We did not have additional seeds of *D. furcatus* to run a repeat, but were able to carry out a partial replicate of Experiment 1 on *D. seguieri* using a subset of 10 of the tested pathogen isolates (Table 2, asterisk, Table S1). We inoculated each isolate onto 10-day old *D. seguieri* plants using greenhouse-reared seed from the two original *D. seguieri* populations. We used the same inoculation method detailed above, and recorded infection rate at flowering.

Experiment 2: Host population by pathogen lineage

To determine whether the pathogen lineage specificity measured in Experiment 1 was consistent across a broader range of host populations we collected seed from eight additional *D. seguieri* populations from across the study region in 2013 (Table 1B, Fig. S1) and inoculated these with a single isolate of Lineage 1 originating from *D. pavonius* (44.2529, 7.1151) and a single isolate of Lineage 2 originating from *D. seguieri* (44.2960, 7.1572). Both isolates were collected from the Rio Freddo valley. We focused on *D. seguieri* in this second experiment because this host species showed strong lineage specific susceptibility in Experiment 1; in particular, Lineage 1 was unable to infect *D. seguieri*, whereas Lineage 2 infected at a high rate.

Data analysis

A total of 57% of the inoculated plants in Experiment 1 flowered within 2 years and were scored for infection (N=420; Table 1). Isolate 5 did not produce a single infection and was removed from all subsequent analyses.

We used generalized linear mixed models, with a binomial link function, to determine the effect of host species and pathogen lineage on infection rate ('glmer' function in 'lme4' package, R 3.6.1). Host species and pathogen lineage were fixed effects in the model, with pathogen isolate nested as a random effect within lineage. Since several combinations of host population by pathogen isolate had fewer than 10 flowering individuals (Table 1A), we pooled host populations from the same species. We confirmed that host population did not have a significant effect within each lineage-by-host species combination using Chi-squared and Fisher exact tests (Table S3). Fisher tests also confirmed that replicate isolates from the same lineage and host-of-origin did not differ significantly in infectivity (Table S4).

To examine the effect of pathogen host-of-origin on infectivity, we ran binomial glm models with host species and host-of-origin within the two lineages (Lineage 2 and 4) that had replicate isolates collected from at least two different hosts.

To test the hypothesis that specialist and generalist pathogens differ in fitness-related traits on their shared host, we used hierarchical random effects models to determine the effect of pathogen lineage on flowering date (phenology), inflorescence number, and spore production on the most commonly shared host. We used the 'lme' function in R 3.6.1 to estimate the variance components of pathogen lineage and pathogen isolate (nested in lineage) and used likelihood ratio tests to determine the effect of pathogen lineage. We carried out a similar analysis to determine the effect of host-of-origin. Pathogen fitness-related traits were analyzed using only the set of plants that flowered in the first year. We also used linear regression, weighted by sample size, to examine the relationship between infection ability of individual *Microbotryum* isolates on different host species and spore production.

A total of 650 *D. seguieri* plants flowered and were scored in the repeat inoculation trial (Table S1). To test for repeatability between the two replicate runs on *D. seguieri* we used a glm model with experimental replicate, pathogen lineage and the interaction of experiment and lineage as fixed effects. We used a quasibinomial error structure, with an estimated dispersion parameter because there was evidence of significant overdispersion in a strict binomial model. For this analysis we only included the subset of Experiment 1 isolates that were tested in both inoculation trials (Table 2, asterisks).

For Experiment 2 we used a glm model with binomial error structure to examine the main effects of pathogen lineage and host population. To determine whether there was an interaction effect between pathogen lineage and population: we multiplied the infection rate of each population when inoculated with Lineage 2 by the overall average reduction in infection by Lineage 1, to generate the expected infection rate of each population by Lineage 1. We multiplied the expected infection rate by the observed sample size for each population and used a Chi-squared contingency test to evaluate whether the expected number of infected Lineage 1 plants deviated from the observed number. We did not do the test in other direction (e.g., predicting Lineage 2 infections from Lineage 1 infection rates) because several populations inoculated with Lineage 1 had zero infections.

RESULTS

Experiment 1.

A total of 370 inoculated plants (out of 735 transplanted individuals) flowered in the first year of the experiment. An additional 50 inoculated plants flowered and were scored in the two subsequent years. Inoculation had a significant, positive effect on flowering rate (See Supplemental Results, S5).

Infectivity:

Host specificity of pathogen lineages: Infection rate was significantly affected by pathogen lineage (X^2=9.801, df=3, p=0.0203) and host species (X^2=169.48, df=2,

p<0.0001). There was also a significant pathogen lineage by host interaction effect (X^2=40.8, df=6, p<0.000l). Lineage 1 showed the highest host specificity and was only able to infect *D. pavonius* (Fig. 2A). Lineage 4 was able to infect both *D. pavonius* and *D. seguieri,* but the latter to a lesser degree. Lineages 2 and 3 caused disease on all three host species. Exclusion of the two isolates with lineage assignment scores below 80% (isolates 12 and 15) had little effect on the results (Table S2).

Dianthus pavonius was the most susceptible of the three host species (Fig. 2A, pink bars) and infection rates did not differ significantly among the four lineages on this host species (X^2=5.547, df = 3, p-value = 0.135). *Dianthus seguieri* and *D. furcatus* were infected at a lower rate, and only then by a subset of lineages (Fig. 2A, green and blue bars). The three pathogen lineages that were able to infect *D. seguieri* differed significantly in their infectivity on that host (X^2= 56.415, df = 3, p<0.0001). Lineage 2 had the highest infection rate on *D. seguieri* (75%), while the other three lineages infected at much lower rates (0, 6, and 13%). In *D. furcatus,* only Lineage 2 and 3 were able to cause disease and there was no significant difference in infectivity between these two lineages (X^2= 0.014205, df = 1, p-value = 0.9051).

Overall, there was a strong concordance of the experimental infection rates (Fig. 2A) with the realized host range of these four lineages in the field (Fig. 2B). Lineage 1, which was only found on *D. pavonius* in the field (Fig. 2B), was only able to infect this host species in Experiment 1 (Fig. 2A). In addition, Lineage 2, which was found on all three host species in the field, was able to infect all three species in Experiment 1.

Host-of-origin of the pathogen: Within the most generalist Lineage 2, where isolates were available from each of the three host species, the host-of-origin had a significant main effect on infection rate (X^2=8.37, df=2, p=0.0152). There was also a significant host-of-origin by host species interaction effect (X^2= 18.41, df=4, p=.0010). Pathogen isolates performed the best, or equal to the best on their host-of-origin (Fig. 3). The case was most extreme for Lineage 2 isolates originating from *D. pavonius;* these infected *D. pavonius* at a high rate but were unable to infect *D. furcatus* and had low infectivity on *D. seguieri* (Fig. 3). Lineage 2 isolates originating from *D. seguieri* had the highest infectivity on *D. seguieri* and *D. pavonius,* but low infectivity on *D. furcatus.* The single Lineage 2 isolate collected from *D. furcatus* had high infectivity on *D. furcatus* and *D. pavonius,* but lower infectivity on *D. seguieri* (Fig. 3).

Isolates from Lineage 4 were collected from two different host species in the field (*D. pavonius, and D. seguieri;* Fig. 1A). However, within this lineage there was no significant effect of host-of-origin (X^2=0.002, df=1, p=0.964). Isolates of Lineage 4 collected from *D. pavonius* and *D. seguieri* had equally high infection on *D. pavonius* (93% and 80%, respectively), and equally low infection on *D. seguieri* (4% and 5%, respectively).

Effects of host population and isolate locality: We did not detect significant population differences among host populations within any of the three tested species in Experiment 1 (Table S3). Nor did we find evidence of variation in infectivity among pathogen isolates from the same lineage and host-of origin (Table S4).

Repeatability of D. seguieri *results.*—When we compared the infection rate of the same subset of isolates on *D. seguieri* in both experiments, we found a significant main effect of pathogen lineage (χ^2=68.2, df=3, p=0.034), and differences between experiments that approached significance (χ^2=23.7, df=1, p=0.061); the infection rate was lower in this repeat experiment (12%, N=432) than in Experiment 1 (40%, N= 67). However, there was no significant interaction between pathogen lineage and experiment (χ^2 =11.78, df=3, p<0.684) and the rank order of infectivity among the four pathogen lineages was consistent across the experiments: pathogen Lineages 2 and 3 were better at infecting *D. seguieri* than Lineages 1 and 4 (Fig. S2A). However, within the replicate experiment, there was a significant interaction between pathogen lineage and host population (X^2=8.71, df=3, p=0.0334; Fig. S2B), which was not detected in the original experiment.

Floral and spore traits

We measured spore production, flowering time and inflorescence number on 212 *D. pavonius* plants that flowered diseased in the first year. Among these diseased plants, pathogen isolate explained a significant amount of the total variation in flowering date (5.7%), inflorescence number (15.8%), and spore production (11%). However, there was no significant effect of pathogen lineage, or host-of-origin on any of these traits (Table 3). Lineage 1, which is specialized to *D. pavonius*, did not have higher spore production on this host species than the other pathogen lineages (Fig. 4).

We did not find any evidence of negative correlation between infection ability of individual *Microbotryum* isolates and spore production on *D. pavonius* (Fig. S3). In weighted linear models, neither infection rate on *D. pavonius* (t=0.363, p=0.722), nor infection rate on other *Dianthus* hosts *pavonius* (t=1.590, p= 0.133) was a significant predictor of spore production on *D. pavonius*. However, we urge caution interpreting this result, as the sample size for estimating infection rates of individual isolates was low.

Experiment 2: Variation among *Dianthus seguieri populations*

In the assessment of lineage specificity on multiple *D. seguieri* populations (Experiment 2), a total of 733 plants flowered. There was a significant effect of pathogen lineage on infection rate (X^2=389.26, df=1, p<0.0001). The Lineage 2 isolate (from *D. seguieri*) had a much higher overall infection rate (72.5%) on *D. seguieri* than the Lineage 1 isolate (from *D. pavonius*) (9.8%), similar to the results obtained in Experiment 1. There was also significant variation in infection rate among host populations by both the Lineage 2 isolate (X^2=35.49, df=7, p<0.0001) and the Lineage 1 isolate (X^2=74.2, df=7, p<0.0001; Fig. 5). There was also a significant interaction effect between pathogen lineage and host population; observed infection rates of Lineage 1 across populations differed from expected values based on Lineage 2 values (X^2=70.79, df=7, p<0.0001). Only the three *D. seguieri* populations, all from the eastern region (Populations 6, 36, 38) showed substantial susceptibility to the pathogen Lineage 1 (Fig. 5).

DISCUSSION

Variation in Pathogen Host range

We found that the infectivity of the four *Microbotryum* lineages on *Dianthus* was highly concordant with observed patterns of their distributions among natural populations of the host species. Lineage 1, which has only ever been found on *D. pavonius* in nature (Petit et al. 2017), does indeed appear to be a true specialist to this host. Replicate isolates from this lineage were able to infect *D. pavonius* at high rate (89%) but had very low infection rates (0–5%) on the other *Dianthus* species. In contrast, Lineage 2 which Petit et al. (2017) found on all three *Dianthus* species in the field, was also able to infect all three host species at high levels in the lab, indicating that it is indeed a true generalist. Lineage 3 was also able infect all three *Dianthus* species, although it had only previously been reported on *D. pavonius* and *D. seguieri.* Lineage 4 was found on *D. pavonius* and *D. seguieri* in the field, and was able to infect only these two species, but not *D. furcatus,* in the lab. Moreover, the lineage-specific infectivity that we found here was generally robust to variation among pathogen isolates within lineages, and among host populations within a species. These results therefore show that the wide host range of these *Microbotryum* lineages on *Dianthus* are not simply the result of occasional host-shifts or spillover events, and that these lineages are likely maintained as viable populations on multiple *Dianthus* host species.

These results with anther-smut fungi of *Dianthus* differ from patterns of host specificity of *Microbotryum* on host species in the genus *Silene* where phylogeographic studies have shown that most species of *Microbotryum* are specialized to a single host species (Lutz et al. 2005; Le Gac et al. 2007). While host shifts can and do occur between closely related species (Antonovics et al. 2002; Refrégier et al. 2008; Tyson et al. 2018), there has been little evidence to suggest a true generalist species in *Silene* that is actively maintained on two or more hosts. Indeed, patterns of host-specificity among *Silene*-infecting *Microbotryum* are present even in communities with sympatric host species (Van Putten et al. 2005; Gladieux et al. 2010; Hood et al. 2019) and overlapping floral phenologies (Tang et al. 2019). One possible explanation for the contrast in host breadth between *Microbotryum* on *Silene* and those pathogen lineages on *Dianthus* is that the *Dianthus* species studied here were part of very recent radiation that occurred during the early Pleistocene (Valente et al. 2010). Consequently, *Dianthus* species are less differentiated than older *Silene* species (a phenomenon noted by Darwin 1869, p. 311). More generally, phylogenetic distance is known to be an important predictor of host-pathogen specificity (Gilbert and Webb 2007; Refrégier et al. 2008; de Vienne et al. 2009; Streicker et al. 2010; Antonovics et al. 2013; Parker et al. 2015).

The discovery that there are *Microbotryum* lineages that are actively maintained on multiple host species of *Dianthus* raises new questions about the ecology of this group of anther-smut pathogens. The dynamics of multi-host pathogens can fundamentally differ from those of single-host pathogens (Woolhouse et al. 2001; Dobson 2004). For example, transmission from highly susceptible host species can maintain the disease on less susceptible species (Power and Mitchell 2004). Generalist pathogens can also affect host community structure through apparent competition (Tompkins et al. 2003; Mordecai 2011) and are more likely

to drive hosts to extinction than specialist pathogens (Woolhouse et al. 2001; Dobson 2004). However, the extent to which the disease dynamics of these multi-host lineages of *Microbotryum* may differ from the dynamics of specialist lineages is currently unknown. The only population where we have studied the transmission dynamics in depth contains a single host (*D. pavonius*) and a single pathogen lineage, Lineage 1 (Bruns et al. 2017). However, *Dianthus* species do occur in sympatry in other regions of the broader Western Alps (Bruns et al. 2018) and harbor multiple pathogen lineages (Petit et al. 2017), indicating the potential for multi-host interactions in nature.

Trade-offs

We expected to find evidence of a cost to generalism in the form of lower infectivity or spore production of the generalist lineages relative to the specialists on their shared host species, consistent with results from several other host-pathogen systems (Straub et al. 2011; Medeiros et al. 2014). However, we found no evidence of such trade-offs: Lineage 1, which was restricted to *D. pavonius* was not significantly better than any of the generalist lineages at infecting *D. pavonius*, nor did it have higher spore production following infection. This result was consistent across the two tested populations of *D. pavonius,* as well as replicate isolates of *Microbotryum* within each lineage. In contrast to the present study, *Microbotryum* species from *Silene* have been shown to have lower fitness on closely-related novel hosts than on their host-of-origin, producing fewer spores (Biere and Honders 1996) and abnormal flowers (Antonovics et al. 2002), and resulting in lower levels of sterility (Sloan et al. 2008).

The lack of strong trade-offs in the *Microbotryum* lineages on *Dianthus* may be one reason why generalist lineages are maintained in *Dianthus*-associated but not in *Silene*-associated *Microbotryum* lineages. Without trade-offs, generalist pathogens should have higher fitness than specialist through access to a broader range of transmission opportunities (Jaenike 1990; Joshi and Thompson 1995; Fry 1990). Why then is the specialist Lineage 1 so abundant on *D. pavonius*? One potential explanation is geographic variation in host species distributions. Petit et al. (2017) showed that the highest incidence of the *D. pavonius* specialist Lineage 1 was found within the eastern region where *D. pavonius* is largely allopatric from *D. furcatus* (See Fig. 1A), even though they grow sympatrically in other parts (Fig. 1B). *Dianthus seguieri* is generally not found in direct sympatry with *D. pavonius*, as it grows at lower elevations (Bruns et al. 2018). The specialist lineage could easily be maintained in these allopatric populations of *D. pavonius*. However, a lack of opportunity for cross-species transmission is unlikely to be the only explanation since Lineage 1 is also found throughout other parts of the hosts' range, including sympatrically with the other four lineages (Petit et al. 2017).

Another possibility is that there are trade-offs between host breadth and other pathogen traits we did not measure in this experiment, such as competitive ability or infection ability at later host life-stages. Previous work has shown that pathogen isolates vary in their ability to colonize, and drive out other *Microbotryum* isolates within a host (Hood 2003). If the specialist lineage is a superior competitor on *D. pavonius*, this could help explain its high abundance. It is also possible that costs of generalism are only evident when hosts are older.

For example, in birds, Medeiros et al. (2014) showed that specialist blood parasites were more common on the adults of shared host species than generalist parasites, but did not differ in relative abundance on juveniles. In this study, we only measured infection ability at the seedling stage, but in *D. pavonius* resistance to anther-smut increases substantially with age (Bruns et al. 2017; Bruns 2019). It could be that the specialist lineage has an infection advantage over the generalist on older *D. pavonius* plants.

Variation in susceptibility among host species

The three host species differed substantially in their overall susceptibility. *Dianthus pavonius* was a universally susceptible host to all the pathogen lineages and was infected at much higher rates than the other two species. This finding is consistent with observations from nature where we have found all four *Microbotryum* lineages on *D. pavonius* (Petit et al. 2017). Moreover, the high relative susceptibly of *D. pavonius* was consistent across two tested host populations, despite significant differences in disease prevalence observed in the field (the Valle Pesio population had a prevalence of 41%, while the Ferrere population was completely healthy). The potential epidemiological consequences of this variation in susceptibility among *Dianthus* species are unclear. In nature, all three host species have similar average disease incidence (59%, 55%, and 44%) and prevalence (17%, 20%, and 18% for *D. furcatus, D. pavonius,* and *D. seguieri*, respectively; Bruns et al. 2018).

There was substantial asymmetry in the species-specific susceptibility and infectivity of host and pathogen species. The pathogen with the most constrained host range (Lineage 1) was only found on the most susceptible host species (*D. pavonius*). A similar pattern was found by de Vienne et al. (2009) in a cross-species inoculation experiment with six *Microbotryum* species associated with: *Silene latifolia, S. dioica, S. vulgaris, S. paradoxa, Saponaria officinalis,* and *Dianthus carthusianorum*. They showed that host species varied in their susceptibility, and that the pathogens originating from more susceptible hosts had more restricted hosts ranges. For example, *D. carthusianorum* was susceptible to all six pathogen species, but the pathogen originating from *D. carthusianorum* was only able to infect *D. carthusianorum*. A similar pattern has been found in animals; Vázquez et al. (2005) showed that specialist parasites of fish and small mammals tended to infect hosts species that harbored the highest parasite richness. De Vienne et al. (2009) hypothesize that this type of asymmetric pattern could be caused by differences in strength of coevolution among different host-pathogen pairs. Strong coevolutionary interactions within one pair should lead to the evolution of greater host defenses and pathogen counter-defenses. Pathogens from these coevolutionary 'hot-spot' hosts may be better equipped to infect other, less well defended host species. In contrast, pathogens co-evolving with highly susceptible hosts, such as *D. pavonius,* may not evolve as many counter-offenses, making host shifts less likely.

The high abundance and universal susceptibility of *D. pavonius* may make it an open target for host shifts and lead to subsequent specialization. Indeed, our data shows that within the generalist Lineage 2, isolates from *D. pavonius* had lower infectivity on the other two host species compared with pathogens from the same lineage that were collected from either *D. furcatus* or *D. seguieri.* The phylogeny of the four *Microbotryum* lineages studied here has not been fully resolved, so we cannot infer the evolutionary history of the lineages. Gene

trees from the two nuclear loci indicate that Lineage 3 is monophyletic and basal, but fail to fully resolve Lineages 1, 2, and 4 (Petit et al. 2017). Further genomic work resolving these evolutionary relationships could help determine whether the specialist Lineage 1 is indeed recently derived from generalists, or vice versa.

***Variation in infectivity among D. seguieri* populations**—The host-species specificity of *Microbotryum* lineages varied among populations within a host species, in that the eight *D. seguieri* populations differed in their infection rate by two pathogen lineages. As expected, Lineage 2 (the generalist pathogen) was consistently better than Lineage 1 (the *D. pavonius* specialist) at infecting *D. seguieri* populations, but there were some interesting deviations. Lineage 2 had lower infectivity on host populations 6 and 7 from the San Bernardo di Mendatica locality in the southeast, and population 6 was also more susceptible to Lineage 1 (Fig. 4). Interestingly, Petit et al. (2017) found that Lineage 2 is rare in this region and Lineages 1 and 4 account for most of the *Microbotryum* samples from this locality. One possibility is that higher levels of host resistance in the southern *D. seguieri* populations has prevented the spread of Lineage 2 into this locality. Another possibility is that Lineage 2 has recently arrived in the south and has not yet had time to locally adapt. In either case, further information on the phylogeographic history of these *Microbotryum* lineages is needed.

CONCLUSIONS

The results of this study illustrate the complexity of host-parasite interactions during their "early" evolution, when host and pathogen species are young. Most macro-evolutionary models of host-parasite divergence assume specialization is an inherent trait of the pathogen (Brooks and McLennan 1991, de Vienne et al. 2007), and a shift onto a new host is followed by pathogen diversification and speciation. Indeed, this type of host-shift driven diversification has been clearly demonstrated for *Microbotryum* on *Silene* as evidenced by discordant phylogenies (Refrégier et al. 2008). However, in the *Dianthus-Microbotryum* system, the concordance between the experimental infectivity of each pathogen lineage and its observed distribution on several *Dianthus* host species in nature, has shown that *Microbotryum* lineages on *Dianthus* vary in host range from specialists on a single species to generalists capable of infecting several species. Specialist pathogen lineages can often co-occur with generalists, and do not necessarily have higher infectivity or spore production on their specialized host species. The infection ability of generalist and specialist pathogen lineages even varies across host populations within a host species.

This variation in host range is likely maintained by a combination of low costs of generalism, and heterogeneity in the degree of sympatry between different host species in different regions, which may allow the persistence of both specialist and generalist lineages. A further issue we have not addressed is that both the host species and the pathogen lineages are capable of hybridizing, with the latter appearing to be common (Petit et al. 2017). Therefore, an important future goal will be to integrate these newly described host range patterns into a broader understanding of speciation processes in host-pathogen systems.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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DATA ACCESSIBILITY

All the data described in the manuscript has been archived in the Dryad repository: https://doi.org/10.5061/dryad.q2bvq83jj

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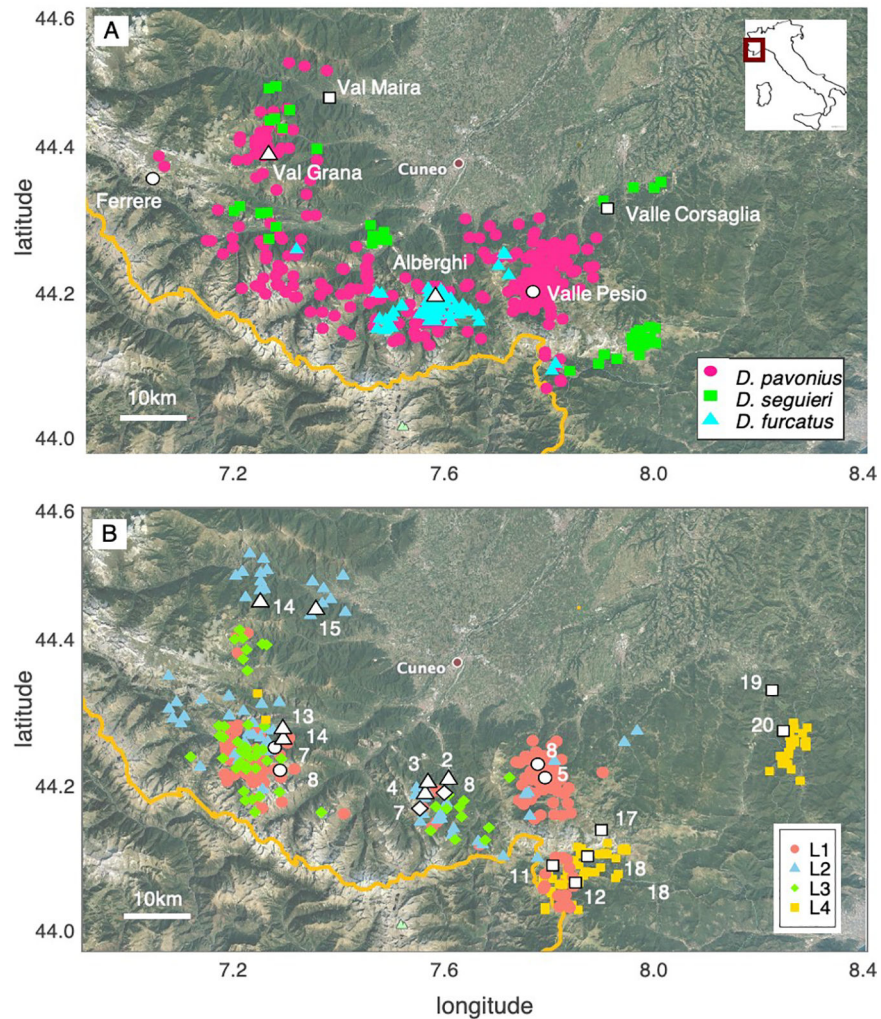
Fig. 1.

Distribution and sampling locations for pathogen and host accessions used in Experiment 1. **A)** Distribution of three *Dianthus* host species (data from Bruns et al. 2018). Colors and shapes indicate host species identity. Points were jittered randomly to minimize overlap. The six locations where seeds were collected are shown in the brighter outlined shapes and labeled by locality. The yellow line is the border with France (where we do not have distribution data). The inset shows the general location in Italy. The closest city, Cuneo, is marked for reference. Inset: the general location in Italy. **B)** Distribution of the four *Microbotryum* lineages (L1-L4) in the Mediterranean Alps (data from Petit et al. 2017). The location of the 19 *Microbotryum* isolates tested in Experiment 1 are shown in white (see Table 1).
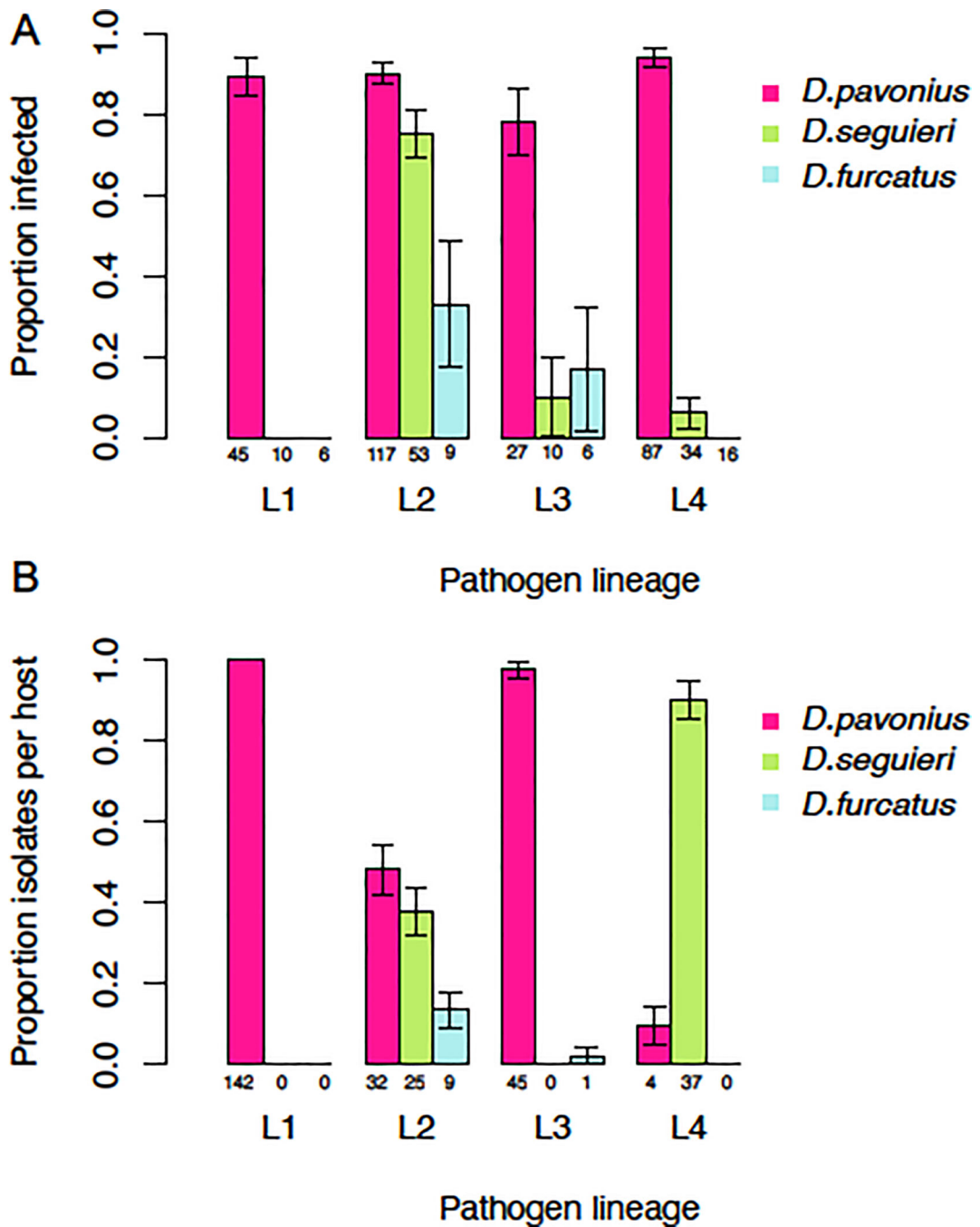
Fig. 2.
Comparison of the A) potential and B) realized host range of four *Microbotryum* lineages (L1-L4). A) Infection results from Experiment 1. Bars show the proportion of each host species infected by pathogen lineages L1-L4. Colors indicate host species: *Dianthus pavonius* (pink), *D. seguieri* (green), or *D. furcatus* (blue). Numbers indicate the total number that flowered and were scored. B) Field collections of the distribution of *Microbotryum* lineages on *Dianthus* species are shown for reference (data redrawn from Petit et al. 2017). Bars show the proportion of isolates from each lineage that were found on

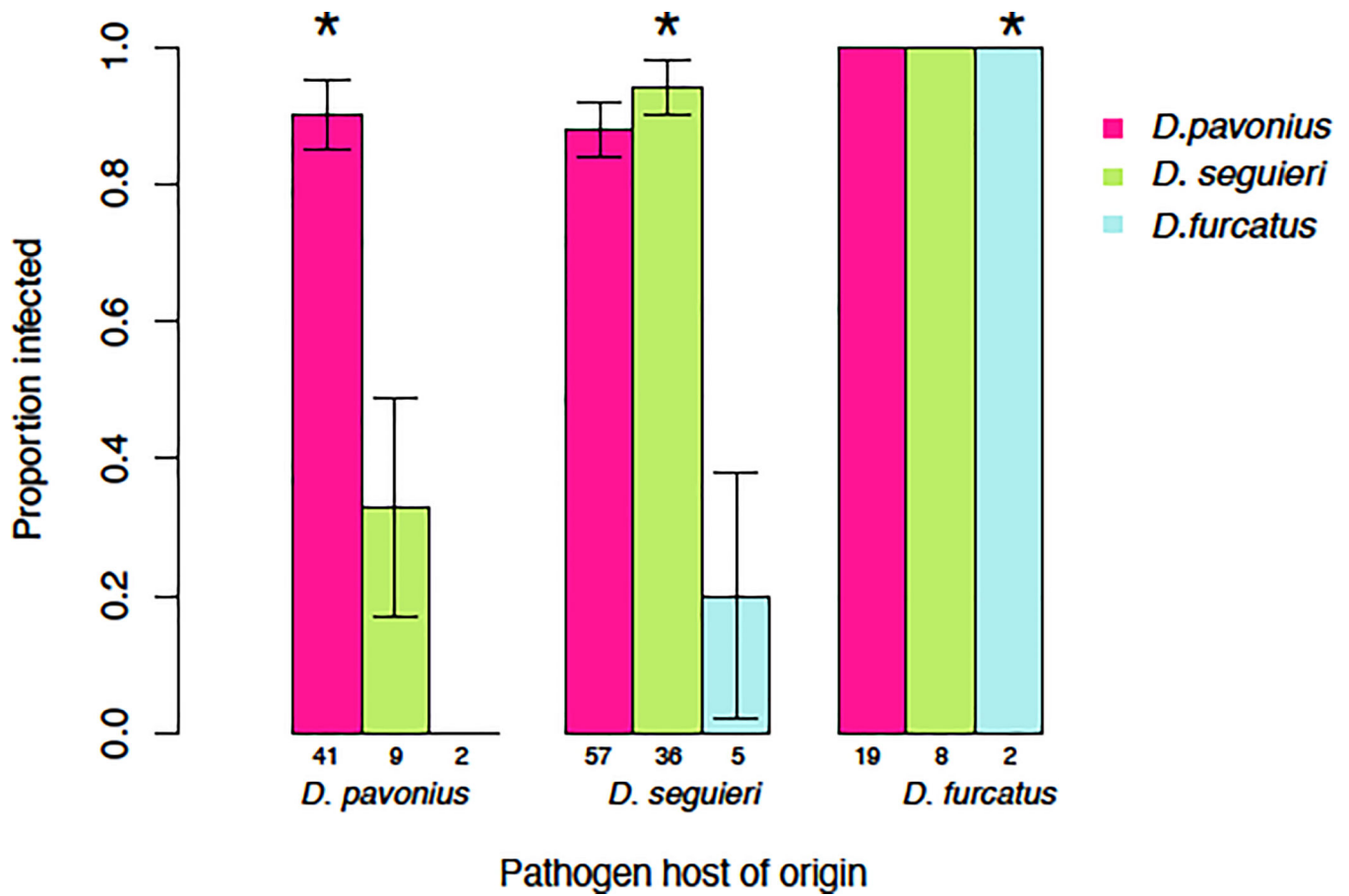each host. Numbers of isolates in each category are indicated at the bottom. Error bars are 1 SEM.

Fig. 3.
Infectivity of Lineage 2 isolates collected from three different hosts-of-origin in Experiment 1. Asterisks indicate combinations where the pathogen was tested on its own host-of-origin. Numbers indicate the total number of inoculated plants that flowered and were scored for disease. Error bars 1 SEM.
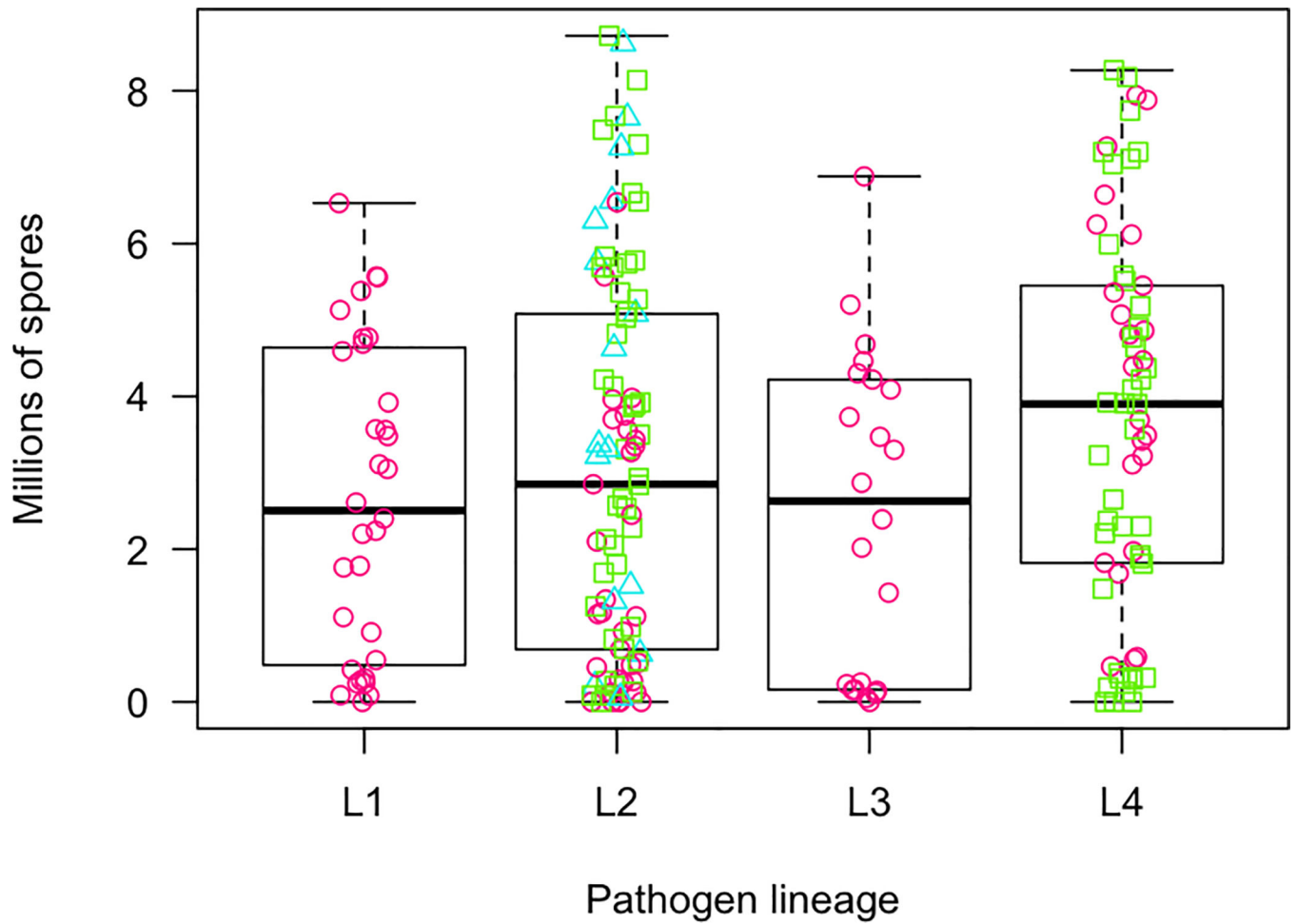
Fig. 4.
Spore production of four pathogen lineages on *Dianthus pavonius* of infected plants in Experiment 1 that flowered in the first year. Colors and shapes indicate host-of-origin: Pink circles-*Dianthus pavonius,* Green squares- *D. seguieri,* Blue triangles-*D. furcatus.* Neither lineage nor host-of-origin was a significant predictor of spore production (Table 3).

Fig. 5.
Infectivity of a single isolate of Lineage 1 (pink) and a single isolate of Lineage 2, (blue) on eight different *D. seguieri* populations in Experiment 2. Large numbers indicate the population ID number (Table 1B), small numbers indicate the sample size of each treatment. Error bars are 1 SEM.

Table 1.

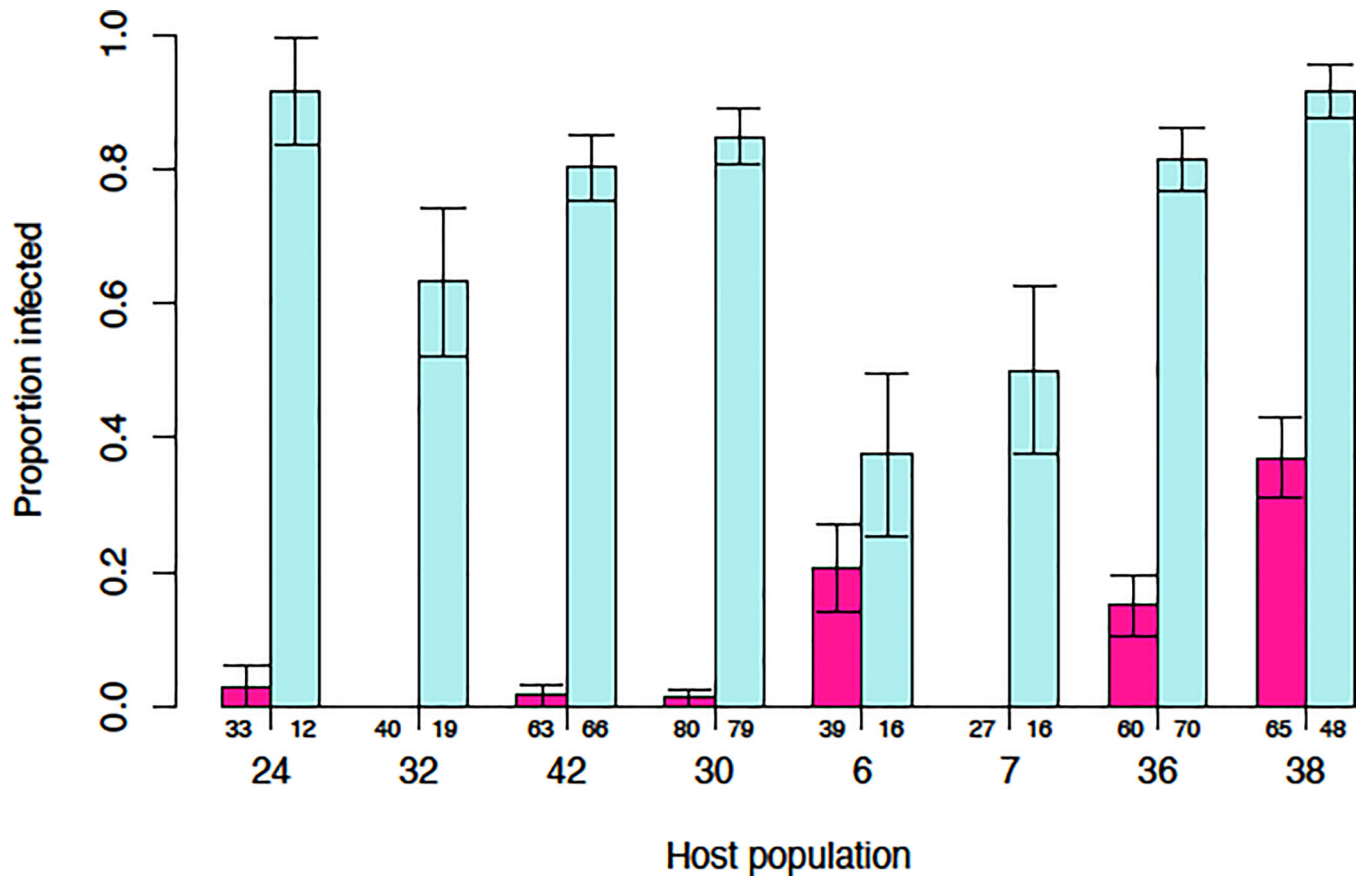Design and sample size (number of plants that survived and flowered) of the two cross-inoculation experiments. **A)** Experiment 1. **B)** Experiment 2, two pathogen lineages inoculated onto eight different populations of *D. seguieri.*

A) Experiment 1

Host population locations					Number of surviving plants that flowered			
					Line age 1	Lineage 2	Line age 3	Lineage 4
Host species	Host pop	Lat.	Long.	Num. Families sampled	(6,7,8)[1]	(2,3,4,13,14,15,16)	(9,10)	(11,12,17,18,19,20)
Dianthus pavonius	Valle Pesio	44.18 986	7.68 991	20	30	81	17	53
	Ferrere	44.36 022	6.92 607	8	15	34	8	32
Dianthus seguieri	Val Maira	44.48 215	7.28 111	5	6	27	3	27
	Valle Corsaglia	44.31 532	7.83 958	3	2	24	7	7
Dianthus furcatus	Alberghi	44.18 235	7.49 404	4	3	5	2	8
	Valle Grana	44.39 608	7.15 869	6	3	4	4	8

B) Experiment 2

Dianthus seguieri host population locations				Number of surviving plants that flowered	
Population number	Location	Latitude	Longitude	Lineage 1[2]	Lineage 2
24	Val Maira	44.4447	7.1784	33	12
32	Valle Grana	44.3638	7.2077	40	19
42	Val Maira	44.4121	7.2633	63	66
30	Valle Gesso	44.2689	7.4075	80	79
6	San Bernardo di Mendatica	44.1128	7.9199	39	16
7	San Bernardo di Mendatica	44.1078	7.923	27	16
36	Valle Corsaglia	44.3397	7.9434	60	70
38	Valle Corsaglia	44.3395	7.9434	65	48

[1] The identity of replicate *Microbotryum* isolates within each lineage are listed in parenthesis. Information on these isolates is found in Table 2

[2]Only a single isolate of each Lineage 1 and Lineage 2 were used in Experiment 2 and these differed from the ones used in Experiment 1 above. See text for details.

Table 2.

Lineage identity, host of origin, and collection location for 19 *Microbotryum* isolates used in the cross-inoculation studies on three host species: *Dianthus pavonius, D. seguieri,* and *D. furcatus.*

Path. Lineage	Isolate[1]	Lineage assignment score[2]	Host of origin	Location name	Host collected from same location	Lat	Lon
Lineage 1	5	0.982	*D. pavonius*	Valle Pesio	Yes	44.2085	7.7182
	6[*]	0.979	*D. pavonius*	Valle Pesio	Yes	44.2288	7.7035
	7[*]	0.824	*D. pavonius*	Rio Freddo	No	44.2196	7.1855
	8	0.979	*D. pavonius*	Rio Freddo	No	44.2538	7.1751
Lineage 2	2[*]	0.976	*D. furcatus*	Alberghi	Yes	44.1745	7.4955
	3[*]	0.96	*D. pavonius*	Alberghi	Yes	44.1705	7.4957
	4	0.917	*D. pavonius*	Alberghi	Yes	44.1705	7.4957
	13[*]	0.975	*D. seguieri*	Val Maira	Yes	44.4822	7.2811
	14	0.983	*D. seguieri*	Val Maira	Yes	44.5087	7.1289
	15[*]	0.747[*]	*D. seguieri*	Rio Freddo	No	44.2960	7.1571
	16	0.965	*D. seguieri*	Rio Freddo	No	44.2960	7.1592
Lineage 3	9[*]	0.885	*D. pavonius*	Alberghi	Yes	44.1677	7.4953
	10	0.9479	*D. pavonius*	Alberghi	Yes	44.1677	7.4953
Lineage 4	11[*]	0.872	*D. pavonius*	San Bernardo di Mendatica	No	44.0511	7.7472
	12	0.789[*]	*D. pavonius*	San Bernardo di Mendatica	No	44.0566	7.7360
	17[*]	0.98	*D. seguieri*	San Bernardo di Mendatica	No	44.0936	7.8278
	18	0.976	*D. seguieri*	San Bernardo di Mendatica	No	44.0953	7.8035
	19	0.975	*D. seguieri*	Lago di Osiglia	No	44.3106	8.2013
	20[*]	0.988	*D. seguieri*	Lago di Osiglia	No	44.3106	8.2013

[1] Asterisks indicate isolates that were also used in Experiment 2

[2] Probability of belonging to the assigned lineage in Structure analysis (Petit et al. 2107).

Asterisks indicate isolates with scores <80%, that may be hybrids.

Table 3.

Proportion of the variation in flowering and spore traits on *Dianthus pavonius* explained by either pathogen lineage or host of origin, accounting for the variation among isolates. Likelihood ratio tests and p-values show the overall significance of including lineage or host origin in the model.

	Among lineage or origin	Among isolate	LR.test	p
Days to first flower				
Pathogen lineage	<0.01%	14.60%	1.8E-07	0.9997
Host of origin	5%	10.60%	1.9E+00	0.1630
Log inflorescence number				
Pathogen lineage	<0.01%	5.70%	1.3E-07	0.9997
Host of origin	0.2%	5.54%	1.3E-07	0.9997
Total spores				
Pathogen lineage	<0.01%	11.30%	6.9E-07	0.9993
Host of origin	2.8%	9.40%	0.4654	0.4951