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Research

The 'black box' of plant demography: how do seed type, climate and seed fungal communities affect grass seed germination?

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Summary

- Demographic studies measure drivers of plant fecundity including seed production and survival, but few address both abiotic and biotic drivers of germination such as variation in climate among sites, population density, maternal plants, seed type and fungal pathogen abundance.
- We examined germination and microbial communities of seeds of *Danthonia californica*, which are either chasmogamous (external, wind-pollinated) or cleistogamous (internal, self-fertilized) and *Festuca roemeri*, which are solely chasmogamous. Seed populations were sourced across environmental gradients. We tested germination and used high-throughput sequencing to characterize seed fungal community structure.
- For *F. roemeri*, maternal plants significantly influenced germination as did climate and pathogens; germination increased from wetter, cooler sites. For *D. californica*, the main drivers of germination were maternal plant, seed type and pathogens; on average, more chasmogamous seeds germinated. Fungal communities depended largely on seed type, with fewer fungi associated with cleistogamous seeds, but the communities also depended on site factors such as vapor pressure deficit, plant density and whether the seeds had germinated.
- Putative pathogens that were negatively correlated with germination were more abundant for both *D. californica* and *F. roemeri* chasmogamous seeds than *D. californica* cleistogamous seeds. In *D. californica*, cleistogamous and chasmogamous seeds contain vastly different fungal communities.

Introduction

The fate of seeds in the seedbank often is not measured (e.g. Morris & Doak, 1998; Fréville & Silvertown, 2005; Lamichhane *et al.*, 2018); which creates a 'black box' within plant demography studies. Nonetheless, the gap between the number of seeds a plant produces, and the number of resulting seedlings, is often considered a key demographic bottleneck (e.g. James *et al.*, 2011; Reed *et al.*, 2020). There are numerous biotic and abiotic forces that interact to determine the fate of seeds. For example, the environmental conditions a maternal plant experiences influences seed size, which affects germination (Schmitt *et al.*, 1992; Kolodziejek, 2017; Larios & Venable, 2018); simultaneously, the abiotic environment can influence the abundance and composition of fungi at a location (Roy *et al.*, 2004; Spear *et al.*, 2015), and fungi can directly affect seed viability and germination (Blaney & Kotanen, 2001; Nelson, 2018). Studies document seed–fungal interactions in relation to germination rates in the seed bank, but seed fungal assemblages before dispersal into the soil environment are not well-characterized and also can affect germination (Nelson, 2018).

The composition and abundance of fungi, including pathogens, that reside in the soil, air and on plant tissues is driven by the abiotic environment, density of neighboring plants and maternal plant. Complex assemblages of fungi can interact with seeds (Shade *et al.*, 2017), ultimately affecting seed viability (Meyer *et al.*, 2010), dormancy (Blaney & Kotanen, 2001; Willis *et al.*, 2014) and germination (Blaney & Kotanen, 2001; Nelson, 2018). Fungi reside both on and inside seeds; some are transmitted vertically (such as endophytic fungi from the mother) and others, horizontally (dispersed from external environment) (Lamichhane *et al.*, 2018; Nelson, 2018). Seed exposure to different types of fungi can result in many different outcomes, ranging from seed death (Meyer *et al.*, 2010; Mordecai, 2013) to improved germination by means of fungal protection against other pathogens. For example, clavicipitaceous endophytic fungi such as *Epichloë* can protect grass seeds against fungal pathogen attack (Saikkonen *et al.*, 2016). Epiphytic seed fungi, however, have not been studied as extensively as seed endophytes, despite there being evidence that seed epiphyte communities are

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dominated by fungi that are known plant pathogens (Links *et al.*, 2014). The environmental and maternal factors that influence which fungi are transmitted to seeds are therefore important when considering germination success, and no studies to date have examined how native seed fungal communities differ among seeds from different climates, hosts and seed types concurrently. Furthermore, although seedborne pathogenic fungi often have been studied in ornamental and crop plants, the role of seed-associated fungal pathogens in determining the fate of seeds in natural systems remains unclear (Munkvold, 2009).

Germination is affected directly by the environment the seed is germinating in, but also indirectly by the environment the seed is produced in (Roach, 1987; Herman *et al.*, 2012). For instance, seeds from maternal plants experiencing stress resulting from temperature, drought or low resource availability can shift in germination time and dormancy phenotypes (Herman *et al.*, 2012). Biotic aspects of the maternal environment, such as density of neighboring plants, also can affect seed dormancy phenotypes and seedling establishment through ecological processes such as resource competition and facilitation (Ellner, 1986; Dyer *et al.*, 2000; Leverett & Shaw, 2019) as well as disease spread (reviewed in Nelson, 2018; Comita & Stump, 2020).

The most common mode of reproduction for plants is production of chasmogamous seeds, where seeds are produced from flowers that are external to the plant, exposed to the air and rain (Culley & Klooster, 2007; Baskin & Baskin, 2017). These flowers have the potential to be outcrossed either via wind, water or insects depending on the species, although in small populations inbreeding still may occur as a consequence of mating among close relatives (Howard, 1993). Some plants have evolved an additional strategy, in which cleistogamous flowers and seeds remain within the plants, and are thus never exposed to the air or water, and are self-fertilized (Culley & Klooster, 2007; Baskin & Baskin, 2017). Cleistogamy is uncommon, with only about 700 cases known among the > 250 000 species of angiosperms (Culley & Klooster, 2007), but of these ≥ 300 are grasses (Baskin & Baskin, 2017). On average, cleistogamous seeds have higher germination than chasmogamous seeds (Baskin & Baskin, 2017). This may be the result of cleistogamous seeds' ability to avoid air- and water-dispersed pathogens on the seed surface. However, cleistogamous seeds more often are infected by endophytic fungi, which can grow into the seeds from the maternal plant's tissues (Clay, 1994; Nelson, 2018). Although some endophytes have been shown to be beneficial, particularly the clavicipitaceous ones, there is a strong likelihood that many of the seed endophytes are latent pathogens (Geisen *et al.*, 2017). Plants that produce both chasmogamous and cleistogamous seeds present an unique opportunity to better understand the interactive effects of abiotic environment, plant density and seed-associated fungi on seed germination and dormancy.

Seed germination is the crucial first step for plant establishment. In this study, we investigated seed germination of two important native bunchgrasses (Poaceae), *Festuca roemeri* (Pavlick) E. B. Alexeev and *Danthonia californica* Bolander, which dominated presettlement prairies in the Pacific Northwest (PNW) (Noss *et al.*, 1995; Christy & Alverson, 2011) and are now being widely planted in restorations (Schultz, 2001; Applestein *et al.*, 2018; Lindh *et al.*, 2018). To understand the natural rates of seed production and germination, we recently completed a study of the demography of these grasses in remnant natural populations, and found that both measured environmental factors and unmeasured 'site' effects, potentially including local fungal communities, influenced population growth rates (DeMarche *et al.*, 2021). Here we begin to open the 'black box' of plant demography by examining how different abiotic and biotic drivers affect seed fungal community composition and seed germination of these grasses. Specifically, we examined the effects of seed type (cleistogamous and chasmogamous), grass species identity and density across a latitudinal climatic gradient in the PNW, USA from southern Oregon to Whidbey Island in the Northern part of Washington State. *Danthonia californica* produces both chasmogamous and cleistogamous seeds whereas *F. roemeri* is solely chasmogamous.

In order to test the germination of pre-dispersal seeds, we made a 'common garden' of Petri dishes in the laboratory, where seeds of each species were exposed to uniform conditions of moisture and temperature. Epiphytic (surface) and whole-seed fungal communities were characterized using microscopy combined with culturing and high-throughput sequencing, respectively, to address the following questions: (1) How does the maternal plants' environment affect seed germination in these grasses? (2) Which fungi are associated with the seeds? Specifically, what are the putative pathogens? (3) What are the relationships between fungal pathogen community composition and seed type, host species, and maternal environmental factors such as climate and conspecific plant density? (4) How do seed type, host species, environmental factors, and fungal pathogen composition affect seed germination? We expected germination to be negatively correlated with the relative abundances of pathogenic fungi, which are themselves directly influenced by climate, plant density and seed type. We expected lower germination from denser host populations if pathogen abundances are positively correlated with host density, reflecting higher transmission (Burdon & Chilvers, 1982; Katz & Ibanez, 2016). We also expected chasmogamous seeds to have greater abundance of pathogenic fungi than cleistogamous seeds, owing to their exposure to the external environment, and we expected higher pathogen abundance to result in lower germination.

Materials and Methods

The hosts

For focal host species, we chose two native, cool-season, C_3 bunchgrasses:

Danthonia californica and *Festuca roemeri*, which are key components of Pacific Northwest prairies (Christy & Alverson, 2011; Stanley *et al.*, 2011). *Danthonia californica* has variable dormancy resulting from seed coat-imposed dormancy, embryo-induced dormancy, no dormancy or all three (Darris & Gonzalves, 2019), and requires cold, moist stratification for best germination (Trask & Pyke, 1998; Russell, 2011; Darris & Gonzalves, 2019). *Festuca*

roemeri germinates best after 2 wk of cold, moist stratification (Wilson & Kaye, 2002; Darris *et al.*, 2012) but tends to have low germination in general (Russell, 2011; Darris *et al.*, 2012). We could find no information on what kind of dormancy *F. roemeri* has, except that it can be overcome by stratification (Wilson & Kaye, 2002; Darris *et al.*, 2008; Wilson *et al.*, 2008; Applestein *et al.*, 2018) and may have an environmental component (Wilson & Kaye, 2002).

Study areas

In June 2018, seeds were collected from six populations in Washington and Oregon of *F. roemeri* and nine populations of *D. californica* (Supporting Information Table S1); when they co-occurred, both species were collected from the same sites. These populations are on an aridity gradient, with less rainfall to the south and more to the north. All populations, except for *D. californica* from the Jefferson, Sublimity and Whidbey sites, were part of a demographic study initiated in 2015 to examine fitness across a climatic gradient running from southern Oregon to Northern Washington (DeMarche *et al.*, 2021). For *D. californica*, both cleistogamous and chasmogamous seeds, when present, were collected for each sampled plant. Seeds were collected from 10–25 individuals with multiple stems per site; the plants were selected haphazardly ≥ 1 m apart along the 1-m-wide transects used in the demography study (Reed *et al.*, 2020; DeMarche *et al.*, 2021); transect length in the demography study was variable and depended on plant density but contained ≥ 200 plants. The starting and ending latitude and longitude were recorded for each transect (Table S1). All seeds were kept at room temperature from the time of collection in June 2018 through the germination trials, which started in October 2018.

Environmental data

The environmental data for all of the sites are presented in Table S2. Climate data (latitude, elevation, precipitation, temperature, dewpoint and vapor pressure deficit (VPD)) were obtained using the continuously updated Parameter-elevation Regressions on Independent Slopes Model (PRISM) database (PRISM, 2018). We focused on the 2018 growing season to correspond to the year of seed production. We averaged means across spring (March–June) and winter (November–February), as these are expected to limit which plants and microbes can grow at a site (more closely than maximums or minimums with regard to temperatures). However, the winter data were so strongly correlated with spring data ($r > 0.70$) that winter variables were dropped from all analyses. The PRISM data were downloaded for the first transect of every site, as the transects were < 20 m apart. Plant density data were gathered *in situ* by counting individuals along a 1-m-wide transect until 200 plants were identified; then we calculated the average density of plants per m^2 for each site. Total soil nitrogen (N) measurements were determined by loss-on-ignition on a Costech Elemental Analyzer as described in Hendricks (2016).

Assessment of germination

We tested *c.* 50 seeds per maternal family per species; the numbers varied slightly owing to availability (mean ± SD 46 ± 10 seeds/maternal family for *F. roemeri* and 44 ± 6 seeds/maternal family for *D. californica,* of which half were chasmogamous and half cleistogamous). A diagram of the entire process that we followed, from seed collection to germination to sequencing of seed-associated fungi, including numbers of seeds at each step, is shown in Fig. 1. Importantly, all seeds were collected during the same time period and treated identically from collection to storage to germination trial. Sterile Petri dishes (90 mm) were lined with sterile filter paper and ≤ 25 seeds/seed type/maternal family seeds per dish were arranged to reduce contact, with *D. californica* seeds separated into different dishes according to their seed type. Hands, forceps and countertop were cleaned with alcohol between seed families to reduce carryover of seed fungi. All Petri dishes were watered to dampness with distilled water, placed immediately into cold storage at 4°C for 4 wk to break seed dormancy, and watered when necessary to keep damp but not wet. After cold stratification the seeds were kept at room temperature. Germinated seeds were recorded and removed every two days and Petri dishes re-watered as needed. Watering and seed removal took place under nonsterile conditions, but the dishes were open for a short time and all dishes were treated the same. To determine whether seeds were viable, we utilized a tetrazolium salt stain (Soares *et al.*, 2016) on a subset of 75 extra *F. roemeri* seeds unused in the germination trial (five seeds per mother, three to seven mothers per site, see Table S3). Tetrazolium stains only viable embryos and has been shown to be very effective in grasses (Soares *et al.*, 2016). The viability testing was performed nine months after the germination experiment was completed and is thus a conservative estimate.

Assessing seed epiphytic communities

When the number of newly germinating seeds approached zero (*c.* 6 wk after removal from 4°C), all ungerminated seeds ($n = 5414$), were examined with a dissecting microscope. Microbes on the seeds were categorized as fungi, bacteria or unknown. When possible, fungi were keyed to genus. A small subset of seeds that germinated ($n = 27$) also were examined for microbes.

In order to identify the fungi growing on ungerminated seeds to species and to verify the accuracy of morphology-based genus-level identifications, we isolated the fungi on culture media before extracting and sequencing DNA. The seeds were cultured on 90-mm-diameter plates of 2% water agar medium supplemented with 20 ml l^{-1} of an antibiotic solution (5 g l^{-1} penicillin, 5 g l^{-1} streptomycin and 1.5 g l^{-1} chloramphenicol) to inhibit bacterial growth. Fungi growing from the cultured seeds were isolated and maintained on 2% malt agar plates. A representative sample of each morphotype was subcultured in 2% malt liquid media and incubated at *c.* 25°C for 1–2 wk to ensure sufficient mycelial growth for DNA extraction. DNA extraction and Sanger sequencing protocols followed Thomas *et al.* (2016) Sequences

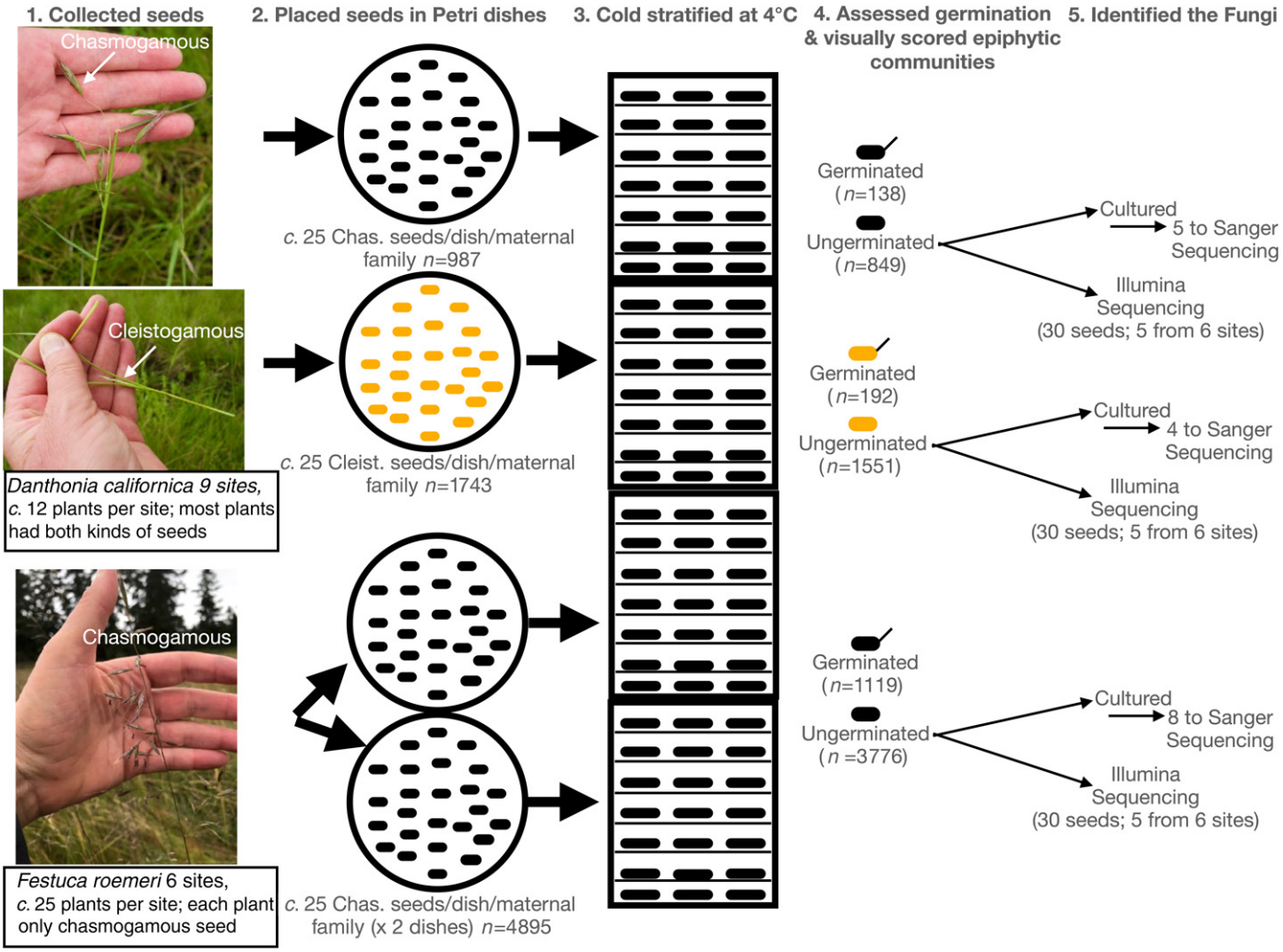

Fig. 1 Experimental design indicating the number of seeds used in each treatment.

then were aligned against the UNITE fungal database using the BLAST algorithm (Altschul *et al.*, 1990; Abarenkov *et al.*, 2010) to obtain species hypotheses (Koljalg *et al.*, 2013).

Assessing whole-seed communities with Illumina sequencing

We examined 90 ungerminated seeds (of 5414) from the germination experiment for whole-seed communities (Fig. 1). For ease of comparing the communities on the two host species, we used seeds from all the sites that had both *Festuca* and *Danthonia*, and those that had both species near to each other (e.g. the Table Rocks and Hazel Dell sites). At the conclusion of the germination experiment, one ungerminated seed was taken from each of five individual maternal plants of *F. roemeri* from each of six populations (French Flat, Upper Table Rock, Horserock, upper Hazel Dell, Upper weir and Whidbey) for a total of 30 seeds. For *D. californica*, one seed of each type (chasmogamous and cleistogamous) was taken from five maternal plants from each of six populations (French Flat, Lower Table Rock, Horserock, lower Hazel Dell, Upper Weir and Whidbey) for a total of 60 seeds.

DNA was extracted from whole single seeds with a DNeasy PowerPlant Pro Kit (Qiagen) following the manufacturer's protocol and stored at −20°C. The internal transcribed spacer region 1 (ITS1) was PCR-amplified in duplicate for all seed extracts using primer pair ITS1F/ITS2 adapted for the Illumina platform (Bokulish & Mills, 2013). Dual indexing of primers using Nextera barcoded primers (TAAGGCGA and CGTACTAG) between the forward Illumina adapter and primer pad permitted multiplexing with other projects. PCRs were performed in 25-μl reaction mixtures containing 12.5-μl GoTaq Green Master Mix (Promega Corp.), 0.5-μl ITS1F forward primer, 0.5-μl ITS2 reverse primer, 0.05-μl bovine serum albumin (BSA), 9.95-μl PCR-grade water and 1-μl template DNA. PCRs were run on a BioRad T100 thermal cycler (Bio-Rad). The following thermal cycler conditions were used: (1) initial denaturation at 94°C for 1 min, followed by 35 cycles of denaturing at 94°C for 30 s, annealing at 55°C for 30 s, and extension at 68°C for 30 s, (2) final extension at 68°C for 7 min, (3) hold at 4°C. Duplicate reactions were pooled and run on a 1% agarose gel to confirm amplification and expected amplicon length. Successful PCR products were quantified on a SpectraMax M5E Microplate

Reader (Molecular Devices, San Jose, CA, USA) with Quant-iT™ PicoGreen dsDNA Assay Kit (Invitrogen). Amplicons were pooled in equimolar concentrations and purified using the QIAquick PCR Purification kit (Qiagen). Paired-end sequencing (250 bp) on the Illumina MiSeq™ platform was carried out at the University of Oregon Genomics and Cell Characterization Core Facility (Eugene, OR, USA).

Bioinformatics and sequence processing

In order to characterize seed fungal community composition, we generated raw sequence data using Illumina MiSeq PE250 and demultiplexed using QIIME v.1.9.1 (Caporaso *et al.*, 2010). All reads were quality-filtered and assembled into amplicon sequence variants (ASVs) using the DADA2 pipeline v.1.10.1 (Callahan *et al.*, 2016), which does not utilize sequence clustering and thus accounts for common issues associated with traditional OTU approaches such as cryptic diversity and falsely detected taxa (Callahan *et al.*, 2017). Briefly, primers and adapters were removed from demultiplexed sequences using CUTADAPT v.1.10.1 (Martin, 2011) and reads were filtered for low quality using DADA2 FILTERANDTRIM with standard filtering parameters (Callahan *et al.*, 2016). Next, sequences were dereplicated and denoised, and paired end reads were merged with ≥ 12 bp overlap and 100% sequence similarity. Finally, chimeric sequences were removed and taxonomy was assigned using the UNITE database (Abarenkov *et al.*, 2010). To normalize differences in fungal amplicon sequence variant (ASV) count data across samples, we used a variance stabilization approach that incorporates a Bayesian mixture model and scales samples accordingly in the R packages PHYLOSEQ and DESEQ2 (Anders & Huber, 2010; McMurdie & Holmes, 2013). This method of normalization was chosen because it is more sensitive to detecting differential abundances and avoids taxon abundance biases introduced by traditional rarefying methods (McMurdie & Holmes, 2014). The resulting ASV table was used to identify putative functional groups of fungi using the FUNGUILD program (Nguyen *et al.*, 2016) (Table S4).

Analysis of germination

We assessed germination using a binomial GLMM in R/LME4 (Bates *et al.*, 2015). The random effects were site- and maternal family-nested within site, and the fixed effects were principal components axes for the environmental factors (see below). For *Danthonia*, we also included seed type as a fixed effect.

We used principal component analysis (PCA), fit to all of the sites for each species, to construct axes of environmental variation for each species using the prcomp function in R v.3.6.1 (R Core Development Team, 2019). We included the following variables in each PCA: latitude, elevation, spring precipitation (PPT), spring minimum temperature (minT), spring mean temperature (meanT), spring maximum temperature (maxT), spring mean dewpoint temperature (meanD), spring minimum vapor pressure deficit (minVPD), spring maximum vapor pressure deficit (maxVPD) and plant density. We also included soil % N (soil N) for *Festuca*, but excluded this variable for *Danthonia* owing to incomplete sampling (missing two sites) and its strong correlation with latitude with the sites we did have ($r = 0.95$).

For both species, the first three principal components explained > 90% of variation and were included as fixed effects in analyses of germination. For *Festuca*, these axes are (mostly): PC1 (50.1%), increasing PPT, decreasing meanT and increasing minVPD; PC2 (25.6%), decreasing maxVPD, decreasing maxT and increasing latitude; and PC3 (15.1%), decreasing plant density, increasing minVPD, and increasing minT (see Fig. S1 for biplot). For *Danthonia*, these are: PC1 (51.5%), increasing PPT, decreasing meanT and increasing minVPD; PC2 (24.8%), decreasing latitude, increasing maxT and increasing maxVPD; and PC3 (16.9%), increasing plant density and decreasing minVPD (see Fig. S2 for biplot).

We tested the significance of random effects of site and maternal plant nested within site using likelihood ratio tests, and the significance of fixed effects using Wald Z tests (Bolker *et al.*, 2009). For one *Danthonia* site (Whidbey Island), seeds were mistakenly pooled during collection and we were unable to separate maternal families. Excluding this site from analyses did not alter conclusions about the significance of maternal or site effects, so we retained it in analyses to increase power for testing effects of seed type and environmental variables on germination.

Analysis of fungal communities

All statistical analyses of whole-seed fungal communities were performed in R v.3.6.1 with the variance-stabilized ASV table. To assess compositional shifts in whole-seed fungal communities, we used the Bray–Curtis dissimilarity index and nonmetric multidimensional scaling (NMDS) plots in R/PHYLOSEQ (McMurdie & Holmes, 2013). Statistical significance of shifts in community composition across site, seed type and host species were tested using a PERMANOVA analysis with the 'adonis' function in R/VEGAN (Dixon, 2003). Sequences assigned as putative plant pathogens by FUNGUILD were subsetted for further analyses; we also appended this dataset with other ASVs not assigned as pathogens by FUNGUILD that we considered potential pathogens. All ordination and PERMANOVA analyses were repeated for the fungal pathogen dataset. Correlation between shifts in our whole fungal community composition, and shifts in the subsetted fungal pathogen communities was calculated using a Mantel test on the Bray–Curtis dissimilarity matrices. Correlations between seed fungal pathogen community composition and individual environmental variables were measured using vector fitting with the function 'envfit' in R/VEGAN package; these vectors were fitted to the NMDS ordination for fungal pathogen composition across seed types. To uncover relationships between germination, plant density and specific fungal pathogens, we calculated the Spearman correlations between these variables and individual fungal ASVs in the pathogen dataset. For this analysis, the ASV count data were Hellinger-transformed using the 'decostand' function in R/VEGAN, and correlations were calculated using the 'rcorr' function in R/HMISC (Harrell, 2016). ASVs that were significantly correlated ($P < 0.05$) with plant density or germination were subsetted for each seed type and visualized using the 'ggcorr' function. Differentially abundant ASVs (Table S5) were

identified using DESEQ with a cutoff of $P < 0.05$; this package utilizes negative binomial generalized linear models to estimate log-fold changes in sequence count data (Anders & Huber, 2010). All data visualization and figures were generated using R/GGPLOT2 (Wickham, 2009).

Raw sequence data for the high-throughput Illumina sequence analyses are publicly available through the Sequence Read Archive, BioProject accession number PRJNA627690.

Results

Does the maternal environment of growth affect seed germination?

***Festuca roemeri* germination** There was strong support for an effect of the first environmental PC (Fig. 2a); germination increases with increasing PC1 (0.71, $z = 6.47$, $P < 0.0001$; increasing precipitation, wetter minVPD and cooler meanT). Neither the second nor third PC axes had significant effects on seed germination. There also was no support for additional variance explained by site after accounting for environmental effects (var. = 0.13, likelihood ratio test: $X^2 = 1.41$, df = 1, $P = 0.24$). However, there was significant variation among *F. roemeri* maternal plants within sites (Fig. 2b) in germination success (var. = 2.29, likelihood ratio test: $X^2 = 549.94$, df = 1, $P < 0.0001$). The full model is shown in Table S6. Leftover seeds, which were not used in the germination trial (unwatered and stored at room temperature), were still viable after the trial had concluded (Fig. S3; Table S3).

***Danthonia californica* germination** There was significant variation in germination by maternal plant within site (var = 0.28, likelihood ratio test: $X^2 = 13.43$, df = 1, $P < 0.001$; Fig. 2c) and by site (var = 0.54, likelihood ratio test: $X^2 = 35.63$, df = 1, $P < 0.001$; Fig. 2c). However, there were no significant additional effects of the environmental PC variables on germination. There was a significant effect of seed type ($z = -2.26$, $P = 0.0236$), with greater germination of chasmogamous seeds, on average (Fig. 2c). Full model shown in Table S7.

Which fungi are associated with the seeds?

On the seed surfaces of ungerminated seeds, we could visually differentiate four fungal genera, a small group of unidentified fungi and bacteria under a dissecting microscope (Figs 3, S4; Table S8). Sanger sequencing of seed-surface cultures and BLAST

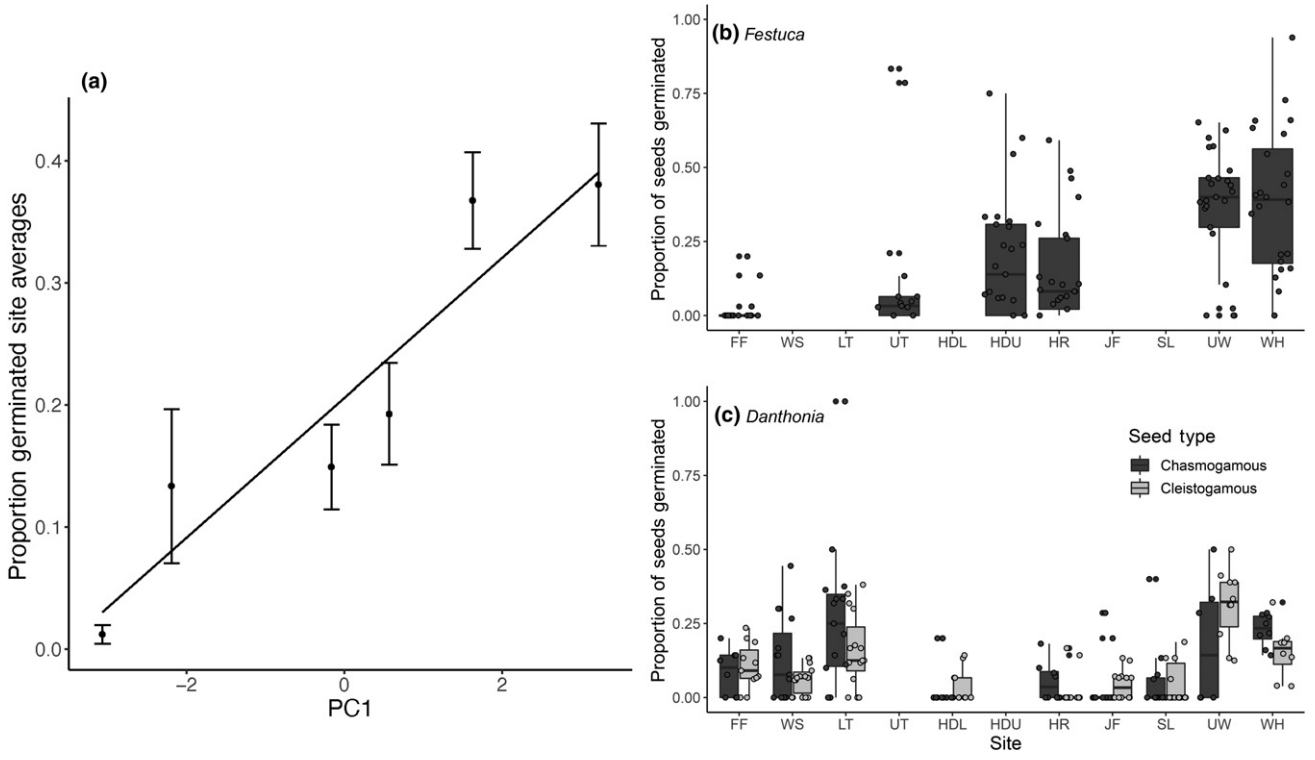

Fig. 2 *Festuca roemeri* significant regression of germination against the first principal components axis (PC1) of environmental variation (a). PC1 runs from drier to wetter minVPD, warmer to cooler maxT, and lower to higher precipitation (VPD, vapor pressure deficit). Points show the mean (±SE) germination across maternal families in each site. Numbers of seeds germinated per site, in order from lower left to upper right in the graph: FF = 674, UT = 630, HDU = 528, HR = 943, UW = 1094, WH = 1020). Box plots of germination showing the variation by maternal family and seed type within each site for *F. roemeri* (b) and *Danthonia californica* (c). In (b) and (c) each dot represents a maternal family mean ($n = 2$–59/maternal family). The sites on the *x*-axes are ordered from south (left) to north (right). Number of seeds germinated per species, seed type and site are summarized in Supporting Information Table S10. Although germination was tested for *D. californica* from the WH site, it was not included in this analysis because the seeds were not kept separate by maternal family. FF, French Flat; WS, Whetstone; LT, Lower Table Rock; UT, Upper Table Rock; HDL, Hazel Dell Lower; HDU, Hazel Dell Upper; HR, Horse Rock Ridge; JF, Jefferson; SL, Sublimity; UW, Upper Weir; WH, Whidbey.

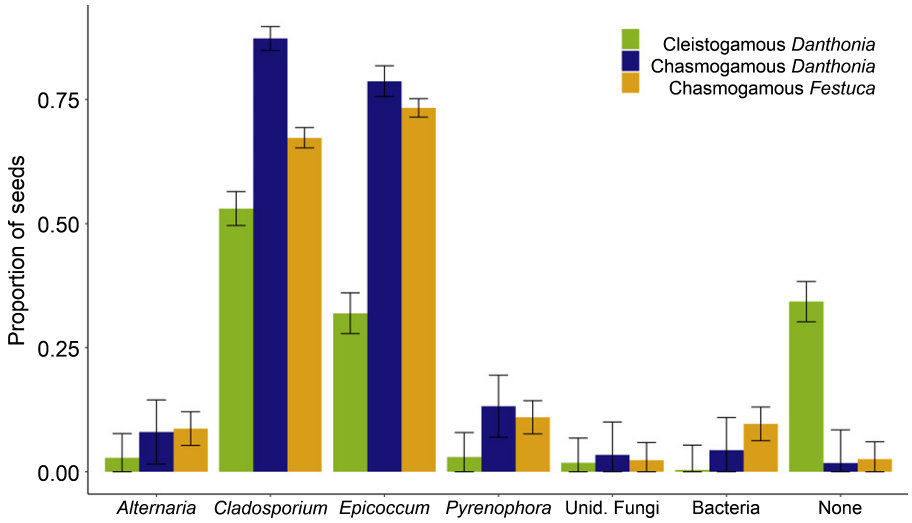

Fig. 3 Proportion of seeds with particular epiphytic microbes present or absent (none that were visually apparent) on ungerminated *Danthonia californica* and *Festuca roemeri* seeds. Unid, unidentified. Proportions ± 95% confidence interval.

in the UNITE Fungal Database yielded the four fungal genera identified morphologically plus three others (*Bjerkandera, Botrytis* and *Sistotrema*), and enabled species hypotheses (Table S9). The only genus that we identified morphologically whose sequences were not unambiguously assigned a firm species hypothesis was *Pyrenophora*. Instead, the UNITE database automatically assigned our sequences to Pleosporaceae because there were many different species names associated with identical and similar (1–3% divergent) sequences. However, these were almost all species of *Pyrenophora* or its anamorph (asexual state), *Drechslera*. For this reason, and because we had morphology for identification as well, we include *Pyrenophora* in Table S9.

From the whole seeds, a large number (6648) of ASVs were uncovered, including all of the genera found earlier on the seed surfaces (list of taxa assigned to genus or species is in Table S4 and the full sequence dataset is in NCBI, Bioproject accession no. PRJNA627690). There were significant shifts in whole fungal community composition across seed type, site and host species (Fig. S5; PERMNOVA $P \leq 0.01$). A total of 832 fungal ASVs were identified by FUNGuild as being putative plant pathogens (Table S4). *Botrytis* and *Hygrocybe* were not included as pathogens in the original FUNGuild output, but we added them to the putative pathogen dataset based on established trophic modes of these genera from grasslands in previous studies (Dean *et al.*, 2012; Halbwachs *et al.*, 2018). We also added three other genera to the putative pathogen subset for exploratory graphing and statistics because they were found in the morphological study: *Bjerkandera, Penicillium* and *Sistotrema*. The Bray–Curtis dissimilarity matrices for the whole fungal community dataset and pathogen dataset were strongly correlated (Mantel statistic $r = 0.8261$, $P = 0.001$ with 999 permutations).

What are the relationships between fungal pathogen composition and seed type, host species and maternal environmental factors?

We found that the proportion of cleistogamous seeds infected was lower than of the chasmogamous seeds for all groups of epiphytic microbes identified on the surface of seeds (Fig. 3). Furthermore, 34% of cleistogamous seeds had no visually discernible fungi on their surfaces vs only 1% of chasmogamous seeds (Fig. 3; Table S8).

At the whole-seed level, we found significant shifts in pathogenic fungal community composition across seed type (Fig. 4, seed type PERMANOVA $P = 0.001$). Additionally, pathogen community composition depended more on site and seed type than host species (site and seed type PERMANOVA $P \leq 0.001$, Fig. S6); chasmogamous seeds from two different host species were more similar to each other than either was to the cleistogamous seeds (pairwise PERMANOVA cleistogamous vs chasmogamous $P \leq 0.001$; chasmogamous *Danthonia* vs chasmogamous *Festuca* $P = 0.15$). Our vector-fitting analysis revealed that shifts in fungal pathogen composition correlated significantly with minimum Spring vapor pressure deficit ($R^2 = 0.27$, $P = 0.001$) and was less influenced by mean Spring dewpoint temperature and plant density ($R^2 = 0.06$ and 0.07, respectively; $P < 0.1$).

In order to further illustrate how dependent fungal communities were on seed type, we graphed the observed abundances of fungal ASVs identified to a pathogenic (biotrophic) genus, *Hygrocybe*, which is important in grasslands (Griffith & Roderick, 2008; Halbwachs *et al.*, 2013a) (Fig. 5). Again, seed type had an effect on fungal abundances ($P < 0.001$, Fig. 5b), as did site ($P = 0.017$). For example, *Hygrocybe nigrescens* was dominant in *D. californica's* cleistogamous seeds (top panel, Fig. 5a) but was rare in chasmogamous seeds of both host species (middle and lower panels, Fig. 5a), whereas *Hygrocybe noninquinans* was dominant in the chasmogamous seeds of both host species and rare in the cleistogamous *D. californica*. Furthermore, correlation analyses showed that *H. nigrescens* in *D. californica's* cleistogamous seeds was negatively correlated with plant density, whereas *Hygrocybe occidentalis* was negatively correlated with germination of *D. californica* chasmogamous seeds (Fig. 6).

How do seed type, host species, environmental factors and fungal pathogen composition affect seed germination?

For the epiphytic microbes, our sample of germinated seeds was small (14 chasmogamous, 10 cleistogamous), so statistics could

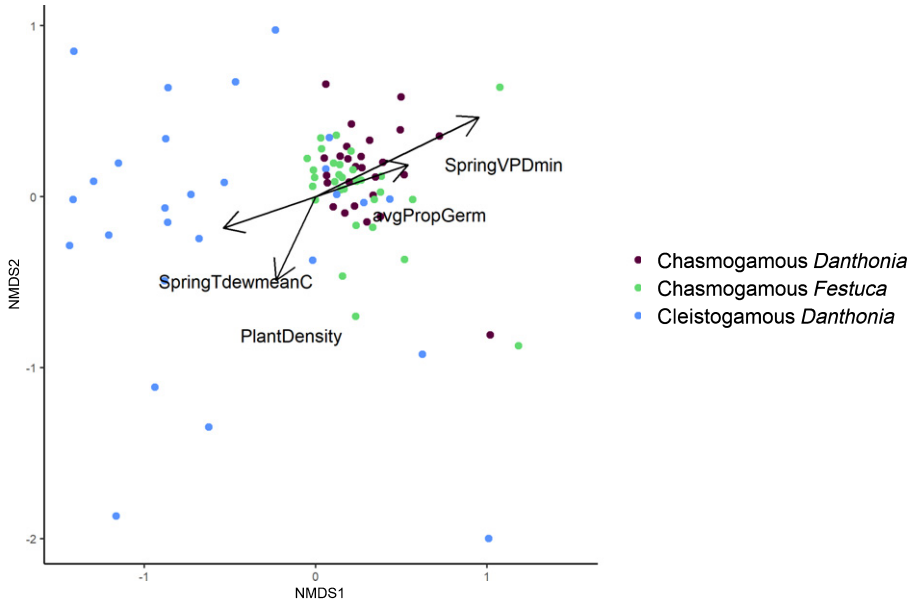

Fig. 4 Nonmetric multidimensional scaling (NMDS) of the whole-seed pathogenic fungal communities plotted with environmental vectors fitted for explanatory variables with at least marginal significance ($P < 0.1$) for each site: climate (Spring vapor pressure deficit (VPD) minimum $R^2 = 0.27$, $P = 0.001$; Spring dewpoint temperature mean $R^2 = 0.06$, $P = 0.08$), plant density ($R^2 = 0.07$, $P = 0.055$) and the proportion of seeds that germinated ($R^2 = 0.10$, $P = 0.01$); each point represents the fungal pathogen communities in an individual seed, and points closer together in space represent more similar communities. Seed fungal pathogen community composition varied significantly across seed type (PERMANOVA $F = 5.76$, $R^2 = 0.13$, $P = 0.001$) and host species (PERMANOVA $F = 3.03$, $R^2 = 0.04$, $P = 0.001$). Cleistogamous *Danthonia californica* $n = 27$, chasmogamous *Danthonia californica* $n = 26$, chasmogamous *Festuca roemeri* $n = 30$.

not be calculated, but the results were qualitatively similar to the ungerminated percentages given above, with the exception of *Pyrenophora*, which was more common on germinated chasmogamous seeds (57%) than ungerminated chasmogamous seeds (13%) (Table S8).

The mean proportion of seeds that germinated was significantly correlated with shifts in fungal pathogen composition by vector analysis (proportion germinated $R^2 = 0.10$, $P = 0.01$; Fig. 4). We also calculated Spearman's correlations for individual pathogenic fungal ASVs with germination and plant density within each seed type; cleistogamous *Danthonia* seeds had the largest number of these fungi (25 ASVs) that were significantly correlated with plant density and germination. However, when considering only the ASVs that were negatively correlated with the proportion of seeds germinated, there were more fungal pathogen ASVs that were negatively correlated with germination of chasmogamous seeds than cleistogamous seeds (Fig. 6). Differential abundance analyses identified three of these fungal pathogen ASVs as being significantly more abundant when independently comparing cleistogamous *Danthonia* seeds with both types of chasmogamous seeds: *Epicoccum nigrum, Alternaria metachromatica* and *Sistotrema brinkmanii* (Table S5). There were no ASVs that were significantly differentially abundant when comparing the two chasmogamous seed types.

Discussion

Seed germination is an inherently multivariate problem. We found that the environmental conditions maternal plants experience (site factors), plant species, maternal plant, seed type and seed fungal communities all influenced germination. That said, seed type had – by far – the largest effects on both germination and seed fungal communities. It is a strength of our study that both cleistogamous and chasmogamous seeds were gathered at the same time from the exact same plants (or in a very few cases, nearby maternal plants in the same population) and subsequently stored in identical conditions for identical lengths of time; thus, we do not suspect that disparities in seed collection and storage were responsible for the differences in our results. Furthermore, they experienced similar environmental conditions in the field; at four of our sites both *Festuca roemeri* and *Danthonia californica* occurred together and at two others (Hazel Dell and Table Rocks) they were separated only by a few hundred meters.

The relative germination success of both *Festuca* and *Danthonia* seeds appears to be environmentally dependent. Our germination results for *Festuca* seeds revealed close relationships between maternal environment (precipitation and temperature) and dormancy/germination success; this is consistent with a previous study testing *Festuca* seeds, which suggested that maternal environment and post-harvest conditions contribute to variation in dormancy and germination (Wilson & Kaye, 2002). Our data from uniform conditions in the laboratory show a pattern of greater germination of chasmogamous seeds than cleistogamous seeds in *D. californica*. However, in a concurrent field study that used seeds from the same maternal families of *D. californica* we found that when seeds were placed away from their natal sites the cleistogamous seeds germinated better than the chasmogamous

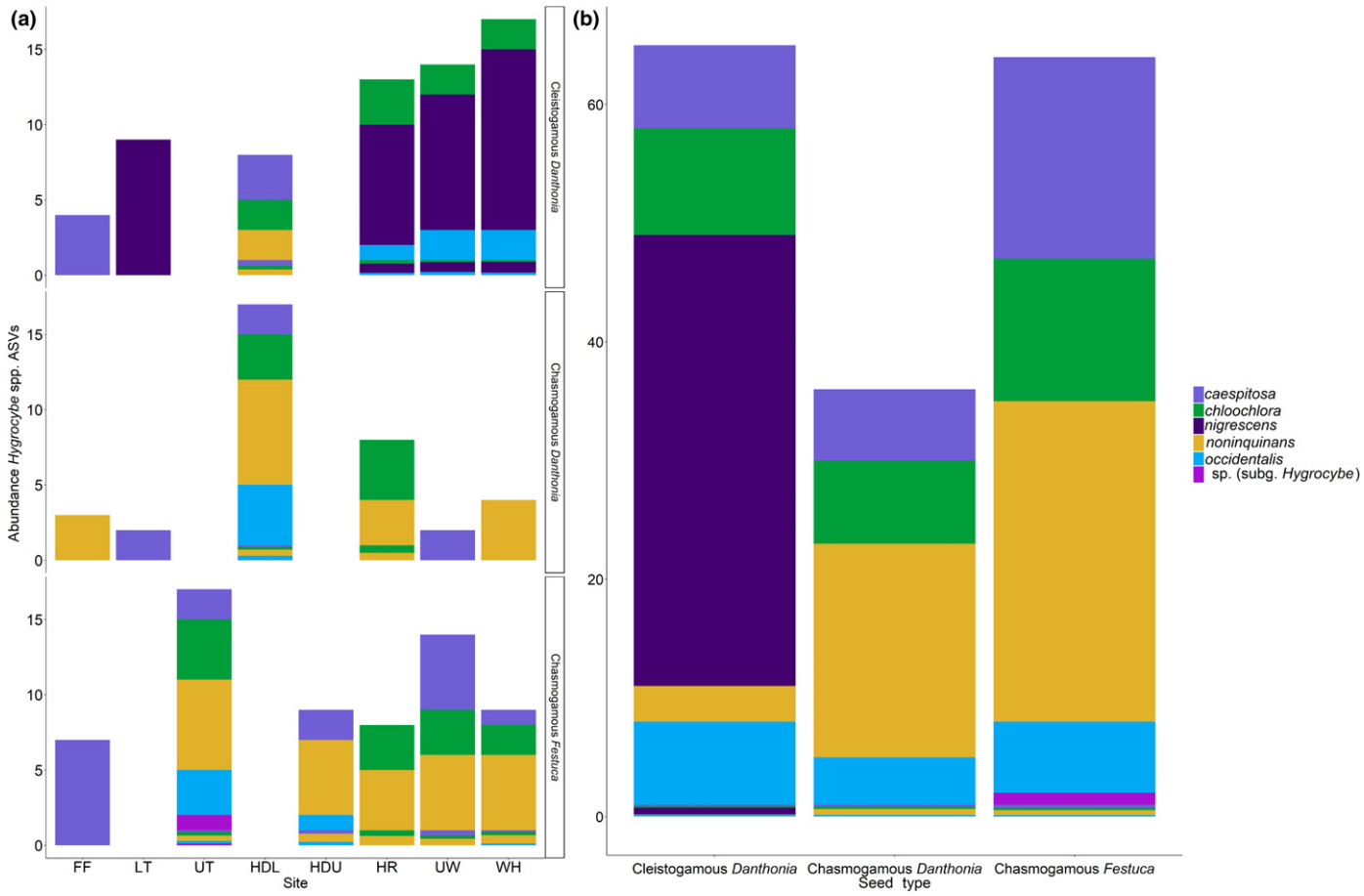

Fig. 5 Abundances of biogenic *Hygrocybe* from whole seeds by site, seed type and species (a), and by seed type and species (b). All of the main effects in a PERMANOVA model were significant: site ($F = 1.73$, $R^2 = 0.17$, $P = 0.017$), seed type ($F = 8.23$, $R^2 = 0.11$, $P < 0.001$), and host species ($F = 4.66$, $R^2 = 0.06$, $P < 0.001$). There was no site-by-host interaction ($F = 1.04$, $R^2 = 0.04$, $P = 0.391$), but there was an interaction between site and seed type ($F = 1.65$, $R^2 = 0.11$, $P = 0.028$). FF, French Flat; LT, Lower Table Rock; UT, Upper Table Rock; HDL, Hazel Dell Lower; HDU, Hazel Dell Upper; HR, Horse Rock Ridge; UW, Upper Weir; WH, Whidbey.

seeds, but at the natal site, the reverse occurred (H. Jones et al. unpublished). One possible explanation for these conflicting results is pathogens. If chasmogamous seeds have higher germination, but then germinants are more likely to be killed by their more abundant pathogens, in the field you would find more cleistogamous seedlings during censuses, but in the laboratory you would see more chasmogamous germination because it is easier to see and count all germination.

Cleistogamous and chasmogamous seeds vary in their germination success depending on the abiotic and biotic conditions in the maternal environment. Plants that produce both types of seeds such as *D. californica* may bet-hedge risks in germination by favoring cleistogamy in environments where climatic variability is high. For instance, in the Pacific Northwest, winter and spring temperatures and precipitation depend cyclically on the Pacific Decadal Oscillations (PDO), causing interannual variability and thus the potential for more germination of cleistogamous seeds (Svejcar *et al.*, 2017; Zhao *et al.*, 2017; Ehleringer & Sandquist, 2018; Lindh *et al.*, 2018). These shifts in environmental conditions also may indirectly affect germination success through structuring communities of seed-killing pathogens present in the external environment.

A major difference between cleistogamous and chasmogamous seeds in *Danthonia* is the exposure (or lack thereof) to the external environment. Specifically, chasmogamous seeds have the potential to be colonized by air- and wind-dispersed microbes, including fungal pathogens. In seasons or sites where air-dispersed seedborne disease is common, cleistogamous seed production and germination may be favored. As such, we expected that epiphytic fungi would be substantially reduced in abundance on cleistogamous seeds compared to chasmogamous seeds. Our culture data show a large, $\leq 50\%$, decrease of epiphytes and pathogens on cleistogamous seeds, and our sequence data show patterns of lower abundance of any fungal pathogen taxa in cleistogamous seeds compared with chasmogamous seeds. Although our correlation analyses detected more fungal pathogen amplicon sequence variants (ASVs) on cleistogamous seeds, the majority of these relationships were positive correlations with plant density. This makes sense as these fungi are likely vertically transmitted, and cleistogamous seeds contained in the stems of maternal plants rarely fall far from their mother or siblings. Although differences in the abundance of fungal pathogens across seed types likely affects germination, the composition of pathogenic fungi in and on seeds can affect seed viability and germination as well

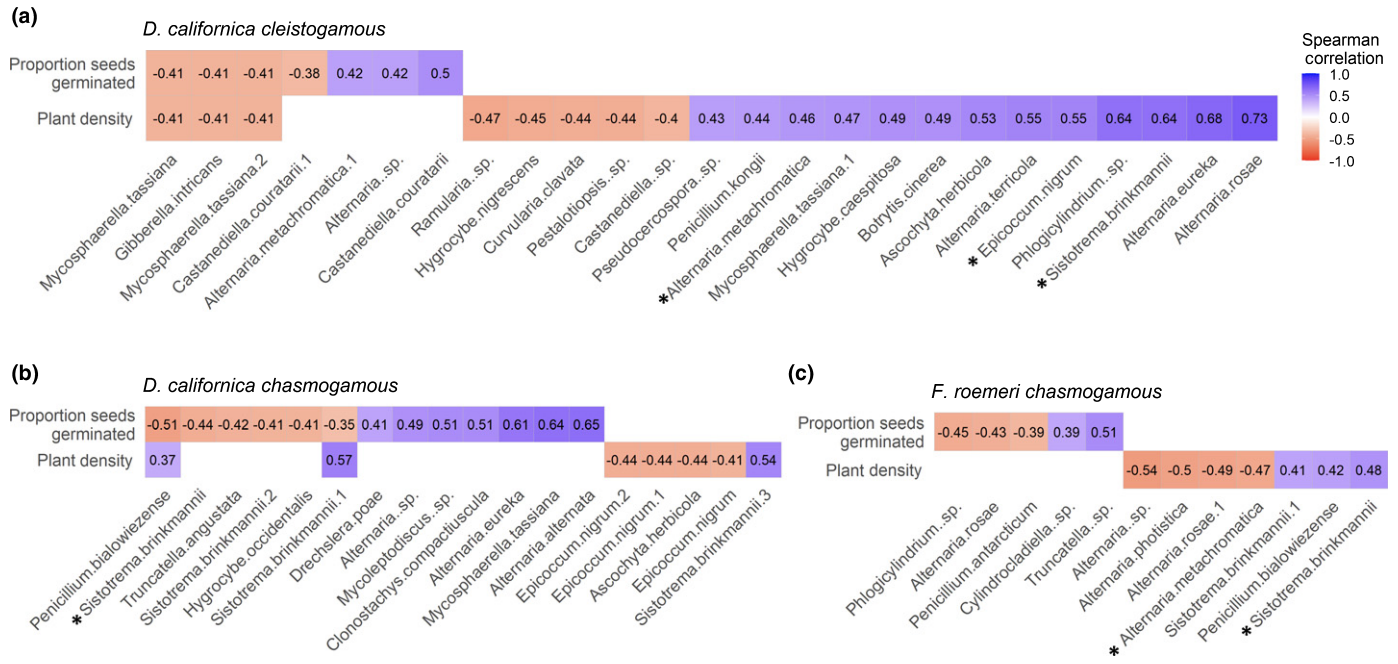

Fig. 6 Spearman correlation matrices for fungal pathogen amplicon sequence variants (ASVs) that were significantly correlated ($P \leq 0.05$) with proportion of seeds germinated or plant density for *Danthonia californica* cleistogamous (a) and chasmogamous (b), and *Festuca roemeri* chasmogamous (c) seeds. ASVs are ordered by the strength of the correlations with germination, then plant density, as a 'heat map' with more negative (red) to the left, moving to more positive (blue) to the right. Asterisks indicate ASVs that were identified as significantly more abundant in chasmogamous seeds than cleistogamous seeds by differential abundance analyses ($P \leq 0.01$).

(Blaney & Kotanen, 2001; Meyer *et al.*, 2010; Nelson, 2018). The marked shifts in epiphyte composition depending on seed type are further supported by our whole-seed fungal data, where seed type most strongly influenced shifts in fungal community composition. Furthermore, shifts in fungal pathogen composition correlated strongly with the proportion of seeds germinated (Fig. 4). This finding suggests that fungal pathogen community composition influenced the germination success and/or dormancy of the seeds, through shifts in the relative abundance or presence/absence of particular fungal genera. For example, *Epicoccum, Sistotrema* and *Alternaria* were differentially more abundant on both grass species of chasmogamous seeds than on cleistogamous *Danthonia* seeds. The greater abundance of these specific groups of fungi on chasmogamous seeds rather than cleistogamous seeds may have influenced germination, but determining the effects of specific seed-associated fungi on the seeds of a particular host plant will require following Koch's postulates – isolation and re-inoculation (Rodriguez *et al.*, 2009).

We identified fungal ASVs for which their relative abundances correlated negatively with seed germination across seed type, but the specific taxa that were correlated was different across cleistogamous and chasmogamous seeds. A number of these taxa have been shown to have negative effects on seed germination in previous studies; for instance, the negative relationship between *Gibberella intricans* and germination of cleistogamous *Danthonia* seeds in this study is consistent with experimental tests of *G. intricans* and germination of garden cress seeds (Ogorek, 2016). Other taxa identified here as negatively correlated with seed germination (such as *Alternaria*, *Mycosphaerella* and *Truncatella* spp.) are known endophytic and pathogenic fungi that may opportunistically infect plant tissues depending on host and environmental contexts (Thomma, 2003; Dean *et al.*, 2012; Wilson *et al.*, 2014; Raimondo *et al.*, 2019), but their effects on seed germination warrant further investigation in controlled inoculation studies.

We found evidence that different sequence variants (ASVs) that were assigned the same fungal taxonomy (e.g. *Sistotrema brinkmanii* with three variants) can be differentially correlated with germination, potentially indicating cryptic speciation. Indeed, this is one of the arguments Callahan *et al.* (2017) used for keeping ASVs separate instead of fusing them into operational taxonomic units (OTUs) based on an arbitrary sequence similarity. We echo Tedersoo *et al.* (2010) in warning that assigning trophic status to fungal genera, such as *Sistotrema*, can be problematic because of cryptic or undescribed variation. It is a strength of our study that we examined correlations with germination at the ASV level, as it is these fitness-level differences that expose potential pathogenic relationships. Although we acknowledge the context-dependency of many fungal trophic modes, much of this variation remains uncharacterized; one of the major aims of our study was to unpack the relationships between putative fungal pathogens and maternal environment, seed type and germination. Using FUNGuild as a starting point to parse apart the fungi that could act as pathogens on and in the seeds and investigating how they vary across seed type, environment and with germination is not explicitly delineating an ecological role but, rather, showing how these fungi vary across conditions on specific host.

Evidence has been accumulating that species of *Hygrocybe* are biotrophic pathogens, that is, they feed on living cells (Halbwachs *et al.*, 2013a,b, 2018). *Hygrocybe* species are, for the most part, forest species (Halbwachs *et al.*, 2013a), but a subset are key components of grasslands in the UK, where many are endangered (McHugh *et al.*, 2001; Griffith & Roderick, 2008). Two aspects of our *Hygrocybe* data add intriguing information to this emerging story. First, we found that *H. occidentalis* decreased germination of chasmogamous seeds but not cleistogamous seeds. How does this level of seed specialization arise and how is it maintained in the host plant? Second, we discovered that otherwise tropical fungi are living in plant hosts geographically distant from where the fungi sexually reproduce (mushrooms, or fruiting bodies, are evidence of sex) (i.e. *H. occidentalis, H. noninquinans* and *H. chloochlora*); for example, the closest place to Oregon that any of these species have been found fruiting is *H. chloochlora* in the southern Appalachians (D. Jean Lodge, pers. comm.). These interesting differences in where mushrooms fruit vs where the fungus lives asexually should be investigated. How general is this biogeographical pattern? Are the tropical fungi remnants of another warmer time, or are they evidence of expansion from the tropics? Do we know anything about the distributions of mushrooms if many of these fungi also are often endophytic?

In addition to the pathogenic *Hygrocybe*, we found two species of *Pyrenophora* at low frequency. These fungi are commonly known as the 'black-fingers-of-death' (Fig. S4) and are well-known seed pathogens that reduce fitness (Meyer *et al.*, 2008). If *Pyrenophora* species are significant seed pathogens, then an outstanding question is why the proportion of germinating seeds infected by them was higher than that of nongerminating seeds. The answer may be that fast germination aids in fungal escape. Beckstead *et al.* (2007) showed that when infected seeds of *Bromus tectorum* germinated quickly, they escaped from *Pyrenophora* death (Beckstead *et al.*, 2007). Climate-induced dormancy thus increases the probability of loss in the seed bank to these pathogens.

We expected that cleistogamous seeds would have a greater probability of infection from clavicipitaceous endophyte infections being vertically transmitted from the maternal plant, some of which may be mutualistic (Clay, 1984; Clay, 1994). However, we had very low abundances of clavicipitaceous fungi in general, with no evidence of *Atkinsoniella* in any of our *Danthonia* seeds ($n = 52$), and infrequent infection for *Epichloë/Neotyphodium* in the *F. roemeri* (8%, two of 25 tested). Our data thus support Afkhami's hypothesis (Afkhami, 2012), based on the California flora, that Mediterranean climates do not favor clavicipitaceous endophytes.

Implications

Restoration of both *D. californica* and *F. roemeri* populations from seed is problematic owing to variation in germination and dormancy (Busby & Southworth, 2014; Applestein *et al.*, 2018; Darris & Gonzalves, 2019). Our work indicates that for *F. roemeri*, germination is likely to decrease as the climate warms and dries. For *D. californica*, although germination rates were variable, climate at the site of origin was not a significant predictor of germination, as also was found by Trask & Pyke (1998). However, our work suggests that cleistogamous seeds may yield better germination rates when some seed pathogens are prevalent. For both species, viability testing with tetrazolium should be combined with germination studies in the future to understand patterns of dormancy as well as germination.

Assemblages of plant-associated microbes are not random, and environmental conditions such as climate, soil type and plant density/identity simultaneously influence microbial community structure (Compant *et al.*, 2016). Understanding the relationship between seed surface microbial community structure and plant or ecosystem function, such as seed germination, is in its infancy, despite the potential importance of these linkages to interpretation of ecological research. We found that the most important environmental factor that influenced fungal pathogen community composition of whole seeds was vapor pressure deficit (VPD), which is a measure of humidity. This is consistent with previous findings of VPD and relative humidity as strong predictors of fungal abundance and richness in the air and on leaf surfaces (Talley *et al.*, 2002). In addition, shifts in atmospheric humidity directly influence water availability for microbes growing on and in maternal plants, with moisture controlling the growth and proliferation of fungal pathogens (Aung *et al.*, 2018). In line with our predictions, plant density was positively associated with seed microbial abundance, indicating that horizontal transmission of microbes is facilitated by host closeness, a common finding for plant-associated fungi (Parker & Gilbert, 2018).

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Author contributions

All authors contributed to writing and editing. HCM drafted the sections on germination and epiphytes and was involved in all

the morphological data collection; KLS performed the final Illumina run and did the next-generation sequencing (NGS) analysis and writing; TET collaborated on the germination and epiphyte data collection; KCE prepped the NGS samples and did the initial (failed) Illumina run; KLM enabled the Illumina sequencing and advised on analysis; MLD and LMH advised on statistics and graphing; and BAR designed the project and coordinated, advised and wrote at all stages. HCM and KLS are co-first authors.

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Supporting Information

Additional Supporting Information may be found online in the Supporting Information section at the end of the article.

Fig. S1 Biplot of site environmental factors for *F. roemeri*.

Fig. S2 Biplot of site environmental factors for *D. californica*.

Fig. S3 Germination vs. viability for *F. roemeri*.

Fig. S4 Photographs of the microbial taxa counted on the seed surfaces.

Fig. S5 NMDS of whole-seed fungal communities and stacked bar chart of functional group assignments for all fungal ASVs.

Fig. S6 NMDS of whole-seed pathogen communities by site.

Table S1 Locality information for seed collection sites.

Table S2 Site environmental data.

Table S3 Viability of *F. roemeri* seeds.

Table S4 Illumina data; Taxonomy from UNITE V8 and Trophic Mode from FUNGUILD.

Table S5 Differential abundance analysis.

Table S6 GLMM Statistics for *F. roemeri* germination.

Table S7 GLMM Statistics for *D. californica* germination.

Table S8 Seed surface microbial survey.

Table S9 Seed surface fungi and GenBank accession numbers.

Table S10 Number of seeds used.

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