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Research

Does host outcrossing disrupt compatibility with heritable symbionts?

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Vertically transmitted microbes are common in macro-organisms and can enhance host defense against environmental stress. Because vertical transmission couples host and symbiont lineages, symbionts may become specialized to host species or genotypes. Specialization and contrasting reproductive modes of symbiotic partners could create incompatibilities between inherited symbionts and novel host genotypes when hosts outcross or hybridize. Such incompatibilities could manifest as failed colonization or poor symbiont growth in host offspring that are genetically dissimilar from their maternal host. Moreover, outcrossing between host species could influence both host and symbiont reproductive performance. We tested these hypotheses by manipulating outcrossing between populations and species of two grasses, *Elymus virginicus* and *E. canadensis*, that host vertically transmitted fungal endophytes (genus *Epichloë*). In both greenhouse and field settings, we found that host–symbiont compatibility was robust to variation in host genetic background, spanning within-population, between-population and between-species crosses. Symbiont transmission into the F_1 generation was generally high and weakly affected by host outcrossing. Furthermore, endophytes grew equally well in planta regardless of host genetic background and transmitted at high frequencies into the F_2 generation. However, outcrossing, especially inter-specific hybridization, reduced reproductive fitness of the host, and thereby the symbiont. Our results challenge the hypothesis that host genetic recombination, which typically exceeds that of symbionts, is a disruptive force in heritable symbioses. Instead, symbionts may be sufficiently generalized to tolerate ecologically realistic variation in host outcrossing.

Keywords: *Elymus*, *Epichloë*, genetic incompatibility, hybridization, vertical transmission

Introduction

Most multicellular organisms host heritable microbes that can be vertically transmitted from maternal host to offspring (Funkhouser and Bordenstein 2013). Vertical transmission intimately links host and symbiont fitness via host reproduction and is expected to favor the evolution of mutualism through the mechanism of partner fidelity

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feedback, wherein fitness changes in one partner affects the fitness of the other partner (Ewald 1987, Sachs et al. 2004). Indeed, heritable microbes can, in many contexts, benefit hosts by defending against biotic and abiotic stress in exchange for protection and regeneration (Oliver et al. 2005, Singh et al. 2011, Pérez et al. 2013).

A potential consequence of vertical transmission is the evolution of specialization, such as host–symbiont genotype matching (Poisot et al. 2011). Vertically transmitted symbionts may specialize on host species or host genotypes and consequently become incompatible with genetically novel hosts (Chong and Moran 2016, Goodrich et al. 2016). Much of the evidence for the genetic incompatibility hypothesis comes from interspecific cross-inoculations. For instance, fungal and bacterial symbionts experimentally introduced to novel plant and insect host species experienced reduced vertical transmission (the fraction of symbiotic host offspring that inherit the symbiont), while hosts had shortened lifespans and reduced fertility relative to hosts with native symbionts (Christensen 1995, McGraw et al. 2002, Kageyama et al. 2006). While these previous experiments inform hypotheses about the evolution of symbiosis, few studies have investigated the consequences of novel interactions between host and symbiont genotypes that occur as a consequence of host reproduction and on ecologically relevant timescales (Gundel et al. 2010, Saikkonen et al. 2010).

One ecologically relevant mechanism hypothesized to create genetic incompatibilities between symbiotic partners is genetic exchange between hosts, such as outcrossing or hybridization (Saikkonen et al. 2004, Gundel et al. 2012, Gibert and Hazard 2013). Specifically, incompatibility between host and symbiont may arise from their contrasting reproductive modes: many heritable symbionts reproduce asexually during transmission from parent to offspring, while many hosts readily outcross (Saikkonen et al. 2004). This mismatch in reproductive mode means that inherited symbionts may encounter a different genetic background in the offspring they colonize compared to the genetic background of the parental (typically maternal) host from which they came.

Host outcrossing could affect host–symbiont compatibility in at least two ways. First, the symbiont could experience reduced vertical transmission by failing to transmit into outcrossed offspring that differ from the maternal genotype (Gundel et al. 2011, 2012). Second, if the symbiont successfully transmits, it may fail to grow or grow poorly within incompatible hosts. Reduced within-host symbiont density or performance could decrease host fitness or limit transmission into subsequent generations. For example, bacterial symbiont titer abundance in pea aphids varied in response to host genetic background (Chong and Moran 2016), and heritable endophytic fungi either died or did not grow in planta after inoculation into novel grass host species (Leuchtmann 1992, Christensen 1995, Ryan et al. 2015). The timing of symbiont colonization of host offspring may determine when incompatibilities arise. For example, if vertical transmission occurs before host outcrossing (i.e. the symbiont colonizes ovules prior to fertilization), as in some *Epichloë* endophyte-grass systems (Majewska-Sawka and Nakashima 2004, Zhang et al. 2017), symbionts may successfully transmit to F_1 offspring but could exhibit poor growth in F_1 adults and low transmission to F_2 offspring. Alternatively, if transmission occurs after fertilization, symbionts could exhibit low transmission to F_1 offspring.

Exploring the ways in which host outcrossing may disrupt symbiont transmission is important because imperfect vertical transmission is widely documented across plant and animal hosts, even for symbioses that are thought to be strongly beneficial (Afkhami and Rudgers 2008, Bright and Bulgheresi 2010, Gundel et al. 2011, Sneck et al. 2017), but the causes of this variation are poorly understood. Additional work is needed on several fronts. First, past studies treated outcrossing qualitatively (outcrossed or not), when in fact it is a continuum ranging from genetic exchange within a population to between species. Second, neutral markers used to measure genetic distance may fail to account for non-neutral differences between populations that can arise from local adaptation. Lastly, even without reduced compatibility, outcrossing may influence host fitness positively (heterosis) or negatively (outbreeding depression) in ways that have downstream effects on heritable partners. To our knowledge, effects of outcrossing on host reproductive performance have not been previously studied in combination with effects on host–symbiont compatibility, although both are important for the long-term dynamics of symbiosis (Gundel et al. 2010).

Here, we used seed-transmitted fungal endophytes (genus *Epichloë*, Clavicipitaceae) and two species of host grasses (*Elymus virginicus* and *E. canadensis*) to experimentally determine the effects of host outcrossing on symbiont vertical transmission, symbiont growth in planta and components of host and symbiont fitness. This system is well-suited to answer questions regarding the effect of host outcrossing on symbiosis because both host species are reproductively labile: they can self-pollinate, outcross with conspecifics and interspecifically hybridize (Church 1958). We manipulated gene flow between parental (P_1) hosts of varying genetic distances to generate outcrossed seeds (F_1 generation) and quantified both endophyte vertical transmission and germination success for F_1 seeds. We then transplanted F_1 seedlings into a common garden, where we quantified fungal endophyte density in planta and vertical transmission to F_2 seeds. We determined the influence of outcrossing on host fertility by quantifying reproductive tiller and seed production of symbiotic F_1 hosts. Lastly, we integrated our results into composite estimates of host and symbiont fitness in response to outcrossing. Importantly, our crossing design allowed us to quantify the genetic distance of mating pairs in two ways: as a continuous distance measured at neutral loci and as qualitative differences defined by the type of cross: within-population, between-population or between-species (hybridization). Here, we addressed the following questions: 1) Does host outcrossing reduce endophyte vertical transmission and does the effect differ between F_1 and F_2 generations? 2) Does

host outcrossing reduce endophyte density in F_1 plants? 3) Does F_1 endophyte density in planta predict transmission to F_2 seeds? And 4) Does outcrossing have positive or negative effects on host (and thereby symbiont) fitness?

Methods

Study system and plant material

Elymus virginicus and *E. canadensis* are perennial, cool season grasses that harbor intermediate to high frequencies of fungal endophytes (*Epichloë* spp.), which can grow asymptomatically in above-ground host tissues (Sneck et al. 2017). *Epichloë* endophytes are known to provide mutualistic benefits to cool-season grass hosts, including our focal host species *E. virginicus* (Rudgers and Swafford 2009), though host fitness benefits are often context-dependent. These endophytes are predominantly or exclusively vertically transmitted from maternal plants to seeds (Cheplick and Faeth 2009). While *Epichloë* spp. found in *E. virginicus* can horizontally transmit (Schardl and Leuchtmann 1999), we did not observe horizontal transmission (indicated by sexual stromata) in this experiment. Controlling outcrossing between a pollen donor and recipient is possible in this system because *Elymus* anthers are relatively large and can be removed prior to stigma emergence, thereby preventing self-pollination. We chose *E. virginicus* as the focal host species for within-species crosses and the pollen recipient in inter-specific crosses to reflect previously described patterns of inter-specific outcrossing, which is mainly from *E. canadensis* pollen donors to *E. virginicus* recipients in populations where they co-occur (Nelson and Tyrl 1978, Saha et al. 2009).

To act as the parental (P_1) generation, we collected ~40 open-pollinated seeds each from individual plants in natural populations of *E. virginicus* (49 plants from 9 populations) and *E. canadensis* (22 plants from 5 populations) in the spring of 2013. Collections ranged throughout the Southern Great Plains, USA (for collection details Sneck et al. 2017; Supplementary material Appendix 1 Table A1). Seeds collected from an individual plant represented a maternal family (half- to full-siblings).

To screen for endophyte-positive (E+) plants, 20 seeds per maternal family were surface sterilized in 5% bleach, cold stratified in 10% agarose in Parafilm-sealed petri dishes at 4°C for two weeks, and then germinated in the greenhouse at Rice University in February 2014 and 2015 with peat-based potting soil. We checked for endophyte presence in at least two tillers using light microscopy under 200× magnification (Bacon and White 1994).

All E+ seedlings were transplanted into 1.8 l pots, fertilized as needed, and vernalized outside for ~2 winter months to promote spring flowering. In total, 107 E+ plants from 89 maternal families were reared over two years (2014 and 2015) to act as the P_1 generation for the greenhouse crossing experiment. Our previous work (Sneck et al. 2017) determined the genotypes of *Epichloë* sp. occupying the maternal families used in the present experiment by amplifying 18 fungal genetic markers. Most endophytes were categorized into two 'genotypes' based on genetic loci associated with genes encoding steps for alkaloid production.

Estimating genetic distance between P_1 plants

We estimated the neutral genetic distance between all pairs of maternal families using eight previously developed microsatellite markers (Saha et al. 2009). Within each population, we genotyped multiple plants per maternal family (median = 2, min = 1, max = 6) and estimated the genetic distance between each pair of families by tallying the number of non-identical alleles across all microsatellite loci (min = 1, max = 18), where higher numbers indicate greater genetic distance (Huff et al. 1993). Given that multiple plants per family were genotyped, we observed slight variations in genotypes within families. To accommodate this variation, we calculated the mean distance between two families as the average number of exclusive alleles across all pairs of individuals between families. Because our focus was between-family rather than between-population genetic distances, we lacked the resolution to calculate population genetic metrics such as F_{ST} or isolation by distance. A full description of the molecular and mathematical approaches used to estimate genetic distance can be found in the Supplementary material Appendix 1.

Crossing experiment

To manipulate gene flow, we made three types of experimental crosses (n = 156 total; Supplementary material Appendix 1 Table A1) between P_1 plants from: the same population 'within-population': n = 20), from different populations ('between-population': n = 86), or from different species ('hybrid': n = 50). We used single plants as both pollen donors and recipients, with individual recipient plants (always *E. virginicus*) treated as blocks (n = 77 maternal blocks) and crosses assigned to individual inflorescences (flower heads) within a plant, which typically had 10–20 inflorescences. To prevent unintended pollination (including selfing), recipient inflorescences were emasculated with fine-tipped forceps then covered with micro-perforated plastic bags. Within one–three days of emasculation, we added a pollen donor inflorescence to the bag and agitated it to facilitate pollination (Dewey 1971). Donor inflorescences with intact anthers were removed from donor plants and placed in 14-ml water-filled centrifuge tubes attached to a bamboo rod in the recipient pot. To verify that our methods were effective in pollinating recipient plants, we quantified seed production in all crosses and compared it to 41 inflorescences that were bagged and allowed to naturally self-pollinate. We also verified that crosses produced offspring of the intended parentage using the microsatellite markers described above to genotype a subset of experimentally crossed (n = 33) and naturally selfed offspring (n = 7).

Endophyte transmission and germination in the F_1 generation

We harvested mature seeds (n = 1348) during summer 2014 (n = 124 crosses) and 2015 (n = 32 crosses). Seeds were cold stratified for two weeks as described above. Then, we tracked seed germination by placing them under 32-Watt aquarium lights with 10 h of light daily. Endophyte status of seedlings was determined by light microscopy as described above. We also assayed endophyte status in seeds that failed to germinate after two months by soaking them in 5% NaOH solution overnight, then squashing and staining with aniline blue (White 1987). This stain binds to fungal hyphae regardless of seed or fungal viability. We scored endophyte status for an average of 7.9 seeds and seedlings per cross (min = 1, max = 29) for a total of 1231 scores. Plant-level transmission was not strongly correlated with number of seeds and seedlings assayed (Spearman rank correlation = 0.11, p = 0.09).

Common garden experiment

We estimated endophyte hyphal density in the F_1 adults and endophyte transmission into the F_2 offspring in a common garden field setting using a subset of E+ offspring from the three cross types: within-population (n = 12 F_1 plants), between-population (n = 49 F_1 plants), and between-species (n = 24 F_1 plants) from across 25 maternal blocks (Supplementary material Appendix 1 Table A1). We transplanted F_1 offspring into 6 l plastic pots in early November 2015 and vernalized outdoors. In February 2016, pots were sunk into 20-cm deep holes at 1 m spacing at a semi-natural field site in Houston, TX (29°65'N, 95°44'W). Each pot received 16 g of Osmocote fertilizer and was watered daily for one week. Ambient vegetation was mowed as needed to reduce light competition. Upon flowering in summer 2016, we bagged three immature inflorescences to force self-pollination. To standardize paternity, vertical transmission into F_2 offspring was estimated using only seeds from bagged inflorescences, which produced fewer seeds (mean = 13.2) than unbagged inflorescences (mean = 24; $t_{167} = -3.51$, p = 0.0006). To confirm F_1 E+ status and detect imperfect endophyte retention from the seedling stage, we scored two tillers per plant for endophyte presence before transplanting into the common garden (fall 2015) and again after F_2 seed production (summer 2016) using a high-throughput antibody immunoblot membrane that narrowly targets *Epichloë* endophyte proteins (Sneck et al. 2017).

Endophyte growth in F_1 adult plants

Hyphal density was estimated in a subset of F_1 E+ plants in the common garden (between-population: n = 44; hybrid n = 19) in June 2016. We were unable to sample within-population crosses because plant tissues senesced earlier than expected. Endophyte presence was sampled non-destructively from multiple tillers per plant (total tillers = 126, mean tillers sampled per plant = 2.5, min = 1, max = 4) using light microscopy. Thin sections of the inner leaf sheath were taken from the same tillers from which endophyte transmission was estimated. In addition, we accounted for within-leaf sheath hyphal density variation by analyzing images of multiple leaf sheath views per tiller, captured with a microscope camera (mean views per tiller = 3.25). From these images, we estimated hyphal density by tracing all visible hyphae using the image processing software ImageJ, where hyphal length (μm) was measured as the number of scaled pixels (288 pixels = 10 μm) within a 200× magnification field of view. Hyphal density values were averaged across all views from a single tiller. Mean log-transformed hyphal density was independent of the number of views per tiller (Spearman rank correlation = −0.15, p = 0.23).

Endophyte transmission into the F_2 generation

In August 2016, we harvested F_2 seeds from self-pollinated inflorescences to estimate vertical transmission with an antibody immunoblot assay. We tested multiple seeds per inflorescence (mean seeds per inflorescence = 10, min = 1, max = 16) and multiple inflorescences per plant (total inflorescences = 637, mean inflorescences per plant = 2.8, min = 1, max = 3) for a total of 1596 seeds assayed.

Fertility of F_1 plants

Because we knew that bagging affected seed production, we collected one open-pollinated (non-bagged) inflorescence per plant to estimate F_1 seed production per inflorescence. Additionally, we counted the total number of inflorescences per plant to estimate overall F_1 reproductive output.

Data analyses

For each of the response variables (F_1 and F_2 endophyte transmission, endophyte hyphal density, seed germination and adult plant inflorescence and seed production), we fit a candidate set of generalized linear mixed-effects models (R package lme4, Bates et al. 2015) that included neutral genetic distance, cross type, neither and both as additive and interactive effects (Table 1). Including cross type, which classifies crosses as within- and between-populations and between-species (hybrid), allows us to compare the influence of neutral (genetic distance, as estimated by microsatellites) versus potentially non-neutral (cross type) sources of host–symbiont genetic incompatibility. We used AIC-based model selection to quantify support for these competing models. Cross type co-varied with neutral genetic distance, as expected, but there was enough overlap in genetic distance among cross types that we were able to fit both variables as fixed effects. We ranked models using bias-corrected Akaike information criterion (AIC_c). To accommodate uncertainty in rankings, we applied model-averaging of all models within $\Delta AIC_c \leq 2$ of the top-ranked models.

For all analyses, we standardized neutral genetic distance to mean zero and unit variance. We explored the possibility of non-monotonic relationships between genetic distance and each response variable by including a quadratic term; however, it received little support and was removed from final analyses to simplify model selection. Also, we do not consider endophyte genotype and endophyte status of pollen

Table 1. AIC model rankings. Top models and models within $\Delta AIC_c \leq 2$ (which were averaged) are shown in bold font.

Response	Fixed effects	ΔAIC_c	AIC_cwt
F_1 E+ transmission	cross type × genetic distance	**0.0**	**0.76**
	null	4.3	0.089
	genetic distance	4.4	0.085
	cross type + genetic distance	6.1	0.036
	cross type	6.7	0.027
F_2 E+ transmission	null	**0.0**	**0.607**
	genetic distance	**1.9**	**0.231**
	cross type	3.5	0.103
	cross type + genetic distance	5.5	0.039
	cross type × genetic distance	6.7	0.021
F_1 hyphal density	null	**0.0**	**0.370**
	genetic distance	**1.1**	**0.220**
	cross type + genetic distance	**1.7**	**0.150**
	cross type	**1.8**	**0.150**
	cross type × genetic distance	2.4	0.110
F_1 seed germination	cross type × genetic distance	**0.0**	**0.76**
	genetic distance	3.3	0.15
	cross type + genetic distance	5.9	0.04
	cross type	6.6	0.028
	null	6.8	0.026
F_1 reproductive tiller production	cross type + genetic distance	**0.0**	**0.420**
	cross type × genetic distance	**0.6**	**0.310**
	genetic distance	**0.8**	**0.270**
	cross type	55.2	<0.001
	null	56.1	<0.001
F_1 seed production	genetic distance	**0.0**	**0.595**
	cross type + genetic distance	**1.3**	**0.308**
	cross type × genetic distance	3.6	0.098
	null	69.1	<0.001
	cross type	73.2	<0.001

donors as explanatory variables, following preliminary analyses in which both variables received little statistical support. All models included random effects of maternal and paternal identity. An additional random effect of cross identity was added to models for F_2 transmission to account for non-independence of common garden plants that came from a single crossing event. Models for F_1 and F_2 transmission used a logit link function, where 'trials' were given by the total seeds and seedlings scored per plant and 'successes' were given by E+ scores. The F_1 and F_2 data came from different environments (greenhouse and common garden, respectively) and for this reason we analyzed them separately. Germination of F_1 seeds was similarly modeled as a binomial response, with total seeds assayed per plant as the number of 'trials'. Endophyte hyphal density in F_1 plants was log transformed and modeled as a Gaussian response, with plant identity as a random effect. F_1 fertility in the common garden included two response variables that were well described by a Poisson distribution: the number of inflorescences per plant and the number of seeds per inflorescence. We verified model goodness-of-fit by comparing scaled-residuals to predicted values (R package DHARMa, Hartig 2017).

To determine the association between endophyte density and transmission success, we calculated the Spearman rank-order correlation coefficient between endophyte hyphal density in F_1 adult plants and endophyte vertical transmission success into F_2 seeds at both the individual tiller and plant levels.

Host and symbiont fitness estimation

We integrated the response variables into composite measures of host and symbiont fitness. To accomplish this, we calculated values for F_1 transmission, germination, inflorescence production, seed production per inflorescence and F_2 transmission for each unique cross. Many crosses were represented by multiple offspring in the common garden, in which case we calculated mean values across all offspring. First, we calculated a composite metric of F_1 host annual fitness as the product: germination rate × number of inflorescences × seeds per inflorescence. This quantity approximates the expected number of seeds produced by a single F_1 seed over one year. Second, we calculated annual symbiont fitness in F_1 hosts as the product: F_1 transmission × F_1 host fitness (above) × F_2 transmission. This quantity approximates the expected number of E+ seeds produced by a single seed from an E+ maternal plant. Hyphal density was excluded from this calculation because we assumed that endophyte fitness was derived entirely from host fitness, in which case the consequences of hyphal density were captured by seed production and endophyte transmission.

Data deposition

Data are available from the Github Digital Repository: <https://github.com/texmiller/ELVI_cross_ms> (Sneck et al. 2018).

Results

Effectiveness of crossing experiment

If experimental crosses were effective, offspring would be equally genetically distant from maternal and paternal parents, and more genetically distant from their maternal parent than self-pollinated offspring. Consistent with these expectations, the genetic distance of F_1 offspring to maternal versus paternal plants was not significantly different ($t_{59} = 1.72$, $p = 0.091$), and outcrossed offspring were more genetically distant from maternal plants than self-fertilized offspring ($t_{12} = 3.95$, $p = 0.002$) (Supplementary material Appendix 1 Fig. A3). Additionally, genetic distance between cross types tracked genetic distance of the microsatellite markers as expected: on average, between-population crosses were more genetically distant than within-population crosses ($z = 2.16$, $p = 0.031$), and hybrid cross types were significantly more genetically distant than between-population crosses ($z = 5.42$, $p < 0.0001$), though there was overlap among groups (Fig. 1).

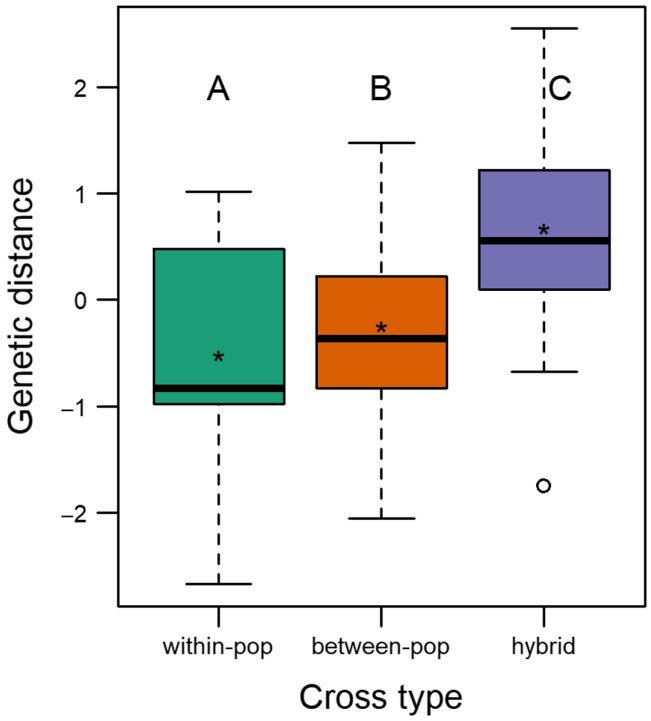

Figure 1. Boxplots of estimated scaled mean genetic distance (pairwise count of microsatellite allele differences, standardized to mean zero and unit variance) between outcrossed host parents from each cross type within-populations, between-populations, and hybrid (between-species). Letters indicate significant difference between mean genetic distances (*$\alpha < 0.05$). Bold horizontal bars indicate the median while the upper and lower portions of the box indicate the 25th and 75th percentiles, or the interquartile range (IQR). The 'whiskers' encompass data within 1.5× of the IQR.

Vertical transmission in response to host outcrossing

We found little support for the hypothesis that endophyte vertical transmission declines with host outcrossing (Fig. 2). For the F_1 generation, transmission differed among crosses in ways that could not be fully explained by neutral genetic distance alone (Table 1). Endophyte transmission for within- and between population crosses increased with genetic distance, from 61% at the lowest genetic distance to 96% at the greatest genetic distance. In contrast, for hybrid crosses, transmission was high at low genetic distances (mean transmission = 100%) and declined at the higher genetic distances (mean transmission = 62%). However, differences related to cross type were most pronounced at genetic distances that were not well represented in our dataset. Over the range of genetic distance that was well sampled, predicted transmission was similarly high among cross types (Fig. 2a–b). Results for the F_2 generation differed from F_1, and the null model (no effect of outcrossing) received the most statistical support (AIC_cwt = 60.7%, Table 1, Fig. 2c–d). A model with a weak negative effect of genetic distance was ranked second ($\Delta AIC_c = 1.9$, AIC_cwt = 23.1%), although this effect is imperceptible in the model-averaged predictions (Fig. 2c). Overall, vertical transmission was lower and more variable in the F_1 generation (mean ± SD: 78% ± 30) than in the F_2 generation (96% ± 13.3).

Endophyte hyphal density in F_1 plants

Variation in endophyte hyphal density within F_1 hosts was not strongly related to cross type (between-population versus inter-specific hybrid) or genetic distance between parents (Fig. 3a–b). The null model received the most statistical support, but models including genetic distance and cross type were within $\Delta AIC_c \leq 2$ (Table 1). Model-averaged predictions showed a positive response of hyphal density to genetic distance and lower density in hybrid versus between-population hosts, but these effects were weak relative to variation in the data (Fig. 3a). Furthermore, hyphal density was not associated with vertical transmission into the F_2 generation at the scale of individual tillers (Spearman's correlation coefficient = −0.08, p = 0.55), nor was it correlated with transmission at the whole plant-level (Spearman's correlation coefficient = −0.06, p = 0.61).

The effect of host outcrossing on host and symbiont fitness components

F_1 seed germination

F_1 seed germination responded to both the genetic distance between parents and cross type (AIC_cwt = 76.0%, Table 1, Fig. 4). The top-ranked model predicted that each cross type experienced different effects of genetic distance on germination, declining with greater genetic distance for hybrid and within-population offspring and increasing for between-population offspring (Fig. 4a). Across groups, post-hoc comparisons revealed that, on average, hybrid offspring germinated at the highest rates (mean = 51.7%) compared to both within-population (mean = 28.5%, Tukey's HSD: z = 2.05, p = 0.04) and between-population offspring (mean = 34.4%, Tukey's HSD: z = 1.69, p = 0.09) (Fig. 4b).

Fertility

Outcrossing negatively affected F_1 fertility (Fig. 5). The best-fit model for reproductive inflorescence production included an additive combination of cross type and neutral genetic distance (AIC_cwt = 42.0%, Table 1), and the interactive model was a close second-best. Model-averaged predictions show that the response of inflorescence production was dominated by qualitative differences among cross types (Fig. 5a–b), with hybrids producing fewer inflorescences (mean ± SD: 24.5 ± 13.5) than within-population (33.6 ± 19.0) or between-population (31.6 ± 14.6) crosses (Fig. 5b), a 23–28% reduction for hybrids relative to within-species crosses. Second, outcrossing also significantly reduced F_1 seed production per inflorescence (Fig. 5c). Seed production was best explained by a negative effect of genetic distance alone and the second-best model included an additional contribution of cross type (Table 1), with hybrid seed production lower than expected based on neutral distance alone (Fig. 5c).

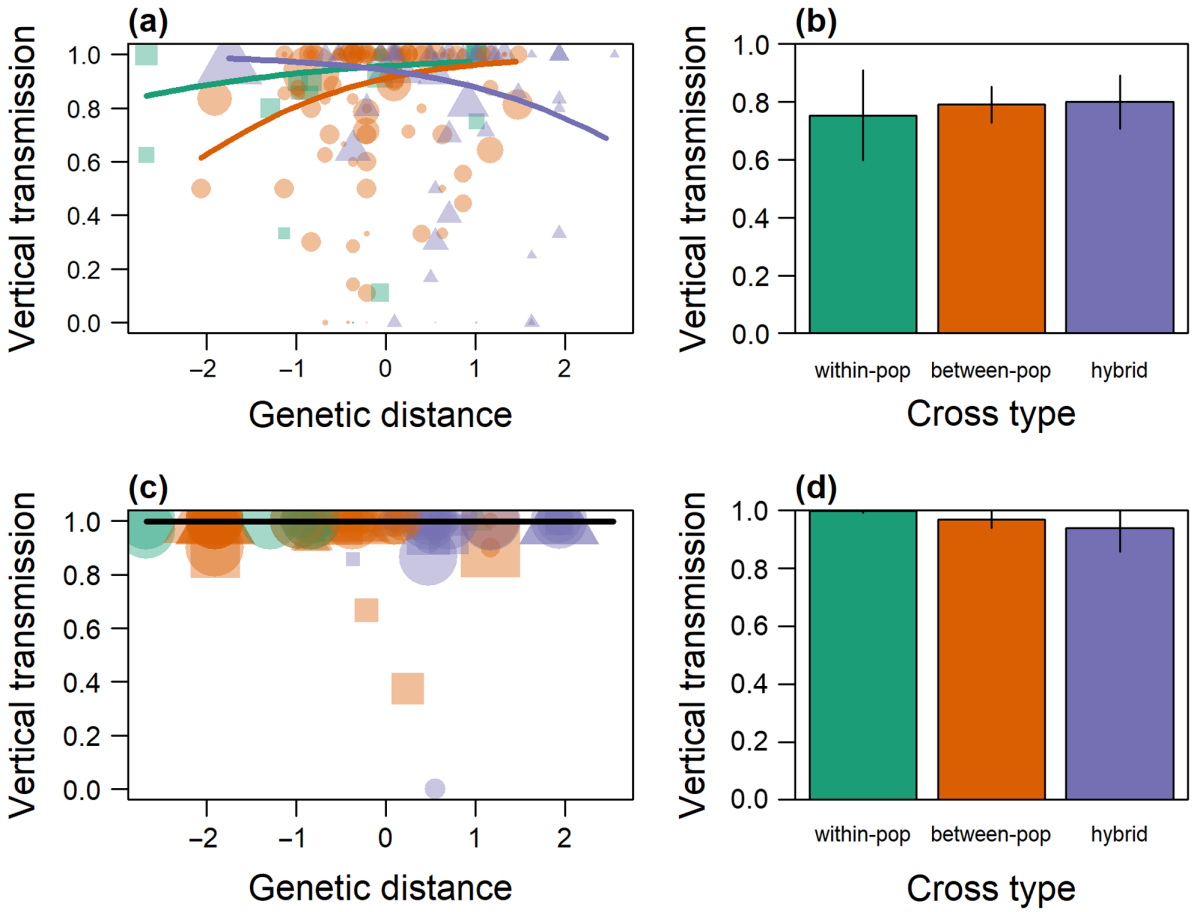

Figure 2. Endophyte vertical transmission (fraction of seeds from E+ plants that are also E+) in F_1 (a–b) and F_2 (c–d) host generations. Transmission is shown (a, c) with respect to neutral genetic distance of experimental crosses (pairwise count of microsatellite allele differences, standardized to mean zero and unit variance), and (b, d) as group means of qualitative cross type. Cross types also shown in (a) and (c) as colors/shapes (within-population = square/green, between-population = circle/brown, hybrid = triangle/purple). In (a) and (c), points sizes are proportional to the number of seeds scored in the transmission assay (range: 1–31). Lines show best-fit model for F_1 transmission and model-averaged predictions for F_2 (Table 1). Error bars in (b, d) show 95% confidence intervals on the means.

On average, inter-specific hybrids experienced reduced seed production relative to within-species crosses (within-population: 16.3 ± 7.52, between-population: 15.8 ± 13.9, hybrid: 8.7 ± 10.1; Fig. 5d), a 45–47% reduction for hyrbids relative to within-species crosses.

Host and symbiont annual fitness

The integrated annual fitness measure showed that, overall, genetically distant, hybrid crosses were associated with a 35–65% reduction in host fitness (average seeds per seed over one year) relative to within-species crosses (Fig. 6). While some hybrid crosses achieved fitness on par with within-species crosses, 42.9% of hybrids had a fitness value of zero, primarily due to complete reproductive failure of F_1 offspring (Fig. 5c). In contrast, 0.0% and 4.5% of within-population and between-population crosses, respectively, had zero fitness. Within-species crosses between host populations had greater fitness than those within populations, on average, suggesting an intermediate optimum of genetic distance between mating partners (Fig. 6). However, this pattern was driven by three between-population crosses with unusually high fitness values. Dropping these outliers resulted in similar mean annual fitness between the two groups of within-species crosses (within-population: 181.1 seeds/seed/year, between-population: 180.1 seeds/seed/year; Fig. 4a). Endophyte annual fitness, or the number of E+ seeds produced per E+ host per year, closely tracked host fitness (Fig. 6). For most crosses, endophyte fitness was lower than host fitness due to failed transmission events.

Discussion

Host outcrossing is hypothesized to create genetic incompatibilities between symbiotic partners that could reduce symbiont vertical transmission and viability (Saikkonen et al. 2004, Cheplick and Faeth 2009, Gundel et al. 2010). Overall, our experimental results fail to support this hypothesis. Instead, we found that outcrossing, especially interspecific hybridization, depressed host reproduction, with

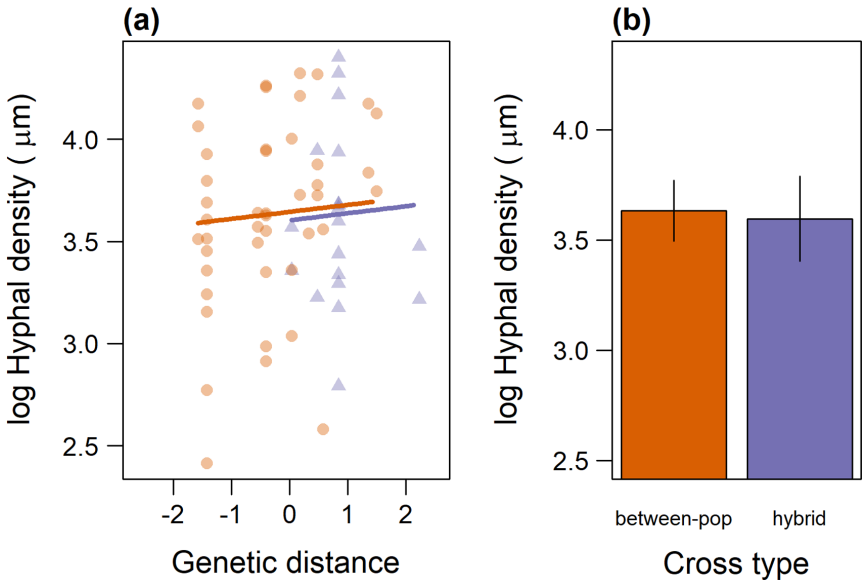

Figure 3. Endophyte hyphal density (µm) in F_1 hosts following experimental crosses. Hyphal density is shown (a) with respect to neutral genetic distance (b) and group means for cross types (cross types also shown as colors/shapes in a, c). Within-population crosses are not represented here because they senesced in the field prior to sampling. Lines in (a) show model-averaged predictions (Table 1). Other plot elements as in Fig. 2.

concomitant reductions in the reproduction of heritable symbionts. Put another way, genetically distant host outcrossing was bad for symbionts, but no worse than it was for the hosts themselves.

Endophyte transmission was generally robust to host outcrossing. In the F_1 generation, there was statistical support for a complex response of transmission to the interaction between cross type and neutral genetic distance (Fig. 2a–b, Table 1). Given that transmission was generally high and differences between cross types were small, especially for well-sampled genetic distances, a conservative interpretation is that vertical transmission was not strongly affected by crossing treatment. Similarly, endophyte growth in planta was not strongly responsive to host genetic background over the range of variation we considered (Fig. 3) and did not correlate with variation in transmission to the F_2 generation. Given that these *Elymus* spp. have diverged relatively recently and continue to experience interspecific gene flow (Saha et al. 2009), selection may favor generalist endophytes that occupy a diversity of host genotypes (Leuchtmann and Clay 1993, Saha et al. 2009, Sun 2014). While, our past work uncovered a link between vertical transmission and seven distinct

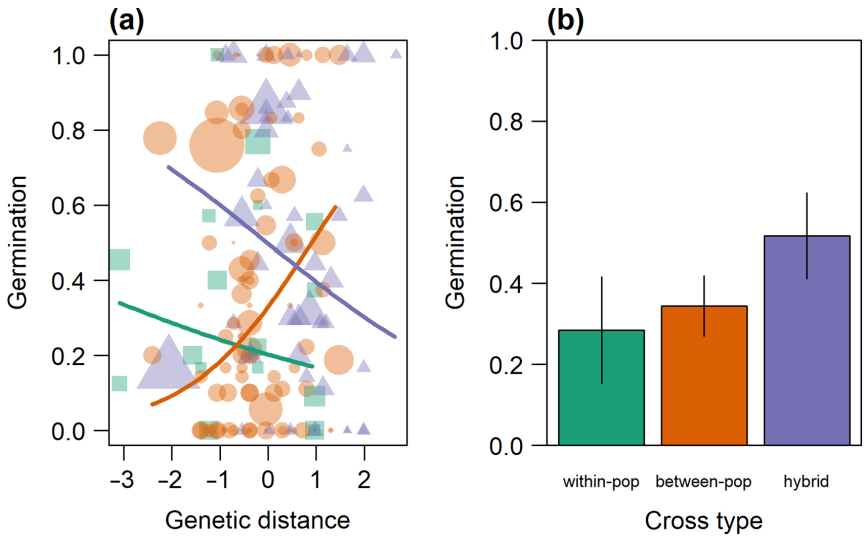

Figure 4. Germination of F_1 seeds following experimental crosses that varied in neutral genetic distance (a) and cross type (b). In (a), point sizes are proportional to number of seeds assayed. Solid lines in (a) show best-fit model (Table 1). Other plot elements as in Fig. 2.

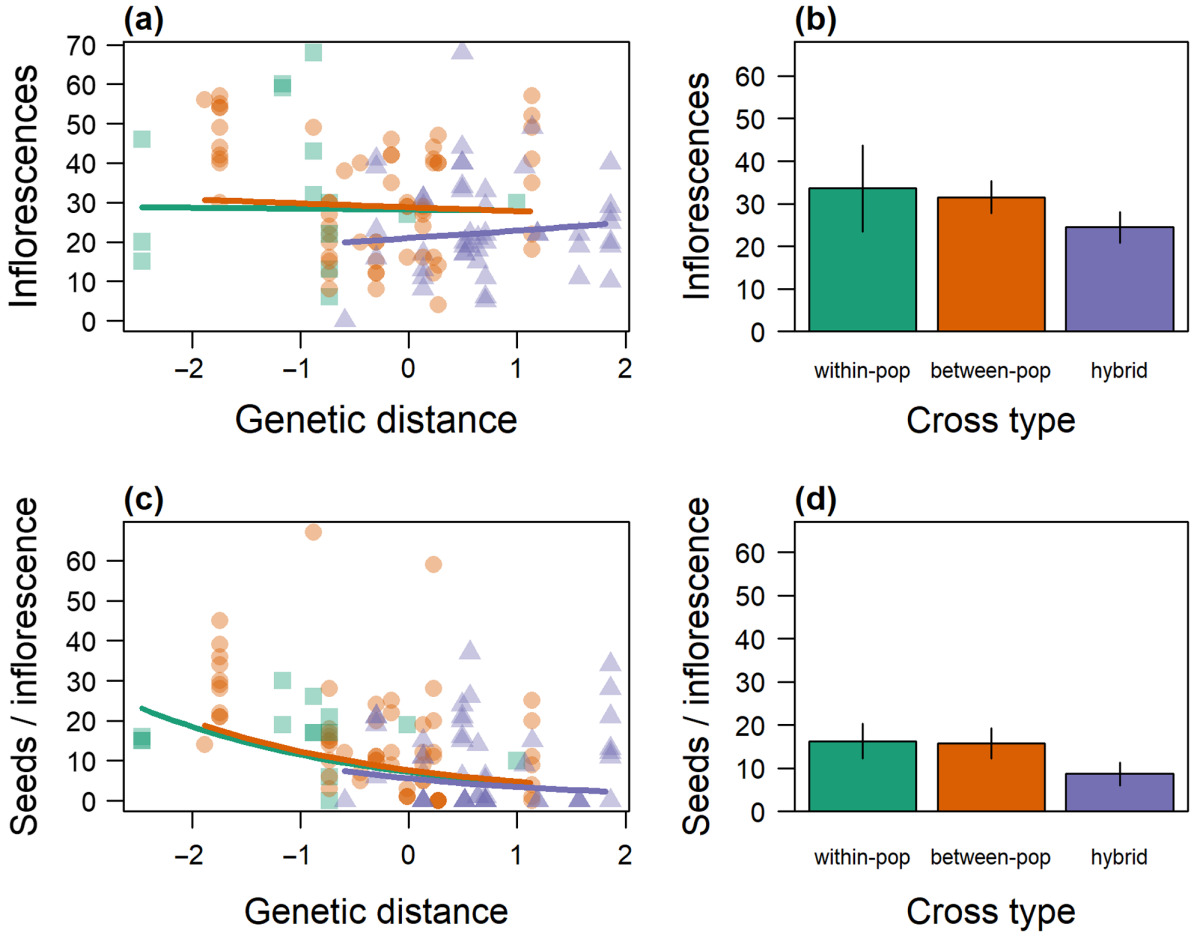

Figure 5. Reproductive fitness of F_1 hosts (inflorescence production (a–b) and seeds per inflorescence (c–d)) following experimental crosses that varied in neutral genetic distance (a, c) and cross type (b, d). Solid lines show model-averaged predictions (Table 1). Other plot elements as in Fig. 2.

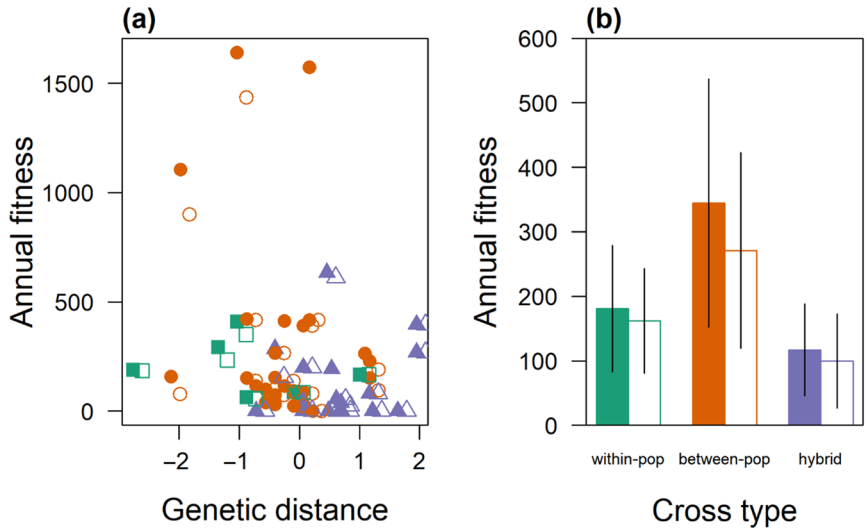

Figure 6. Integrated annual fitness estimates for hosts (expected number of seeds per seed per year; filled shapes/bars) and symbionts (expected number of E+ seeds per seed from an E+ plant; open shapes/bars), shown with respect to neutral genetic distance (a) and qualitative cross type (b). In (a), symbiont fitness values are jittered to the right of host fitness, for visibility. Other plot elements as in Fig. 2.

endophyte alkaloid genotypes (Sneck et al. 2017), the present study only included two of these genotypes and observed no difference in their transmission success. Therefore, any differences in endophyte transmission reported here, likely reflect an effect of host genotype rather than endophyte genotype. In addition, our results contrast with inoculation experiments that observed reduced or abnormal growth of symbionts in novel hosts (Christensen 1995, Saikkonen et al. 2010). On the other hand, our results are consistent with a previous study that found no change in endophyte transmission following experimental crosses between host populations (Gundel et al. 2012). Collectively, the available literature and our new results suggest that experimental inoculation studies that force novel host–symbiont associations may overestimate the potential for genetic incompatibility relative to approaches that measure symbiont responses to ecologically realistic outcrossing events, particularly within the grass–endophyte system.

Even though symbionts can occupy and grow in genetically distant hosts, we found that outcrossing can have strong reproductive consequences for both symbiotic partners. For instance, germination rates were highest for hybrid seeds (Fig. 4), revealing a potential benefit to both symbiotic partners due to heterosis. On the other hand, we also observed strong fertility costs to host outcrossing (inflorescence number and seed production), which was lowest in hybrid offspring (Fig. 5). Integrating these costs and benefits into a single fitness metric revealed an overall cost of between-species outcrossing for hosts and, consequently, their heritable symbionts (Fig. 6). The high frequency of complete reproductive failure among hybrids suggests that these are often a dead-end for vertically transmitted symbionts. At the same time, evidence for introgression of *Elymus canadensis* alleles into natural populations of *E. virginicus* (Saha et al. 2009) suggest that F_1 hybrids should be occasionally viable, as our results confirm. There was mixed support for an intermediate genetic distance yielding maximum reproduction of F_1 hosts, and thus symbionts, depending on whether or not three highly productive outlying crosses were included. Previous studies support the idea that between-population (but not between-species) outcrossing may have fitness benefits for hosts and symbionts (Gundel et al. 2012). Our results suggest that the degree to which hosts and symbionts may benefit from between-population mating may be sensitive to the identity of the populations involved.

Qualitative outcrossing categories generally tracked neutral genetic distance at microsatellite loci, as expected, meaning the genetic distances between species and populations were greater than those from within-populations (Fig. 1). However, our analysis revealed some host responses that could not be predicted by neutral distance alone. For example, germination results suggested that between population crosses, but not other cross types, may respond positively to genetic distance between populations, whereas fertility results showed that hybrid performance was lower than expected based on neutral genetic distance. These idiosyncratic results hint at a complex genetic architecture that underlies host reproductive traits, and reinforce the idea that neutral metrics can miss components of isolation between populations and species that may be important for understanding how outcrossing can jointly influence hosts and symbionts.

Observed differences in endophyte transmission between F_1 and F_2 host generations may have multiple causes. First, different endophyte detection methods were used to quantify endophyte transmission into the F_1 and F_2 host generations. While past studies have demonstrated that these methods produce similar results (Hiatt et al. 1999), small differences in detection accuracy could have contributed to the observed differences in transmission into the F_1 and F_2 host generations. Second, the F_1 generation was produced by experimental selfing/outcrossing, which reduced seed set compared to naturally self-fertilized individuals in the same greenhouse environment. Therefore, our pollination treatments may have induced pollen limitation. In contrast, the F_2 generation was produced by natural self-fertilization in the common garden, where pollen was likely more abundant. Given that endophytes can manipulate host reproduction by allocating resources to maternal (seed) over paternal (pollen) functions (Gorischek et al. 2013), it is possible that endophyte transmission also responds to pollen load. Lastly, endophytes transmitting into F_1 seeds in the greenhouse were exposed to a different abiotic environment than F_2 seeds in the common garden. Although past experiments did not detect differences in the transmission of *Epichloë* sp. due to host resource availability (Davitt et al. 2011), our previous work demonstrated that natural variation in endophyte transmission can co-vary with the environment (Sneck et al. 2017). Therefore, differing abiotic environments could have caused differences in endophyte transmission between greenhouse and common garden hosts.

In order to focus on endophyte growth and transmission efficiency, our study included only E+ hosts; naturally E− hosts were excluded at our initial screening. It is possible that reduced host reproduction due to outcrossing may indeed reflect some form of incompatibility, if benefits of symbiosis break down under inter-specific hybridization. An important next step to test this hypothesis will be to contrast E+ and E− host fitness of varying genetic backgrounds, to test whether benefits of symbiosis are sensitive to host genotype.

In conclusion, this study provides new experimental evidence that outcrossing between genetically distant hosts does not necessarily disrupt symbiont growth or transmission. Our study was the first, to our knowledge, to create ecologically realistic levels of gene flow and test effects on both symbiont vertical transmission and host and symbiont reproductive fitness, factors that are theorized to drive symbiont prevalence in host populations (Saikkonen et al. 2002, Gundel et al. 2008, Bibian et al. 2016). Importantly, we demonstrate that host outcrossing can have strong fitness effects for hosts and the symbionts they harbor, with inter-specific hybrids as likely dead-ends.

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Supplementary material (available online as Appendix oik-06182 at <www.oikosjournal.org/appendix/oik-06182>). Appendix 1.