

Natural History Note

Symbiosis Lost: Imperfect Vertical Transmission of Fungal Endophytes in Grasses

Michelle E. Afkhami* and Jennifer A. Rudgers†

Department of Ecology and Evolutionary Biology, Rice University, Houston, Texas 77005

Submitted August 27, 2007; Accepted April 2, 2008;
Electronically published July 25, 2008

Online enhancement: appendix.

ABSTRACT: Vertically transmitted symbionts associate with some of the most ecologically dominant species on Earth, and their fixation has led to major evolutionary transitions (e.g., the development of mitochondria). Theory predicts that exclusive vertical transmission should favor mutualism and generate high frequencies of symbiosis in host populations. However, host populations often support lower-than-expected symbiont frequencies. Imperfect transmission (i.e., symbiont is not transmitted to all offspring) can reduce symbiont frequency, but for most beneficial symbionts it is unknown whether vertical transmission can be imperfect or during which life-history stage the symbiont is lost. Using quantitative natural history surveys of fungal endophytes in grasses, we show that transmission was imperfect in at least one stage for all seven host species examined. Endophytes were lost at all possible stages: within adult plants, from adult tillers to seeds, and from seeds to seedlings. Despite this loss, uninfected seeds failed to germinate in some species, resulting in perfect transmission to seedlings. The type and degree of loss differed among host populations and species and between endophyte genera. Populations with lower endophyte frequencies had higher rates of loss. Our results indicate new directions for understanding cooperation and conflict in symbioses and suggest mechanisms for host sanctions against costly symbionts.

Keywords: *Neotyphodium*, *Epichloë*, mutualism, Poaceae, herbivory, drought.

Vertically transmitted symbioses—that is, when symbionts are inherited by offspring—feature as some of the most important interactions in evolutionary history. Most notably, organelles in plants and eukaryotic animals resulted from the fixation of vertically transmitted symbionts (Margulis and Neelson 1989; McFadden 2001). Vertically transmitted enteric bacteria have allowed animals ranging in size from aphids to mammals to utilize resources and occupy ecological niches that would otherwise be inaccessible to them (Moran 2001; Bäckhed et al. 2005; Haine 2008). Similarly, the evolution of symbioses between coral and vertically transmitted *Symbiodinium* results in the development of keystone habitats for diverse marine communities (Stachowicz 2001).

Theory predicts that the way in which symbionts are transmitted will affect their costs and benefits to hosts and, ultimately, their frequency within host populations (Lipsitch et al. 1996; Kover and Clay 1998). The rate of vertical transmission is a key element in mathematical models of symbiosis and can influence both the evolution of virulence and the stability of host-symbiont dynamics (Yamamura 1993; Lipsitch et al. 1995, 1996; Lively et al. 2005). Exclusive vertical transmission is expected to favor mutualism (low virulence) and to generate high frequencies of symbiosis in host populations (Fine 1975; Ewald 1987). Models have also shown that imperfect vertical transmission (i.e., the symbiont is not transmitted to all offspring) can maintain host populations with <100% symbiosis, even when fitness benefits of the symbiont are strong (Lipsitch et al. 1995; Ravel et al. 1997; Gyllenberg et al. 2002; Saikkonen et al. 2002; Gundel et al. 2008). In antagonistic symbioses, the effect of selection on the host should act to reduce vertical transmission; indeed, imperfect vertical transmission is widespread and well documented (e.g., insect-*Wolbachia* associations; Newell 1998; Hurst et al. 2001; Stewart et al. 2005). However, we are aware of only one study that has investigated imperfect vertical transmission for a symbiosis that can be mutualistic: in aphids, vertical transmission of enterobacteria was imperfect when

* Corresponding author. Present address: Evolution and Ecology, University of California, One Shields Avenue, 2320 Storer Hall, Davis, California 95616; e-mail: meafkhami@ucdavis.edu.

† E-mail: jrudgers@rice.edu.

aphids supported multiple types of bacteria (Moran and Dunbar 2006). Although it is possible that neither partner can control transmission rates, the existence of imperfect vertical transmission could generate conflict within the symbiosis and selection for control mechanisms. For example, hosts could sanction against costly symbionts by reducing transmission to offspring.

We assessed natural rates of imperfect transmission in symbioses between fungal endophytes and plants. Vertical transmission of foliar fungi is widespread in seed plants and occurs in several plant families, including the grasses (Poaceae; Petrini 1991; Braun et al. 2003; Ernst et al. 2003; Kucht et al. 2004). We focus on *Neotyphodium* and *Epichloë* endophytes (Ascomycota: Clavicipitaceae), which are facultative symbionts in an estimated 20%–30% of grass species (Leuchtman 1992). Endophytes receive carbon from host plants (Thrower and Lewis 1973) and can increase a host's resistance to herbivory and pathogens, as well as improve nutrient uptake and drought tolerance (Bazely et al. 1997; Malinowski et al. 2000; Clay and Schardl 2002; Clay et al. 2005), although benefits are often conditional (Faeth et al. 2004; Saikkonen et al. 2006).

Imperfect transmission provides a potential mechanism for the maintenance of variation in symbiont frequency in nature. If the benefits of vertically transmitted symbionts are consistently strong, selection should favor symbiotic hosts and lead to fixation of the symbiosis. However, surveys of natural symbioses often report <100% symbiotic hosts. For example, 47% of 267 endophyte-infected populations of a native grass (*Lolium perenne*) displayed <25% symbiont frequency (Lewis et al. 1997). Variation in symbiont frequency may be maintained by spatiotemporal conditionality in symbiont benefits (Saikkonen et al. 1998; Ahlholm et al. 2002). For example, a *Neotyphodium* endophyte in *Lolium arundinaceum* increased host biomass under high nutrient conditions but reduced biomass when nutrients were low (Cheplick et al. 1989). Imperfect transmission is a far less investigated mechanism for the maintenance of variation in symbiont frequency (Gundel et al. 2008) and could mediate the influence of spatiotemporal conditionality by generating greater variation in endophyte frequency through symbiont loss.

Imperfect vertical transmission of endophytes may occur in several ways. First, although endophytes are often systemic, infected plants may produce some uninfected seed-bearing stems (i.e., tillers). Second, the endophyte may not grow in all seeds produced by an infected tiller. Finally, the endophyte in maternal seed tissue may become inviable or unable to infect the embryo. The frequency of imperfect vertical transmission has not been documented for natural host populations, and it is unclear at which life-history stage the endophyte may be lost. Furthermore, rates of vertical transmission may vary among types of

endophytes and host species. In grasses, *Epichloë* endophytes can be transmitted both vertically through seeds and horizontally through “choke” disease, in which the fungus produces sexual stromata that suppress all or some host seed production (fig. 1; White 1988; Schardl et al. 2004). Despite this cost to the plant, some *Epichloë* endophytes could still provide a benefit by increasing plant growth or survival (Pan and Clay 2003). In contrast, *Neotyphodium* endophytes are exclusively vertically transmitted and are some of the most beneficial endophytes; the lack of sexual reproduction is often associated with heteroploidy caused by hybridization (Schardl and Selosse 2007). The lack of horizontal transmission in *Neotyphodium* may select for higher rates of vertical transmission compared with *Epichloë* (however, see Lipsitch et al. 1995). In our study, we use quantitative natural history surveys to address three specific questions about symbiont transmission: (1) Is vertical transmission of fungal endophytes imperfect in natural populations of grasses? (2) At which life-history stage is the endophyte lost? (3) How variable is endophyte loss across endophyte genera, host species, and populations?

Methods

Study Sites and Plant Collection

From May through August of 2005 and 2006, we collected tissue from seven native grass species in natural populations in Indiana (table 1), including 12 collections from five locations. In three species (*Poa alsodes*, *Elymus virginicus*, and *Festuca subverticillata*), endophytes are known to increase host growth, biomass, or survival under some conditions (Kannadan and Rudgers 2008; Rudgers and Swafford, forthcoming; M. E. Afkhami and J. A. Rudgers, unpublished data). Populations were arbitrarily chosen and represented a range of natural frequencies of the endophyte (15%–100% of plants infected; table 1). For each population, we haphazardly collected one to three seed-bearing tillers from each of 20 randomly selected plants. We placed seeds from each tiller in an envelope to dry at room temperature for 2–5 days and then stored them at 4°C to prevent endophyte mortality caused by high heat and humidity (Siegel et al. 1984). We collected tillers after seed production occurred so that laboratory conditions did not influence endophyte transmission among tillers or from tillers to seeds. Because logistic constraints precluded examination of the seed-to-seedling transition in nature, we grew seedlings in the greenhouse (~23°C, with daily watering and no supplemental light). Seeds were cold stratified at 4°C for 2–4 weeks before planting in 1.2% water agar or pots (Ray Leach Conetainers, Stuewe and Sons, Corvallis, OR) containing Metromix 200 soil (Sun Gro

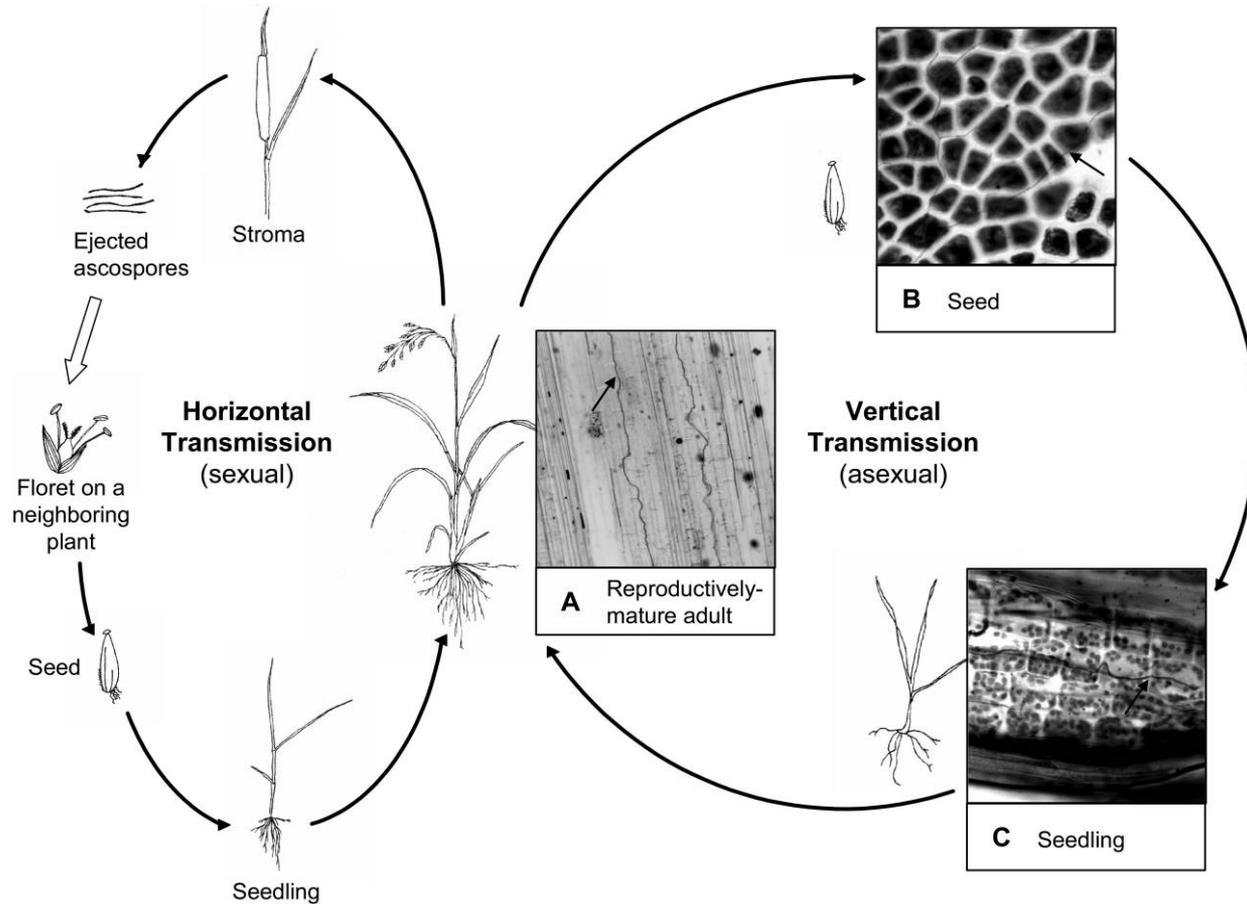


Figure 1: Life cycle diagram of grass-endophyte symbioses. *Neotyphodium* endophyte hyphae in a tiller (A), seed (B), and seedling (C) of *Poa alsodes* taken at $200\times$ magnification on a Leica DFC-480 digital camera attached to a Leica DM-2500 compound microscope (Leica Microsystems, Wetzlar, Germany). Both *Epichloë* and *Neotyphodium* endophytes can undergo vertical transmission from parent plants to seedlings through the seeds. However, only *Epichloë* endophytes also undergo horizontal transmission in which a fungal stroma “chokes” a developing inflorescence, thereby suppressing seed formation and producing fungal ascospores instead (Schardl et al. 2004). Ascospores are ejected and can infect florets of neighboring plants.

Horticulture Distribution, Bellevue, WA). Although endophyte loss from the seed-to-seedling stage could be influenced by laboratory conditions, we expect that our estimates of endophyte loss were more conservative than what would occur in nature because field conditions can be harsh (e.g., high heat and humidity).

Endophyte Detection in Parent Plants

We scored each tiller for presence of endophyte by staining leaf sheaths or nodes with lactophenol cotton blue (Clark et al. 1983), which detects the endophyte regardless of its viability. For each plant, we examined stained tissue under a compound microscope (fig. 1A) and determined the proportion of endophyte-infected tillers. Horizontal transmission of endophytes occurs to florets of neighboring

plants (fig. 1); thus, incomplete systemic infection is not expected to result from horizontal transmission to adults followed by insufficient time for colonization of the entire host. We used staining methods rather than culturing methods because the growth of other fungi on nutrient agar can interfere with endophyte detection. Staining is widely used for grass endophytes (Bacon and White 1994) and typically gives similar results to immunoassay- or polymerase chain reaction-based methods (Dombrowski et al. 2006; Trento et al. 2007). However, because endophyte hyphae are dispersed throughout leaf tissues and because only small sections of tissue are examined, the possibility exists for obtaining false negatives. Nonetheless, we rarely observed false negatives (i.e., when a tiller was scored as endophyte free but produced infected seeds or seedlings; three of 685 tillers [$<0.5\%$ of all samples]). Fi-

Table 1: Collection information and conditional endophyte transmission rates in tiller, seed, and seedling stages

Grass species, endophyte species, and location	Population location (latitude, longitude)	Percentage of infected					Germination (%)
		Plants	Tillers	Seeds	Seedlings	NG seeds	
<i>Elymus hystrix:</i>							
<i>Epichloë elymi:</i> ^a							
IUG	39°11'25"N, 86°30'35"W	47.4 (19)	68.5	50.0 ^{AB}	8.0 ^A	73.6 ^B	20.2 ± 4.2
LDW	39°14'42"N, 86°13'07"W	15.0	100	50.0 ^b	0 ^b	NA	21.5 ± 5.0
<i>Elymus riparius:</i>							
<i>Epichloë</i> species:							
IUG	39°11'25"N, 86°30'35"W	68.8 (16)	97.2	83.3 ^A	76.6 ^A	NA	58.2 ± 5.7
PJYD	39°11'53"N, 86°29'09"W	47.1 (17)	60.0	NA	41.1 ^A	43.4 ^A	27.8 ± 4.3
<i>Elymus virginicus:</i>							
<i>Epichloë elymi:</i> ^a							
BAYLES	39°13'12"N, 86°32'22"W	80.0	85.4	76.9 ^A	82.8 ^A	61.1 ^A	43.3 ± 4.0
PJYD	39°11'53"N, 86°29'09"W	15.0	44.4	0 ^b	88.9 ^b	NA	38.0 ± 5.7
<i>Festuca subverticillata:</i>							
<i>Neotyphodium starrii:</i> ^c							
BAYLES	39°13'12"N, 86°32'22"W	95.0	100	83.7 ^A	75.9 ^B	NA	59.6 ± 3.6
LDW	39°14'19"N, 86°13'04"W	100	100	69.5 ^A	100 ^B	NA	21.0 ± 3.7
RIES	39°13'34"N, 89°29'22"W	100	100	99.5 ^A	77.3 ^B	NA	89.9 ± 2.9
<i>Poa alsodes:</i>							
<i>Neotyphodium</i> species:							
LDW	39°14'39"N, 86°13'07"W	100	100	67.5 ^A	99.7 ^B	33.3 ^C	79.7 ± 5.1
<i>Poa sylvestris:</i>							
<i>Neotyphodium starrii:</i> ^c							
LDW	39°14'22"N, 86°12'55"W	65.0	94.9	83.0 ^b	0 ^b	NA	18.9 ± 9.9
<i>Sphenopholis nitida:</i>							
<i>Epichloë</i> species/ <i>Neotyphodium</i> species: ^d							
RIES	39°13'34"N, 89°29'22"W	92.9 (14)	97.2	66.8 ^b	NA	NA	NA

Note: Mean percentage of infected tillers was calculated using only infected plants, and mean percentages of infected seeds, seedlings, and nongerminating (NG) seeds per tiller were calculated using only infected tillers. Percent plant infection and percent seed germination (\pm SE) were calculated using infected and uninfected plants. For the percentage of plants that were infected (no. of infected plants divided by no. of all plants collected), $n = 20$ in all cases except those indicated with a number in parentheses. Significant differences among seeds, seedlings, and NG seeds from randomization analyses are distinguished by different uppercase letters. IUG = Griffy Lake Indiana University Research and Teaching Preserve (IURTP); LDW = Lilly-Dickey Woods IURTP; PJYD = Pulliam property; BAYLES = Bayles Road IURTP; RIES = Riesberg property. IUG, LDW, PJYD, BAYLES, and RIES represent separate populations found in different locations within the study area; the specific locations of these populations are not relevant to the interpretation of the data.

^a Schardl and Leuchtman 1999.

^b Sample sizes were too low to perform a randomization analysis. For some species, certain stage(s) could not be accessed (NA) and/or statistically analyzed as a result of biological limitations: low infection frequencies and/or low seed production limited the number of seeds that were scored, low germination limited seedling data, and seed contamination limited for which species NG seeds could be scored.

^c Leuchtman and Clay 1990.

^d Clay and Leuchtman 1989.

nally, in many cases, we examined all tillers produced by a plant. However, when subsampling was necessary it made estimates of imperfect transmission more conservative: our methods could not detect cases where all subsampled tillers from an infected plant were endophyte free.

Endophyte Detection in Seeds

We scored a subset of three to 15 seeds per tiller using aniline blue stain, which detects the endophyte regardless of its viability (thereby making it superior to culturing methods; Bacon and White 1994; fig. 1B). False negatives for seeds are unlikely because all tissue in which the endophyte could reside is examined microscopically. For each parent tiller, we calculated the proportion of endophyte-infected seeds.

Endophyte Detection in Seedlings

We planted a subset of seeds from each tiller in the greenhouse. After 1–3 months of growth, seedlings were scored using rose bengal stain following Belanger (1996; fig. 1C). For each parent tiller, we calculated the proportion of endophyte-infected seedlings. Additionally, we examined a subset of seeds that had not germinated after 3–4 months (using aniline blue stain) to compare infection frequencies between seedlings and the subset of seeds that had failed to germinate.

Statistical Analyses

Is Vertical Transmission of Fungal Endophytes Imperfect? At Which Life-History Stage Is the Endophyte Lost? We evaluated endophyte loss in two ways: using conditional analysis and using population-level analysis. For the conditional analysis, we determined the percentage of tillers infected per plant for only the subset of plants that were known to be infected (i.e., analysis was conditional for the plant being infected). Similarly, we calculated the percentage of seeds and seedlings infected, conditional for infection of the parental tiller (table 1). Because infection percentage was calculated only for infected plants (or tillers), any value <100% provides evidence for imperfect vertical transmission (table 1; bootstrapped 95% CI in the appendix in the online edition of the *American Naturalist*). Because scoring seeds is a destructive procedure, it was not possible to track an infected seed as it became a seedling. Therefore, we compared conditional infection frequencies between seeds and seedlings using distribution-free randomization tests (with 9,999 iterations; Manly 1991; ANOVA embedded in an SAS randomization macro, Cassell 2002; residuals from ANOVA could not be normalized). Models included the fixed factor of life-history

stage (seed or seedling), the random factors of population and plant (nested within the population), and the stage \times population interaction (SAS Institute 2004). When seeds that failed to germinate could be recovered, we included “nongerminating seed” as a third level of stage and used Tukey HSD tests within the randomization macro. Population-level analyses included both infected and uninfected plants (i.e., all data collected; fig. 2), and they predicted how infection frequency will change in the population over time. We applied distribution-free randomization tests for mixed-model ANOVA but also included “tiller” as a level of stage. We present actual and adjusted *P* values for population-level analyses: we Bonferroni-adjusted for the number of species examined and, within a species, for the number of populations examined.

How Variable Is Endophyte Loss across Endophyte Genera, Host Species, and Populations? We examined variation in endophyte loss by calculating the difference in endophyte frequency between each life-history transition, using only the subset of plants that were endophyte infected. We used this difference as the response variable (“loss”) in a mixed-model ANOVA that included the fixed factors of endophyte genus (*Epichloë* or *Neotyphodium*) and stage transition (plant–tiller, tiller–seed, or seed–seedling), and the random effects of plant species (nested within endophyte genus) and population (nested within plant species and endophyte genus; Proc Mixed, SAS Institute 2004). Endophyte genus was designated on the basis of prior morphological and phylogenetic analyses (table 1). Two host species (*Poa sylvestris* and *Sphenopholis nitida*) were not included because they produced too few seeds or seedlings. Loss was rank transformed (Conover and Iman 1981). A significant endophyte genus \times stage transition interaction would indicate that endophyte genera differed in the endophyte loss stage. When this interaction was significant, we tested a priori planned contrasts of *Epichloë* versus *Neotyphodium* at each stage.

Finally, if imperfect transmission is an important source of variation in endophyte frequency, we predicted that populations with lower natural endophyte frequencies would exhibit greater rates of endophyte loss. We examined the relationship between infection frequency and the degree of endophyte loss from adult plants to seedlings using a Pearson product moment correlation (SAS Institute 2004; $n = 11$ populations). Because comparisons made across species risk pseudoreplication if phylogeny is ignored (Felsenstein 1985; Harvey and Pagel 1991), we also obtained phylogenetically independent contrasts (details presented in the appendix).

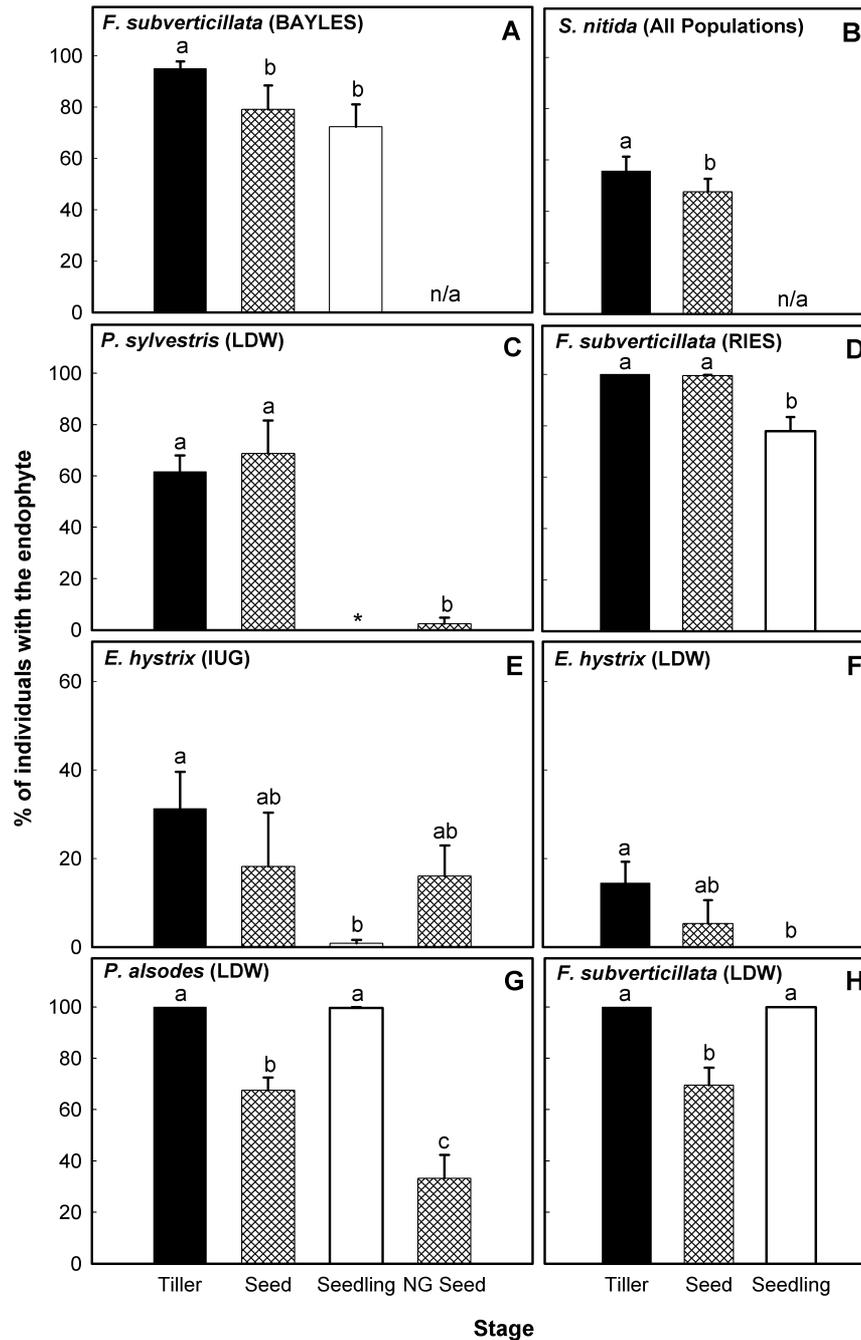


Figure 2: Population-level variation in endophyte frequency for eight of the 12 grass populations. Grass species identities are provided on each graph, followed by the population code in parentheses. A, B, Cases of endophyte loss between the tiller and seed stages. C–F, Cases of endophyte loss between the seed and seedling stages. G, H, Cases where loss between the tiller and seed stage was compensated in the seedlings, in part because of the lower germination rate of endophyte-free seeds. Within a graph, bars indicate mean \pm SE, and significant differences among stages according to randomization Tukey tests are denoted by different letters ($P < .05$). See appendix for full statistical results. Asterisk indicates that all *Poa sylvestris* (Lilly-Dickey Woods Indiana University Research and Teaching Preserve [IURTP; LDW]) seedlings that germinated were uninfected, but statistical results were omitted because of the very low sample size ($n = 5$ seedlings). BAYLES = Bayles Road IURTP; RIES = Riesberg property; IUG = Griffy Lake IURTP. LDW, BAYLES, RIES, and IUG represent separate populations found in different locations within the study area; the specific locations of these populations are not relevant to the interpretation of the data.

Results

Is Vertical Transmission of Fungal Endophytes Imperfect?

Imperfect vertical transmission of endophytes occurred in all seven grass species and all 12 populations (table 1). In total, we scored >4,500 samples, including 685 tillers, 1,897 seeds, 1,482 seedlings, and an additional 477 seeds that failed to germinate. The degree of endophyte loss was substantial but varied among stages, populations, and species (table 1), as described below.

At Which Life-History Stage Is the Endophyte Lost?

Imperfect vertical transmission occurred at all three life-history transitions: among tillers, from tillers to seeds, and from seeds to seedlings. However, endophytes were most commonly lost during the tiller-to-seed transition.

Imperfect Transmission among Tillers. The endophyte was not present in some tillers of infected plants; the percentage of infected tillers ranged from 44% to 100% (conditional analysis; table 1). Imperfect infection among tillers occurred in five of seven grass species: *Elymus hystrix*, *Elymus riparius*, *Elymus virginicus*, *Poa sylvestris*, and *Sphenopholis nitida* (table 1).

Imperfect Transmission to Seeds. All seven species and 12 populations displayed imperfect transmission to seeds in the conditional analysis (table 1). Seeds produced by tillers that were known to be infected ranged from 0% to 99.5% infected (table 1). The more conservative population-level analysis showed significantly reduced infection for three grass species spanning four populations (fig. 2A, 2B, 2G, 2H; table A2 in the online edition of the *American Naturalist*). Significant population-level loss ranged from a 15% lower infection frequency in seeds than tillers for *S. nitida* to a 33% lower infection frequency for *Poa alsodes*. In all cases, reduced symbiosis in the seed stage resulted from the endophyte growing into some, but not all, seeds produced by an infected tiller. However, in the Bayles Road Indiana University Research and Teaching Preserve (IURTP; BAYLES) population of *Festuca subverticillata* (fig. 2A), the endophyte did not grow into any of the seeds for ~12% of the infected tillers.

Imperfect Transmission to Seedlings. In the conditional analysis, seedlings grown from infected tillers ranged from 0% to 100% infected. There was reduced transmission from seeds to seedlings in the conditional analysis for two of three populations of *F. subverticillata* (table 1) and, in the more conservative population-level analysis, for one of three populations (fig. 2A, 2D, 2H). In *P. sylvestris*, seedlings were all uninfected, although very few seeds and

seedlings were produced per tiller (total scored: $n = 46$ seeds, $n = 5$ seedlings), giving low confidence in this result (table 1; fig. 2C). In other work, we have found that *P. sylvestris* can produce infected seedlings (Rudgers et al., forthcoming). In *E. hystrix*, the frequency of seedling infection was significantly lower than it was for tiller infection, but the frequency of seed infection did not differ significantly from the frequency of tiller or seedling infection at the population level (fig. 2E, 2F).

Perfect Transmission to Seedlings. Two grass species lost endophyte infection at the tiller-to-seed transition but displayed significantly higher frequencies of infection in seedlings than in seeds (table 1; fig. 2G, 2H). Both *P. alsodes* and *F. subverticillata* (Lilly-Dickey Woods IURTP [LDW] population) had a ~30% lower infection frequency in seeds than tillers, but seedlings were virtually 100% infected. For *P. alsodes*, seeds that did not germinate after 4 months had significantly lower frequencies of infection than at any other stage ($P < .0001$; fig. 2G; table 1), suggesting that endophyte presence may increase germination rates. *Elymus virginicus* showed a similar pattern to *P. alsodes*, with lower infection frequencies in nongerminating seeds relative to seedlings; however, this difference was nonsignificant in both analyses (table 1; table A2).

How Variable Is Endophyte Loss across Endophyte Genera, Host Species, and Populations?

We predicted that, because *Neotyphodium* is exclusively vertically transmitted (and possibly more beneficial), imperfect vertical transmission would be less common for this genus than for *Epichloë*. When averaged across the three life-history transitions, *Neotyphodium* experienced significantly less loss (mean loss, $8.1\% \pm 1.3$ SE) than did *Epichloë* (mean loss, $25.8\% \pm 4.2$ SE; mixed-model ANOVA, $F = 7.4$, $df = 1, 5$, $P = .04$). However, the effect of endophyte genus varied among life-history transitions (endophyte genus \times transition, $F = 5.8$, $df = 2, 332$, $P = .003$), with significant differences observed between *Neotyphodium* and *Epichloë* at the plant-to-tiller transition only (plant-tiller transition: $P < .0001$, mean loss \pm SE for *Neotyphodium*, $0.0\% \pm 0.0\%$ SE, $n = 4$ populations, mean loss \pm SE for *Epichloë*, $23.4\% \pm 8.8\%$ SE, $n = 6$ populations; tiller-seed transition: $P = .4$; seed-seedling transition: $P = .8$). In addition, the overall frequency of the endophyte in natural host populations was higher for hosts with *Neotyphodium* (tillers, $88.1\% \pm 7.0\%$ SE; seeds, $76.8\% \pm 6.0\%$ SE; seedlings, $87.5\% \pm 7.2\%$ SE) than for hosts with *Epichloë* (tillers, $36.7\% \pm 11.3\%$ SE; seeds, $30.6\% \pm 14.3\%$ SE; seedlings, $20.9\% \pm 10.3\%$ SE). Surprisingly, variation in endophyte loss among host species

was not significantly greater than variation among host populations ($F = 0.6$, $df = 3, 5$, $P = .7$).

Across the populations we examined, the amount of endophyte loss from plants to seedlings was negatively correlated with natural frequencies of endophyte infection (fig. 3). This pattern is consistent with the hypothesis that imperfect vertical transmission can maintain variation in endophyte frequencies among populations in nature. The correlation remained after correcting for phylogenetic relatedness among the host species and populations (fig. 3; fig. A1 in the online edition of the *American Naturalist*).

Discussion

Symbiosis Lost

Despite the theory suggesting that rates of vertical transmission should be high for symbionts that can be mutualistic, our results show that imperfect transmission is not a rare event in facultative grass-endophyte symbioses. We detected endophyte loss in at least one life-history stage for all seven grass species examined. Although theory regarding the importance of transmission to host-symbiont dynamics has been well developed (Yamamura 1993, 1996; Lipsitch et al. 1995, 1996; Kover and Clay 1998), our work is among the first to document rates of vertical transmission for symbioses toward the mutualism-parasitism continuum (see also Moran and

Dunbar 2006). Given that imperfect transmission in grass-endophyte symbioses appears to be common, studies exploring other potentially beneficial symbioses (e.g., between insects and gut flora or between corals and algae; Stachowicz 2001; Hongoh et al. 2005) may uncover similar patterns. Collectively, data on natural variation in transmission will inform models by providing limits on the acceptable range of values for rates of vertical transmission.

How Many Ways to Lose a Symbiont?

We were surprised to discover several pathways for imperfect transmission. Previous endophyte studies have noted some amount of imperfect infection among tillers (Clay et al. 1989; Kover and Clay 1998) and imperfect transmission to offspring (4 uninfected seeds out of 14 scored from infected *Danthonia* parents [Clay 1994] and some uninfected seedlings produced by 2 of 8 infected *Elymus hystrix* parents [Tintjer 2005]) but have not surveyed rates of loss or investigated all life-history transitions. We found that the endophytes could be lost at all three possible life-history transitions: among tillers within plants, from tillers to seeds, and from seeds to seedlings. In addition, populations with lower infection frequencies experienced higher rates of loss, which may explain the common observation of <100% endophyte frequency in species that are known to benefit from endophytes (e.g., White 1987; Lewis et al. 1997; Saikkonen et al. 2000).

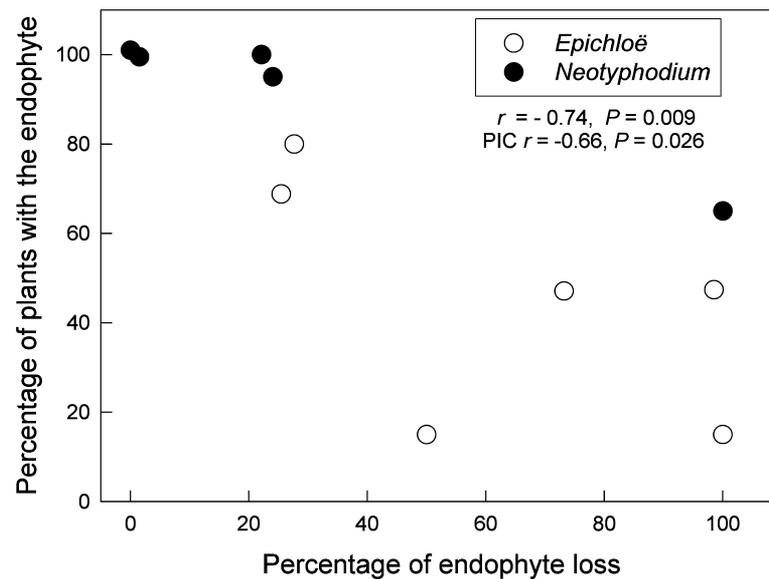


Figure 3: Correlation between the percentage of plants with the endophyte and the percentage of endophyte loss from endophyte-infected plants to seedlings. Each symbol represents a host population. *Filled circles*, populations hosting *Neotyphodium*; *open circles*, hosts of *Epichloë*. The Pearson product moment correlation is presented (r), as well as the correlation coefficient from the analysis of phylogenetically independent contrasts (PIC r ; see also appendix and fig. A1 in the online edition of the *American Naturalist*).

Imperfect vertical transmission may be an especially important mechanism to maintain variation in *Neotyphodium* frequencies because individual hosts cannot regain the endophyte once it is lost.

In some species, uninfected seeds failed to germinate, resulting in perfect transmission from tillers to seedlings despite a loss of the endophyte from tillers to seeds. Selection may favor endophytes that trigger rapid seed germination because endophyte viability in seeds can decline with extended periods of high heat and humidity (Siegel et al. 1984; Welty et al. 1987). However, earlier work on the effect of endophytes on germination has been equivocal (Clay 1987; Novas et al. 2003; Faeth et al. 2004; Gundel et al. 2006). In our study, the endophyte may have decreased seed dormancy, increased seed viability, selectively grown into high-quality seeds, or reduced the plant's investment in endophyte-free seeds. If the endophyte can alter host investment in nonsymbiotic offspring, then grass-endophyte symbioses may function like insect-*Wolbachia* interactions, where the exclusively female-transmitted symbionts appear to reduce investment in male offspring (Werren 1997; Charlat et al. 2003). Experimental manipulations of endophyte presence are needed to determine how endophytes affect germination. It would also be useful to track seeds for more than 4 months to assess long-term dormancy and viability.

Phylogenetic and Geographic Variation in Transmission Rates

The type and frequency of endophyte loss varied significantly among host species and populations and between endophyte genera. Rates of imperfect transmission were significantly higher for *Epichloë* (which displays both vertical and horizontal transmission) than they were for *Neotyphodium* (which is exclusively vertically transmitted), and this difference was mainly explained by a greater rate of loss among tillers within plants. This result is consistent with the hypothesis that plants may reduce vertical transmission of potentially less beneficial symbionts such as *Epichloë* relative to the potentially more beneficial *Neotyphodium*, but experiments are required to test this directly.

In our survey, among-population variation in transmission was as great as among-host species variation. However, studies including host species that span a broader clade would likely detect more variation among species. The substantial population variation supports the geographic mosaic theory of coevolution (Thompson 1994, 2005; Sullivan and Faeth 2004), which underscores the importance of variation among populations in the evolution of interspecific interactions. It would be valuable to investigate whether this geographic variation results

from local plant or endophyte genetic differences and/or variability in local environmental conditions. In fact, a recent mathematical model predicted that genetic differences in imperfect vertical transmission can reduce the minimum transmission rate required for symbiont survival, suggesting an important role for genetic variation (Sanchez et al. 2000).

Controls on Vertical Transmission?

It remains unclear whether hosts or symbionts can exert control over the rate of vertical transmission. Importantly, host control combined with imperfect transmission could allow for sanctions against costly cheater endophytes. Like vertical transmission, host sanctions (i.e., in which the host can respond on the basis of the behavior of the symbiont) have been suggested as a key mechanism for maintaining mutualism in symbioses (Ferdy and Godelle 2005; Sachs and Simms 2006). For example, legumes can reduce photosynthates to sanction rhizobia that do not produce sufficient nitrogen (Denison 2000; Kiers et al. 2003). Similarly, if an endophyte becomes costly, the host could exclude it from some or all seeds. Host sanctions would be especially effective against *Neotyphodium* because the endophyte cannot spread contagiously. In addition, *Epichloë* that function as parasites by reducing plant reproduction could also be sanctioned by host control of vertical transmission.

If exclusively vertically transmitted endophytes (i.e., *Neotyphodium*) can control transmission, selection should favor endophytes that grow into all seeds. However, environmental conditions could interact with symbiont control. For example, stressful conditions can reduce hyphal densities (Mack and Rudgers 2008), which may preclude endophyte infection of all seeds or reduce hyphal densities in seeds (possibly reducing endophyte transmission to seedlings). Under these conditions, selection may favor endophytes that preferentially grow into ovules that are likely to become high-quality or compatible seeds. For example, a closely related endophyte, *Atkinsonella*, can manipulate plants to produce only self-fertilized seeds, which are likely to be the most compatible host genotypes (Clay and Jones 1984). Partner choice could explain the higher frequencies of infection in germinating versus nongerminating seeds, which we observed in three of seven host species.

Conclusion

In our study, we showed that imperfect vertical transmission of a facultative symbiosis is widespread in nature, occurs at several life-history transitions, varies geographically, and is correlated with symbiont frequency. Consideration of the rates and causes of imperfect vertical trans-

mission refines our understanding of how variation in symbiont frequency is maintained and helps to predict when symbionts are most likely to become fixed. Future experiments that assess the relative contributions of host, symbiont, and environmental conditions in altering rates of vertical transmission are needed. We hope this research sparks an interest in how imperfect vertical transmission shapes the dynamics of symbioses, informs mathematical models, and generates new directions for study in this symbiosis and in others.

Acknowledgments

We thank K. Clay, N. Gerardo, J. N. Holland, N. Moran, C. Schardl, J. Strassmann, J. White, K. D. Whitney, H. Wilkinson, and two anonymous reviewers for their invaluable comments on the manuscript, and O. Bartlett, C. A. Baskett, S. Hammer, P. J. Pulliam, M. Rúa, and W. Valencia for their assistance with data collection. This research was funded by National Science Foundation grant DEB 054278 to J.A.R. and a Houston Rodeo Scholarship to M.E.A.

Literature Cited

- Ahlholm, J. U., M. Helander, S. Lehtimäki, P. Wali, and K. Saikkonen. 2002. Vertically transmitted fungal endophytes: different responses of host-parasite systems to environmental conditions. *Oikos* 99: 173–183.
- Bäckhed, F., R. E. Ley, J. L. Sonnenburg, D. A. Peterson, and J. I. Gordon. 2005. Host-bacterial mutualism in the human intestine. *Science* 307:1915–1920.
- Bacon, C. W., and J. F. White Jr. 1994. Stains, media, and procedures for analyzing endophytes. Pages 47–56 in C. W. Bacon and J. F. White Jr., eds. *Biotechnology of endophytic fungi of grasses*. CRC, Boca Raton, FL.
- Bazely, D. R., M. Vicari, S. Emmerich, L. Filip, D. Lin, and A. Inman. 1997. Interactions between herbivores and endophyte-infected *Festuca rubra* from the Scottish islands of St. Kilda, Benbecula and Rum. *Journal of Applied Ecology* 34:847–860.
- Belanger, F. C. 1996. A rapid seedling screening method for determination of fungal endophyte viability. *Crop Science* 36:460–462.
- Braun, K., J. Romero, C. M. Liddell, and R. Creamer. 2003. Production of swainsonine by fungal endophytes of locoweed. *Mycological Research* 107:980–988.
- Cassell, D. L. 2002. A randomization-test wrapper for SAS PROCs. I. SAS Institute, ed. *Proceedings of the Twenty-Seventh Annual SAS Users Group International Conference*.
- Charlat, S., G. D. D. Hurst, and H. Merçot. 2003. Evolutionary consequences of *Wolbachia* infections. *Trends in Genetics* 19:217–223.
- Cheplick, G. P., K. Clay, and S. Marks. 1989. Interactions between infection by endophytic fungi and nutrient limitation in the grasses *Lolium perenne* and *Festuca arundinacea*. *New Phytologist* 111:89–98.
- Clark, E. M., J. F. White, and R. M. Patterson. 1983. Improved histochemical techniques for the detection of *Acremonium coenophialum* in tall fescue and methods of in vitro culture of the fungus. *Journal of Microbial Methods* 1:149–155.
- Clay, K. 1987. Effects of fungal endophytes on the seed and seedling biology of *Lolium perenne* and *Festuca arundinacea*. *Oecologia* (Berlin) 73:358–362.
- . 1994. Hereditary symbiosis in the grass genus *Danthonia*. *New Phytologist* 126:223–231.
- Clay, K., and J. P. Jones. 1984. Transmission of *Atkinsonella hypoxylon* (Clavicipitaceae) by cleistogamous seed of *Danthonia spicata* (Gramineae). *Canadian Journal of Botany* 62:2893–2895.
- Clay, K., and A. Leuchtman. 1989. Infection of woodland grasses by fungal endophytes. *Mycologia* 81:805–811.
- Clay, K., and C. Schardl. 2002. Evolutionary origins and ecological consequences of endophyte symbiosis with grasses. *American Naturalist* 160(suppl.):S99–S127.
- Clay, K., G. P. Cheplick, and S. Marks. 1989. Impact of the fungus *Balansia henningsiana* on *Panicum agrostoides*: frequency of infection, plant growth and reproduction, and resistance to pests. *Oecologia* (Berlin) 80:374–380.
- Clay, K., J. Holah, and J. A. Rudgers. 2005. Herbivores cause a rapid increase in hereditary symbiosis and alter plant community composition. *Proceedings of the National Academy of Sciences* 102: 12465–12470.
- Conover, W. J., and R. L. Iman. 1981. Rank transformations as a bridge between parametric and nonparametric statistics. *American Statistician* 35:124–129.
- Denison, R. F. 2000. Legume sanctions and the evolution of symbiotic cooperation by rhizobia. *American Naturalist* 156:567–576.
- Dombrowski, J. E., J. C. Baldwin, M. D. Azevedo, and G. M. Banowitz. 2006. A sensitive PCR-based assay to detect *Neotyphodium* fungi in seed and plant tissue of tall fescue and ryegrass species. *Crop Science* 46:1064–1070.
- Ernst, M., K. W. Mendgen, and S. G. R. Wirsal. 2003. Endophytic fungal mutualists: seed-borne *Stagonospora* spp. enhance reed biomass production in axenic microcosms. *Molecular Plant-Microbe Interactions* 16:580–587.
- Ewald, P. 1987. Transmission modes and evolution of the parasitism-mutualism continuum. *Annals of the New York Academy of Sciences* 503:295–306.
- Faeth, S. H., M. L. Helander, and K. T. Saikkonen. 2004. Asexual *Neotyphodium* endophytes in a native grass reduce competitive abilities. *Ecology Letters* 7:304–313.
- Felsenstein, J. 1985. Phylogenies and the comparative method. *American Naturalist* 125:1–15.
- Ferdy, J. B., and B. Godelle. 2005. Diversification of transmission modes and the evolution of mutualism. *American Naturalist* 166: 613–627.
- Fine, P. 1975. Vectors and vertical transmission: an epidemiological perspective. *Annals of the New York Academy of Sciences* 266: 173–194.
- Gundel, P. E., P. H. Maseda, M. M. Vila-Aiub, C. M. Ghersa, and R. L. Benesch-Arnold. 2006. Effects of *Neotyphodium* fungi on *Lolium multiflorum* seed germination in relation to water availability. *Annals of Botany* 97:571–577.
- Gundel, P. E., W. B. Batista, M. Texeira, M. A. Martinez-Ghersa, M. Omacini, and C. M. Ghersa. 2008. *Neotyphodium* endophyte infection frequency in annual grass populations: relative importance of mutualism and transmission efficiency. *Proceedings of the Royal Society B: Biological Sciences* 275:897–905.
- Gyllenberg, M., D. Preoteasa, and K. Saikkonen. 2002. Vertically transmitted symbionts in structured host metapopulations. *Bulletin of Mathematical Biology* 64:959–978.

- Haine, E. R. 2008. Symbiont-mediated protection. *Proceedings of the Royal Society B: Biological Sciences* 275:353–361.
- Harvey, P. H., and M. D. Pagel. 1991. *The comparative method in evolutionary biology*. Oxford University Press, New York.
- Hongoh, Y., P. Deevong, T. Inoue, S. Moriya, S. Trakulnaleamsai, M. Ohkuma, C. Vongkaluang, et al. 2005. Intra- and interspecific comparisons of bacterial diversity and community structure support coevolution of gut microbiota and termite host. *Applied and Environmental Microbiology* 71:6590–6599.
- Hurst, G. D. D., F. M. Jiggins, and S. J. W. Robinson. 2001. What causes inefficient transmission of male-killing *Wolbachia* in *Drosophila*? *Heredity* 87:220–226.
- Kannadan, S., and J. A. Rudgers. 2008. Endophyte symbiosis benefits a rare grass under low water availability. *Functional Ecology* 22:706–713.
- Kiers, E. T., R. A. Rousseau, S. A. West, and R. F. Denison. 2003. Host sanctions and the legume-rhizobium mutualism. *Nature* 425:78–81.
- Kover, P. X., and K. Clay. 1998. Trade-off between virulence and vertical transmission and the maintenance of a virulent plant pathogen. *American Naturalist* 152:165–175.
- Kucht, S., J. Gross, Y. Hussein, T. Grothe, U. Keller, S. Basar, W. A. König, U. Steiner, and E. Leistner. 2004. Elimination of ergoline alkaloids following treatment of *Ipomoea asarifolia* (Convolvulaceae) with fungicides. *Planta* 219:619–625.
- Leuchtman, A. 1992. Systematics, distribution, and host specificity of grass endophytes. *Natural Toxins* 1:150–162.
- Leuchtman, A., and K. Clay. 1990. Isozyme variation in the *Acremonium/Epichloë* fungal endophyte complex. *Phytopathology* 80:1133–1139.
- Lewis, G. C., C. Ravel, W. Naffaa, C. Astier, and G. Charmet. 1997. Occurrence of *Acremonium* endophytes in wild populations of *Lolium* spp. in European countries and a relationship between level of infection and climate in France. *Annals of Applied Biology* 130:227–238.
- Lipsitch, M., M. A. Nowak, D. Ebert, and R. M. May. 1995. The population dynamics of vertically and horizontally transmitted parasites. *Proceedings of the Royal Society B: Biological Sciences* 260:321–327.
- Lipsitch, M., S. Siller, and M. A. Nowak. 1996. The evolution of virulence in pathogens with vertical and horizontal transmission. *Evolution* 50:1729–1741.
- Lively, C. M., K. Clay, M. J. Wade, and C. Fuqua. 2005. Competitive co-existence of vertically and horizontally transmitted parasites. *Evolutionary Ecology Research* 7:1183–1190.
- Mack, K. M. L., and J. A. Rudgers. 2008. Balancing multiple mutualists: asymmetric interactions among plants, arbuscular mycorrhizal fungi, and fungal endophytes. *Oikos* 117:310–320.
- Malinowski, D. P., G. A. Alloush, and D. P. Belesky. 2000. Leaf endophyte *Neotyphodium coenophialum* modifies mineral uptake in tall fescue. *Plant and Soil* 227:115–126.
- Manly, B. F. J. 1991. *Randomization and Monte Carlo methods in biology*. Chapman & Hall, New York.
- Margulis, L., and K. H. Neelson. 1989. Symbiosis as the source of evolutionary innovation. *Endocytobiosis and Cell Research* 6:235–239.
- McFadden, G. I. 2001. Primary and secondary endosymbiosis and the origin of plastids 37:951–959.
- Moran, N. A. 2001. The coevolution of bacterial endosymbionts and phloem-feeding insects. *Annals of the Missouri Botanical Garden* 88:35–44.
- Moran, N. A., and H. E. Dunbar. 2006. Sexual acquisition of beneficial symbionts in aphids. *Proceedings of the National Academy of Sciences of the USA* 103:12803–12806.
- Newell, M. 1998. Mechanisms and timing of mother-to-child transmission of HIV-1. *AIDS* 12:831–837.
- Novas, M. V., A. Gentile, and D. Cabral. 2003. Comparative study of growth parameters on diaspores and seedlings between populations of *Bromus setifolius* from Patagonia, differing in *Neotyphodium* endophyte infection. *Flora* 198:421–426.
- Pan, J. J., and K. Clay. 2003. Infection by the systemic fungus *Epichloë glyceriae* alters clonal growth of its grass host, *Glyceria striata*. *Proceedings of the Royal Society B: Biological Sciences* 270:1585–1591.
- Petrini, O. 1991. Fungal endophytes of tree leaves. Pages 179–197 in J. H. Andrews and S. S. Hirano, eds. *Microbial ecology of leaves*. Springer, New York.
- Ravel, C., Y. Michalakis, and G. Charmet. 1997. The effect of imperfect transmission on the frequency of mutualistic seed-borne endophytes in natural populations of grasses. *Oikos* 80:18–24.
- Rudgers, J. A., and A. Swafford. Forthcoming. Benefits of a fungal endophyte in *Elymus virginicus* decline under drought stress. *Basic and Applied Ecology*.
- Rudgers, J. A., M. E. Afkhami, M. A. Rúa, A. J. Davitt, S. Hammer, and V. M. Huguet. Forthcoming. A fungus among us: broad patterns of endophyte distribution in the grasses. *Ecology*.
- Sachs, J. L., and E. L. Simms. 2006. Pathways to mutualism breakdown. *Trends in Ecology & Evolution* 21:585–592.
- Saikkonen, K., S. H. Faeth, M. Helander, and T. J. Sullivan. 1998. Fungal endophytes: a continuum of interactions with host plants. *Annual Review of Ecology and Systematics* 29:319–343.
- Saikkonen, K., J. Ahlholm, M. Helander, S. Lehtimäki, and O. Niemeläinen. 2000. Endophytic fungi in wild and cultivated grasses in Finland. *Ecography* 23:360–366.
- Saikkonen, K., D. Ion, and M. Gyllenberg. 2002. The persistence of vertically transmitted fungi in grass metapopulations. *Proceedings of the Royal Society B: Biological Sciences* 269:1397–1403.
- Saikkonen, K., P. Lehtonen, M. Helander, J. Koricheva, and S. H. Faeth. 2006. Model systems in ecology: dissecting the endophyte-grass literature. *Trends in Plant Science* 11:428–433.
- Sanchez, M. S., J. Arnold, and M. A. Asmussen. 2000. Symbiont survival and host-symbiont disequilibria under differential vertical transmission 154:1347–1365.
- SAS Institute. 2004. SAS, version 9.1.3. SAS Institute, Cary, NC.
- Schardl, C. L., and A. Leuchtman. 1999. Three new species of *Epichloë* symbiotic with North American grasses. *Mycologia* 91:95–107.
- Schardl, C. L., and M. A. Selosse. 2007. Fungal endophytes of grasses: hybrids rescued by vertical transmission? an evolutionary perspective. *New Phytologist* 173:452–458.
- Schardl, C. L., A. Leuchtman, and M. J. Spiering. 2004. Symbioses of grasses with seedborne fungal endophytes. *Annual Review of Plant Biology* 55:315–340.
- Siegel, M., D. R. Varney, M. C. Johnson, W. C. Nesmith, R. C. Buckner, L. P. Bush, P. B. Burrus, and J. R. Herdison. 1984. A fungal endophyte of tall fescue: evaluation of control methods. *Phytopathology* 74:937–941.
- Stachowicz, J. J. 2001. Mutualism, facilitation, and the structure of ecological communities. *BioScience* 51:235–246.

- Stewart, A. D., J. M. Logsdon, and S. E. Kelley. 2005. An empirical study of the evolution of virulence under both horizontal and vertical transmission. *Evolution* 59:730–739.
- Sullivan, T. J., and S. H. Faeth. 2004. Gene flow in the endophyte *Neotyphodium* and implications for coevolution with *Festuca arizonica*. *Molecular Ecology* 13:649–656.
- Thompson, J. N. 1994. *The coevolutionary process*. University of Chicago Press, Chicago.
- . 2005. *The geographic mosaic of coevolution*. University of Chicago Press, Chicago.
- Thrower, L. B., and D. H. Lewis. 1973. Uptake of sugars by *Epichloe typhina* (Pers. Ex Fr.) Tul. in culture and from its host, *Agrostis stolonifera* L. *New Phytologist* 72:501–508.
- Tintjer, T., A. Leuchtmann, and K. Clay. 2008. Variation in horizontal and vertical transmission of the endophyte *Epichloë elymi* infecting the grass *Elymus hystrix*. *New Phytologist* 179:236–246.
- Trento, S., S. Elias, A. Garay, and J. Zavala. 2007. Comparison of endophyte detection in fescue and ryegrass seeds using an immunoblot assay and a microscopic method. *Seed Science and Technology* 35:65–74.
- Welty, R. E., M. D. Azevedo, and T. M. Cooper. 1987. Influence of moisture content, temperature, and length of storage on seed germination and survival of endophytic fungi in seeds of tall fescue and perennial ryegrass. *Phytopathology* 77:893–900.
- Werren, J. H. 1997. Biology of *Wolbachia*. *Annual Review of Entomology* 42:587–609.
- White, J. F., Jr. 1987. Widespread distribution of endophytes in the Poaceae. *Plant Disease* 71:340–342.
- . 1988. Endophyte-host associations in forage grasses. XI. A proposal concerning origin and evolution. *Mycologia* 80:442–446.
- Yamamura, N. 1993. Vertical transmission and evolution of mutualism from parasitism. *Theoretical Population Biology* 44:95–109.
- . 1996. Evolution of mutualistic symbiosis: a differential equation model. *Researches on Population Ecology* 38:211–218.

Natural History Editor: Henry M. Wilbur