DIVERSITY, HOST AFFINITY, AND DISTRIBUTION OF SEED-INFECTING FUNGI: A CASE STUDY WITH *CECROPIA*

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Abstract. Recruitment limitation has been proposed as an important mechanism contributing to the maintenance of tropical tree diversity. For pioneer species, infection by fungi significantly reduces seed survival in soil, potentially influencing both recruitment success and adult distributions. We examined fresh seeds of four sympatric *Cecropia* species for evidence of fungal infection, buried seeds for five months in common gardens below four *C. insignis* crowns in central Panama, and measured seed survival and fungal infection of inviable seeds. Seed survival varied significantly among species and burial sites, and with regard to local (Panama) vs. foreign (Costa Rica) maternal seed sources. Fresh seeds contained few cultivable fungi, but >80% of soil-incubated seeds were infected by diverse Ascomycota, including putative pathogens, saprophytes, and endophytes. From 220 isolates sequenced for the nuclear internal transcribed spacer region (ITS), 26 of 73 unique genotypes were encountered more than once. Based on the most common genotypes, fungal communities demonstrate host affinity and are structured at the scale of individual crowns. Similarity among fungal communities beneath a given crown was significantly greater than similarity among isolates found under different crowns. However, the frequency of rare species suggests high fungal diversity and fine-scale spatial heterogeneity. These results reveal complex plant–fungal interactions in soil and provide a first indication of how seed survival in tropical forests may be affected by fungal community composition.

Key words: Ascomycota; Cecropia; diversity; fungi; ITS; pioneer species; tropical forest; recruitment limitation; richness.

INTRODUCTION

Constraints imposed on resource allocation to seed production and dispersal, coupled with high rates of seed predation, can significantly limit initial recruitment success of tree populations (Harms et al. 2000, Dalling et al. 2002). In turn, recruitment limitation provides an important mechanism facilitating species coexistence by slowing competitive exclusion of species in a community (Hurtt and Pacala 1995, Hubbell 2001). One estimate suggests that half of the seeds produced by 90% of tree species in tropical forests are consumed by animals and fungi (Janzen and Vázquez-Yanes 1991). While patterns of mammalian and insect seed predation have been studied in detail (e.g., Janzen 1968, Wright 1983, Forget et al. 1994, Silman et al. 2003), basic information on the magnitude and distribution of seed losses to fungal pathogens is still lacking (Gilbert 2002).

For species with rapid seed germination, infection by pathogens at the seedling stage is likely to be more important than infection of seeds (e.g., Augspurger

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1984). However, for pioneer species that recruit from a persistent soil seed bank, a critical stage for recruitment limitation may occur after seeds are dispersed and before gap formation triggers seed germination. During this period, most seed losses are attributable to insect predators that attack seeds on the soil surface (Alvarez-Buylla and Martínez-Ramos 1990). The small fraction of seeds that survive and are incorporated into soil are the source of recruits in gaps that form months or years after seed dispersal (Murray and Garcia 2002).

While in the soil seed bank, seeds are at risk from infection by fungi. Fungicide treatment of buried seeds of two common tropical pioneer tree species (*Cecropia insignis* and *Miconia argentea*) increased survival by 40–45% in the forest understory in Panama (Dalling et al. 1998), and by 36% for *C. insignis* in a greenhouse study in Costa Rica (R. Gallery, *unpublished data*). Similarly, fungicide treatment of buried seeds of a temperate shade-intolerant species (*Betula papyrifera*) more than doubled seed survival in the forest understory, while this effect was less significant in open habitats (O'Hanlon-Manners and Kotanen 2004). Together these results demonstrate the potential for high seed mortality from fungal infection in soil, which can alter seed shadows in the understory and ultimately restrict recruitment of light-demanding species.

Infection of seeds can occur at pre- and post-dispersal stages and may include endophytic, saprophytic, and pathogenic fungal species. Pre-dispersal infection by endophytes (fungi that colonize and live within living plant tissues without causing disease; see Arnold and Lutzoni 2007) may have important consequences for seed persistence in soil, as endophytes have been shown to confer increased resistance to subsequent infection by pathogens (Arnold et al. 2003). Once in soil, opportunities increase for seed infection. Tropical soils contain highly diverse assemblages of fungi (e.g., Lodge 1997, Persiani et al. 1998). The prevalence of saprophytic, pathogenic, or endophytic fungi, the ways in which they interact, their specificity, and the degree to which they demonstrate spatial structure at local, regional, and geographic scales remain largely unknown.

Here, we use a combination of field experiments and molecular tools to characterize a tropical seed-infecting fungal community. We focus on *Cecropia*, a common genus of neotropical pioneer trees represented by four species in lowland forests of central Panama. Specifically we ask: Are seeds commonly infected by fungi prior to dispersal? How diverse are communities of fungi that infect seeds in soil? At what spatial scale are fungal communities structured, and do they show host affinity? Are differences in seed mortality rates among *Cecropia* species the result of differences in susceptibility to infection by particular fungal taxa?

METHODS

Study site and species

We investigated fungal communities associated with seeds of four *Cecropia* species (Urticaceae; Sytsma et al. 2002; Table 1) that grow in seasonally moist lowland forest in the Barro Colorado Nature Monument (BCNM) in central Panama (9°9′ N, 79°51′ W; see Leigh et al. 1996). *Cecropia* comprises 61 pioneer species restricted to the neotropics (Berg et al. 2005). Seeds of *Cecropia* (fruit, after Lobova et al. [2003], but hereafter referred to as seeds) are borne in a fleshy, catkin-like perianth and are dispersed by birds, bats, and primates (Lobova et al. 2003). Seeds are common in the soil seed bank, where they typically persist for one to two years (Alvarez-Buylla and Martínez-Ramos 1990, Dalling et al. 1998, Murray and Garcia 2002; but see Holthuijzen and Boerboom [1982] for a report of longer seed persistence).

Seed incubation experiment

In June 2003, seeds were collected from five individuals (maternal sources) of each of four *Cecropia* species growing in BCNM, and from four individuals of *C. insignis* growing in tropical wet forest at the La Selva Biological Station (LS), Costa Rica (10°26′ N, 84°00′ W; 475 km northwest of BCNM). Immediately after collection from seed traps or from the canopy, seeds were removed from infructescences, rinsed in 10% bleach (0.5% sodium hypochlorite) solution for two minutes to remove surface contaminants, and surface

dried under sterile conditions in a darkroom. Surface contaminants were removed from all seeds to control for initial infection rates. Seeds were sorted by maternal source into lots of 30, mixed with 10 g of sterilized forest soil (autoclaved at 115°C for two hours), and enclosed within nylon mesh bags (0.5 mm mesh size). The nylon bags are effective at excluding most seed-predating arthropods, while allowing seeds to be infected by fungi, bacteria, and viruses, and to be damaged by nematodes.

Bags were buried 3 cm beneath the soil surface and 30 cm apart in one 5 × 5 m plot directly beneath the crown of each of four mature (42–50 cm dbh) female *C. insignis* at Barro Colorado Island (>50 m apart, within BCNM). *C. insignis* trees used as burial sites also served as maternal seed sources and were similar in terms of annual fruit production and soil moisture levels (R. Gallery, *unpublished data*). We did not bury seeds beneath crowns of the other *Cecropia* species because these individuals primarily occur at the forest margin. Four replicate bags of 30 seeds from each maternal source were buried at each site (96 bags below each crown). A total of 375 bags were successfully excavated after five months (December 2003), which typically corresponds to 50% seed mortality for *C. insignis* (Dalling et al. 1998; R. Gallery, *unpublished data*). Contents of each bag were transferred onto sterile filter paper in Petri dishes, watered with sterile water, sealed with one layer of parafilm, and incubated for two months in a shadehouse under 30% full sun and high-red : far-red irradiance to induce germination. Parafilm minimizes evaporation from the dishes but still permits gas exchange (Hummel et al. 2004). Seed survival was measured as the proportion of 30 seeds that germinated (radicle and cotyledon emergence), adjusted for the initial viability of the maternal source, which was measured at the onset of the experiment through germination trials with 100 seeds per maternal source.

Culturing and molecular characterization of fungi from soil-incubated seeds

Seeds from four randomly selected maternal sources per species were sampled evenly from three randomly chosen below-crown plots to test for fungal infection. Four seeds that failed to germinate were chosen haphazardly from each mesh bag, surface-sterilized by sequential immersion in 95% ethanol (10 s), 10% bleach (2 min), and 70% ethanol (2 min), and placed intact on slants of 2% malt extract agar (MEA), which encourages growth of diverse microfungi (Fröhlich and Hyde 1999). Cultures were incubated for up to six months at room temperature and examined weekly for evidence of fungal growth. All filamentous fungi recovered were isolated to pure culture and deposited as vouchers at the Robert L. Gilbertson Mycological Herbarium (University of Arizona, Tucson, Arizona, USA).

Filamentous fungi were observed in cultures from 389 of 480 seeds (81.0%). Because cultures lacked diagnostic reproductive structures, provisional morphotypes were

TABLE 1. Characteristics of four focal *Cecropia* species (Croat 1978), including incidence, richness, and diversity of filamentous fungi recovered from inviable *Cecropia* seeds after soil incubation beneath *C. insignis* crowns at Barro Colorado Nature Monument, Panama.

Species (authority)	Geographic dsitribution	Local distribution (abundance)	Fruiting period	Seed mass (mg)†	Population
C. insignis (Lieb.)	Nicaragua–Colombia	mature forest (common)	Apr–Jun	0.42	Panama
C. insignis (Lieb.)	Nicaragua–Colombia	mature forest (common)	Apr–Jun	0.42	Costa Rica
C. longipes (Pitt.)	Panama	secondary forest (rare)	Jul–Sep	0.83	Panama
C. obtusifolia (Bertol.)	Mexico–Venezuela	mature/secondary forest (occasional)	all year	0.57	Panama
C. peltata (L.)	Mexico–Venezuela	secondary forest (rare)	all year	0.55	Panama
Total					

† Average dry seed mass (including endocarp) was measured by combining 20 seeds from the five maternal sources per species ($n = 100$ per species) in aluminum foil and drying seed lots at 70°C for 48 h.

assigned on the basis of mycelial characteristics (Arnold et al. 2000). Total genomic DNA was extracted directly from cultures following Arnold and Lutzoni (2007). The nuclear ribosomal internal transcribed spacer region (ITS), a ~600 base pair region frequently used in fungal systematics at the species level, was amplified by PCR for 220 isolates representing the most distinctive morphotypes associated with each *Cecropia* species. PCR conditions followed Arnold and Lutzoni (2007) with primers ITS1F and ITS4. Automatic sequencing in both directions was performed using an ABI 3700 (Applied Biosystems, Foster City, California, USA) for all PCR products that demonstrated single bands when visualized on 1% agarose gels treated with SYBR Green (Applied Biosystems) in TAE buffer.

Sequence data were assembled and edited in Sequencher 4.2 (Gene Codes Corporation, Ann Arbor, Michigan, USA) and consensus sequences submitted to BLAST searches of the NCBI GenBank database for provisional identification at higher taxonomic levels (database available online).[6] Sequencher 4.2 was used to assemble genotype groups on the basis of 99% ITS sequence similarity, reflecting the number of unique genotypes while conservatively accounting for up to 1% of variation (~6 base pairs) as a result of sequencing error. Genotype groups based on 99% ITS sequence similarity are not meant to approximate species boundaries, but rather to serve as functional taxonomic units for comparisons of fungal communities among hosts and crowns.

Fungi from canopy-collected seeds

To investigate the potential for fungi to infect seeds before soil contact, fresh seeds were collected in May–June 2004 from the canopy of each focal species, or from mesh seed traps that were checked daily. Forty seeds from each of nine maternal sources were examined for fungal infection by culturing on 2% MEA following surface-sterilization (20 seeds) or incubation in sterile water (24 h) followed by surface sterilization (20 seeds). In each case, 10 seeds were cut in half before culturing, and 10 were cultured intact. No filamentous fungi were recovered.

[6] ⟨http://www.ncbi.nlm.nih.gov/BLAST/⟩

To assess the potential association of unculturable fungi with seeds, we extracted total genomic DNA directly from five surface-sterilized seeds for each of three *C. insignis* maternal sources at BCNM, and from sterile water in which seeds were rinsed following surface-sterilization (control). Products of PCR using the fungal specific primer ITS1F and universal primer ITS4 were ligated to a cloning vector using the Invitrogen TOPO TA cloning kit, and competent cells of *E. coli* transformed using heat shock. Individual clones were cultured on LB medium for 16 h, isolated, subjected to a secondary PCR, and positive results sequenced in both directions using primers ITS1F and ITS4.

Statistical analyses of seed incubation experiment

The percent of initially viable seeds that germinated after soil incubation was analyzed using analysis of variance (ANOVA) with type III sums of squares (SAS Institute 2003). We used a mixed effects ANOVA model to test the variation in seed survival among four sympatric *Cecropia* species, and a separate model to test the effect of seed provenance (BCNM vs. LS) for *C. insignis* only. Prior to analysis, germination data were logit-transformed to approximate normality. The denominator degrees of freedom were adjusted based on the Satterthwaite approximation for unequal variance. Because the four bags per maternal source incubated at a given below-crown site could not be considered independent (see *Results: Molecular characterization of fungi from soil-incubated seeds*), mean survival was used in each model (see Appendix A for full ANOVA tables). Crown (burial location) was treated as a fixed effect in these models because an objective of this study was to determine whether variation in fungal community composition among below-crown sites influences seed survival. Maternal source (nested within species) was treated as a random effect and was used to test species (fixed). Tukey-Kramer tests were used for a posteriori comparisons among species and below-crown sites (alpha = 0.05).

RESULTS

Seed incubation experiment: four sympatric Cecropia spp.

Seed survival differed among the four *Cecropia* species from BCNM (Fig. 1, Appendix A; $F_{3,16} = 9.99$,

TABLE 1. Extended.

Proportion of seeds infected	No. isolates sequenced	ITS genotypes	Proportion of singletons	Fisher's α
0.80	45	25	0.68	20.8
0.60	29	16	0.56	14.7
0.85	38	26	0.77	35.7
0.95	65	22	0.59	11.6
0.81	43	23	0.70	19.9
	220			

$P < 0.001$). *Cecropia longipes* demonstrated the highest survival across all crowns (69.8% ± 3.7% [mean ± SE]), whereas *C. insignis* experienced lower overall survival relative to other species (32.8% ± 3.1%, significant only between *C. insignis* and *C. longipes*; $P < 0.05$). Seed survival differed significantly among crowns ($F_{3,48} = 3.41$, $P < 0.05$), with higher average survival beneath crown 1 (54.2% ± 3.5%) vs. the other three crowns (40.1% ± 3.1%, 42.5% ± 3.2%, 42.9% ± 3.6%; respectively; $P < 0.05$). There was no significant interaction of species and crown ($P = 0.165$).

Survival of *C. insignis* seeds was significantly influenced by an interaction of crown and provenance (LS vs. BCNM; $F_{3,20} = 8.03$, $P < 0.01$), within which both crown and provenance were significant (see Appendix A). Maternal source, nested within provenance, did not significantly influence seed survival ($P = 0.961$).

Molecular characterization of fungi from soil-incubated seeds

Seventy-three ITS genotypes were recovered among 220 sequenced isolates from inviable, soil-incubated
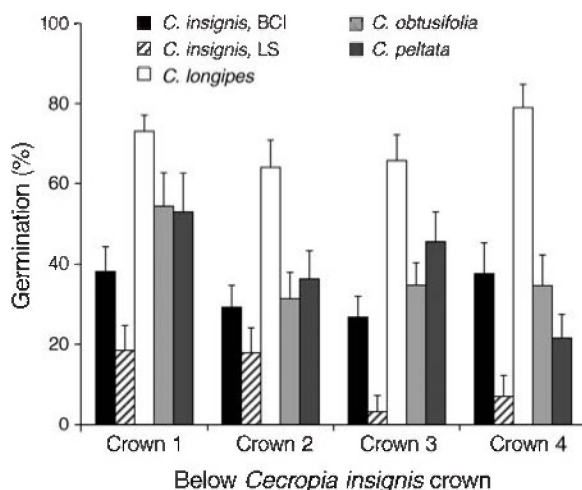

FIG. 1. Differences in seed germination among *Cecropia* species at sites below four crowns of *C. insignis* trees at Barro Colorado Nature Monument, Panama. Bars represent the mean percentage (+SE) of germination success of 20 samples from each species at a given site. Abbreviations are: BCI, Barro Colorado Island; LS, La Selva.
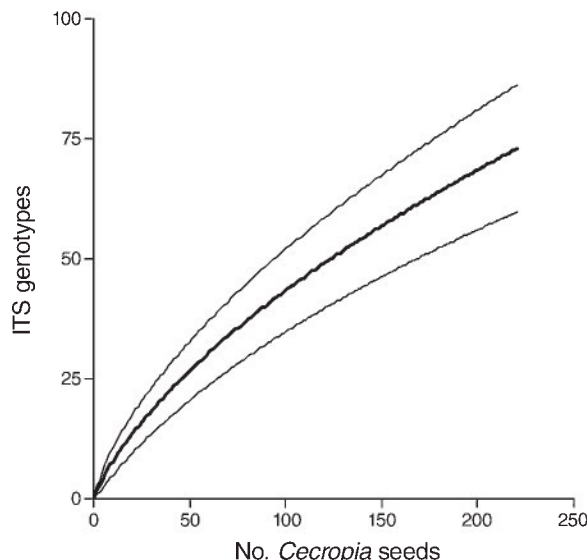

FIG. 2. Mean accumulation of nuclear ribosomal internal transcribed spacer region (ITS) genotypes, defined by 99% ITS sequence similarity, per *Cecropia* seed sampled. Analyses were conducted in EstimateS ⟨http://viceroy.eeb.uconn.edu/EstimateS⟩. The curve incorporates all seeds for which fungal isolates were sequenced; the heavy line indicates the mean ITS accumulation of 50 randomizations of seed order derived from the observed data, and the other two lines indicate 95% confidence intervals around the mean.

seeds. Twenty-six genotypes were isolated from more than one seed; the remainder (64.3%) were recovered only once (Appendix B). Genotypic richness is non-asymptotic (Fig. 2), with high diversity values: Simpson's index = 15.2, Shannon index = 3.45, and Fisher's alpha = 38.2. *C. longipes* showed highest diversity (Fisher's alpha) of seed infecting fungi and the highest number of singleton isolates (Table 1). The fewest isolates were recovered from *C. insignis* from La Selva, which also was characterized by low diversity (Table 1).

The four most commonly isolated ITS genotypes account for 45.4% of the total sample and were the only isolates recovered from all *Cecropia* spp. Hypocrealean species were especially common (Appendix C). Numerous isolates showed only limited affinity for known fungi in GenBank, suggesting a large contribution of previously unknown or unsequenced taxa. When compared against a database of 2007 ITS sequences for endophytes from tropical, temperate, boreal, and arctic sites (A. E. Arnold, *unpublished data*), 29% of genotypes showed ≥95% concordance with foliar endophytic fungi, including representatives of the Ascomycota lineages Diaporthales, Xylariales, Hypocreales, and Botryosphaeriaceae.

Fungal community similarity was significantly greater among bags of seeds beneath a given crown than between crowns ($P \le 0.01$, based on comparison of mean within-crown similarity, measured with Jaccard's index, with 1000 randomizations of between-crown similarity values). Pairwise similarity was significantly
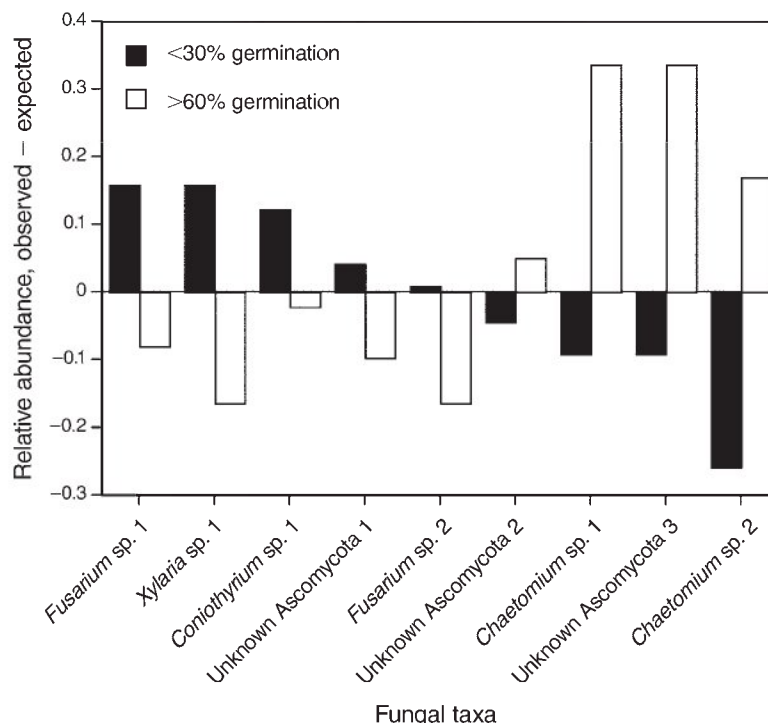
FIG. 3.   Relative abundance of dominant fungi recovered from seed bags of all *Cecropia* species with low (<30% germination) and high (>60% germination) survival. The observed frequency reflects the proportion of isolates from all low (and high) germination bags that is represented by a given genotype. The null hypothesis (expected values) predicts that genotypes will be equally distributed in low- and high-germination bags. The *x*-axis shows a gradient of potential seed-antagonistic (taxa on left) and seed-beneficial (taxa on right) fungi in the soils of Barro Colorado Nature Monument (BCNM).

higher among crowns (0.595 ± 0.108) than among *Cecropia* species (0.370 ± 0.089; Wilcoxon signed-rank test, *P* = 0.03): 92% of nonsingleton genotypes occurred in more than one burial site, relative to 69% occurring in more than one *Cecropia* species. *C. insignis* seeds from the two provenances (BCNM and LS) shared more fungal genotypes with one another than either shared with any other species (two-tailed *t*-test, *t* = −7.06, *P* < 0.05).

Molecular characterization of seedborne fungi before soil contact

In contrast to culturing, direct PCR recovered evidence of fungi in association with at least one seed from each maternal source. No fungal sequences were recovered from the water in which seeds were rinsed following surface-sterilization (control). A total of five ITS genotypes was recovered from *C. insignis* seeds, including Ascomycota (Xylariales: Xylariaceae and Amphisphaeriaceae; Phyllachorales: Phyllachoraceae); and Basidiomycota (Stereales: Stereaceae; Tremellales: Exidiaceae). None of these genotypes was recovered in culture from soil-incubated seeds in the present study, although a matching genotype of the phyllachoraceous species was recovered from *C. insignis* seeds incubated in soil in Costa Rica in a separate experiment (Gallery et al. 2007). All genotypes recovered among the Ascomy-

cota have ≥95% ITS sequence affinity with known endophyte species (A. E. Arnold, *unpublished data*).

Discussion

Variation in seed survivorship in *Cecropia*

We found substantial differences in seed survival among the four sympatric *Cecropia* species and between the local (BCNM) and foreign (La Selva) sources of *C. insignis* seeds buried beneath *C. insignis* crowns. Variation in survival was not due to differences in incidence of fungal infection among *Cecropia* species (Table 1). Instead, the high survival of seeds of a locally rare species, *C. longipes,* could reflect higher tolerance to infection, infection from different suites of fungi, or the presence of symbiotic fungi that protect the seed. Fungi associated with seeds of *C. longipes* were more diverse than those associated with other *Cecropia* species (Table 1). The most common genotype in all other *Cecropia* species infected 13.5% to 30.7% of seeds examined, but was recovered among only 7.9% of *C. longipes* seeds (Appendix C). The causes of differential seed survival between La Selva and BCNM *C. insignis* provenances remain unclear. These seeds were infected by more similar fungal communities than those associated with other *Cecropia* species. Lower survival of La Selva seeds may therefore reflect greater susceptibility to one or several fungal taxa to which BCNM seeds are tolerant.

Transmission, diversity, and distribution of seed-infecting fungi

Although we failed to cultivate microfungi from fresh seeds, direct PCR revealed that seeds may become infected with endophytic fungi while in the canopy. The diversity of fungi encountered suggests infection of developing fruits via contagious spread (i.e., aerial spore deposition) rather than maternal transmission of a specialized symbiont via growth into developing ovules (see Oliver et al. 2001, Ernst et al. 2003). Regardless of mode of infection, the presence of known endophytes in canopy-collected seeds leaves open the possibility that persistence in soil may be mediated by fungal infections obtained during seed development.

In contrast to canopy seeds, filamentous microfungi were readily cultivated from soil-incubated seeds. Most of these cultivars were rare: more than half of the 73 genotypes isolated were collected only once, in accordance with previous studies of other fungal guilds in tropical forests (e.g., Arnold et al. 2000). The most common genotypes were recovered from all crowns and host species, suggesting generalism with regard to site- and host-affinities. However, the remaining nonsingleton genotypes showed that fungal communities were more similar within crowns than among crowns, and within *Cecropia* species than among species. Similarly, the large number of singleton genotypes suggests a tremendous richness of rare species that may contribute to crown-level effects. If heterogeneously distributed genotypes include many of the pathogens and endophytes that affect seed fate, then the spatial structure and host preference we observe here may have a significant impact on *Cecropia* recruitment patterns.

Neither taxonomic affinity nor correlative patterns of seed survival are sufficient to infer ecological roles of fungi; inoculation trials are required to examine causality. By considering average seed germination rates associated with particular fungal isolates, this study provides an important tool for identifying putative fungal pathogens and endophytes for further examination (Fig. 3). For example, we find *Fusarium* sp. 1 to be associated with low germination success, whereas *Chaetomium* spp. are associated with high germination. Kirkpatrick and Bazzaz (1979) found similar associations for *Fusarium* spp. and *Chaetomium* spp. with seeds of grassland annuals. However, Shafer and Kotanen (2004) found that *Fusarium oxysporum* was generally associated with high germination (~80% in four temperate grasses). These results demonstrate the need for caution in assigning fungal life histories on the basis of taxonomy alone, as many fungal genera contain both pathogens and endophytes, and a given fungal species can have different effects (ranging from positive to negative) on different hosts.

In lowland forest in Panama, a diverse community of fungi infects seeds both during fruit development and especially within the soil seed bank. Correlations among isolates and seed survival provide a basis for determining the prevalence of pathogenic, saprophytic, and endophytic fungi and the ways in which they interact to influence seed survival. While some fungi appear to be common and widely distributed generalists, preliminary indications of fine-scale spatial variation and host affinities within soil fungal communities suggest an important and intriguing role in shaping the recruitment and distribution of susceptible hosts.

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APPENDIX A

A mixed-model ANOVA table showing fixed effects of seed species and burial location (crown) on seed survival and an ANOVA table showing effects of provenance, burial location, and maternal source on seed survival (*Ecological Archives* E088-036-A1).

APPENDIX B

A figure showing percent abundance of ITS genotypes isolated from seeds of four *Cecropia* species (*Ecological Archives* E088-036-A2).

APPENDIX C

A table showing top BLAST matches (in GenBank) for ITS genotypes of fungi isolated from seeds of four *Cecropia* species following incubation for five months in the forest understory beneath four crowns of *C. insignis* (*Ecological Archives* E088-036-A3).