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Spatial Ecology of the Fungal Genus *Xylaria* in a Tropical Cloud Forest

Daniel C. Thomas[1,*], **Roo Vandegrift***, **Ashley Ludden, George C. Carroll,** and **Bitty A. Roy**

Institute of Ecology and Evolution, University of Oregon, Eugene, OR, U.S.A.

ABSTRACT

Fungal symbioses with plants are ubiquitous, ancient, and vital to both ecosystem function and plant health. However, benefits to fungal symbionts are not well explored, especially in non-mycorrhizal fungi. The Foraging Ascomycete hypothesis proposes that some wood-decomposing fungi may shift life-history strategies to endophytism to bridge gaps in time and space between suitable substrates. To test this hypothesis we examine spatial relationships of *Xylaria* endophytic fungi in the forest canopy with *Xylaria* decomposer fungi on the forest floor. We sampled for fungi of the genus *Xylaria* using a spatially explicit sampling scheme in a remote Ecuadorian cloud forest, and concurrently carried out an extensive culture-based sampling of fungal foliar endophytes. We found 36 species of *Xylaria* in our 0.5 ha plot, 31 of which were found to only occur as fruiting bodies. All five species of *Xylaria* found as endophytes were also found as fruiting bodies. We also tested the relationships of both stages of these fungi to environmental variables. Decomposer fungi were differentiated by species-specific habitat preferences, with three species being found closer to water than expected by chance. In contrast, endophytes displayed no sensitivity to environmental conditions, such as host, moisture, or canopy cover. We found evidence of spatial linkage between life stages in two species. We also demonstrate that direct transmission of endophytes from leaves to woody substrates is possible. These results indicate that endophytism may represent one way for decomposer fungi to escape moisture limitation, and that endophytic fungi may act as sources of dispersal for decomposer fungi consistent with predictions of the Foraging Ascomycete hypothesis.

Abstract in Spanish is available with online material.

Key words: decomposer; endophyte; Foraging Ascomycete hypothesis; host preference; life-history strategy; spatial analysis; tropical cloud forest; Xylariaceae.

Much of the recent interest in the plant microbiome is economic, as awareness grows that the plant microbiome is vital to plant health (Carroll 1988, Berendsen *et al.* 2012, Berlec 2012, Chaparro *et al.* 2012), and may be important in mitigating effects of disease and climate change on human food plants (Köberl *et al.* 2011, Woodward *et al.* 2012). Fungal endophytes, an important component of the plant microbiome, are receiving particular attention (Porras-Alfaro & Bayman 2011, Jones 2013). Fungal endophytes are defined functionally, as those fungi found within living, healthy plant tissues; they make their living by not harming their host enough to induce a defensive reaction (Clay 1990, Rudgers *et al.* 2009). Since their discovery, they have been found to be both ubiquitous and incredibly diverse in plants of all ecosystems (Arnold & Lutzoni 2007, Porras-Alfaro & Bayman 2011).

Although numerous benefits to fitness for host-plant partners in the endophytic symbiosis have been observed, and many more proposed (Rodriguez *et al.* 2009), benefits for the fungal partners remain something more of a mystery. To date, the majority of endophyte research has been on temperate-zone clavicipitaceous endophytes of grasses, which often affect herbivory and host physiology, and are thus both ecologically and economically important (Clay & Schardl 2002, Schardl *et al.* 2004, Saikkonen *et al.* 2006). These fungi infect their hosts systematically and are passed on directly to their host-plant's offspring (Clay 1988). The fitness of these fungi increases with increased health and survival of their plant host. On the other hand, many non-clavicipitaceous fungal endophytes are very closely related to known plant pathogens (Carroll 1988, Freeman & Rodriguez 1993), and are well armed with energetically expensive arrays of enzymes for digestion of plant-tissues (Carroll & Petrini 1983, Schulz *et al.* 1999). Some endophytes have been observed to be latent pathogens or saprotrophs, waiting for host-plant weakness or death to be the first to colonize and digest host tissues (Chapela & Boddy 1988, Osono 2006, Promputtha *et al.* 2007, 2010), an obvious fitness benefit for the fungi involved.

However, many fungal endophytes neither vertically transmit to host-plant offspring, nor act as latent pathogens or saprotrophs of host tissues (Lodge 1997). The benefit of endophytism, if any, for these fungi remains unknown. Endophytism appears on the surface to be detrimental to fitness because these fungi undergo an extended period with reduced metabolic rate (Stone *et al.* 2004), and reduced or non-existent rates of sexual reproduction.

How then could the endophyte life-history strategy, which is observed in hundreds of species of fungi, and every major lineage of non-lichenized Pezizomycotina, possibly be adaptive? There are many potential benefits of endophytism to the fungal partner: the period of quiescence, or reduced metabolic rate (Stone *et al.* 2004), may allow for persistence in the environment. The host plant potentially provides a stable carbon source, and

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*D. Thomas and R. Vandegrift contributed equally to this work.
[1]Corresponding author; e-mail: dthomas@uoregon.edu

the host may provide protection from environmental pressures such as desiccation (Chaves *et al.* 2002) and harmful UV radiation (Krauss *et al.* 1997). Endophytism may also play a role in dispersal, as we examine here

Much discussion has taken place in recent years over questions of microbial dispersal (Green *et al.* 2004, Green & Bohannan 2006, Martiny *et al.* 2006, Hanson *et al.* 2012). Dispersal is defined as any transport of propagules, individuals, or gametes that creates gene flow within or between populations (Ronce 2007, Clobert *et al.* 2012). Historically, microorganisms were thought to be functionally unlimited in their ability to disperse over the planet (Becking 1934, Fenchel & Finlay 2004). Despite this, many recent studies of microbes have uncovered evidence for dispersal limitation, or the inability of a strain or species to access and successfully establish itself in an otherwise suitable habitat (Roy 2001, Green & Bohannan 2006, Telford *et al.* 2006, Grubisha *et al.* 2007, Peay *et al.* 2010, Galante *et al.* 2011). Such dispersal limitation may function to constrain the geographic ranges of some species, or the range of gene flow within or between local populations of a given species; indeed, such constraints on gene flow between populations are theorized as a major driver of speciation over evolutionary time scales (Clobert *et al.* 2012). There is evidence that at least some decomposer fungi are dispersal limited, even at local scales (Norros *et al.* 2012). Dispersal limitation may reduce the fitness of an organism relative to competitors (Hurtt & Pacala 1995), suggesting that fungi may be under selective pressure to increase dispersal at both local and regional scales.

Dispersal involves successful transport and successful establishment of propagules (Clobert *et al.* 2012, Hanson *et al.* 2012, Peay *et al.* 2012). An endophytic life stage may enhance both of these processes: senescent leaves fall farther than the vast majority of spores are predicted to travel unassisted (Roper *et al.* 2010, Galante *et al.* 2011, Figure S1), carrying with them mycelium, avoiding the uncertainty inherent in the germination phase of growth from spores. In evergreen forests, leaves generally fall asynchronously, which provides low propagule density over relatively long periods of time (in tropical cloud forests, leaves live 12 mo to >5 yr; Bruijnzeel & Veneklaas 1998, Reich *et al.* 1991), in contrast to spore dispersal from a fruiting body, which provides high propagule density over relatively short periods of time (<1 yr; Rogers 1979, Whalley 1996). Leaves may enhance colonization rates, by creating a sheltered microclimate favorable to inoculation. Additionally, living leaves may provide refugia for endophytic fungi, where fungi can wait out difficult conditions at low metabolic cost, benefiting from the protection afforded by the leaf tissue (Stone 1987, Schulz & Boyle 2005). The idea of endophytism as a secondary life-history strategy for decomposer fungi to span (*i.e.*, disperse across) the scarcity of primary substrates and challenging environmental conditions in both time and space is known as the Foraging Ascomycete (FA) hypothesis (Carroll 1999).

Here, we attempt to critically examine the FA hypothesis in a cloud forest ecosystem, using the genus *Xylaria* Hill ex Schrank (Xylariaceae, Ascomycota) as an example of typical endophytic fungi that may utilize a FA strategy (Fig. 1). Members of this genus are important saprotrophs, found primarily on decomposing dead wood—and, rarely, on leaves and fruits—on the forest floor (Whalley 1996, Lodge 1997, Rogers 2000). *Xylaria* are visible during sexual sporulation, forming relatively large, macroscopic stromata, or 'fruiting' structures (Bayman *et al.* 1998, Davis & Shaw 2008). *Xylaria* are common in virtually every study that has ever been done on endophytes, especially in tropical ecosystems (see Davis *et al.* (2003) for an extensive list). We focus here on a common endophyte genus to avoid the problem of being swamped in the overwhelming diversity of fungal endophytes in the tropics (Arnold *et al.* 2000, Arnold & Lutzoni 2007). These two life stages in *Xylaria,* leaf endophyte and wood decomposer, have been observed within single, tightly defined clades (Okane *et al.* 2008). Additionally, *Xylaria* grow readily in culture, making them ideally suited for study in laboratory conditions. (Whalley 1996, Bayman *et al.* 1998).

Following the FA hypothesis, we hypothesized that: (1) distributions of wood-decomposing *Xylaria* should be spatially coupled to the distributions of those same *Xylaria* in the endophytic life stage. To test this hypothesis, we used a spatially explicit sampling scheme: we looked for spatial clustering not attributable to environmental gradients or biotic interactions, but indicative of dispersal linkage between life stages. This is in opposition to Beckman's hypothesis that microbes are unlimited in their dispersal abilities; if this is the case, *Xylaria* in both life stages should be distributed randomly and independent of each other, save for the selective impacts of the environment. Additionally, (2) if *Xylaria* endophytes display a FA lifestyle, we would expect endophytic host generalism in the tropics, as host selectivity would interfere with dispersal in systems where most available hosts are present in low densities (May 1991). The FA hypothesis also leads to the hypothesis (3) that endophytes will be released from environmental constraints relative to their corresponding decomposers. Using ITS rDNA barcode sequence comparisons (Gardes & Bruns 1993, Schoch *et al.* 2012), we matched decomposer *Xylaria* with endophytes in leaves from the canopy, and compared habitat characteristics of both. Lastly, we expect (4) the FA strategy to be a specialized survival/dispersal mechanism utilized by a subset of fungi within the genus *Xylaria*. Given the diversity of the genus, we expect variation in species' niches to modulate the selective advantage of endophytism.

METHODS

The study site.—All fieldwork described was performed at Reserva Los Cedros, a private, protected forest preserve in the western slope of the Andes, in northwestern Ecuador (00°18′31.0″ N, 78°46′44.6″ W), at 1000-2700 m asl. The reserve lies within the Andean Chocó bioregion, one of the most biodiverse habitats on the planet (Gentry 1992). The reserve protects approximately 6800 hectares of forest, approximately 80 percent of which is primary, premontane tropical wet and cloud forest. The Reserve also shares a border with the 305,000 hectare government-protected Cotocachi-Cayapas Ecological Reserve. Rainfall averages 2903 ± 186 mm per year (Policha 2014). Humidity is typically high (~100%), and daily temperatures at the

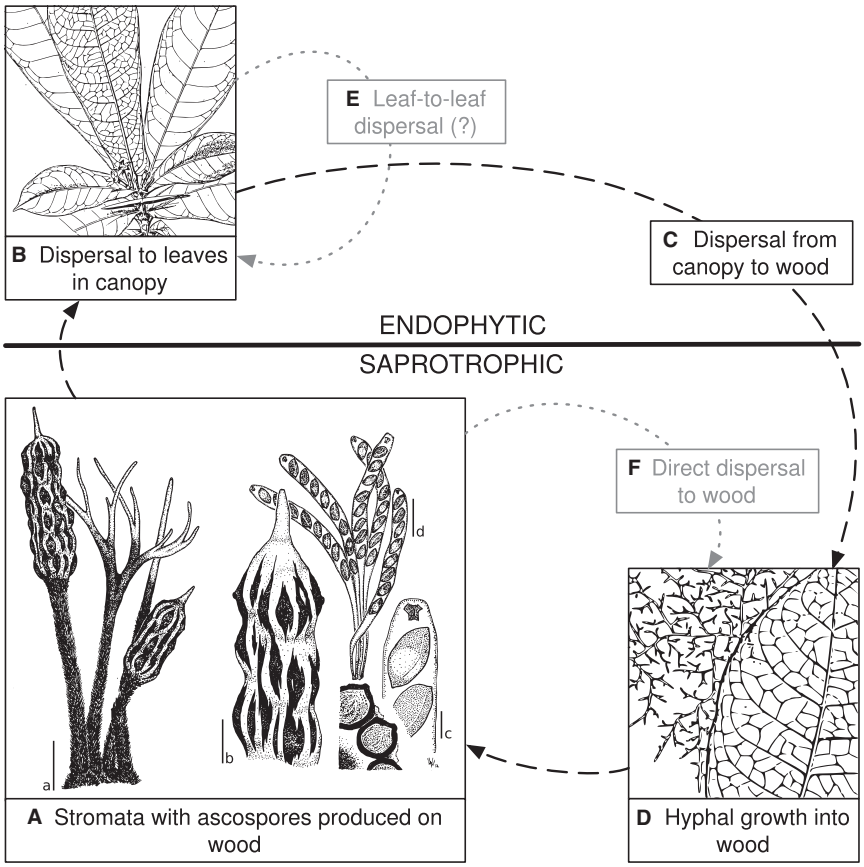

FIGURE 1. Schematic of proposed *Xylaria* life cycle, illustrating the Foraging Ascomycete hypothesis. Stromata (A) are produced on suitable substrate (generally wood); *Xylaria apiculata* Cooke, one of five *Xylaria* species present in both endophytic and decomposer life stages in this study, is illustrated as a typical example of the genus (scale bars: a = 2 mm; b = 1 mm (including stromatal section); c = 10 μm; d = 50 μm). The fungus disperses into the canopy (B) where it initiates endophyte infection; we presume ascospores to be the predominant mechanism of dispersal. When leaves are shed from the canopy (C), they take their endophytes with them; entire leaves may become dispersal vectors. The fungus grows from shed leaves into suitable substrate (D; see also Fig. S8), and the cycle continues. Not explicitly considered in this study are other potential courses of dispersal (in gray): there may be leaf-to-leaf dispersal in the canopy (E), which would maintain endophyte infection even in the absence of sexual reproduction on the forest floor. We find no evidence for this in the literature, however, and expect it to be rare or non-existent. Direct dispersal of ascospores to suitable substrate (F) is undoubtedly a common means of dispersal in this genus. Although an interesting and important mechanism, we do not explicitly examine direct dispersal; this study focuses on elucidating the role of endophytism in the dispersal ecology of *Xylaria*. Panel B redrawn from Seboth (1881).

site range from 15°C to 25°C (Policha 2014). Seasonal variation in climate is minimal. Our sampling occurred during the early part of the wettest season, in January 2012, when fungal fruiting was presumed to be highest.

We sampled within a previously established, 'permanent' tree monitoring plot (Peck *et al.* 2008). Sampling occurred in a primary forest at 1300 m, on the banks of a perennial stream and the surrounding area. The sampling area consisted of 120 individual points, spaced 10 m apart in the east–west direction and 5 m apart in the north–south direction. At each point, the two lowest leaves of the nearest tree or tree-like plant (Table S1) were collected for culturing of endophytes, as well as additional material for host-identification, if necessary. All xylarioid stromata within a 1.2 m radius of the point were collected from the forest floor and any aerial substrate within reach.

Previous environmental data for the plot were inaccessible, so stream mapping and individual point data were recollected later, in March 2014. Site characteristics in the plot are expected to change slowly (Policha 2014). Slope by clinometer, canopy cover by densitometer, and aspect were measured for each point. Our sampling area was small (~0.5 ha) and is presumed to be homogeneous in soil quality and precipitation regime (Policha 2014).

Sample processing.—Leaves were washed gently in a basin of water (~30 sec) to remove epiphyllous debris. Endophytes were recovered from two 2-mm-diameter disks taken from each leaf using a Harris® (GE Healthcare Life Sciences, Pittsburgh, PA, USA) micropunch sampling tool, for a total of 480 individual leaf disks. Disks were surface sterilized by immersion in 70 percent ethanol for 1 min, 5 percent sodium hypochlorite for 2 min, then

rinsed thoroughly in sterile water and placed on water agar (2% agar) petri dishes. Fungi were individually isolated onto MEA plates (2% malt extract, 0.1% yeast extract, acidified to pH 4) as they grew out from the disks of leaf tissue (methods modified from Okane *et al.* 2008). Water agar plates with leaf disks were examined daily for a period of 9 wk, with new isolations made as needed.

All culture work was done in a portable sterile laminar flow hood constructed using a Dayton® Blower (model MG11040581710 10), $^1/_4$ inch Acrylite® (Evonik Industries, Parsippany, NJ, USA), and a Hepa-sep® (HEPA Corporation, Anaheim, CA, USA) filter (model STD12-12-05PEADC50). Power was supplied by a micro-hydrological power plant installed at Reserva Los Cedros.

Cultures were grown on MEA until sufficient hyphae were present for DNA extraction. Under laminar flow, all aerial mycelium were harvested, and then pressed into a Whatman (GE Healthcare Life Sciences, Pittsburgh, PA, USA) FTA® card with the aid of a standard claw hammer (Dentinger *et al.* 2010). Stromata were sampled by removing outer carbonaceous layers using a flame-sterilized scalpel, and preservation of inner tissues in Whatman (GE Healthcare Life Sciences, Pittsburgh, PA, USA) FTA® cards.

ENDOPHYTE TRANSFER EXPERIMENT.—In April 2014, we also collected leaves from a randomly selected tree within the plot (*Nectandra lineatifolia* (Ruiz & Pav.) Mez) for an experiment to examine the transmission of endophytic Xylariaceae to woody substrates. Eight 2-cm sections were cut from each of the 12 leaves, surface sterilized as described above, and placed on sterile (twice-autoclaved) white birch tongue depressors (Puritan, Guilford, Maine, U.S.A.) as a standardized angiospermous woody substrate. Four sections from the same leaf were placed on each tongue depressor. These were incubated at room temperature in EtOH-sterilized Ziploc storage boxes (with an open container of sterilized water to maintain humidity) at the field station for 6 wk, after which time the leaf segments were removed, the tongue depressors were air-dried in open, downward-facing, sterile plastic zipper bags, in which they were then transported back to the United States.

In our lab in Oregon, we started initial cultures from the first three tongue depressors in early August, 2014. We split each tongue depressor into three pieces lengthwise and extracted the middle piece; this was split into 12 equal pieces (~4 mm^2 each), each of which was plated onto water agar for fungal isolation, and incubated indefinitely. Subcultures were made on MEA as described above; cultures were identified to genus by a combination of morphology and DNA sequence.

DNA EXTRACTION.—Lab protocols followed Dentinger *et al.* (2010). Samples were excised from the Whatman (GE Healthcare Life Sciences, Pittsburgh, PA, USA) FTA cards using a 2-mm punch tool and sterilized cutting mat. The punch tool was flame sterilized between uses, and its sterility was confirmed with extraction and PCR tests of DNA from sterile filter paper segments cut by the tool between each use.

Sigma Extract-N-Amp™ Plant PCR Kit reagents were used for extraction from Whatman© (GE Healthcare Life Sciences, Pittsburgh, PA, USA) FTA cards. With each sample disk, 25 μL of extraction reagent was added to each well and incubated for 10 min at 95°C (using an Applied Biosystems© Vereti© (ThermoFisher Scientific, Waltham, MA, USA) model thermal cycler). After incubation, 25 μL of dilution reagent was added to halt further extraction.

PCR AMPLIFICATION.—Template DNA was diluted. Generally, 1:19 dilutions worked best, though optimal dilution ranged from 1:1 to 1:99. DNA amplification was carried out using the fungal-specific ITS1F (5′-CTTGGTCATTTAGAGGAAGTAA-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC3-′) primer sets (Gardes & Bruns 1993). DNA amplification was conducted in a standard 96-well plate with 10-μL reaction volumes (2 μL of template, 5 μL of Sigma Aldrich (St. Louis, MO, USA) Jumpstart™ Taq Readymix™, 2.2 μL sterile water, 0.4 μL 25 mM $MgCl_2$, and 0.2 μL of each primer).

The PCR amplification was done with an Applied Biosystems© Vereti© (ThermoFisher Scientific, Waltham, MA, USA) model thermal cycler with the following parameters: initial denaturation at 95°C for 2 min, five cycles of denaturation at 95°C for 30 sec, annealing at 60°C for 30 sec, and extension at 72°C for 1 min; followed by 25 cycles of denaturation of 95°C for 30 sec, annealing at 55°C for 30 sec, and extension at 72°C for 1 min; a final extension at 72°C for 10 min and a final step of indefinite duration at 4°C.

DNA SEQUENCING AND ANALYSIS.—The PCR products were visualized on a 1 percent agarose gel. Before sequencing, all successful PCR reactions were cleaned by adding 0.4 volumes of a master mix containing 10 percent FastAP© thermosensitive shrimp alkaline phosphatase (Thermo Fisher Scientific©, Pittsburgh, PA, USA) and 1 percent exonuclease I solution (New England Biolabs©, Ipswich, MA, USA) to the PCR product, and incubation for 15 min at 37°C followed by 15 min at 85°C. Samples were then frozen until shipping for sequencing at Functional Biosciences, Inc (Madison, Wisconsin, U.S.A.) on ABI 3730xl instruments using Big Dye V3.1 (ThermoFisher Scientific, Waltham, MA, USA).

Forward and reverse sequences were aligned and curated in Geneious v6.0.3 (Biomatters, Auckland, New Zealand). Sequences were grouped into 97 percent similarity clusters using UClust as implemented in MacQIIME v.1.7.0 (Caporaso 2010) with default settings. Specimens were identified morphologically with the help of Dr. Yu-Ming Ju (Academia Sinica, Taipei, Taiwan, ROC), and sequences were named via confirmed morphological identification wherever possible. In nearly all cases, 97 percent was an adequate cut-off to delineate previously defined morphological species. In one case two species occurred within a grouping (*Xylaria schweinitzii* Berk. & M.A. Curtis and *Xylaria ophiopoda* Sacc.). A maximum likelihood tree was constructed using the PhyML plugin in Geneious, and the two major branches of the tree corresponded perfectly to the two morpho-

logical species. Species groupings were adjusted to accommodate splitting that cluster. Some *Xylaria* species were unable to be identified morphologically due to immaturity or poor condition of specimens. When not in a cluster with identifiable specimens, these were assigned a species identifier, but no name. Finally, a species occurrence matrix was built for all species of *Xylaria*, both endophytes and decomposers.

Statistical methods.—Data were analyzed using R Statistical Software, v. 3.1.0 (R Core Team 2014), including the *sp* (Pebesma & Bivand 2005), *bipartite* (Dormann *et al.* 2008), and *vegan* packages (Oksanen *et al.* 2013). All scripts are publicly available online (Thomas *et al.* 2014).

Estimates of xylariaceous species richness within our plot were estimated using Chao2 and Jacknife1 estimators (Burnham & Overton 1978, Chao 1984, Colwell & Coddington 1994). Sampling effort was visualized with species accumulation curves constructed using the vegan package in R.

Spatial clustering of endophyte and decomposer *Xylaria* life stages of each species was analyzed using nearest neighbor analysis (Clark & Evans 1954) with randomization (Fortin & Dale 2005), using a customized script in R (Thomas *et al.* 2014). Four spatial relationships were examined: clustering of: (1) stromata around stromata; (2) endophytes around endophytes; (3) endophytes around stromata; and (4) stromata around endophytes. For some taxa, not all stages were present; in these cases the subset of possible comparisons was performed.

Spatial clustering of fungal observations around a stream that dissected the plot were also analyzed using custom scripts in R. When all life stages were present, combined life stages (all fungi), stromata alone, and endophytes alone were examined.

The nearest neighbor, with randomization statistic, we employ here is not often utilized in ecology (but see Clark and Evans (1954) and Dixon (1994) for related usages). In each case, a nearest neighbor test statistic was generated using the average of distances of up to five (Liu 2001) nearest neighbor observations from each point, for all observations of a species and life stage. A test-statistic distribution was generated for each species using 20,000 randomly generated sampling areas with the same number of both endophytes and decomposer fungi as the actual sampling area. In each rank of nearest neighbor, or 'distance class', the observed mean nearest neighbor distance ($\bar{d}_o$) was compared to the randomly generated distribution of expected mean nearest neighbor distances ($\bar{d}_e$), and the proportion of $\bar{d}_e$ values lower than the observed were taken as the probability that a given species was spatially under-dispersed significantly more than as predicted by a completely spatially random null model (*i.e.*, that the distance between points is less than that expected by chance; this is often called 'clustering' or 'clumping'). *P* values were considered significant at $P = 0.05$ or below; all nearest neighbor distances are reported in meters.

If the real distance to the nearest neighbor is less than the randomly generated distance to the nearest neighbor more than 95 percent of the time ($P < 0.05$), we take this to mean that the points are significantly clustered. In other words, it is more likely that observations of these species will occur in proximity to other observations of the same species than expected by chance. In the absence of environmental gradients controlling this spatial structuring within a life stage, we take this as evidence of spatial dependence: in the case of life stages clustering to themselves (*e.g.*, endophytes around endophytes), this is likely a signal of 'true' or 'autogenic' autocorrelation (Fortin & Dale 2005), or the tendency of neutral processes to cause organisms to cluster in space and time. In the case of different life stages clustering together (*e.g.*, endophytes around decomposers), we take this as evidence that dispersal is occurring between these different life stages. Tests for spatial correlation ('autocorrelation') of environmental variables were conducted using a Mantel correlogram of environmental dissimilarity of plots against a physical distance matrix. Testing for community turnover, or decay of similarity in *Xylaria* species composition among plots with distance, was done using a Mantel correlogram of *Xylaria* species composition distance matrix against a physical distance matrix of all plots sampled (Fortin & Dale 2005).

In addition to determining if the clusters are non-random, the nearest neighbor metric we employ here allows us to examine the direction of clustering *between* life stages—that is, we can compare the distance to nearest stromata from an endophyte, or vice versa. When determining whether there is clustering of the two life stages, two *P* values are obtained: one for stromata clustering around endophytes, and one for endophytes clustering around stromata.

We examined host preference by endophytes using two methods: (1) we used chi-squared goodness-of-fit tests of host preference by our most common *Xylaria* endophyte (*X. adscendens*) and endophyte preference in the most common host tree (*Faramea* aff. *oblongifolia* Standl.); (2) we used bipartite network analysis to examine the strength of interactions between the host-plants and endophytes.

In the goodness-of-fit analysis of host preference, the null hypothesis was that infection depended only on host commonness, and was generated from the respective ratios of species of all host trees from our plot that were found to host any xylariaceous endophyte. Reciprocally, the null hypothesis for endophyte preference was that the most common host tree would be infected by xylariaceous endophytes in roughly the same frequency that these endophytes were collected from all hosts in the plot. These hypothesized ratios were then compared to the observed ratios of host trees from which *Xylaria adscendens* (Fr.) Fr. was isolated and the frequencies of endophyte species observed solely in *Faramea* aff. *oblogifolia*, using a chi-squared goodness-of-fit test with Monte Carlo simulation (from the base R *stats* package).

Network analysis followed Ikeda *et al.* (2014). Using the *bipartite* package in R (Dormann *et al.* 2008) species interaction matrices were constructed and a network-wide H_2' value (Blüthgen *et al.* 2006) was calculated to characterize the level of preference ('specialization') among host-plants and endophytes. These results were then compared to a null model of network assembly (Vázquez *et al.* 2007), with 10,000 randomization cycles.

Tests for grouping of species by habitat characteristics—slope, canopy, distance-to-water, and aspect (separated into component

northern and eastern exposures)—were done using Permutational Multiple Analysis of Variance (PerMANOVA), with the *adonis* function in *vegan* package in R. These data were visualized with nonmetric multidimensional scaling (NMDS). Differences among the above characteristics for all sites containing a *Xylaria* observation were summarized in an environmental distance matrix as input for the *metaMDS* function in the *vegan* package in R (Oksanen *et al.* 2013); points were then categorized by the species of *Xylaria* observed. The *metaMDS* considers multiple possible solutions using Procrustes analysis and employs Wisconsin double standardization to reduce Kruskal stress in ordination. We considered solutions with stresses below 0.15 to be informative. Linear models of differences in habitat, used for weighting relative importance of habitat variables, were also constructed using the *adonis* function.

RESULTS

Endophytes were isolated from 38 tree species in 19 different families, as well as a species of large fern and several large herbaceous plants when no woody hosts were present within the sampling plot (Table S1). From the 480 total leaf segments, 720 unique cultures were isolated; no leaf segment yielded zero fungi. Of the endophyte isolates, 104 (14.4%) were in the Xylariaceae (19 species in *Xylaria, Hypoxylon, Nemania,* and *Annulohypoxylon*). We collected stromata in two genera of Xylariaceae, *Xylaria* and *Kretzschmaria,* from 79 (65.8%) of the points within the plot. We found 36 species of *Xylaria*, 31 of which were found to only occur as fruiting bodies, and five of which were found as both stromata and endophytes. All five species of *Xylaria* found as endophytes were also found as fruiting bodies; there were no endophytic *Xylaria* not also recovered as stromata (Table 1; Table S2). *Xylaria* leaf endophyte species were found to be a subset of wood decomposer species: all *Xylaria* endophyte species were also recovered as decomposer species. There were species-specific differences in the frequencies of occurrence of the leaf endophyte and decomposer (stromatal) life stages (Table 1): that is, frequency of one life stage does not predict frequency of the other; they are specific to particular species.

Chao2 and Jackknife1 species richness estimators predicted 52.33 (SE = 11.7) and 49.9 (SE = 4.2) *Xylaria* decomposer (stromatal) species, and 5.00 (SE = 0) and 8.0 (SE = 1.7) *Xylaria* endophyte species. This is in agreement with species accumulation curves of our sampling effort indicating that we sampled nearly completely for culturable endophyte species, but that decomposer species remain to be discovered within the plot (Fig. 2).

Five species of *Xylaria* were found both in the leaves and as decomposers. Of these, two species demonstrated non-random clumping of differing life stages (*i.e.*, endophyte-stage fungi were found to clump around decomposer-stage fungi, or *vice versa*): *X.* aff. *curta* ($\bar{d}_o$ (1) = 18.10, $\bar{d}_e$ (1) = 43.90 ± 17.48, P = 0.048) and *X. fissilis_1* ($\bar{d}_o$ (2) = 13.83, $\bar{d}_e$ (2) = 19.94 ± 3.84, P = 0.036) (Table 2; Fig. 3; Figures S2 and S3). For these five species, significant clumping within a life stage was only observed for endophytic *X. adscendens* ($\bar{d}_o$ (2) = 11.91, $\bar{d}_e$ (2) = 13.43 ± 0.89, P = 0.044; Table S3, Figures S4 and S5).

TABLE 1. *List of all* Xylaria *species recovered and the number of points in the study area (out of 120) from which each species was recovered in each life stage. Distinct ITS clusters in otherwise indistinguishable taxa are indicated by an underscore followed by a clade number on the specific epithet.*

Taxa	Points with Stromata	Points with Endophytes
Xylaria adscendens (Fr.) Fr.	3	26
Xylaria anisopleura (Mont.) Fr.	3	
Xylaria apiculata_1 Cooke	9	1
Xylaria apiculata_2 Cooke	1	
Xylaria atrosphaerica (Cooke & Massee) Callan & J.D. Rogers	4	1
Xylaria aff. *comosa* (Mont.) Fr.	5	
Xylaria cristata Speg.	1	
Xylaria cuneata Lloyd	4	
Xylaria curta_1 Fr.	1	
Xylaria curta_2 Fr.	1	
Xylaria aff. *curta* Fr.	2	1
Xylaria enterogena Mont.	11	
Xylaria fissilis_1 Ces.	11	5
Xylaria fissilis_2 Ces.	2	
Xylaria globosa (Pers.) Mont.	5	
Xylaria meliacearum Læssøe	3	
Xylaria multiplex (Kunze) Fr.	3	
Xylaria ophiopoda Sacc.	5	
Xylaria schweinitzii Berk. & M.A. Curtis	16	
Xylaria scruposa_1 (Fr.) Fr.	12	
Xylaria scruposa_2 (Fr.) Fr.	4	
Xylaria subtorulosa Speg.	2	
Xylaria telfairii (Berk.) Sacc.	7	
Xylaria xanthinovelutina (Mont.) Fr.	2	
Xylaria sp. 01	1	
Xylaria sp. 02	1	
Xylaria sp. 03	1	
Xylaria sp. 05	1	
Xylaria sp. 06	1	
Xylaria sp. 07	1	
Xylaria sp. 08	1	
Xylaria sp. 10	1	
Xylaria sp. 11	1	
Xylaria sp. 12	2	
Xylaria sp. 13	2	
Xylaria sp. nov. 2	1	

Of the five *Xylaria* species exhibiting both decomposer and endophytic life stages, three species in the decomposer life stage appear to be closely clustering around the stream present in our sampling area (Fig. 4; Table S4): *X.* aff. *curta* ($\bar{d}_o$ (2) = 29.67, $\bar{d}_e$ (2) = 52.75 ± 12.81, P = 0.016), *X. atrosphaerica* ($\bar{d}_o$ (2,3) = 21.11 and 33.17, $\bar{d}_e$ (2,3) = 34.23 ± 8.01 and 47.30 ± 9.91, P = 0.007 and 0.048), and *X. apiculata* ($\bar{d}_o$ (1,2,3) = 6.85, 12.74, 18.08, $\bar{d}_e$

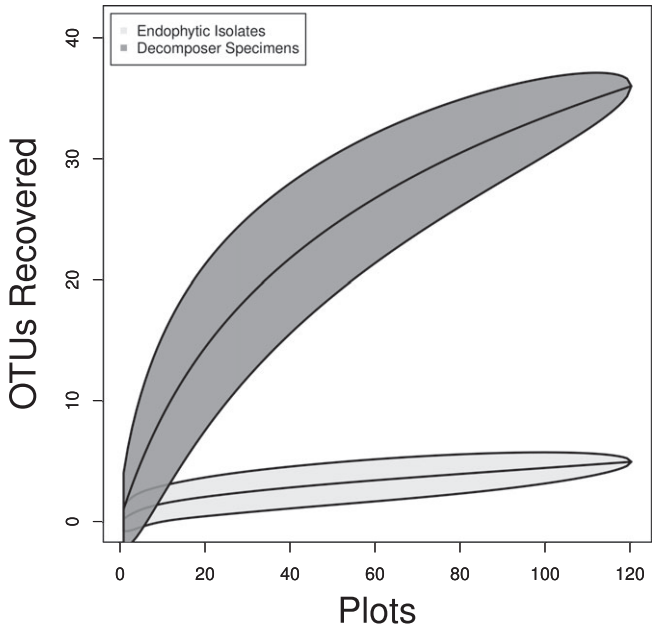

FIGURE 2. Species accumulation/sampling effort curve of both decomposer stromata collected on the forest floor and endophytes cultured from leaves; shaded areas are 95% confidence intervals.

(1,2,3) = 13.49 ± 3.50, 21.62 ± 3.99, 28.17 ± 4.54, $P = 0.006$, 0.002, 0.001). None of the species in the endophytic life stage were clustered around water (Figure S6; Table S4).

Among the 36 species of *Xylaria* detected as decomposers, significant clustering of stromata to stromata was observed in two species (*X. multiplex* and *X. ophiopoda*; Table S5). Significant clustering of stromata around streams was observed in eight species (*X.* aff. *curta*, *X. cuneata*, *X. apiculata_1*, *X. subtorulosa*, *X. multiplex*, *X.* sp. *13*, *X. enterogena*, and *X. atrosphaerica*; Table S5).

Spatial correlation of environmental variables was significant only at distances below 15 m, and variance explained was extremely low (Mantel's $r = 0.06$, $R^2 = 0.004$, $P < 0.05$). *Xylaria* species composition was not found to be significantly autocorrelated on the scale of this study (Mantel's $r = 0.01$, $R^2 < 0.001$, $P = 0.394$).

Habitat preferences were not found to be significantly different among the five *Xylaria* species when we examined combined life stages (PerMANOVA, $F_{4,58} = 1.57$, $R^2 = 0.10$, $P = 0.112$). However, when examined separately, decomposer *Xylaria* may show species-specific habitat preferences (PerMANOVA, $F_{4,24} = 1.84$, $R^2 = 0.23$, $P = 0.07$; Figure S7); endophytic *Xylaria* do not ($F_{4,29} = 0.45$, $R^2 = 0.06$, $P = 0.94$; Figure S7). In decomposer fungi, differences among habitats were defined most strongly by proximity to water (PerMANOVA, $F_{1,23} = 112.42$, $R^2 = 0.44$,

TABLE 2. *Nearest Neighbor analysis of spatial clusters in five species of* Xylaria. *Values shown are the observed mean nearest neighbor distance ($\bar{d}_o$), the expected mean nearest neighbor distance ($\bar{d}_e$) from a Monte Carlo simulation null model assuming complete spatial randomness (CSR), the standard deviation around the expected mean nearest neighbor distance (s_e), and the* P *values, calculated as the proportion of simulations where $\bar{d}_e < \bar{d}_o$. Bold indicates* P < *0.05; italics indicate 0.05* < P < *0.10; dashes indicate insufficient sample size to conduct the analysis at a given neighbor class.*

		Stromata around Endophytes				Endophytes around Stromata			
Taxa	Neighbor class	$\bar{d}_o$	$\bar{d}_e$	s_e	P	$\bar{d}_o$	$\bar{d}_e$	s_e	P
Xylaria aff. *curta*	1	11.2	31.5	18.2	0.152	18.1	43.9	17.5	**0.048**
	2	25.0	56.3	21.5	*0.068*	–	–	–	–
Xylaria apiculata_1	1	20.0	13.9	8.5	0.794	51.2	43.7	10.5	0.771
	2	25.0	22.3	9.3	0.705	–	–	–	–
	3	30.4	29.4	10.4	0.612	–	–	–	–
	4	36.4	36.0	11.6	0.609	–	–	–	–
	5	53.2	42.8	13.0	0.819	–	–	–	–
Xylaria fissilis_1	1	10.8	12.3	3.5	0.354	15.8	19.1	4.3	0.210
	2	13.8	19.9	3.8	**0.036**	28.4	31.5	5.3	0.285
	3	21.1	26.1	4.3	0.108	41.2	43.0	6.0	0.406
	4	26.1	31.7	4.8	0.102	58.0	55.5	8.3	0.634
	5	32.4	37.1	5.3	0.178	76.6	69.6	8.8	0.771
Xylaria adscendens	1	22.1	25.3	5.5	0.302	10.0	7.2	3.0	0.836
	2	46.2	43.4	6.2	0.715	11.9	12.4	2.7	0.494
	3	72.9	62.7	9.6	0.839	16.8	15.9	3.1	0.653
	4	–	–	–	–	19.7	19.0	3.3	0.627
	5	–	–	–	–	23.5	21.9	3.5	0.703
Xylaria atrosphaerica	1	22.4	21.8	12.9	0.639	33.4	43.9	13.4	0.230
	2	25.0	36.3	15.5	0.277	–	–	–	–
	3	36.1	50.6	17.8	0.236	–	–	–	–
	4	50.3	66.8	18.8	0.221	–	–	–	–

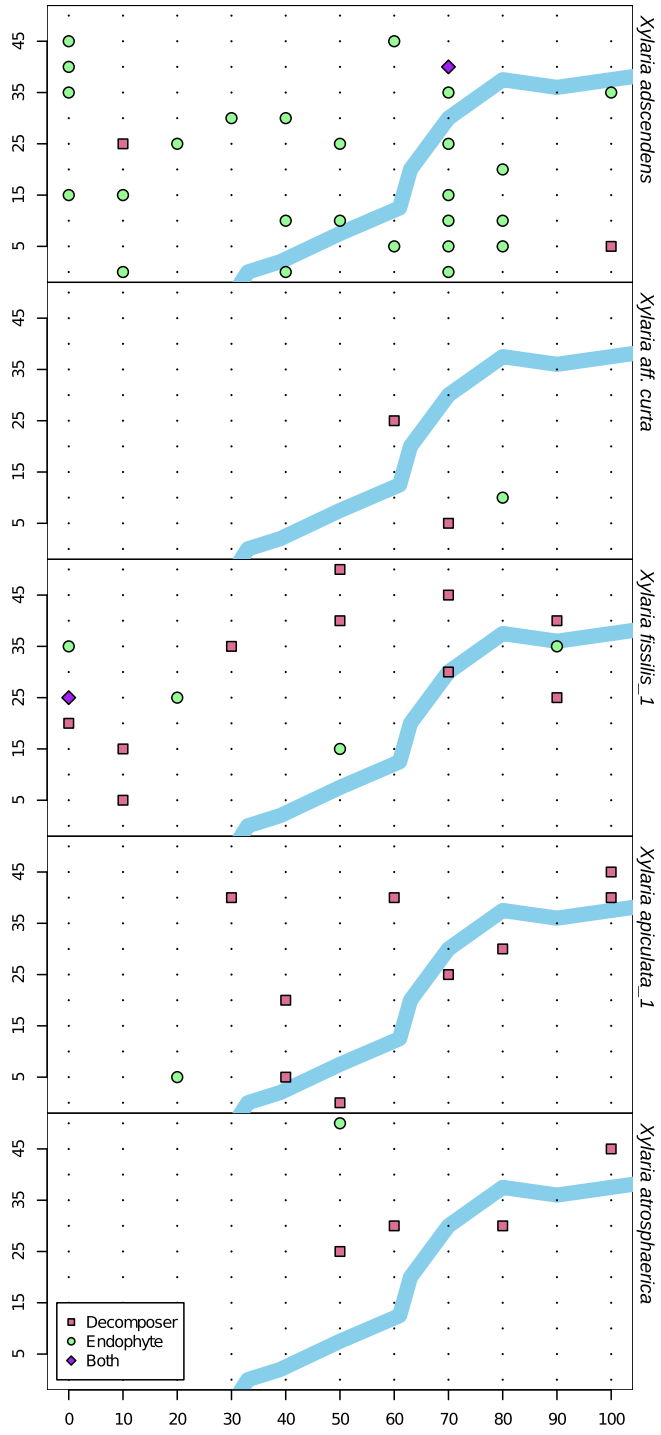

FIGURE 3. Maps of the five species of *Xylaria* displaying both endophyte and decomposer life stages. All collection points are marked; the stream is indicated with a blue line. Scale in meters.

$P = 0.001$), followed by slope ($F_{1,23} = 31.36$, $R^2 = 0.12$, $P = 0.001$), canopy cover ($F_{1,23} = 20.61$, $R^2 = 0.08$, $P = 0.001$), and aspect, in its components of northern and eastern exposure ($F_{1,23} = 11.84$, $R^2 = 0.05$, $P = 0.001$ and $F_{1,23} = 6.20$, $R^2 = 0.02$, $P = 0.006$, respectively).

We found no evidence for host preference by endophytes from the family Xylariaceae. Host trees for the most common endophyte, *Xylaria adscendens,* did not vary from general abundances of host trees within the total plot (χ^2, 10,000 replicates, ($N = 10$) = 2.45, $P = 0.74$). Relative abundances of endophytes recovered from the most common host, *Faramea* aff. *oblongifolia,* did not show a significant difference in endophyte abundances within the entire plot, (χ^2, 10,000 replicates, ($N = 26$) = 19.80, $P = 0.86$). Network specialization did not exceed levels expected by chance alone given abundances of endophytes and host-plants ($H_2' = 0.261$, mean randomized $H_2' = 0.290$, 10,000 cycles, $P = 0.62$; Fig. 5).

ENDOPHYTE TRANSFER EXPERIMENT.—We isolated *Xylaria* from eight of 12 segments from one of the three sampled tongue depressors (22% of segments). By the sixth month, the *Xylaria* had established competitive dominance in these tongue depressor segments, and was observed to initiate fruiting in seven of the eight segments from which it was isolated (Figure S8); all stromatal primordia displayed classic *Nodulisporium* anamorphs. Unfortunately, we have not been able to obtain usable ITS sequence for these isolates, presumably due to co-extraction of PCR inhibiting fungal cell wall polysaccharides.

DISCUSSION

The Foraging Ascomycete hypothesis challenges two classical assumptions about fungal dispersal: first, that fungi are unlimited in their dispersal abilities (Becking 1934, Fenchel & Finlay 2004), and second, that sexual spores of decomposers are the sole major source of dispersal for these fungi (Malloch & Blackwell 1992, Bayman *et al.* 1998, Norros *et al.* 2012). Following these assumptions, endophytism has been supposed by some to be an accidental 'dead end' infection of living plants (Bayman *et al.* 1998). The FA hypothesis proposes that for some fungi, endophytism is not an accidental 'dead end', but an important mechanism of fungal dispersal—an adaptation for bridging temporal or spatial scarcity of primary substrates. Under this model, a host-plant acts as a reservoir of mycelium, distributing fungi across the range of leaffall.

As such, the FA hypothesis yields several testable predictions: (1) A measurable spatial linkage between endophyte and decomposer life stages for fungi utilizing a FA strategy, wherein stromata serve as sources of endophytic infection (in addition to being sources of direct dispersal), but represent relatively short 'bursts' in time, whereas areas of endophytic infection serve as slower, more 'trickling' dispersal centers. (2) A prediction of endophytic host generalism in diverse tropical forests, as strong host preference would interfere with dispersal abilities in systems where the density of any one host species is usually quite low (May 1991). This prediction may not hold in systems where strong dominant hosts are available, as in many temperate forests. (3) The FA hypothesis leads to a prediction that endophytes will be less constrained by environmental conditions than their corresponding decomposers. And, (4) we predict the FA strategy to be a specialized survival/dispersal mechanism utilized by a subset of fungi. Variation in niche or preferred habitat would modulate the

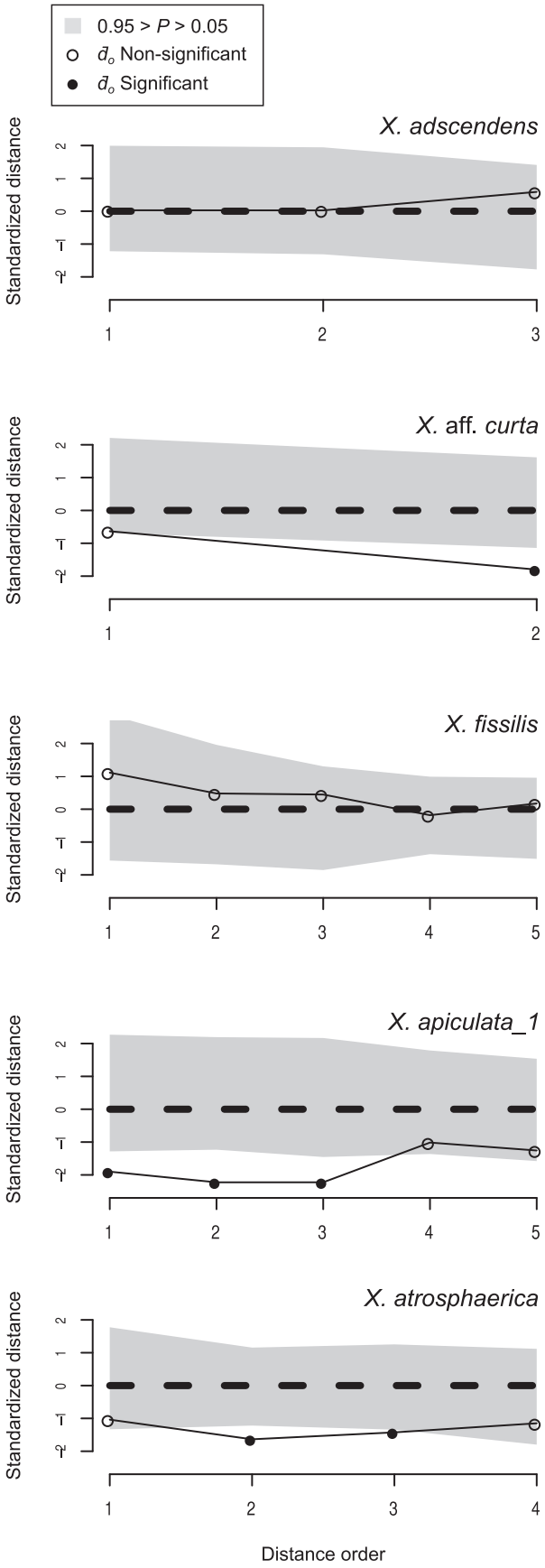

FIGURE 4. Graphical representation of the results of nearest neighbor Monte Carlo-type simulations for clustering of stromata around the stream (see also Table S4). For each species, the standardized mean distance to nearest neighboring point along the stream ($\bar{d}_o$) for all available distance classes is plotted. The dashed line represents the mean distance to points along the stream of the permutations on complete spatial randomness ($\bar{d}_e$), standardized to zero for all distance classes; the *y*-axis units represent deviation from the permutational mean (s_e). Open points are non-significant ($P > 0.05$); closed points are significant ($P < 0.05$); the gray area represents the region where $0.95 > P > 0.05$.

selective advantage of endophytism. Thus, we predict some species in a group to be more adapted to endophytism than others.

We found significant clustering between life stages in two of the five species of *Xylaria* with both life stages, *Xylaria* aff. *curta* Fr. and *Xylaria fissilis* Ces. This suggests spatial linkage of life stages, consistent with prediction (1). It is worth noting that the genetic marker used to link endophytic and decomposer life stages, ITS, has certain limitations. This marker is composed largely of two highly variable introns, and as such is excellent for species identification where reference sequences are available, but is not appropriate for phylogenetic approaches to clustering (Schoch *et al.* 2012), and is not useful for determining relatedness of individuals within a taxon. As we expect that meiotically produced ascospores are the source of endophytic infection, markers that allow the determination of relatedness between isolates, in addition to the identity of isolates, may compliment ITS in future studies. Additionally, the utilization of next-generation sequencing techniques in the elucidation of endophytic communities will allow much greater depth of sampling, regardless of locus selected. Such depth of sampling will be particularly useful in further examination of the environmental constraints and host specificity of fungi suspected of utilizing a FA life history strategy.

Demonstrating the possibility of transfer from endophytic to a decomposer life stage, we have observed endophytic strains of *Nemania serpens* (Xylariaceae)—close relative of *Xylaria* (Hsieh *et al.* 2010)—from conifer needles to colonize dead *Acer macrophyllum* wood in laboratory conditions (G. C. Carroll, unpubl. data). Here, we explicitly tested the ability of endophytic members of the Xylariaceae to successfully transfer from leaves at our Ecuadorian site to dead woody substrates in laboratory conditions. This test conclusively demonstrates the link between endophytic and saprotrophic *Xylaria,* showing that endophytic isolates can colonize dead woody substrates from within leaves (Fig. S8). These observations are contrary to the predictions of Bayman *et al.* (1998), who hypothesize that *Xylaria* endophytes are one-way "dead ends"—purely a sink for dispersal.

Consistent with prediction (2), we did not detect host preference by xylariaceous endophytes. However, the power of our study to detect host preferences may be limited due to the large number of hosts with few samples. Our culture and sampling efforts, though quite extensive, were insufficient to populate multivariate community analyses of host-associated xylariaceous communities (see, for example, Veresoglou & Rillig 2014).

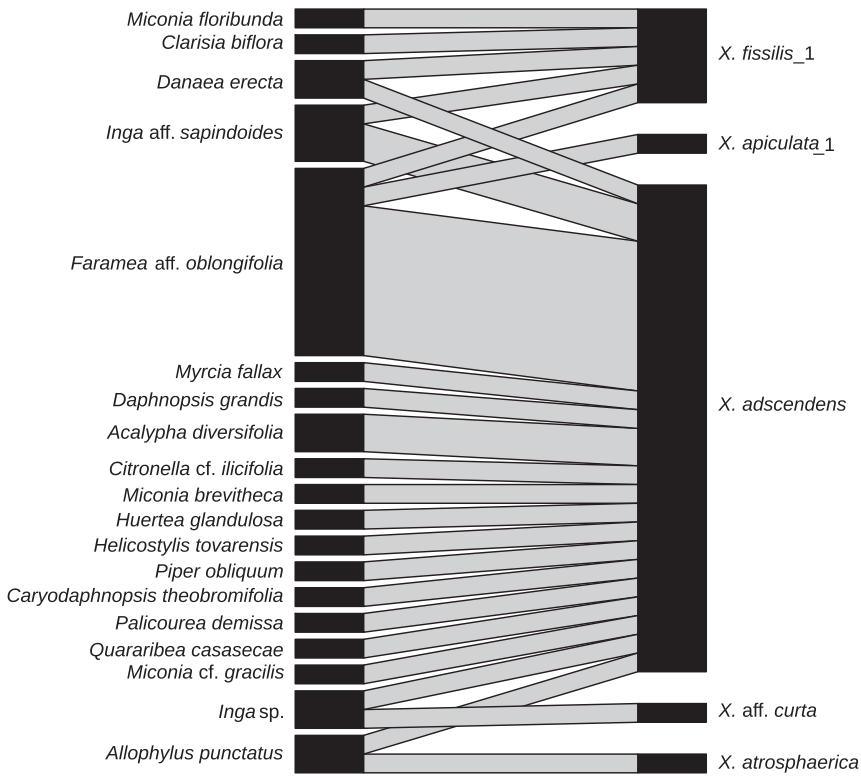

FIGURE 5. Bipartite network visualization of *Xylaria* endophytes (right) and plant-hosts (left). Widths of links are scaled to number of points at which endophytes were isolated from hosts.

Culture-based studies may be particularly disadvantaged when dealing with questions of endophyte host specificity because of culture bias and other limitations of culture-based studies, such as sampling depth (species accumulation curves generally saturate at impractical levels of effort per leaf) (Arnold *et al.* 2000, Arnold & Herre 2003, Lau *et al.* 2013). Some culture-based studies have addressed culture bias through the use of specialized extracts of host-plants in growth medium (Arnold *et al.* 2000, Arnold & Herre 2003, Lau *et al.* 2013), or through direct PCR/cloning methods (Higgins *et al.* 2011), but these approaches are very labor intensive in experiments involving more than a few species or hosts. We chose to work with *Xylaria* species, in particular, because they typically culture readily both from spores and from leaves as endophytes (Bayman *et al.* 1998), reducing potential culture bias. In a direct comparison of direct PCR (using cloning) vs. culturing, Higgins *et al.* (2011), reporting at the order level, found that Xylariales were somewhat more common in cultures (48%) vs. clones (38.9%), but that they were common in both.

When examining questions of host specificity, endophytes are probably best analyzed as multivariate communities within hosts, or as networks of host/endophyte co-occurrences (Peršoh 2013, Higgins *et al.* 2014, Ikeda *et al.* 2014). In future efforts, culture-independent, high-throughput meta-barcode sequencing techniques combined with whole community analysis of endophytes will more adequately address host-endophyte affinities (see, for example, Peršoh 2013).

Nonetheless, our results are in agreement with many studies that indicate that most non-clavicipitaceous tropical foliar endophytes, and especially *Xylaria,* are host generalists (Bayman *et al.* 1998, Cannon & Simmons 2002, Suryanarayanan *et al.* 2002, Arnold & Lutzoni 2007, Higgins *et al.* 2011), and are supportive of the idea that plant-associated fungi in hyper-diverse regions of the tropics will tend toward host generalism (May 1991). Some have suggested that endophyte communities should be regionally unique, due to dispersal limitation (Higgins *et al.* 2014, Vaz *et al.* 2014), and that endophytes of individual plants are predicted as much by location as by host affinities. Higgins *et al.* (2011, 2014), for example, found that tropical forest grass endophyte communities are more similar to the leaves of the nearby woody plants than those of distant grasses.

We found that endophytes are released from environmental constraints as compared to corresponding decomposers, as expected from prediction (3). Decomposers exhibited sensitivity to environmental variables that was not observed in endophytes, particularly to proximity of water. This is not surprising, as moisture is important for spore germination and decomposition by most free-living fungi (Moore 1986, Eveling *et al.* 1990, Gange *et al.* 2007). Indeed, it has been speculated that the evolutionary origins of the Xylariaceae are linked to adaptation for water conservation (Rogers 1979, 2000). Our findings, that *Xylaria* are found fruiting in closer proximity to water sources than expected by chance, seem to indicate a strong role of water use in the ecological and evolutionary constraints for the genus. Endophytic fungi, however, exist in the highly buffered environment of the internal tissues of their host-plants; it is predictable that environmental conditions would have a less direct effect on their distributions. We see this in our spatial

clustering analysis, where endophytes are not constrained by proximity to the stream (Table S4; Fig. 3). The unconstrained endophytic life stage may be a way that these fungi can bridge spatial and temporal gaps in suitable habitat; this is the core of the FA hypothesis, and our results here are consistent with this.

Lastly, in agreement with prediction (4), in our study all endophytic species of *Xylaria* were also recovered as decomposers from rotting wood on the forest floor. The reverse was not true; many decomposers were found only as stromata and were not detected as endophytes. Our diversity estimators and sampling effort curves indicate that we recovered most of the culturable *Xylaria* species from the leaves, but that decomposer *Xylaria* were undersampled. Okane *et al.* (2008) suggest that there may be Xylariaceae that exist solely as endophytes, but did not undertake concurrent systematic stromata collection to verify this. It is clear from our study that there are species-specific differences in the frequencies at which *Xylaria* displaying both life stages were found in the endophytic and saprotrophic phases (Table 1), supporting the notion that there are dispersal or habitat differences among species. Our results suggest that endophytism is a specialist strategy for some members of the genus *Xylaria*.

We observed probable dispersal linkage in the form of spatial clustering of fungi. We also observed release from moisture limitation by two decomposer fungi through endophytism, suggesting that the endophytic life stage may be serving as a method to span dry habitats or persist during times of low moisture. We also directly observed the ability of endophytic *Xylaria* to colonize available woody substrates and initiate stromata formation. Finally, we found no evidence for host preference in endophytic *Xylaria* species. The limitations of a single observational study must be acknowledged: it remains to be seen if similar trends will be observed in some endophytic fungi of temperate zones or outside of montane cloud forests in the tropics. Nevertheless, we find these results to be consistent with the predictions of the Foraging Ascomycete Hypothesis, and a successful first step into the investigation of this intriguing and ecologically important hypothesis.

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SUPPORTING INFORMATION

Additional Supporting Information may be found with online material:

TABLE S1. *Plant host species from which endophytes were isolated.*

TABLE S2. *Herbarium and GenBank accession numbers for specimens and cultures examined in this study.*

TABLE S3–S4. *Nearest Neighbor analysis of spatial clusters in the five species of* Xylaria.

TABLE S5. *Nearest neighbor analysis of all* Xylaria *species recovered as stromata.*

FIGURE S1. A survey of leaffall in two trees at Los Cedros.

FIGURE S2–S6. Graphical representation of results of nearest neighbor Monte Carlo-type simulations.

FIGURE S7. Non-metric multidimensional scaling diagram of habitat characteristics.

FIGURE S8. *Xylaria* sp. grown directly from leaves onto a segment of white birch tongue depressor.

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