

Fungal-Fungal Associations Affect the Assembly of Endophyte Communities in Maize (*Zea mays*)

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Abstract Many factors can affect the assembly of communities, ranging from species pools to habitat effects to interspecific interactions. In microbial communities, the predominant focus has been on the well-touted ability of microbes to disperse and the environment acting as a selective filter to determine which species are present. In this study, we investigated the role of biotic interactions (e.g., competition, facilitation) in fungal endophyte community assembly by examining endophyte species co-occurrences within communities using null models. We used recombinant inbred lines (genotypes) of maize (*Zea mays*) to examine community assembly at multiple habitat levels, at the individual plant and host genotype levels. Both culture-dependent and culture-independent approaches were used to assess endophyte communities. Communities were analyzed using the complete fungal operational taxonomic unit (OTU) dataset or only the dominant (most abundant) OTUs in order to ascertain whether species co-occurrences were different for dominant members compared to when all members were included. In the culture-dependent approach, we found that for both datasets, OTUs co-occurred on maize genotypes more frequently than expected under the null model of random species co-occurrences. In the culture-

independent approach, we found that OTUs negatively co-occurred at the individual plant level but were not significantly different from random at the genotype level for either the dominant or complete datasets. Our results showed that interspecific interactions can affect endophyte community assembly, but the effects can be complex and depend on host habitat level. To our knowledge, this is the first study to examine endophyte community assembly in the same host species at multiple habitat levels. Understanding the processes and mechanisms that shape microbial communities will provide important insights into microbial community structure and the maintenance of microbial biodiversity.

Introduction

Diamond's seminal paper on bird community assembly on islands [13] sparked widespread interest in how communities are formed ([22] and references therein). That is, do species in a community come together by chance or are there common factors that determine which species are present in a community? With a notable exception in microbial communities, community assembly has been studied in a variety of systems [8, 19, 27–29, 35, 40] and focused on factors that can affect the presence or absence of species in a community, ranging from species pools to interspecific interactions [18, 24]. The prevailing hypothesis for microbial species is that “everything is everywhere” (i.e., microbes are not dispersal-limited) and that the environment selects (Baas-Becking hypothesis, as cited in [30]). If so, microbial communities in similar habitats should represent random assemblages of the species pool with the presence of species at different locations determined by chance, e.g., order of species arrival, niche

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availability [30, 36, 38, 42]. On the other hand, if factors such as interspecific interactions drive the assembly of microbial communities, then microbial communities will consist of nonrandom species assemblages.

Hosts, as an environment, can have significant effects on microbial community composition and diversity ([1–3, 5, 20, 22, 25, 34, 39, 41, 42], but see [9]). Host plants are the primary habitat for fungal endophytes, as endophytes live asymptotically in the aboveground tissues of plants and rely completely on host plants for resources [10, 33]. Plant species found in the same geographical site are often infected by and dominated by different endophyte species [9, 21, 39]. Moreover, at a finer scale, individual plants can provide a mosaic of habitats such that different endophyte species can dominate on different plant tissues [32]. Thus, endophyte communities can be considered at multiple habitat levels even within the same host species. Although endophyte community composition and diversity has been the subject of a number of studies [9, 21, 34, 39, 42], we have a limited understanding of the mechanisms that can shape how endophyte communities are formed.

Using recombinant inbred (RI) lines of maize, we examine endophyte community assembly within a single host species on multiple habitat levels, from individual plants as habitat patches to genotypes as genetic habitat patches. Each RI line represents a particular set of alleles, is genetically distinct, and can be considered to be a different genotype. Thus, we can control for and examine the effect of host genetic environment on community assembly by planting replicates of genotypes within and across field sites. We use culture-dependent and culture-independent approaches to characterize the fungal endophyte community. We then analyze species co-occurrences to determine the roles of chance and interspecific interactions (e.g., competition, facilitation) in endophyte community assembly. To our knowledge, this is the first study to examine endophyte community assembly within the same host species on multiple habitat levels.

We hypothesized that negative interspecific interactions, such as competition, dominate the assembly of endophyte communities and are more important than chance [13, 19]. We first use a culture-dependent approach to describe the fungal taxonomic units (OTUs) making up endophyte communities across different maize genotypes and determine patterns of co-occurrence among those OTUs. Separately, we determine patterns of co-occurrence among fungal endophyte OTUs identified in a previous culture-independent study of endophyte communities on maize [34]. Finally, we qualitatively compare results within and between host habitat levels to determine the general role of interspecific interactions on the assembly of fungal endophyte communities. Specifically, we compare species co-occurrence results from genotypes and individual plants

within the culture-independent study and compare results from the culture-dependent study to those from the culture-independent study at the genotype level. We focus on common patterns that emerge from comparisons of the culture-dependent and culture-independent datasets to determine the most important factors affecting the assembly of fungal endophyte communities, factors that should be detected across variation in space and time. Our results show that interspecific interactions can affect endophyte community assembly, but the effects can be complex and depend on habitat level.

Methods

Field Sampling and Fungal Community Assessment—Culture-Dependent Study

The RI lines of maize used in this study were developed as part of a larger study described in Baumgarten et al. [6]. The RI lines were derived from one of two crosses, parent inbred lines A188 × CMV3 or W23 × CMV3, and were previously characterized as strongly resistant or very susceptible to the fungal pathogen *Ustilago maydis*. Samples for the culture-dependent assessment were made from F8 plants representing 11 lines: two A188 × CMV3 and nine W23 × CMV3 lines (R.L. Phillips, Department of Agronomy and Plant Sciences, University of Minnesota, accession numbers 55280-8, 55380-5, 55734-11, 55806-2, 55815-6, 55833-5, 55840-4, 55900-3, 55930-11, 55930-2, 55932). Plants were grown at two University of Minnesota Agricultural Experiment Stations, Saint Paul and Waseca, MN, USA. Many crop species are grown at both sites each year, with maize and soybean (*Glycine max*) being dominant. Our plots at both sites were primarily surrounded by other maize fields. In each of the two agricultural fields, 20 plants from each maize line (hereafter, genotype) were planted into a single row and all rows together constituted a plot; plots in each agricultural field had exactly the same planting scheme [6] (Fig. 1). One hundred forty-seven maize plants from Saint Paul and 46 plants from Waseca were sampled at the end of the growing season in 2002.

From each plant, we sampled four tissue types, the upper leaf, lower leaf, ear, and stalk, to represent the overall endophyte community found on a maize plant. Although we were not always able to sample all of the four tissues from an individual plant, we were able to obtain adequate samples of each tissue type for each genotype. Tissues were placed on ice after collection and during transport and then kept refrigerated until plated. All tissue sections were surface-sterilized using a standard procedure—70% ETOH for 1 min, 50% bleach for 2 min, 70% ETOH for 1 min, and then rinsed in sterile distilled water before trimming to

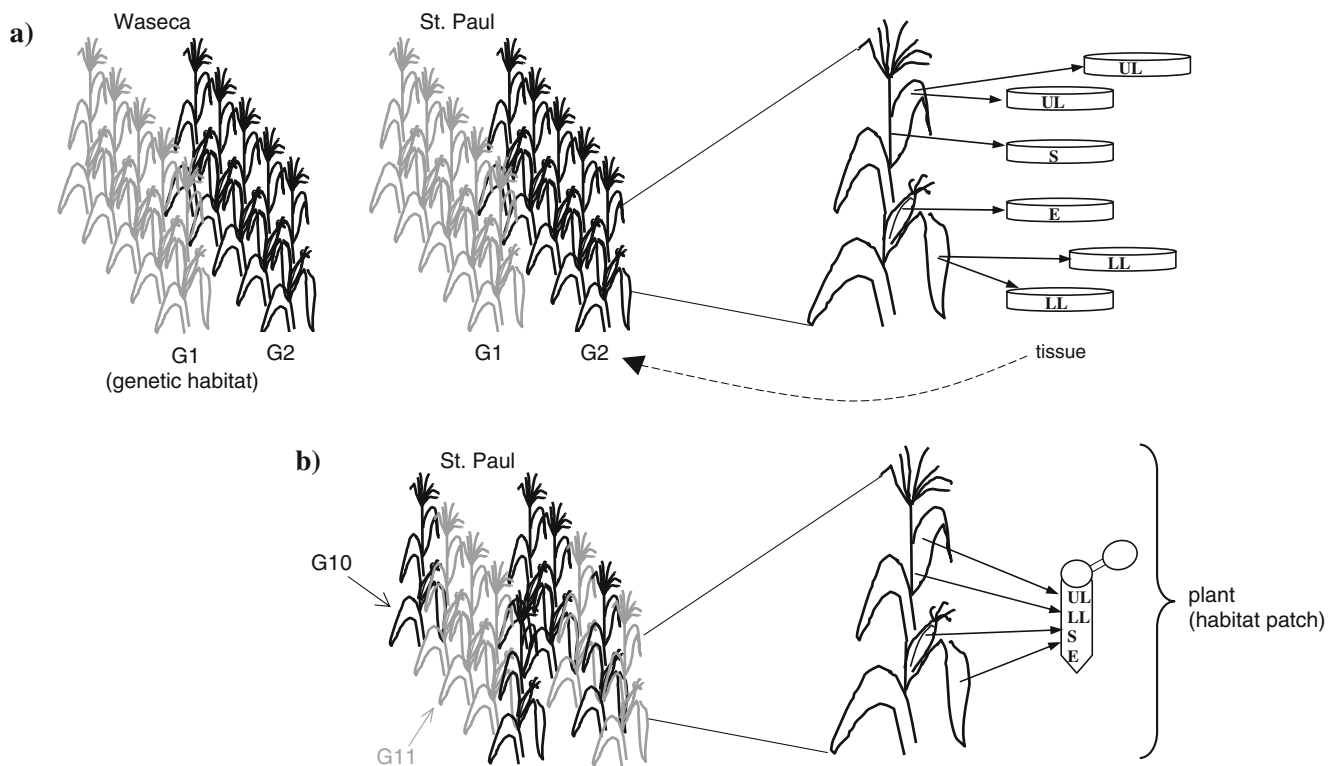


Figure 1 Schematic of the field experiment and sampling scheme for the **a** culture-dependent study (two of 11 genotypes are shown) and **b** culture-independent study (two of six genotypes are shown). In **a**, genotypes, indicated by *G1* and *G2*, are planted in two field sites, Waseca and Saint Paul. From each sampled plant, four different tissues were plated onto media. All of the tissue samples from replicate plants of a genotype were combined to assess the endophyte

community on the genotype. In **b**, genotypes (*G10*, *G11*) were randomly assigned to three positions within a row in the Saint Paul field. Four different tissues were collected per plant and DNA was extracted separately for each tissue. Equal amounts of DNA from each tissue were pooled to produce a composite sample for the plant. For genotype analyses, replicate plants of the same genotype were combined

the appropriate size for plating. Ethanol–bleach sterilization procedures are effective for isolating endophytes [37]. Stalk samples were 0.5×0.5 cm sections that were taken from the center of the stalk (mostly pith). The ear tissue was one kernel from each ear, with the seed coat removed and the kernel trimmed on at least four sides. Two 0.5×0.5 -cm sections were cut and plated for each leaf sampled.

The tissues obtained from a single plant were randomly assigned to one of four media types in order to expose the different tissue types to as many media types as possible. The only exceptions were leaf samples, where we made certain that the two sections from each leaf were not placed onto the same media type. With our large number of tissue samples, this approach was the most efficient method to expose different tissues samples from each maize line to multiple media types and to reduce culturing biases from using a single media type. Four types of media were used for plating, potato-dextrose agar (PDA; BD Difco, Franklin Lakes, NJ, USA), PDA with Rose Bengal (PDA + RB), cornmeal–malt agar (CMM; BD Difco, Franklin Lakes, NJ, USA), and CMM with Rose Bengal (CMM + RB). Rose Bengal was used to retard growth of fast-growing species

and allow slower growing fungi a chance to emerge from tissues. Fifty milligrams per liter each of streptomycin sulfate and penicillin G or chloramphenicol was added to each media to reduce bacterial growth. Each tissue sample was placed onto its own plate and incubated at room temperature for a minimum of 4 months. After plating, plates were examined daily for fungal emergence or contamination; contamination was rare, but any plates that were considered to be contaminated were discarded and not included in our study. As fungal colonies emerged from plant tissues, they were subcultured individually onto PDA plates. Fungi were then grouped into morphological taxonomic units, or morphotypes, based on colony characteristics and morphology (e.g., color, texture, growth rate, etc.). Representatives of each fungal group were deposited in the University of Minnesota, Bell Museum of Natural History, Fungal Culture Collection. We recorded all plates that did not have fungi emerging from tissue samples at the end of incubation period.

Cultures representing different morphotypes were further classified using nuclear rDNA-internal transcribed spacer (ITS) sequence. Fungi were grown in potato dextrose broth,

lyophilized, and the DNA extracted following [7]. Fungal-specific polymerase chain reaction (PCR) primers ITS1F and ITS4 [15] were used to amplify the ITS region of the rDNA (ITS1, 5.8S, and ITS2) from each DNA sample. Each 25- μ l PCR reaction contained the following: 20–30 ng of genomic DNA, 1 \times Takara Ex Taq PCR buffer with MgCl₂, 200 μ M dNTPs, 0.25 μ M of each primer, 1.25 U of Ex Taq (Panvera, Madison, WI, USA), and sterile water. PCR conditions were 94°C for 1 min, 35 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 1.5 min, with a final extension at 72°C for 3 min. Quantity and size of PCR products were determined on 0.8% 0.5 \times Tris–acetate–ethylenediaminetetraacetic acid agarose gels and visualized using ethidium bromide under UV light. PCR products were sequenced in one direction using either the ITS1F or ITS4 primer with the ABI BigDye terminator chemistry on an ABI 3700 capillary sequencer at the University of Minnesota Sequencing Center.

Sequences were edited using Sequencher 3.1 (Gene Codes Corporation, Ann Arbor, MI, USA). Complete full-length sequences varied in length and ranged from ~450 to over 600 bp. Edited full-length sequences were grouped into smaller alignments, or operational taxonomic units, based on sequence similarity using the “assemble automatically” function in Sequencher 3.1 [34]. The “dirty data” assembly algorithm was used for groupings, with a value of 55 (nucleotides) for the minimum overlap and a minimum percent match value of 98 (sequences that were \geq 98% similar to each other). Within a group, alignments minimized gaps and sequences were similar in length.

In order to verify that all sequences were fungal and to determine generic affinities of cultures, edited full-length sequences were compared to the “nr” database in GenBank with Basic Local Alignment Search Tool (BLAST) using the default parameters (filter = “low complexity”, expect = “10”, word size = “11”). Sequences were considered a match with a particular genus if a majority of the query sequence (ITS1, 5.8S, and ITS2) matched the database sequence with an *E* value <0.001 and an alignment score >200.

Nucleotide Sequence Accession Numbers

All unique sequences were deposited in GenBank, accession numbers FJ210470–FJ210642.

Culture-Independent Comparison Dataset

In order to infer general patterns of community assembly, we qualitatively compared the results from the culture-dependent study to results based on data from a previous PCR-based assessment of maize endophytes (see [34] for details). Briefly, at the end of 2003, leaf, ear, and stalk tissues were collected from eight to ten F9 plants from each

of six RI lines. Each of the six maize lines used in the culture independent study was derived from one of the maize lines used in the culture-dependent study [6] and differed from their parents at <0.4% of their loci (G. May, personal communication). Three of the six lines were scored as strongly resistant and three as highly susceptible to *U. maydis*. Lines were planted at the University of Minnesota Agricultural Station in Saint Paul, MN, USA, one of the fields used in the culture-dependent study (Fig. 1b). A total of 58 plants were sampled across the six maize lines.

All sampled tissues were surface-sterilized in the field by rinsing in water, submerging within a 50% bleach solution for 2 min, and rinsing with water again. Surface-sterilized tissues were kept on dry ice in the field and then stored at –80°C until DNA extraction. DNA was extracted from the upper leaves, lower leaves, stem, and kernels, separately, and then combined in equal concentrations to produce a composite sample for each plant. The ITS region of the rDNA was amplified from the total extracted DNA using the same conditions as described above. PCR products were then cloned using the TOPO TA Cloning kit following the instructions provided by the manufacturer (Invitrogen, Carlsbad, CA, USA).

Approximately 50 fungal clones were sequenced for each sampled plant in the culture-independent study [34]. Sequences used in the analyses and comparisons in this paper, GenBank accession numbers EF504318–EF505893, represent fungi that were found throughout the above-ground tissues of maize plants. Sequences were grouped into OTUs based on 90% or 98% sequence similarity. Taxonomic affiliations to genus were determined using BLAST. Since results were similar for the 90% and 98% sequence similarity OTU groupings, we focus on comparisons between the 98% sequence similarity results and the culture-dependent results.

Statistical Analyses

To compare the sampling approaches and the effect of sampling effort, we calculated species (OTU) accumulation curves using EstimateS 7.5 (<http://purl.oclc.org/estimates>; [12]) for the culture-dependent and culture-independent studies.

We utilized two tests to determine patterns of species co-occurrences, Stone and Roberts’ *C* score test and Pearson correlation coefficients. Hereafter, in reference to the endophyte data, species will refer to OTU groupings. For the culture-dependent approach, we compared species co-occurrences at the levels of maize genotype, where all tissue samples from replicate plants of a genotype were combined to represent the fungal community of that genotype, and geographic site, where all plants were

considered replicates of a field site (Fig. 1; Table 5). Preliminary analyses of the endophyte community assembly data indicated that results from the two field sites were concordant with each other and with the results when data from the field sites were combined. Therefore, for ease of presentation, we present only the results for the combined dataset. For the culture-independent approach, we compared species co-occurrences at the genotype and at the plant levels (Fig. 1; Table 5). The genotype level for the culture-independent study is similar to that used in the culture-dependent study, where plants with the same genotype were considered replicates of a genotype.

First, we used Stone and Roberts' *C* score test in Ecosim to determine patterns of species co-occurrence [16, 38]. The *C* score is the mean number of "checkerboard units" found across all pairwise species comparisons between habitats; in this study, habitats were maize plants (culture-independent study) or maize genotypes (culture-dependent and culture-independent studies). Specifically, "checkerboard" units are defined as those pairwise comparisons where two endophyte species do not co-occur in the same habitat [16, 18, 38]. For example, a checkerboard is if species 1 is present on habitat A, but species 2 is not and species 2 is present on habitat B, but species 1 is not. More checkerboards will be detected in communities shaped by negative interactions, e.g., when competition leads to exclusion of one species by another, compared to communities determined by chance. On the other hand, fewer checkerboards will be detected for communities shaped by positive interactions, e.g., where one species may facilitate infection by another species [14, 17, 19, 26, 27, 35, 40].

The data for the *C* score test consisted of presence-absence data for each endophyte OTU. We analyzed two data matrices, one that included all OTUs found in the endophyte community (referred to as the "complete" dataset) and a second data matrix that included only the dominant OTUs ("dominant" dataset). We defined dominant OTUs for the culture-dependent dataset as those OTUs found more or equal to ten times out of all of the sampled tissues and for the culture-independent dataset, dominant OTUs as those OTUs with ≥ 30 sequences ($\geq 1\%$ of total samples). For the culture-dependent data, the complete OTU dataset included 133 OTUs and 13.5% of those were in the dominant OTU dataset. For the culture-independent dataset, the complete OTU dataset included 103 OTUs and the dominant dataset included 8.9% of those in the complete OTU dataset. Comparisons between the complete and dominant datasets allowed us to examine whether measures of species co-occurrences differed for dominant members of the endophyte community compared to when all members were included.

In Ecosim, observed data matrices were compared to 5,000 matrices that were generated under two null models

using the sequential swap algorithm [16]. Data were arranged with habitats in columns and species in rows, so that the occurrence of each species is represented across habitats. The first null model (fixed-fixed, FF) generates random species combinations while maintaining overall differences between habitats, such that reshuffling maintained the total number of endophyte species found per habitat (fixed column totals) and maintained the overall frequency of each endophyte species across all habitats as the original data (fixed row totals). In the second null model (fixed equiprobable, FE), habitats were assumed to have an equal probability of being infected by any endophyte species (equiprobable columns), while the overall frequency of each endophyte species across all habitats was the same as the observed data (fixed row totals). The probability of having the observed data matrix by chance was calculated for each null model matrix constraint (FF and FE). These two constraints were chosen to reduce the probability of a type I error (i.e., falsely rejecting the null hypothesis when it is true) and because they best represent the "everything is everywhere" hypothesis for microbes [16, 30].

Second, in order to examine which species were positively or negatively associated with each other, we used PROC CORR in SAS (version 9.1) to calculate Pearson correlation coefficients for both the culture-dependent and culture-independent datasets. In order to have a reasonable sample size in the datasets, we used the dominant dataset in these analyses. We ran two correlations for each dataset, one examining associations for OTUs and a second where we combined the OTUs by genus in order to determine if there were correlations between genera. Again, we will focus on the 98% sequence similarity results for the culture-independent study since results were similar regardless of the sequence similarity cutoff used. Correlations for plant-level data are presented for the culture-independent study.

Results

Culture-Dependent Data

We collected a total of 738 tissue samples from 102 plants. Out of the 738 samples, a single fungal colony emerged from 521 tissue samples, more than one fungal colony emerged per tissue sample in 136 tissue samples, and no fungal colony emerged from 81 samples. Media type had no effect on fungal emergence as the presence of fungi were equally distributed across the different media ($X^2=4.90$, $df=3$, $p=0.18$) and there was no effect of media on fungal emergence from different tissue types ($X^2=12.96$, $df=9$, $p=0.16$; Table S1).

Fungal Endophyte OTUs

We found that species accumulation curves did not plateau for either the culture-dependent or culture-independent OTUs (Fig. 2), suggesting that more species would be identified if more plants are sampled. However, the curves became less steep as the number of plants sampled increased, indicating that the rate at which new OTUs would be detected was low.

Of the fungi that could be identified to genera using ITS sequences and BLAST searches, many of the common genera were detected in both studies: *Acremonium*, *Alternaria*, *Cladosporium*, *Cochliobolus*, *Curvularia*, *Epicoccum*, *Fusarium*, *Phoma*, *Rhodotorula*, and *Sporobolomyces*. However, six genera were found only in the culture-dependent study: *Hypocrea*, *Mucor*, *Nemania*, *Pichia*,

Preussia, and *Xylaria*. Nine genera were found only in the culture-independent study: *Aureobasidium*, *Bullera/Bulleromyces*, *Colletotrichum*, *Cryptococcus*, *Filobasidium*, *Leptosphaeria/Phaeosphaeria*, *Lewia*, *Puccinia*, and *Ustilago*.

Species Associations—Culture-Dependent Approach

For the culture-dependent dataset, we found that the observed *C* scores at the genotype level were not significantly different from expectations under the FF null model for either the complete or dominant OTU datasets, indicating that associations between OTUs were not different from random associations (Table 1). On the other hand, the observed *C* scores were significantly smaller than expected under the FE model for both the complete and dominant datasets (Table 1; Fig. 3), indicating that endophyte OTUs were not associated with all host genotypes at the same frequency and OTUs positively co-occurred.

All significant Pearson correlation coefficients for the culture-dependent datasets were positive (Table 2; Table S2). Only the presence of *Mucor* was not significantly correlated with the other genera for the genera dataset. A large number of significant correlations were found for the OTUs based on colony morphology. For example, morphotype B was positively correlated with 13 of the other morphotypes and negatively correlated with four (Table S2). Conversely, morphotype O did not have significant correlations with any other morphotypes.

Species Associations—Culture-Independent Approach

At the genotype level, the *C* scores for both the complete and dominant OTU culture-independent datasets were not significantly different from expected for either the FF or FE null model (Table 3), indicating that species co-occurrences

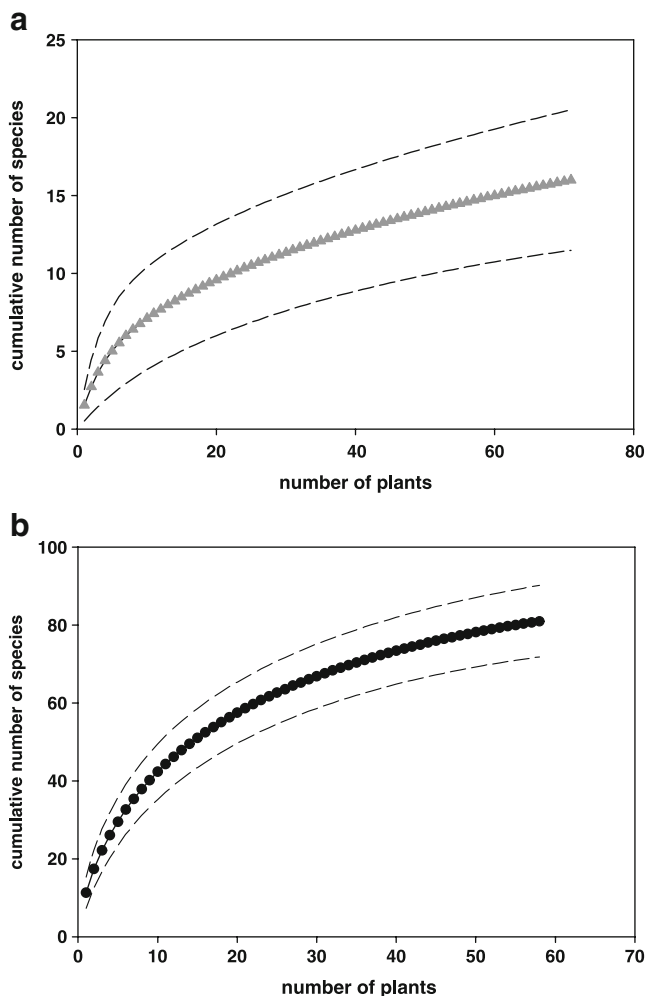


Figure 2 Species (OTU) accumulation curves with 95% confidence intervals (broken lines) for the **a** culture-dependent (triangles) and **b** culture-independent (circles) studies. The number of plants sampled per study is on the x-axis and the cumulative number of unique OTUs on the y-axis. For the culture-dependent study, only morphotypes that could be grouped by sequence similarity were included

Table 1 *C* scores for the culture-dependent dataset (genotype level)

Dataset	Observed <i>C</i> score	Mean of expected <i>C</i> scores	<i>p</i> value
Complete—FF	1.37	1.36	0.33
Complete—FE	1.37	2.97	0.00*
Dominant—FF	1.84	1.88	0.77
Dominant—FE	1.84	4.95	0.00*

Analyses were conducted on the “complete” dataset, which included all morphotypes, and for the “dominant” morphotypes, which only included morphotypes that occurred more or equal to ten times across all samples. All *p* values are for “observed > expected” except those denoted by an “*”, which are *p* values for “observed < expected” and indicate fewer checkerboards than expected and positive species co-occurrences

FF expected *C* scores under the fixed–fixed null model, FE expected *C* scores under the fixed–equiprobable null model

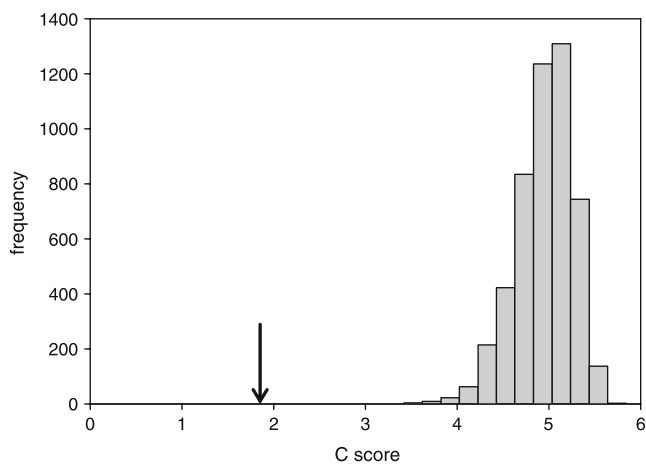


Figure 3 *C* scores based on presence–absence data for the dominant OTUs found in the culture-dependent study in the genotype-level analysis. The *gray bars* represent the 5,000 expected *C* scores generated under the fixed-equiprobable null model. The *arrow* denotes the observed *C* score for the dataset. The observed *C* score is significantly smaller than the expected *C* scores

on different maize genotypes were not different from random co-occurrences.

On the other hand, at the plant level, we found that *C* scores for the complete and dominant datasets were significantly greater than expected under the FF null model (Table 3; Fig. 4), indicating that species had negative co-occurrences and negative interactions. Results were mixed for the FE null model (Table 3). The observed *C* score was greater than expected for the dominant OTUs and gave evidence for negative species co-occurrences. The observed *C* score was not different from expected for the complete dataset under the FE model, indicating that individual host plants were similar environments and thus had the same probability of being infected (Table 3). Results from the FE model suggest that host plants represent different environments for dominant vs. less common fungal species, or that negative species interactions may be stronger between dominant species.

Most of the significant Pearson correlation coefficients between fungal genera and species at the plant level were negative for the culture-independent datasets (Table 4), in agreement with the plant-level species co-occurrence analyses. Interestingly, there were three *Alternaria* OTUs

in the dataset and they did not have identical interactions with the other OTUs. Still, all significant interactions of *Alternaria* with other species were negative. *Alternaria* 1 was negatively correlated with both *Epicoccum* 1 and the negative correlation between *Alternaria* 2 and *Cladosporium* approached significance ($p < 0.07$). Significant interactions for the *Epicoccum* OTUs were also negative, with both *Epicoccum* 1 and 2 negatively correlated with *Phoma*. A significant positive correlation occurred between *Phoma* and *Cladosporium*.

Discussion

We found support for the role of fungal interactions in the assembly of maize fungal endophyte communities (Table 5), in contrast to the expectation that microbial communities are random assemblages and determined by chance [30]. We hypothesized that endophyte community assembly would be dominated by negative species interactions. Support for this hypothesis came only at the plant level in the culture-independent study, where we found patterns of species co-occurrences consistent with the interpretation that the community is dominated by negative species interactions. Contrasting results were found at the plant genotype level in the culture-dependent study, where patterns of species co-occurrences instead suggested that these communities were dominated by positive species interactions, or aggregations of particular species.

In the culture-dependent study, we found fewer checkerboards at the genotype level than expected under FE null model (Table 1), indicating more pairwise species co-occurrences than by chance. Supporting these results, we also found positive correlation coefficients among the dominant taxa (Table 2). Observations of positive species co-occurrences are usually attributed to positive species interactions, particularly facilitation. Facilitation in the fungal endophyte community could occur if host infection by one species leads to the host becoming more vulnerable to infection by another species. In animal parasite systems, positive parasite co-occurrences have been partially attributed to immunodepression in animal hosts [27, 35, 40]. However, positive co-occurrences between species can also

Table 2 Pearson correlation coefficients for the dominant genera found in the culture-dependent study

Dominant genera were found more or equal to ten times out of all samples. Significant correlations ($p \leq 0.05$) are in bold and correlations approaching significance are in italics ($p < 0.07$).

Genus	Fusarium	Cochliobolus	Alternaria	Preussia	Epicoccum/Phoma	Mucor
Fusarium	1	0.9489	0.9786	0.9719	0.9481	0.549
Cochliobolus		1	0.9692	0.9421	0.9791	0.3471
Alternaria			1	0.9572	0.9796	0.4738
Preussia				1	0.9425	0.5827
Epicoccum/Phoma					1	0.4004
Mucor						1

Table 3 *C* scores for the 98% culture-independent dataset

Dataset	Observed <i>C</i> score	Mean of expected <i>C</i> scores	<i>p</i> value
Genotype, complete—FF	1.09	1.08	0.17
Genotype, complete—FE	1.09	1.08	0.50
Genotype, dominant—FF	0.32	0.20	0.17
Genotype, dominant—FE	0.32	0.25	0.38
Plants, complete—FF	11.49	11.13	0.00
Plants, complete—FE	11.49	11.33	0.13
Plants, dominant—FF	106.82	99.56	0.00
Plants, dominant—FE	106.82	86.51	0.00

Analyses were conducted for the genotype and plant level. All *p* values are for “observed > expected”. Significant *p* values are in bold and indicate more checkerboards than expected and negative species interactions

FF expected *C* scores under the fixed-fixed null model, *FE* expected *C* scores under the fixed-equiprobable null model

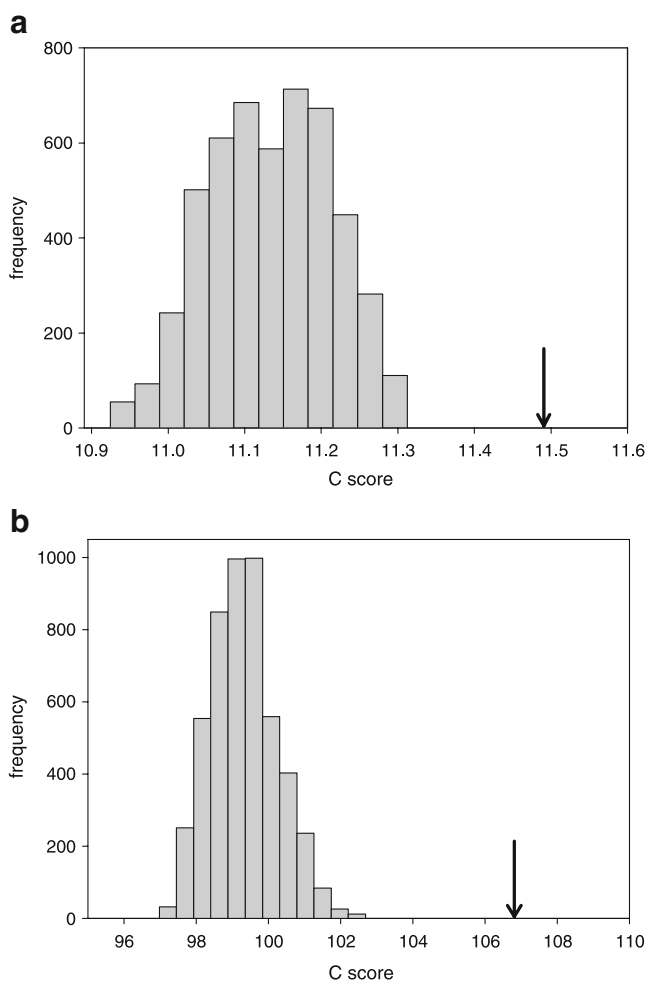


Figure 4 *C* scores based on presence–absence data for the **a** complete and **b** dominant OTUs found in the culture-independent study in the plant-level analyses. The *gray bars* represent the 5,000 expected *C* scores generated under the fixed-fixed null model. The *arrow* denotes the observed *C* score for the dataset. The observed *C* scores are significantly larger than the expected *C* scores

be attributed to different species having similar host requirements and a lack of competitive exclusion. To distinguish between these two possibilities, future studies could track endophyte communities through time to see if the pattern and chronology of endophyte arrival is consistent with facilitation or whether certain species are consistently found on particular host genotypes.

In the culture-independent approach, the complete and dominant datasets had significantly larger *C* scores than expected under both null models at the plant level, indicating that fungal endophyte taxa negatively co-occurred (Table 5). Negative co-occurrences were further supported by the prevalence of negative correlations for fungal OTUs and genera OTUs (Table 4). Negative species co-occurrences have been attributed to competitive interactions, where less competitive species are excluded or reduced. Negative species co-occurrences have been found in a number of communities, such as mycorrhizal fungi [26], lichens [29], plants [8], and animals [17, 19]. Potential mechanisms of fungal-fungal competition leading to negative species co-occurrences include priority effects, rapid fungal growth leading to site preemption within hosts [4, 10], and the production of inhibitory chemicals that negatively affect other fungi [11, 31]. Although negative species co-occurrences are often attributed to competitive interactions, other mechanisms can also lead to negative co-occurrences, e.g., historical or phylogenetic processes [17, 18, 23] and indirect effects mediated through the plant host [22, 31]. Within maize, there is evidence that direct interactions between fungi can affect the outcome of species interactions (Lee et al., in preparation).

The relative importance of interspecific interactions in endophyte community assembly in maize may depend on how habitat patches are defined for communities. Fungal interactions that involve contact or close proximity will occur on individual host plants. On the other hand,

Table 4 Pearson correlation coefficients for the 98% sequence similarity OTUs from the culture-independent study

	Alternaria1	Alternaria2	Alternaria3	Cladosporium	Epicoccum1	Epicoccum2	Lewia	Phoma
Alternaria1	1	0.1934	0.2237	-0.0444	-0.327	-0.0288	0.1077	-0.1031
Alternaria2		1	-0.214	-0.256	-0.1682	0.0128	0.1477	-0.1184
Alternaria3			1	-0.1235	-0.367	-0.1007	0.1109	-0.2267
Cladosporium				1	-0.1998	-0.022	0.0023	0.4118
Epicoccum1					1	0.2046	-0.1598	-0.272
Epicoccum2						1	0.1057	-0.4069
Lewia							1	-0.0059
Phoma								1

Only OTUs that had ≥ 30 sequences were included in the analysis. Significant correlations ($p \leq 0.05$) are in bold and correlations approaching significance are in italics ($p < 0.07$)

common characteristics of habitats, such as genotype, can be important environmental filters for species [34]. Direct comparisons between habitat levels, i.e., plant vs. genotype, could only be made within the culture-independent study because this was the only study where there were reasonable samples numbers at both levels. In contrast to the plant-level results, we found that species co-occurrences were not significantly different from expected under either null model at the genotype level, indicating that endophyte communities on the six maize genotypes were not different than random assemblages. The genotype level, to a certain extent, represents a pooled sample of the endophyte community and community interactions. Communities on individual plants were determined by sequencing 50 clones; species from those clones were found on the same plant and likely to have interactions within the host. On the other hand, communities on genotypes were determined from eight to ten replicate plants, which all represented genetically identical, but spatially separate habitats, with 400–500 sequenced clones. Species that were rare would be counted as present in the community of at the genotype level, even though they may have few direct interactions between fungi. This could lead to species co-occurrence patterns in host genotypes that are not seen within individual plants and reduce the number of checkerboards, resulting in species co-occurrences that are not different from random.

We compared the results for the culture-dependent and culture-independent studies at the host genotype level, the only level in common between the two studies. Interestingly,

we found contrasting results for the culture-dependent study, where we detected positive species co-occurrences, and the culture-independent study, where species co-occurrences were not different from those predicted under random models (Table 5). In addition to inherent differences between the two approaches [23, 28], the different outcomes may be due to the ability to detect different suites of fungi. Culturing may favor the recovery of common species that grow rapidly or grow well under the same resource conditions. We used four different media types and found that most of the culturable fungi were detected on all media types, suggesting that the detected fungi had similar resource requirements. Since species with similar resource requirements are likely to be detected together, they will have high co-occurrences. In contrast, culture-independent approaches, such as sequencing, have a greater ability to detect and resolve taxa and detection does not depend on the taxa's resource requirements [23]. Thus, results from the culture-dependent approach may be useful for understanding community assembly for particular suites of fungi from similar niches, while the culture-independent approach may provide a better whole community estimate since species from all niches are detected.

Understanding the mechanisms and processes that contribute to community assembly has been the subject of numerous studies [4, 8, 17–19, 23, 26, 27, 29, 40]. Although chance may play a role in community assembly, our results clearly demonstrate that fungal endophyte communities on maize were not just random assemblages; interspecific interactions among fungi play a role in

Table 5 Summary table of the sampling scheme and species co-occurrence results for each approach

Approach	Level of analysis	Data from	Results from co-occurrence analyses
Culture dependent	Genotype	Tissues	Positive interspecific interactions (FE)
Culture independent	Genotype	Individual plants	Random
	Individual plant	Fungal clones	Negative interspecific interactions (FF, FE)

The null model is in parentheses for the co-occurrence analyses

endophyte community assembly. Moreover, the habitat level at which endophyte communities were evaluated is important. Thus, the addition or loss of fungal species in communities can have unforeseen consequences on fungal species co-occurrences and community composition. Endophyte community assembly is complicated and using a combination of culture-dependent and culture-independent approaches may provide the most comprehensive assessment of community assembly.

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