Fungal diversity in galls of baldcypress trees

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ARTICLE INFO

ABSTRACT

The baldcypress midge (*Taxodiomyia cupressi* and *Taxodiomyia cupressiananassa*) forms a gall that originates from leaf tissue. Female insects may inoculate galls with fungi during oviposition, or endophytes from the leaf tissue may grow into the gall interior. We investigated fungal diversity inside of baldcypress galls, comparing the gall communities to leaves and comparing fungal communities in galls that had successful emergence versus no emergence of midges or parasitoids. Galls of midges that successfully emerged were associated with diverse gall fungal communities, some of which were the same as the fungi found in surrounding leaves. Galls with no insect emergence were characterized by relatively low fungal diversity.

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1. Introduction

Plants and insects interact frequently with bacteria and fungi, often forming symbioses with outcomes that can be positive, negative or neutral for either party (Vega et al., 2008). Fungal endophytes are fungi that live symbiotically inside of plant tissue without showing any sign of disease, and as long as they remain asymptomatic, their interaction with the plant is considered neutral or positive (Wilson, 1995a). The plant-fungal symbiosis may have a positive outcome if the fungi help protect plants from insects or other fungal pathogens (Carroll, 1988; Arnold et al., 2003; Estrada et al., 2013). For example, fungal endophytes in plant tissue may also be entomopathogens (Marcelino et al., 2008). Several previous studies have documented the presence of fungi inside of insect galls (reviewed in Lawson et al., 2014). In oak trees, culturable fungi were associated with dead cynipid wasps in galls, but it was not always clear whether the insect died because of an entomopathogenic infection or because fungi killed the gall tissue thus starving the insect (Wilson, 1995b). It was also possible that the insects died for unrelated reasons, and the fungal community colonized the gall secondarily. In each case, the outcome of a dead

insect may indirectly benefit trees because gall-making insects are generally considered to be plant parasites (Price et al., 1987).

Galling insects parasitize the baldcypress tree (*Taxodium distichum*), a tree species that plays key roles in increasing structural stability in coastal wetlands, decreasing storm damage, and providing habitat for plants and animals (Krauss et al., 2009; Shaffer et al., 2009). The commonly observed galling insects are the baldcypress midges, *Taxodiomyia cupressi* and *Taxodiomyia cupressiananassa,* which lay their eggs primarily on baldcypress leaves, and the galls that are formed around the developing larvae originate from plant tissue (Chen and Appleby, 1984). Since baldcypress leaves also host fungal endophytes, it is possible that some fungal endophytes from leaves may enter the galls and directly or indirectly influence the success of the gall midges (Wilson, 1995b; Lawson et al., 2014). The fungal communities inside of galls, however, could also be shaped by fungi that enter during oviposition (Wilson, 1995b). The first steps towards addressing these alternative scenarios are to describe the fungal communities inside of baldcypress galls and leaves. Post-mortem studies of fungi associated with dead midges inside of galls will also help clarify the role of fungi in baldcypress-gall interactions.

In this study, we sought to: (1) verify that fungi inhabit the interior of baldcypress galls, and describe their diversity; and compare fungal communities (2) in galls with insect emergence

and no insect emergence, (3) in galls collected across three sites in Southeastern Louisiana, and (4) in galls made by two different species of baldcypress midge. We further compared the fungi found in galls with fungal endophytes that inhabit baldcypress leaf tissue. We predicted that galls with healthy midges would support a greater diversity of fungi than the dead midge fungal community, which may become overrun by entomopathogens or saprotrophs. We predicted that the three sites would have unique fungal communities since the sites were several kilometers apart. This work leads to increased understanding of how the cryptic fungal community of baldcypress trees interacts with parasites.

2. Methods

2.1. Study sites and collections

Our study organisms were the fungal communities found inside the galls made by baldcypress midges, particularly the two species *T. cupressi* and *T. cupressianassa*. We collected baldcypress midge galls in late October 2014 from 25 trees across three locations: John Lafitte National Historical Park and Preserve (29°37′44″N, 90°08′39″W), Tickfaw State Park (30°23′04″N, 90°39′03″W), and a private residence in Hammond, Louisiana (30°35′01″N, 90°26′02″W). Because the midges that parasitize baldcypress are bivoltine in Louisiana (and univoltine, depending on latitude, elsewhere), we collected the second cohort that has a second emergence in the mid-summer. This cohort copulates and the females, within one day of emergence, lay their eggs on living leaves of baldcypress. The adults die shortly after. The hatched larvae feed and burrow into the leaves, causing a gall to form, and then over-winter on the ground (Chen and Appleby, 1984). We clipped galls from baldcypress leaves before the galls dropped with the foliage in November and December. We sampled 10 trees each at John Lafitte Park and Tickfaw. An additional five trees were sampled at the private residence in Hammond. Each of these locations had large expanses of baldcypress habitat. The sampled trees ranged from having their base being completely submerged in water, to partially or seasonally submerged, to being on dry land.

The two species of baldcypress midge, *T. cupressi* and *T. cupressianassa*, produce different types of galls. We collected equal numbers of each gall type at each location. Some trees contained both types of galls, but the majority of individual trees had only one or the other gall type. At each infested tree, we collected ~50 galls from the trees. We recorded the gall type and density for the trees from which we sampled. The sampling heights ranged from 2 to 6 m above the ground.

After we collected the galls, we prepared them for over-wintering. We placed groups of 10 galls from the same tree and of the same species into plastic screen mesh pouches. We took these pouches to the private residence in Hammond, LA, where we attached them to lengths of stretchable grafting tape and wrapped them around the trunks of baldcypress trees. We placed them so that they were very close to the ground and not touching one another. They were left to overwinter from late October 2014 to late February 2015.

On February 22, 2015 we collected the gall pouches and prepared them for emergence monitoring in New Orleans, LA. We transferred the galls to large Petri dishes. Each dish had 10-20 galls of the same tree and gall type, contained a cotton ball soaked in deionized water and was closed but not sealed to allow air transfer. We arranged Petri dishes in large, open plastic boxes in a greenhouse and monitored them daily for insect emergence. The greenhouse was not climate controlled, and so was at ambient temperatures. Emerged midges or parasitoid wasps were placed in a 95% ethanol solution with a drop of 50% glycerol. Galls with emergence holes were taken back to the lab to culture the fungi. The galls were checked for emergences daily for 6 weeks. After a week without new emergences of gall midges or parasitoids, we assumed that the rest of the galls had no living insects. At 7 weeks we took the galls without emergence back to the lab for fungal culturing.

2.2. Fungal cultures from galls

To culture fungi from inside of galls, we first surfaced sterilized the galls. Using a tea strainer, the galls were submerged into 95% ethanol solution for 10 s, 10% Clorox solution for 2 min and then 70% ethanol solution for 2 min. We tested this surface sterilization method by pressing some galls on plates to be sure that no fungi from the outside surface of the gall were present. After surface-sterilization, we placed the galls in a sterile Petri dish and cut them into several small pieces with sterilized tools. We transferred all pieces of each opened gall to its own sterile microtube, and added ~1–2 ml of sterile water with 1% Tween (Fisher Scientific, Fair Lawn, New Jersey). They were then vigorously mixed together by shaking for ~20 s. The solution was transferred with a pipette to a large Petri dish of 2% malt extract agar (MEA) and the gall was discarded. The plates were allowed to sit for 48–60 h and then we recorded the number of colony forming units (CFUs). After counting CFUs, we haphazardly selected four fungal isolates per gall plate to transfer into smaller plates of MEA, creating four axenic cultures per gall. The isolates were chosen haphazardly from separate locations on the plates. After 2–3 weeks of growth, we sorted them into morphotype groups based on visual similarities in hyphal structure and color. Further characterization with molecular methods is described below.

2.3. Fungal cultures from leaves

We collected baldcypress leaves in October and November of 2014 from John Lafitte National Historical Park and Preserve from and Tickfaw State Park, at the same time galls were being collected. At each site, we sampled several healthy leaves from each of 12 trees. Using the same surface sterilization techniques as above for galls, we haphazardly selected and subsequently plated 4 mm pieces of baldcypress leaves (needles) onto plates with MEA. After 3–4 d, we transferred fungi emerging from leaf pieces into smaller plates to create axenic cultures, and the leaf endophyte cultures were characterized using molecular techniques along with gall fungal cultures (see below). Leaf endophyte isolation rates were low, and will be addressed more fully in a future publication. For the comparative purposes in the present paper, we include sequence data for 59 foliar endophyte cultures to relate to fungi observed in galls. These were the only 59 cultures (out of 123 total cultures from 24 individual trees) that were identified using sequencing from those sites; the low number of sequences was due to contamination by mites in the leaf culture collection.

2.4. Molecular methods

Since we observed two morphotypes that were clearly dominant and represented 166 cultures isolated from galls, we sequenced 10 isolates from each of these two morphotypes, from both emergence and non-emergence galls (i.e. 40 cultures were sequenced for these 2 morphotypes). For the rest of the morphotypes, all isolates were sequenced. Total genomic DNA was extracted from each of the selected isolates using the UltraClean Microbial DNA Isolation (MoBio Laboratories, Inc.) following the TissueLyser protocol. We amplified the nuclear ribosomal internal transcribed spacer (nrITS) and partial large subunit using primers

ITS1F (5′-CTTGGTCAT TTAGAGGAAGTAA) or ITS5 (5′-GGAAG-TAAAAGTCGTAACAAGG) for the forward reaction and LR3 (5′-GGTCCGTGTTTCAAGAC) or ITS4 (5′-TCCTCCGCTTATT GATATGC) for the reverse reaction. Our cycling protocol for amplification followed Arnold and Lutzoni (2007).

After visualization confirmation with SYBR on 1% agarose gels, we sent the PCR products to Beckman Coulter Genomics (Boston, MA) for Sanger sequencing. We sent 164 PCR products from individual cultures of gall or foliar fungi. Due to low quality in the sequences, 20 samples were removed before analyses, leaving 144 sequences (see Supplementary Material, Data in Brief). The sequences were assembled and edited in Sequencher v5.0 (Gene Codes Corp., Ann Arbor, MI) with support from Mesquite. Operational taxonomic units (OTUs) were formed from sequences assembled based on 97% similarity (Table S1). These OTUs were compared to the NCBI archives through BLAST searches to assign putative taxonomic identities based on the sequence similarity. For each OTU described in Table 1, we include the accession and strain number of a representative strain from our collection, the accession number of the nearest match in the NCBI databank, percent identity and query cover information in a Supplementary Table (Data in Brief). Voucher cultures were archived in the Van Bael laboratory at Tulane University and are available from the author by request. Sequences were archived in the NCBI database with accession numbers KY765150 - KY765293.

2.5. Statistical analyses

In total, fungal communities were described from 20 galls with insect emergence and 42 galls with no insect emergence. Due to differences in sample sizes, we used permutational analyses throughout, with up to 999 permutations of the data where possible. To analyze CFU counts, we used a linear model with CFUs as the response variable (square root transformation) and site, gall type (midge species), and insect status (emergence vs. non-emergence) as the explanatory variables (R Core Team, 2015). Our first model included all interactions, but none were significant so we excluded interactions from the final model. To estimate and compare species diversity, we calculated a Fisher's alpha value for the emergence and non-emergence gall fungal communities, site location and midge species based on OTU counts (PRIMER v 7, 2015). To analyze fungal community composition, we used permutational multivariate analysis of variance (PERMANOVA) with the fungal community composition as the response variable and gall species, site, and insect status (emergence vs. non-emergence) as the explanatory variables in PRIMER. The Bray-Curtis method was used to assign dissimilarities among fungal community samples (Clarke and Gorley, 2015), and an overall transform of the distance-based matrix ($\log(x+1)$) was used before PERMANOVA analyses. The first PERMANOVA model included all interactions among the explanatory variables. Since no interactions were significant, we presented a simplified model without interactions. For explanatory variables that described a significant amount of variation ($p < 0.05$), we used canonical analysis of principle components (CAP) ordination to illustrate the fungal species composition differences among gall samples. The PERMANOVA and CAP analyses used fungal communities from galls only, while the comparisons in Table 1 include foliar endophytic fungal OTUs as well.

Table 1
Relative frequency (%) of fungal OTUs[a] ranked from most to least common, with sample sizes (n) indicating the number of sequences in a given category.

Class	Genus species	Gall fungi		Leaf fungi	All fungi
		No insect emerged (n = 33)	Insect emerged (n = 52)	Foliar Endophytes (n = 59)	Total (n = 144)
Sordariomycetes	Sordariomycetes sp.1	0.0	3.5	18.1	21.5
Sordariomycetes	Pestalotiopsis sp.	11.8	4.9	0.0	16.7
Sordariomycetes	Sordariomycetes sp.2	0.0	9.0	4.9	13.9
Sordariomycetes	Pestalotiopsis maculiformans	4.9	2.8	0.0	7.6
Dothideomycetes	Botryosphaeria dothidea	2.1	2.8	0.0	4.9
Dothideomycetes	Neofusicoccum parvum	0.7	2.8	0.0	3.5
Sordariomycetes	Sordariomycetes sp.3	0.0	3.5	0.0	3.5
Dothideomycetes	Phyllosticta elongata	0.0	0.0	2.1	2.1
Unknown	Uncultured fungus 1	1.4	0.7	0.0	2.1
Sordariomycetes	Sordariomycetes sp.4	0.0	0.0	2.1	2.1
Sordariomycetes	Nigrospora oryzae	0.7	1.4	0.0	2.1
Unknown	Unidentified fungus 1	0.0	0.0	1.4	1.4
Unknown	No match available	0.0	0.0	1.4	1.4
Sordariomycetes	Sordariomycetes sp.5	0.0	0.0	0.7	1.4
Sordariomycetes	Sordariomycetes sp.6	0.0	0.7	1.4	1.4
Sordariomycetes	Xylariaceae sp.1	0.0	0.0	1.4	1.4
Unknown	No match available	0.0	0.0	0.7	1.4
Unknown	Unidentified fungus 2	0.0	0.7	0.7	0.7
Sordariomycetes	Trichoderma sp.	0.0	0.0	0.7	0.7
Unknown	No match available	0.0	0.0	0.7	0.7
Sordariomycetes	Sordariomycetes sp.7	0.0	0.0	0.7	0.7
Unknown	No match available	0.0	0.0	0.7	0.7
Unknown	Unidentified fungal endophyte	0.0	0.0	0.7	0.7
Sordariomycetes	Diaporthe sp.	0.0	0.0	0.7	0.7
Sordariomycetes	Xylariaceae sp.2	0.0	0.0	0.7	0.7
Dothideomycetes	Phyllosticta sp.1	0.0	0.0	0.7	0.7
Sordariomycetes	Colletotrichum aeschynomenes	0.0	0.0	0.7	0.7
Sordariomycetes	Sordariomycetes sp.8	0.0	0.7	0.0	0.7
Sordariomycetes	Sordariomycetes sp.9	0.0	0.7	0.0	0.7
Dothideomycetes	Aureobasidium sp.	0.7	0.0	0.0	0.7
Eurotiomycetes	Periconia sp.	0.0	0.7	0.0	0.7
Eurotiomycetes	Penicillium sp.	0.7	0.0	0.0	0.7
Sordariomycetes	Sordariomycetes sp.10	0.0	0.7	0.0	0.7
Dothideomycetes	Dothideomycetes sp.	0.0	0.7	0.0	0.7

[a] See Supplementary Table (Data in Brief) for number of sequences in each OTU group and BLAST information on query cover and nearest matches in the NCBI database.

3. Results and discussion

3.1. Abundance in galls

Baldcypress galls hosted an abundant and diverse fungal community. The abundance of fungi ranged from 3 to 189 CFUs, and was similar for galls of different types. Specifically, in a model that compared the effects of gall species, insect status and site, CFU counts did not differ by gall species ($F_{1,57} = 1.7$, $p = 0.19$) or by insect status (emergence versus non-emergence; $F_{1,57} = 0.1$, $p = 0.99$). The model showed, however, that counts of CFUs differed by site ($F_{2,57} = 4$, $p = 0.022$), with Jean Lafitte showing a greater abundance of fungal CFUs than Tickfaw or Hammond (CFU mean ± 1 standard error; John Lafitte 82 ± 10, Tickfaw 53 ± 7, Hammond 39 ± 10). When galls were opened for culturing, no visual signs of fungal mycelium were noted inside of emergence galls or non-emergence galls.

3.2. Diversity

The fungal community inside of baldcypress galls and leaves was diverse. Our total of 144 combined (leaf and gall) sequences was represented by 34 OTUs (Table 1, Table S1). Of the 34 OTUs, 17 were singletons, as they only occurred in our collection once. We identified three classes of fungi in the Ascomycota: Dothideomycetes, Eurotiomycetes, and Sordariomycetes. Approximately 68% of our isolates were from these classes, while we could not identify the remainder of the isolates using the NCBI database (Table 1, Supplementary Material, Data in Brief). The Fisher's alpha values were 3.26 for non-emergence gall fungi, 8.12 for galls with insect emergence, 2.04 for *T. cupressi*, 8.59 for *T. cupressiananassa*, 1.92 for Hammond, LA, 0.65 for Jean Lafitte National Park, LA, and 2.34 for Tickfaw State Park, LA.

We observed that there was greater overall fungal diversity in the emergence gall community compared to the non-emergence community. There was an approximate 150% increase in fungal diversity (from 3.26 to 8.12 Fisher's index value) between galls without versus with an insect emerging. The most common OTU observed in galls was a species of *Pestalotiopsis,* representing a sequence that was found in non-emergent galls 17 times. This OTU

was also found 7 times in galls with live insects but never from leaf samples. The genus *Pestalotiopsis* includes many plant pathogens and endophytes, and one previous study described *Pestalotiopsis microspora* as an endophyte from baldcypress bark (Li et al., 1996). Recent work, however, has shown that strains of *Pestalotiopsis* can also be entomopathogenic to conifer-infesting insects (Lv et al., 2011). It is unclear from our current study whether strains of *Pestalotiopsis* were implicated in the lack of insect emergence, and the fungi could have originated in leaf tissue or with insect oviposition. Previous work has suggested that insects may already be inoculated with fungi before interacting with plants. For example, a finding of greater endophyte diversity associated with leaf-mining insects supports the idea that larval insects bring fungi with them when they burrow into plant tissues (Faeth and Hammon, 1997). Lawson et al. (2014) also found patterns consistent with the possibility that aphids brought some fungi into their galls.

3.3. Community composition

We observed few putative fungal species in both the galls and leaf tissue. Specifically, four OTUs (out of 34) were isolated from galls and leaves (Table 1). Two of these overlapping OTUs were Sordariomycetes. Interestingly, overlap among galls and leaf tissue OTUs was only observed from galls with emerged insects, with no overlap from galls without emerged insects (Table 1). Due to the low leaf isolation rate and low overlap with galls (Table 1), we did not analyze the leaf community composition further.

The emergence status of the gall was the most important factor for explaining variation in fungal communities (Fig. 1). Our PERMANOVA test showed that insect status, or whether or not an insect emerged, was a significant predictor of the fungal community composition ($F_{1,19} = 5.4$, $p = 0.002$). The two other factors were not significant (gall species, $F_{1,19} = 1.4$, $p = 0.21$; site $F_{2,19} = 1.4$, $p = 0.17$). This differs from a previous study showing that three gall forming species had distinct fungal community composition in their galls (Lawson et al., 2014). Moreover, the lack of a site effect did not support our second prediction, that the study sites several kilometers apart would differ in their gall fungal communities. In other systems, foliar endophyte communities have been observed to differ significantly across distances as short as a few meters apart
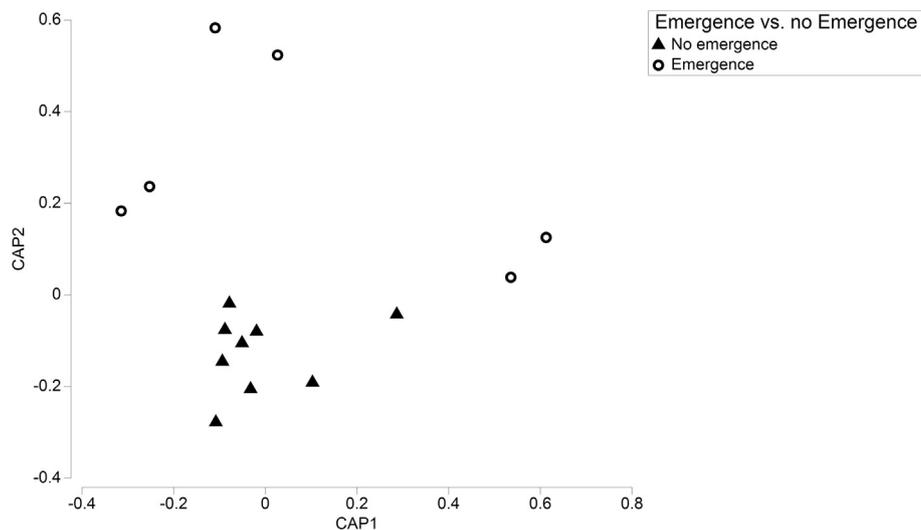

Fig. 1. Fungal communities had different OTU composition in galls with and without insect emergence. The plot shows an ordination using the canonical analysis of principle coordinates (CAP) with CAP1 and CAP2 representing the axes. The gall fungal communities were more similar to one another in galls where no insect emerged (see clustering of dark triangles), suggesting that a pathogenic or saprotrophic fungus dominated them. The ordination represents communities of 24 galls (6 with insects emerged, 18 with no emergence), but one of the closed triangles is representative of 9 overlapping gall samples with the same fungal community.

(Hartley and Gange, 2009). An important consideration for our study, however, is that we allowed all of the galls to overwinter in the same outdoor location. We cannot assess whether gall fungal communities were indicative of their sampling site, their over-wintering site, or a combination. A way to control for this in the future would be to have the gall samples overwintering at their own sites.

4. Conclusions

Our study of fungal communities inside of baldcypress galls and leaves showed that: (1) baldcypress galls host a diversity of fungi; (2) there is low overlap among gall and leaf putative fungal species; and (3) the diversity and community composition of fungi differed for galls with versus without insect emergence. Overall, galls from which an insect did not emerge appeared to be dominated by a less diverse fungal community. It has been shown previously that fungal endophytes can benefit the plants by being detrimental to the gall forming insects (Wilson and Carroll, 1997; Hartley and Gange, 2009), and, conversely, that fungal endophytes can have no noticeable effect on the gall forming insects or their plant hosts (Wilson, 1995b; Faeth and Hammon, 1997). The interactions between endophytes and gall forming insects are myriad and complex, and the potential costs or benefits of endophytic fungi to the host plant and/or galling insects may depend on the species involved. In the case of baldcypress gall fungi, this study provides a foundation for further work on how these organisms interact to influence the health of baldcypress trees.

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