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Fungal diversity in galls of baldcypress trees

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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

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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. cupressiananassa*. 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 overwinter 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. cupressiananassa*, 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 overwintering. 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

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