SCALE INSECT INFESTATION OF *PHRAGMITES AUSTRALIS* IN THE MISSISSIPPI RIVER DELTA, USA: DO FUNGAL MICROBIOMES PLAY A ROLE?

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INTRODUCTION

Species interactions are often studied as two-way relationships; however, it is well known that third parties can play a critical role in the outcome of these interactions. A vast array of microbes inhabits most plant tissues and affect plant growth, physiology, nutrient quality, defense, and immune status in ways that influence how a plant interacts with competitors, mutualists, pathogens and predators (Friesen, 2013; Hardoim et al., 2015; Porras-Alfaro and Bayman, 2011). There is also growing evidence that microbes are important in mediating plant interactions with insect pests (Cardoza et al., 2012; Price et al., 1980; Shikano et al., 2017). For example, several studies have linked microbes inhabiting plant tissue with the ability of plants to withstand and deter herbivory (Clay et al., 2005; Hartley and Gange, 2009; Rudgers and Clay, 2008).

Pathogenic and mutualistic microbes can have both positive and negative outcomes on plant-herbivore interactions. Microbes which benefit plants can also indirectly benefit herbivores, for example, foliar application of the commercially available product EM·1® stimulates growth of tomatoes, which in turn, enhances the growth and survival of some tomato pests (Megali et al., 2014). On the other hand, there are many cases in which plant symbionts (Choudhary and Varma, 2016; Hartley and Gange, 2009; Pieterse et al., 2014; Pineda et al., 2015, 2013) and pathogens (Kruess 2002, Al-Naemi and Hatcher 2013) negatively impact herbivore performance by producing or stimulating the production of defensive compounds that deter herbivores, thereby decreasing herbivory. In other cases, pathogens may change plant chemistry in ways that increase herbivore performance; for example, white mold (*Sclerotium rolfsii)*-infected peanut plants contain higher soluble sugars and lower starch and phenolics,

which leads to higher survival and growth of Beet Army Worm larvae (Cardoza et al., 2003).

Microbes can also play a role in insect outbreaks and associated plant host die-offs. Insects can carry microbes that are pathogenic toward plants, such that plants incur damage from both insect herbivory as well as microbial pathogen attack. For example, Mountain Pine Beetle larvae attack the inner bark (phloem) of some western pine species and carry the blue stain fungus, which infects and blocks the xylem and phloem of the tree (Safranyik, L., Shrimpton, D.M., Whitney, 1975). Microbial symbionts carried by insects have also been known to increase the invasive potential of an insect. One example is the invasive red bay ambrosia beetle whose fungal symbiont, which causes laurel wilt disease, is extremely virulent towards plant hosts only in its invaded range, resulting in insect outbreak and forest decline in North America (Fraedrich et al., 2008, 2007). A cause for increased pathogenicity or virulence of insect microbial symbionts towards the host plant is genotypic differences between native and introduced lineages of a plant (Cardoza et al., 2012; Fraedrich et al., 2007).

Here we test whether fungal microbes within plant tissues play a role in the plant-insect system of *Phragmites australis* (common reed) and *Nipponaclerda biwakoensis* (a scale insect), in which the insect is associated with massive plant die-offs in the Mississippi River Delta. *Phragmites australis* (Cav.) Trin. Ex Steud. (Poaceae) is a cosmopolitan marsh species with both native and invasive haplotypes in North America. In the Gulf Coast, there are several known haplotypes which occur sympatrically, representing more recent introductions as well as a haplotype with an ancient introduction or potentially native origin (Hauber et al., 2011; Lambertini et al., 2012; Meyerson et al.,

2012; Saltonstall, 2002). The two haplotypes on which this study focuses are both invasive, though their life histories, time of introduction, and invasive status differ.

Haplotype M (hereafter "European haplotype") was introduced to the Northeastern United States from Europe (Lambertini et al., 2012) approximately 150 years ago (Saltonstall, 2002). It has spread rapidly westward across North America and is the cause of much conservation concern due to its highly aggressive monotypic growth (Chambers et al., 1999; Martin and Blossey, 2013; Silliman and Bertness, 2004). Only within the last 60 years has it began to appear in the MRD (Saltonstall, 2002). Haplotype M1 (hereafter "Delta haplotype") is also a fairly recent introduction from the Northern Africa/Mediterranean region. Its range is restricted to the Gulf Coast, and it is the dominant type seen in the Mississippi River Delta.

Though highly invasive elsewhere, *P. australis* is a foundational plant species whose system of deep roots and rhizomes act to decrease erosion, control floodwaters and increase accretion in the MRD, an area that is otherwise experiencing rapid land loss. *P. australis* is one of the first plants to colonize new areas of land created in the delta mouth. It also plays an integral role in the MRD in the face of sea level rise, because its dense belowground network of roots and rhizomes promotes soil accumulation and prevents erosion brought on by wave action and storm surges along the Louisiana coast (Coops et al., 1996; Gedan et al., 2011). *P. australis* also has the ability to grow at water depths unsuitable for other marsh plants, which makes it a focal species for restoration (J. Cronin, *personal communication*). Thus, disruptions and die-offs in this foundational plant species will likely have negative consequences for the MRD.

Nipponaclerda biwakoensis (Aclerdidae) has origins in China and Japan where it does not seem to detrimentally impact *P. australis* because its numbers are kept in check by parasitic wasps, and insectivorous birds (Gan et al., 2010; Kaneko, 2005, 2004). The mode and timing of introduction to North America is unknown, though it was first discovered in 2016 in vast numbers (Knight et al., 2018). This scale insect settles under the leaf sheaths of *P. australis* stems, tending to concentrate around the leaf nodes, though they can be found along the entirety of the internode in some cases (*personal observation*). Similar to aphids, *N. biwakoensis* bores into the stem tissue and extracts sugars from the phloem of the plant. Once the insect has inserted its mouthparts into the stem material, it forms a waxy coating surrounding its body and remains in that place for the duration of their life cycle (Knight et al., 2018).

The appearance of *N. biwakoensis* coincided with widespread *P. australis* die-offs of over 100,000 acres in the Mississippi River Delta (Ramsey III and Rangoonwala, 2017), and experimental evidence suggests the scale is part of the cause (Knight et al., *in review*). The European and Delta haplotypes of *P. australis* are closely related to one another, though they are experiencing an almost 8-fold difference in infestation by *N. biwakoensis* (Knight et al., 2018). Several factors may contribute to the differences in scale infestation between the European and the Delta haplotypes of *P. australis,* such as preference for particular genotypic traits like softer stem tissue, or differences in chemical defenses like total phenolics (Cronin et al., 2015), but here we posit that the internal fungal communities play a role. For example, the European type may possess a community rich in beneficial endophytic fungi, and the Delta type may have a community composition containing more pathogenic fungi, thereby weakening its

herbivory defenses. Furthermore, because of the extent and rapid pace of the die-off, we were interested in investigating if pathogenic fungi may be proliferating and working in concert with scale insect damage to contribute to die-off. To explore this, we will first test the hypothesis that, consistent with the observations by Knight et al. (2018), European and Delta haplotypes differ in severity of infestation by *N. biwakoensis*. To determine whether there is a difference between the haplotypes in mortality that may be linked to infestation by *N. biwakoensis,* we will also assess whether the numbers of living or dead stems differs among haplotypes. Second, we test the hypothesis that haplotypes differ in the fungal communities present in their stem and leaf tissue. And third, we test whether scale infestation is associated with changes in fungal community structure.

MATERIALS AND METHODS

Site and sample collection

In September 2018, 20 samples of each haplotype (European and Delta) were collected at two separate sites located at 29° 14’ 37.4” N 89°15’ 5.7” W (Site 1, European), 29° 14’ 36.3” N 89° 15’ 3.1” W (Site 1, Delta), 29° 13’ 98.3” N 89°19’ 17.9” W (Site 2, European) and 29° 14’ 0.9” N 89°15’ 15.7” W (Site 2, Delta) in the Birdfoot Delta of the Mississippi River, Louisiana, USA, for a total of 80 individuals. The paired haplotype plots at each site were identified through Sanger sequencing by researchers at Louisiana State University. Individual tillers of *Phragmites* were cut at the surface of the water if the plot was flooded (because scale insects do not occur under water) and at the soil in dry plots and placed individually in bags on wet ice for transport. Information on stand density and number of dead and living tillers was determined for each individual using a circular quadrat which covers an area of ¼ m^2 placed with the selected tiller in the

center. On the day of collection, samples were placed in a 4°C refrigerator for holding. Within 24 hours, small subsamples of stem and leaf tissue from each of the 40 individual tillers collected were placed in a -80°C freezer for metagenomic analysis. Using the remaining portions of the individuals, number of scale insects per plant was determined by counting the number of scales every third internode on the plant and extrapolating to the whole plant. Because the internode lengths differ within one individual, as well as between haplotypes, this value was standardized by the overall above-water height of the individual to determine a scale density per centimeter of height.

Surface sterilization

For metagenomic analysis, 10 individuals from each plot were selected by arranging all collected individuals in order of lowest scale density to highest. Starting with the lowest, every-other individual was chosen. This ensured we had a range of infestation represented for each plot and each haplotype. A 10 cm section of healthy leaf tissue and a 4 cm internodal section of stem from each sample were selected and placed in a tea strainer. Plant tissue was sequentially soaked in 95% ethanol for 10 seconds, 0.5% sodium hypochlorite solution for 2 minutes, 70% ethanol for 2 minutes, and rinsed in sterilized deionized water for 2 minutes. Samples were then dried on UV sterilized KIMTECH Kimwipes before placing in gamma-sterilized cryovials for storage at -80°C.

DNA extraction

Following surface sterilization, extractions for the leaf samples were done following Qiagen DNeasy ® PowerPlant® Pro Kit protocol using Phenolic Separation Solution and 250µl of Solution IR. Leaf samples were eluted using 100µl of Solution EB. For stem samples, 75mg of plant tissue was used and elution was done using 50µl of Solution EB

in addition to the changes noted previously. Extraction concentrations were determined using a ThermoFisher Scientific Qubit fluorometer. All samples above a concentration of 10ng/µl were standardized by mixing extraction product with additional Solution EB so they reached a concentration of 10ng/µl.

PCR

Sequencing libraries were created in a two-step process following U'ren and Arnold (2017). PCR1, or amplification, was done in duplicate using a mastermix of Phusion Flash (ThermoFisher F548), 20mg/mL Bovine Serum Albumin (ThermoFisher NEF cat#F9000), ITS Forward and Reverse primers, and DNAse-free dWater. The primer sequences used were ITS1F 5' – CTT GGT CAT TTA GAG GAA GTA – 3' and ITS2R 5' – GCT GCG TTC TTC ATC GAT GC – 3'. Thermal cycler conditions for amplification were: 5 minutes at 95.0° C, 20 seconds at 98.0° C, 15 seconds at 54.0° C, 30 seconds at 72.0° C, repeat 30 times, 5 minutes at 72.0° C and then stored at 4.0° C. After confirming amplification was successful using gel electrophoresis, sample duplicates were pooled and forward and reverse indexing primers were added so that no two samples contained the same combination of indexing barcodes. Thermal cycler conditions for indexing primer attachment were: 10 seconds at 95.0° C, 15 seconds at 98.0° C, 5 seconds at 50.0° C, 20 seconds at 72.0° C, repeat those steps 9 times, 1 minute at 72.0° C, and then stored at 4.0° C. Barcode attachment was then confirmed through gel electrophoresis and samples were pooled into a common library. Four negative controls were also processed and included in the library for identification of possible contaminant sequences. 5ng of DNA from each sample was added to the library. The pooled library was purified and concentrated using Agencourt AMpure XP Beads with Dynamag-2

Magnet. Pooled libraries were then sent to Duke University for Illumina MiSeq high-throughput sequencing (300 bp paired reads).

Sequence analysis

Amplicon sequence variants (ASV) were identified following the QIIME2 version 2018.2 pipeline (Bolyen et al., 2019). Primers and adapters were trimmed in QIIME2 and data was then denoised and paired reads joined using DADA2 version 1.8. No further trimming was done to the sequences because DADA2 is robust to low-quality sequences through the incorporation of read quality information into its error model. One sample was dropped due to very low reads compared to the remainder of the dataset. Taxonomy was assigned using UNITE 7.2 (Nilsson et al., 2018) and functional guild assignments were obtained using FUNGuild (Nguyen et al., 2016). Using the negative controls included in the pooled library sent for sequencing, 7 possible contaminant sequences were removed using the decontam package in R (Davis et al., 2018). Prior to analysis, data were rarefied to an even sampling depth of 3401 reads and subsampled without replacement, which removed 630 ASV sequences.

Statistical analysis

All statistical analyses were done using R (R Core Team, 2019). To determine whether there was a difference in scale densities, stand densities, and ratio of dead to living tillers per ¼ m^2 between haplotypes, we used the lme() function in the package nlme (Pinheiro et al., 2020) to create linear mixed models with site as a random effect. While site was never a significant term in the models (models with the random effect were compared to ones without using a likelihood ratio test), we retained site in the

models to account for any minor differences among sites. Type III ANOVAs were then performed using anova() in R.

ASV richness (Chao1), Shannon diversity, and evenness were calculated for all samples using phyloseq (McMurdie and Holmes, 2013). To test the effect of haplotype, tissue type, and their interaction on alpha diversity and ASV richness, linear mixed effects models and ANOVAs were performed as above with site as a random effect. Similar to the between-haplotype analyses above, site was never a significant term in the models, but it was still retained in the models to account for any minor differences among sites. Pairwise differences between treatments were tested using Tukey *post hoc* tests with glht() from the multcomp package (Hothorn et al., 2008).

Scale density covaried so strongly with haplotype (Fig. 1a) that we were unable to test their effects on fungal diversity together in a single model. Therefore, we assessed the effect of scale density on fungal diversity metrics separately for each haplotype. We fit a linear mixed effects model testing the effect of scale density on the different diversity metrics, with site as a random effect, and performed a type III ANOVA for each haplotype individually.

The effects of haplotype and tissue type on fungal community composition were evaluated using distance-based redundancy analyses (dbRDA) with Bray Curtis distance in the vegan package (Oksanen et al., 2019). Site was found to be weakly significant ($P=0.013$ in a model only including site as an explanatory variable), so it was used as a conditioning variable in the analysis. Permutation tests were used to test significance of the constraining variables. Within-haplotype effect of scale density and tissue type was then assessed with separate dbRDAs and permutation tests for each haplotype.

We also assessed whether ASV community composition between haplotypes and tissue types varied in community structure through analysis of dispersion, a metric of variability. Dispersion was calculated using Bray Curtis distance in the betadisper() function in the vegan package and tested with a permutation test. All plotting was done using the ggplot2 package (Wickham, 2016).

RESULTS

Phragmites scale infestation and die-off

Scale density, stand density, and proportion of dead individuals did not differ between sites, however, there were substantial differences between the haplotypes. The Delta haplotype contained nearly twice the number of scale insects per individual, and 50% higher scale density (per cm stem) (Fig. 1a). The density of individual tillers per ¼ m^2 did not differ significantly between the two haplotypes, though the ratio of dead to alive individuals showed the Delta haplotype to have significantly more dead individuals than the European haplotype (Fig. 1b,c).

Fungal Diversity

The European haplotype had significantly higher fungal Chao1 richness and Shannon diversity than the Delta haplotype in both leaf and stem tissues (Fig. 2a,b), but haplotypes had similar evenness (Fig. 2c). Leaves generally had higher richness, Shannon diversity, and evenness compared to stems; the only exception was that the Delta haplotype had similar richness in leaves and stems (Fig. 2).

Because scale density covaried so strongly with haplotype, the effect of scale density on diversity metrics was tested within each haplotype separately. Within-haplotype analyses revealed no significant effect of scale density on Chao1 richness

(European haplotype: F = 0.253, P = 0.617, Delta haplotype: F = 0.022, P = 0.882) Shannon diversity (European: F = 0.096, P = 0.758, Delta: F = 0.807, P = 0.777), and evenness (European: F = 0.831, P = 0.367, Delta: F = 0.977, P = 0.445).

Compositional Differences

Both haplotype (F = 15.943, P < 0.001) and tissue type (F=10.907, P< 0.001) significantly affected fungal endophyte community composition (Fig. 3). Site was also significant (F = 2.62, P = 0.009) but only explained 3% of the variation, while haplotype and tissue type explained 30%. Interestingly, there was considerable overlap in stem communities in the two haplotypes (Fig. 3), though they still significantly differed (F = 4.343, P < 0.001). Leaf communities, on the other hand, had no overlap and were also significantly different from one another (F = 23.305, P < 0.001). Within-haplotype analyses of scale density on fungal community composition showed no significant effects for either the European (F = 0.901, P = 0.489) or Delta (F = 1.045, P = 0.389) haplotypes.

Within-haplotype variance in microbiome composition was slightly but significantly higher in the European haplotype samples (F = 3.973, P = 0.046). When individual tissue types were analyzed, there was no difference in dispersion between leaf and stem tissues (F = 2.820, P = 0.095).

Wickerhamomyces anomalus was the most abundant ASV identified across haplotypes and tissue types, though in European haplotype leaf tissues, it was second to *Nigrospora oryzae* (Table 1). *Fusarium polyphialidicum* was also generally in high abundance though it was more abundant in leaf tissues than stem tissues. *Rhinocladiella similis* and *Meyerozyma caribbica* were two other ASVs of high abundance, appearing within the top five in all tissue types except for European haplotype leaf tissues where

Septoriella hubertusii was uniquely more abundant. Other unique ASVs appearing in high abundance in only one haplotype/tissue type combination are *Leptoxyphium madagascariense* in European haplotype stem tissue, and *Phialemoniopsis curvata* and *Candida orthopsilosis* in Delta haplotype stem tissue.

DISCUSSION

There is growing recognition that microbiomes can play important roles in mediating interactions among plants and their insect herbivores (Clay et al., 2005; Hartley and Gange, 2009; Hubbard et al., 2019; Rudgers and Clay, 2008) and could have consequences for differential infestation of plant haplotypes. Here we find that the *Phragmites australis* Delta haplotype had higher insect infestation, lower fungal diversity, and different fungal community composition in its stems and leaves compared to the European haplotype. However, in our within-haplotype analyses, we did not find any evidence that microbial diversity or composition was specifically associated with scale infestation. Therefore, we conclude that differences in scale infestation are likely due to physiological and morphological differences between haplotypes, rather than fungal microbiomes.

Scale infestation and mortality

The findings of this study provide support for Knight et al. (2018) and Knight et al. (*in review*), which suggest that different *P. australis* haplotypes displayed differing levels of susceptibility to scale infestation with the Delta haplotype having higher infestation rates than the European haplotype, and subsequently, a greater likelihood of experiencing die-back. Knight et al. (2018) report scale densities that are on average 8 times higher in the Delta type compared to the European haplotype (sampled mid-

summer 2017), and Knight et al. (*in review*) report scale densities up to 7 times greater in Delta (sampled summer 2018-19). Our data shows slightly lower numbers; we found scale densities on average 1.5 times higher, but up to 4 times higher in the Delta haplotype (sampled in September 2018). Our sampling locations were near or in the same sites as sampled in the Knight et al. studies, thus the data show that both small-scale spatial and temporal variability in scale densities may exist over time.

Additionally, we found that the Delta type and had nearly three times higher mortality (proportion of dead tillers) compared to the European type, suggesting that infestation by *N. biwakoensis* is a likely cause of *P. australis* dieback. This contrasts with Knight et al.'s (*in review*) mesocosm experiment results, which suggests that the scale insect increases stem mortality similarly across both haplotypes. Interestingly, in our field data the difference in mortality is higher than the difference in infestation, which could indicate that the Delta-type is more sensitive to the effects of infestation than the European-type. Since the Delta-type is the predominant haplotype found in the MRD, this could have grave consequences if *N. biwakoensis* continues to have a foothold in this area.

Microbiome Diversity and Composition

Our results support the growing number of studies that find significant intra-specific variation in plant microbiomes that is explained by plant haplotype or genotype (Bálint et al., 2013; Bodenhausen et al., 2014; Wagner et al., 2016). In *Phragmites*, the microbiome is fairly well-studied (Clay et al. 2016; Kowalski et al. 2015; Soares et al. 2016) and several studies have explored the effect of *Phragmites* haplotype on microbiome diversity and composition. Studies have found differences in rhizosphere

bacterial communities among European (M), Gulf (I), and native haplotypes (Bowen et al., 2017) and differences in root and rhizosphere oomycete communities between European (M) and native haplotypes (Nelson and Karp 2013, Bickford et al 2018). However, one study found that root fungal and bacterial communities in European (M) and native haplotypes did not differ (Bickford et al. 2018). The only study that has compared microbiomes in the closely-related European (M) and Delta (M1) haplotypes was Allen et al. (2020) who found differences in leaf fungal composition, but not diversity or dispersion, between the haplotypes. Here we used metagenomic methods capturing nearly 5 times more taxa and found that European and Delta haplotypes differ in community composition, diversity, and dispersion of their fungal microbiomes, with European haplotypes having greater diversity and dispersion than Delta despite being collected from the same local area.

Many studies in the literature across many plant taxa report that different tissue types vary in the composition and diversity of their microbiomes. (Cregger et al., 2018; De Souza et al., 2016; Jin et al., 2014). Within *Phragmites,* a few studies have investigated this (Clay et al., 2016; Ma et al., 2013; Zhou et al., 2019). Ma et al. (2013), a culture-independent study on bacteria and archaea, found stems and leaves to have the same microbial diversity, while Clay et al. (2016), a culture-based study on fungi, found significantly higher fungal richness and diversity in stem tissue compared to leaf tissue. Both results contrast with our finding that leaves were more diverse than stems. Clay et al (2016) also found greater overlap in community composition (although still significant differences) among tissue types compared to our study in which tissue types had strikingly different compositions. Interestingly, the abundant taxa in the Clay et al. (2016)

study (*Sarocladium strictum*, *Alternaria alternata*, and *Stagonospora neglecta*) were not found in our dataset at all suggesting either differences in culture-based vs. metagenomic methods or the effect of environment on composition (the Clay et al. 2016 study was done in the Midwest vs. the Gulf Coast).

Interestingly, our study is the first to simultaneously assess microbiome differences among haplotypes and tissue types. We found a considerable amount of overlap in stem communities between European and Delta haplotypes, while leaf communities displayed little overlap. This suggests that the extent of intraspecific variation in microbiome communities may depend on what plant compartment is being studied.

While not often investigated as a characteristic of microbial structure, there are still some studies in the literature which show beta dispersion to be a useful metric when exploring microbial community assembly across environments, temporal scales, and tissue types (Ettinger et al., 2017; Shade et al., 2013; Toju et al., 2018), though few that look at dispersion between genotypes/haplotypes (Glasl et al., 2019). We found a significant difference in fungal community variance between haplotypes: the invasive European haplotype had a significantly greater amount of community dispersion compared to the Delta haplotype. This could be due to greater genetic variation or reduced specialization within the European haplotype.

ASVs of Note

Candida orthopsilosis, Fusarium polyphialidicum, Leptoxyphium madagascariense, Meyerozyma caribbica, Nigrospora oryzae, Phialemoniopsis curvata, Rhinocladiella similis, Septoriella hubertusii, and *Wickerhamomyces anomalus* were the

most abundant fungal taxa across haplotypes and tissue types, though little functional information is available on most. *Wickerhamomyces anomalus*, identified by FUNGuild as an undefined saprotroph, was the most abundant ASV in both leaf and stem tissues in both haplotypes, except for European haplotype leaf tissue, where it is ranked second. It appears in all but one sample. *W. anomalus* is an endophytic yeast which has potential as an antifungal biocontrol and outperforms several other endophytic fungi in the inhibition of grey mold in *Eucalyptus dunnii* seedlings (Poitevin et al. 2020). *Meyerozyma caribbica*, also assigned to the undefined saprotroph guild, had the third highest relative abundance across our samples, occurring in all but 8 samples. *M. caribbica* is another endophytic yeast shown by Poitevin et al. (2019) to be inhibitory against grey mold in *Eucalyptus dunnii*. Jan et al. (2019) also showed the potential of *M. caribbica* to mitigate salt stress in *Zea maize*. *Fusarium polyphialidicum,* an animal pathogen, endophyte, plant pathogen, saprotroph, and wood saprotroph, appeared in high abundances in both haplotypes and tissue types and was found by Galgóczy et al. (2013) to produce antifungal polypeptides homologous to the *Penicillium chrysogenum,* antifungal protein (PAF) which inhibits the growth of phytopathogenic fungi (Kaiserer et al., 2003) but may be used by *F. polyphialidicum* in competition for resources with other fungi. It has also been isolated from infected pineapple fruits and found to produce mycotoxins (Abbas and Ocamb, 1995; Shi et al., 2017; Stepień et al., 2013), which could indicate a pathogenic lifestyle. *Nigrospora oryzae*, belonging to the undefined saprotroph functional guild, only appeared in high abundances in leaf tissues, is a known cause of leaf spot in several plant species (Zhai et al., 2013; Zhang et al., 2012; Zheng et al., 2012). Numerous, but not all,

individuals collected in this study exhibited signs of leaf spot, which may be linked to the presence of *N. oryzae.*

No direct link between microbiomes and scale infestation

While we found that haplotypes differed in scale insect infestation, mortality, and microbiome composition, we did not find any significant relationships between scale density and fungal microbiomes in our within-haplotypes analyses. Thus, we suggest that differences in both scale infestation and fungal communities are individually affected by morphological or physiological traits of *Phragmites* rather than directly influencing each other. Further supporting this conclusion is our finding that stem microbiomes were highly similar between haplotypes and the fact that *N. biwakoensis* is a phloem-feeding insect concentrated on the stems and would come into the greatest contact with stem fungi; thus the differing levels of scale infestation on European and Delta haplotypes is likely due to traits other than fungal community composition. Furthermore, we did not find high abundances of an obvious pathogen that might be transmitted by the insect to the plants, thus influencing die-off. It is also possible that scale insects are less likely to introduce pathogens to plants, compared to herbivores like chewing insects or stem boring insects, because the wound is so minor, and the phloem is moving out of the plant to the insect, though there is some evidence of other phloem-feeding hemiptera transmitting phloem-limited bacteria to their plant hosts (Hall et al., 2013; Pitino et al., 2014).

Plant morphology is one factor that likely plays a role in affecting infestation density. Despite being genetically very similar (Lambertini et al., 2012), the Delta-type is much taller in height and has a much thicker, and in turn juicier, stem, which may make it

more appealing for phloem-sucking insects (*personal observation*). The European haplotype, on the other hand, has a much thinner, drier, and more brittle stem, which may make it difficult for *N. biwakoensis* to extract the sugars it needs. Other work has demonstrated differences in leaf traits and herbivory between native and invasive lineages of *Phragmites* in North America, and between European lineages in the U.S. vs. Europe (Cronin et al., 2015; Lambert and Casagrande, 2007; Park and Blossey, 2008). In particular, Cronin et al. (2015) found that in North America, the European lineages (including the European and Delta haplotyes) have greater leaf toughness and experience substantially lower herbivory than native North American lineages. We suggest that further work quantifying morphological differences *within* invasive genotypes would be fruitful. The differences in leaf and stem morphology, along with genetic differences, may also explain why leaf and stem microbiomes differed between the European and Delta haplotypes in our study (Tellez, 2019; Van Bael et al., 2017).

Conclusions

Because *P. australis* is recognized as a foundational plant species that is important for counteracting land loss and erosion in the MRD, die-offs are cause for concern. Thus, finding the reason for these die-offs is the first step in preserving and restoring Louisiana's coastal wetlands. Here, we confirmed that the Delta haplotype was more infested and experienced more significant die-off than the European haplotype. We also demonstrated marked differences between haplotypes in fungal community structure, with the European haplotype differing in composition, and having greater fungal diversity and greater variance in community composition. However, microbiome differences were not related to infestation severity, thus more work is needed to investigate the mechanics

of *N. biwakoensis* infestation and differences in plant traits (e.g. stem toughness and thickness) between haplotypes that might contribute to scale infestation, as well as other stressors such as salinity or drought that might exacerbate die-offs. We also suggest that investigation of bacterial communities and belowground microbiomes could give us a more holistic view of plant health and its relationship to the scale infestation. Furthermore, more work aimed at elucidating the function of the taxa that make up the fungal microbiome is critical for better interpretation of the consequences of differences in microbiome composition among haplotypes for plant growth and herbivore interactions. Overall, our understanding of how microbiomes mediate plant-herbivore interactions is still in its infancy, and understanding these linkages is essential for restoration and conservation of many foundational plant species.

APPENDIX

TABLES

Table 1 (See next page). Rank abundance of the top ASVs identified in each haplotype and tissue type combination. Overall, *Wickerhamomyces anomalus* was the most abundant, appearing in the top two most abundant for every haplotype/tissue type. *Rhinocladiella similis* also appears within the top five, though it is on average lower in rank. *Fusarium polyphialidicum* and *Meyerozyma caribbica* appear within the top five of all sample combinations except for Delta stem tissue and European leaf tissue respectively. *Nigrospora oryzae* and *Septoriella hubertusii* appear in high abundances only in leaf tissues, while *Candida orthopsilosis* and *Leptoxyphium madagascariense* are only highly abundant in stem tissues of separate haplotypes.

ASV Rank Abundance							
		Leaf Tissue		Stem Tissue			
Class	**ASV Identification**	**European**	**Delta**	**European**	**Delta**	**FUNGuild Assignment**	**Additional Notes**
Saccharomycetes	*Wickerhamomyces anomalus*	2	1	1	1	Undefined Saprotroph	grey mold inhibition
Eurotiomycetes	*Rhinocladiella similis*	5	5	2	3	Undefined saprotroph, wood saprotroph	
Saccharomycetes	*Meyerozyma caribbica*	-	3	3	2	Undefined saprotroph	grey mold inhibition, potential for salt stress mitigation
Sordariomycetes	*Fusarium polyphialidicum*	4	2	5	-	Animal pathogen, endophyte, plant pathogen, saprotroph, wood saprotroph	produces antifungal polypeptides
Sordariomycetes	*Nigrospora oryzae*	1	4	-	-	Undefined saprotroph	cause of leaf spot
Dothideomycetes	*Septoriella hubertusii*	3	-	-	-	Fungal parasite, plant pathogen, plant saprotroph	
Dothideomycetes	*Leptoxyphium madagascariense*	-	-	4	-	Undefined saprotroph	
Sordariomycetes	*Phialemonopsis curvata*	-	-	-	4	-	
Saccharomycetes	*Candida orthopsilosis*	-	-	-	5	Undefined saprotroph	

FIGURES

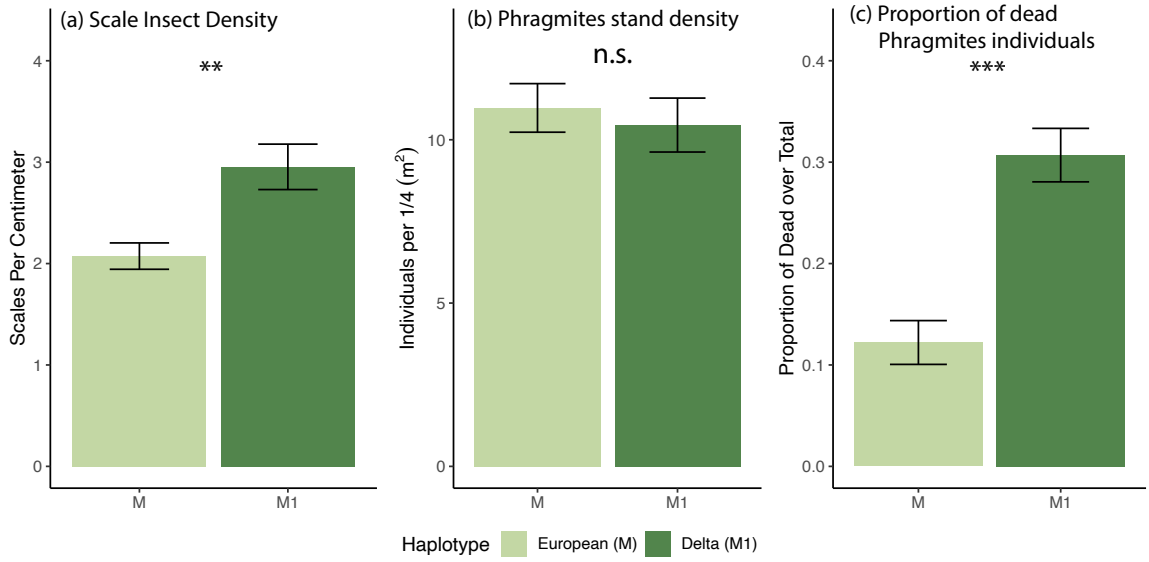

Figure 1. Effect of haplotype on scale density (a), *Phragmites* stand density (b), and proportion of dead *Phragmites* individuals (c). Statistical results shown are from an ANOVA: *†P < 0.1, * P < 0.05, ** P < 0.01, ***P < 0.001*, *n.s. non-significant*. Scale density per centimeter (a) differed significantly between the European and Delta haplotypes (F = 11.659, P < 0.001) with the Delta haplotype having an average of 50% more scale insects per centimeter on each individual. Stand density (number of individuals per ¼ m^2) did not differ between haplotypes (F = 0.235, P = 0.629). The proportion of dead to living individuals per ¼ m^2 differed significantly between haplotypes (F = 30.813, P < 0.0001) with the Delta haplotype having a greater proportion of dead individuals per quadrat.

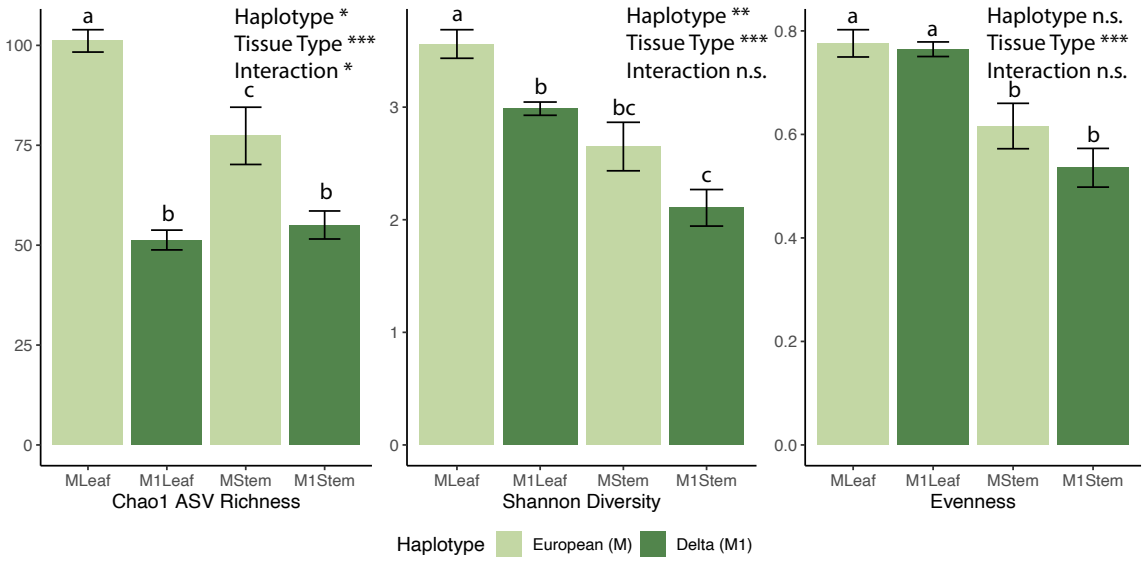

Figure 2. Effect of haplotype and tissue type on fungal microbe ASV Chao1 richness (a), Shannon Diversity (b), and Evenness (c). Statistics shown are from tukey post-hoc pairwise analyses. †P< 0.1, * P < 0.05, ** P < 0.01, ***P < 0.001, n.s. non-significant. Note *y*-axis scales differ across richness/diversity measures.

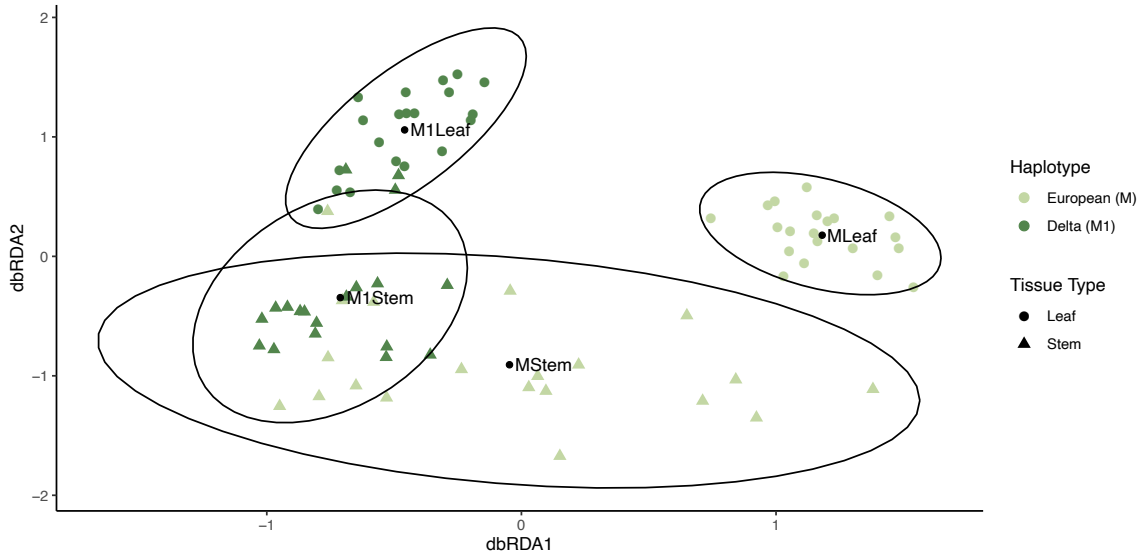

Figure 3. Effect of haplotype and tissue type on fungal community composition. Distance-based redundancy analysis of the effect of haplotype and tissue type on fungal endophyte communities. Samples are colored by haplotype and the different tissue types are shown as filled circles (leaf tissue) and triangles (stem tissue). The centroids for each haplotype x tissue type combination are shown by the black filled circles. Between haplotypes, leaf and stem communities differed significantly ($F = 23.305$, $P < 0.001$, $F = 4.343$, $P < 0.001$ respectively). Within-haplotype communities also differed significantly by tissue type (European: $F = 10.66$, $P < 0.001$, Delta: $F = 6.192$, $P < 0.001$).

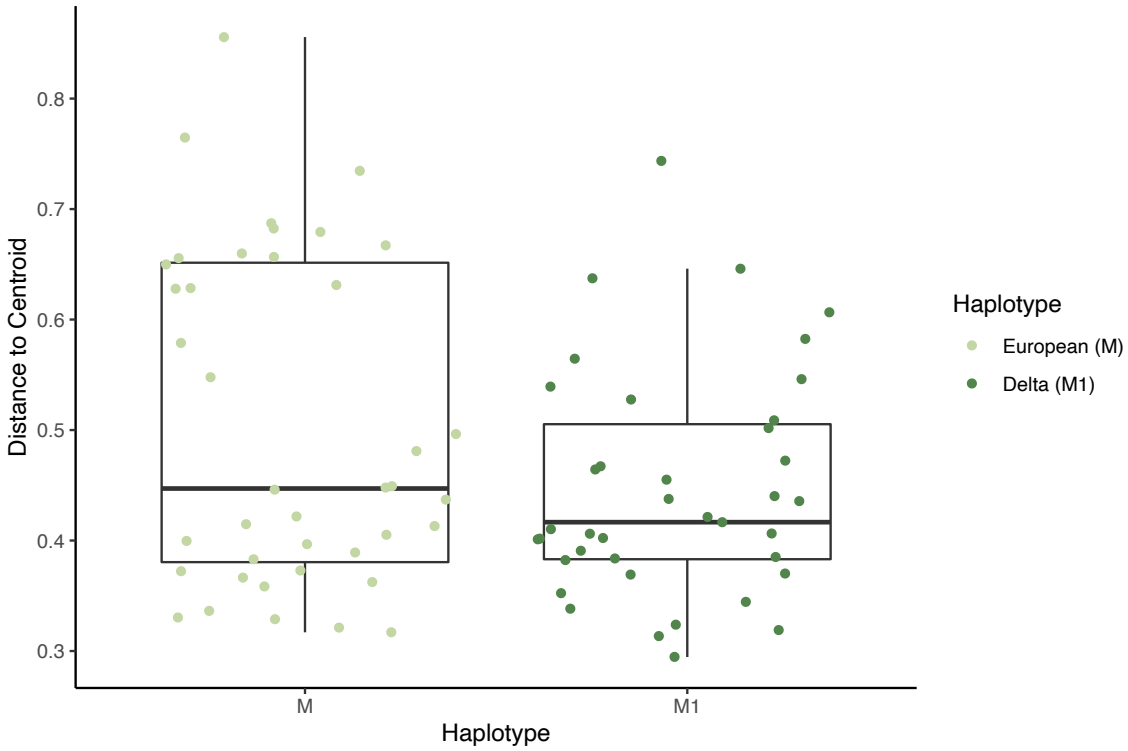

Figure 4 Variability in community composition assessed by analysis of beta dispersion between haplotypes Mean sample distance to centroid differed significantly between haplotypes (F = 3.973, P = 0.046), this difference was slight.

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BIOGRAPHY

Caitlin R. Bumby earned her undergraduate degree in Ecology and Evolutionary Biology at the University of Colorado Boulder where she gained a love for plant and fungal ecology. Driven by her interest in systematics and molecular ecology, she then completed a graduate certificate program in Bioinformatics through Penn State University. She continued her studies at Tulane University where she completed the Thesis-based Master's program in Ecology and Evolutionary Biology. Her education will be continued at the University of New Orleans where she will be pursuing a Ph.D. in Integrative Biology.