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Leaf Mycobiome and Mycotoxin Profile of Warm-Season Grasses Structured by Plant Species, Geography, and Apparent Black-Stroma Fungal Structure

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ABSTRACT Grasses harbor diverse fungi, including some that produce mycotoxins or other secondary metabolites. Recently, Florida cattle farmers reported cattle illness, while the cattle were grazing on warm-season grass pastures, that was not attributable to common causes, such as nutritional imbalances or nitrate toxicity. To understand correlations between grass mycobiome and mycotoxin production, we investigated the mycobiomes associated with five prominent, perennial forage and weed grasses [*Paspalum notatum* Flügge, *Cynodon dactylon* (L.) Pers., *Paspalum nicorae* Parodi, *Sporobolus indicus* (L.) R. Br., and *Andropogon virginicus* (L.)] collected from six Florida pastures actively grazed by livestock. Black fungal stromata of *Myriogenospora* and *Balansia* were observed on *P. notatum* and *S. indicus* leaves and were investigated. High-throughput amplicon sequencing was applied to delineate leaf mycobiomes. Mycotoxins from *P. notatum* leaves were inspected using liquid chromatography-mass spectrometry (LC-MS/MS). Grass species, cultivars, and geographic localities interactively affected fungal community assemblies of asymptomatic leaves. Among the grass species, the greatest fungal richness was detected in the weed *S. indicus*. The black fungal structures of *P. notatum* leaves were dominated by the genus *Myriogenospora*, while those of *S. indicus* were codominated by the genus *Balansia* and a hypermycoparasitic fungus of the genus *Clonostachys*. When comparing mycotoxins detected in *P. notatum* leaves with and without *M. atramentosa*, emodin, an anthraquinone, was the only compound which was significantly different ($P < 0.05$). Understanding the leaf mycobiome and the mycotoxins it may produce in warm-season grasses has important implications for how these associations lead to secondary metabolite production and their subsequent impact on animal health.

IMPORTANCE The leaf mycobiome of forage grasses can have a major impact on their mycotoxin contents of forage and subsequently affect livestock health. Despite the importance of the cattle industry in warm-climate regions, such as Florida, studies have been primarily limited to temperate forage systems. Our study provides a holistic view of leaf fungi considering epibiotic, endophytic, and hypermycoparasitic associations with five perennial, warm-season forage and weed grasses. We highlight that plant identity and geographic location interactively affect leaf fungal community composition. Yeasts appeared to be an overlooked fungal group in healthy forage mycobiomes. Furthermore, we detected high emodin quantities in the leaves of a widely planted forage species (*P. notatum*) whenever epibiotic fungi occurred. Our study demonstrated the importance of identifying fungal communities, ecological roles, and secondary metabolites in perennial, warm-season grasses and their potential for interfering with livestock health.

KEYWORDS mycotoxin, mycobiome, warm-season grasses, forage, fungi, endophytes, hypermycoparasite, *Myriogenospora*, *Balansia*, *Clonostachys*

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Plants harbor a diverse array of microorganisms, including species from the kingdom Fungi (1). The leaf surface and interior provide habitats for epibiotic and endophytic fungi, respectively. While often not causing observable symptoms to the plant hosts, the impacts of these leaf-inhabiting fungi on their host plants are dynamic, ranging from beneficial to neutral to detrimental (2).

Of all leaf-inhabiting fungi, the role that C3 grass-associated endophytes (for example, *Epichloë* spp.) play has been well characterized. These vertically transmitted fungi obtain photosynthates from the plant and produce toxins which deter herbivores (2). While fungally derived metabolites can protect plants against herbivores, they also sometimes cause severe health problems when consumed by livestock (3). In temperate regions where tall fescue (*Festuca arundinacea* Schreb.) grass is a major pasture forage species, fescue toxicosis is a significant problem. This disease is the result of ingestion of ergot alkaloids released by *Epichloë coenophiala* in grazing livestock. The U.S. beef cattle industry alone loses an estimated $2 billion annually from fescue toxicosis-related sequelae, including abortion and weight loss (4).

Perennial, warm-season, C4 grasses contribute significantly to feeding livestock in the southern regions of the United States (5). Considering nutritive value and soil adaptability, a variety of perennial grass forage species are grown across Florida; however, *Paspalum notatum* Flügge (bahiagrass), which is native to South America (approximately −20 to −35 degrees latitude), is one of the most important perennial cultivated forage species. It is easy to establish, can be used for soil stabilization, has good persistence, requires minimal maintenance, and has low susceptibility to diseases and pests (6). Cultivars 'Pensacola' and 'Argentine' are among the most widely planted varieties, but several improved cultivars are increasingly being planted (7).

Depending upon pasture management, less desirable grasses can contaminate pastures and are regarded as weeds. For example, *Paspalum nicorae* Parodi (Brunswickgrass) is considered a weed, since it is much less palatable to cattle and can contaminate *P. notatum* seed production fields (8). Originally from tropical Asia, *Sporobolicus indicus* (smutgrass) is regarded as one of the most troublesome pasture grass weeds (9) since it is adapted to a variety of soil types and often outcompetes other pasture forages (10). *S. indicus* is considered a weed grass due to its low nutritive value and unpalatable nature (11). Although native to the Americas, *Andropogon virginicus* (broomsedge) has infiltrated millions of acres of pastureland across the southeastern United States (12), and it competes for resources with preferred forage grasses (9, 11). *S. indicus* and *A. virginicus* often coinhabit *P. notatum* pastures and, while less palatable, can occasionally be consumed by livestock.

Unlike C3 grasses, perennial, warm-season, C4 grasses have had little delineation of their leaf-associated endophytic and epibiotic fungal communities. Recently, Depetris et al. (13) reported various culturable endophytes from *Paspalum* spp. and demonstrated their positive effects on plant host performance. Many fungi of the Clavicipitaceae family can live as endophytes inside asymptomatic plant tissues (defined here as apparently healthy plant tissues without visible fungal structures) for part or all of their life and may occasionally produce visible reproductive structures on the plant surface (14). While many plant-inhabiting fungi are regarded as endophytes in the Clavicipitaceae, some members of this family adopt an epibiotic existence whereby they live superficially on the exterior of host plants (15). Ergot alkaloids, a group of mycotoxins often produced by clavicipitaceous fungi, have been reported from several C4 and C3 grass species (16). For example, ergot alkaloids were detected *in situ* from three *Balansia epichloë*-infected grass leaves, including *Sporobolus* from the southeastern United States (17). Compared to clavicipitaceous fungi living within C3 grasses, genera of Clavicipitaceae that occur on C4 grass leaves (including *Myriogenospora* and *Balansia*) remain relatively understudied (18, 19). Besides the mostly endophytic genus *Balansia*, the epibiotic genus *Myriogenospora* has been reported multiple times within warm-season grasses, including *P. notatum*; it forms black stromata superficially on the plant leaves (defined as symptomatic here), causing a "tangle-top" symptom (18, 20). Despite its frequent occurrence and phylogenetic similarity to other mycotoxin-producing fungi, the ability of *Myriogenospora* to produce mycotoxins is unknown (21).

Understanding the potential factors that influence mycotoxin production in pastures is critically important for plant and animal health.

Phyllosphere microbiomes, including the epibiotic and endophytic microbial communities, can be impacted by various abiotic and biotic factors, including plant host genetics (e.g., species and genotype), plant traits (e.g., leaf chemical composition), management (e.g., chemical applications), seasonality, and geographic location (22–26). Core microbial members commonly present across multiple plant species and geologic areas may reflect their indispensable interactions with plants (27, 28). In contrast to the core microbes, indicator microbial species found abundantly in particular plants may reflect specific associations and adaptations of certain plant-microbe pairs (25). Overall, by affecting plant traits and plant-microbial interactions, phyllosphere microbiomes can have a direct impact on and potential application for use in agricultural and environmental health (27, 29, 30). Culture-based detection methods have revealed a high abundance of fungi belonging to Ascomycota that are associated with warm-season grasses (13). Recently, culture-independent methods, which can detect more complete assemblies of fungi, have been applied to the biofuel crop *Panicum virgatum* L. (switchgrass), a warm-season, C4 grass. By investigating the mycobiome of *P. virgatum* grown in temperate fields, the grass genotype and seasonality were revealed to significantly affect leaf mycobiome assemblies (31, 32). Additionally, leaf mycobiome had intricate interactions with the bacterial communities living therein (32). However, warm-season grasses growing in warmer regions have not been subjected to culture-independent mycobiome detection. Climate change and anthropogenic activities, including agrotechnology, have raised the concerns of changing plant-fungal interaction and the emergence of new diseases (33, 34). These knowledge gaps call for investigations in the mycobiome and potential mycotoxin production in the perennial, warm-season grasses.

To further our understanding of the effect that plant taxonomy (i.e., species and cultivar) has on microbial community and mycotoxin profiles of warm-season grass-associated fungi across different Florida locations, we investigated the leaf-inhabiting fungal community of two improved perennial forage species, *P. notatum* and *Cynodon dactylon*, and three weed species, *P. nicorae*, *S. indicus,* and *A. virginicus*, which often contaminate improved forage pastures and hay fields across six locations in Florida. Our objectives were to (i) investigate the fungal community assemblies across Florida pastures for cooccurring forage and weed species using high-throughput amplicon sequencing, (ii) cross-compare the foliar fungal communities between grass samples both with and without obvious fungal stromata in *P. notatum* and *S. indicus*, and (iii) detect mycotoxins present in the important forage *P. notatum* via liquid chromatography-tandem mass spectrometry (LC-MS/MS). Our results revealed the core and unique fungal communities across warm-season pastures and the mycotoxin composition of one critical forage species (*P. notatum*) in Florida, providing key insights relevant to plant and cattle health, pasture management, and the emerging concern and potential impact of mycotoxins on livestock production.

RESULTS

In addition to being on the leaf, black fungal structures were also associated with the inflorescence of *P. notatum* (Fig. 1E to H) and were confirmed to be *Myriogenospora atramentosa* by its nuclear ribosomal internal transcribed spacer (nrITS) region (Sanger sequencing, GenBank accession numbers ON678271 to ON678272; specimen code at the University of Florida Herbarium, FLAS-F-63886). DNA amplicon sequencing that targets fungal ITS1 was further applied to identify and compare the mycobiomes living in and on symptomatic and asymptomatic leaf samples. Fungal ITS1 metabarcoding resulted in merged read numbers ranging from 9,805 to 54,194 per grass sample, yielding 989 amplicon sequence variants (ASVs).

Comparison of the fungal composition in asymptomatic with that in symptomatic tissues. The mycobiome of grass samples bearing black fungal stromata, especially symptomatic *P. notatum*, was distinct from that of the asymptomatic ones (Fig. 2A). In the asymptomatic leaves of *P. notatum* and *S. indicus*, taxa in the class Dothideomycetes (*S. indicus*, 61.4% ± 21.1%; *P. notatum*, 60.2% ± 21.1%) had the greatest abundance, followed by Tremellomycetes (*S. indicus*, 18.4% ± 16.5%; *P. notatum*, 19.9% ± 13.4%) and Sordariomycetes (*S. indicus*, 12.4% ± 22.8%; *P. notatum*, 13.4% ± 18.8%) (Fig. 2B),

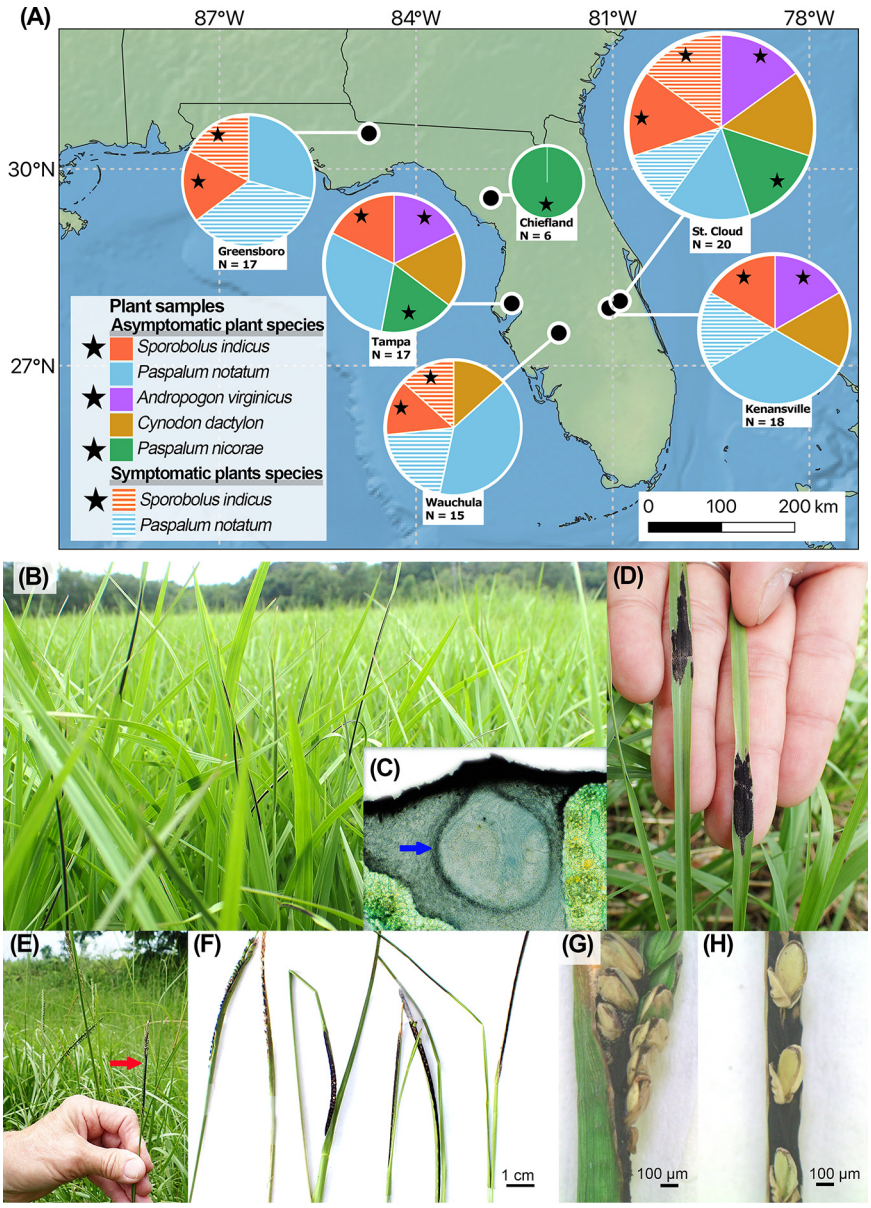

FIG 1 Sampling locations and examples of symptomatic grasses in Florida pastures. (A) Map of collection sites across Florida, USA. Each pie chart corresponds to the sampling of a given site. The color indicates plant species, and the pattern indicates symptom pathology. The size of the pie chart corresponds to the number of samples at each site. Stars indicate plant species considered weeds. (B) *Paspalum notatum* leaves with black fungal structures produced by *Myriogenospora atramentosa*. (C) Cross section of *P. notatum* leaf bearing the superficial fungal structure of *M. atramentosa*. Blue arrow identifies the black *M. atramentosa* perithecium. The surrounding green part is *P. notatum*'s leaf tissue. (D) *Sporobolus indicus* leaf blades with fungal structures produced by *Balansia epichloë*. (E to H) *M. atramentosa* on the seed head of *P. notatum* (red arrow).

regardless of plant species. For both plant species with symptoms, Sordariomycetes were the most prevalent fungal class (*S. indicus*, 62.8% ± 34.9%; *P. notatum*, 82% ± 18.8%), compared to other classes living in and on the same tissue (Fig. 2B). Both *P. notatum* ($P = 0.001$) and *S. indicus* ($P = 0.006$) hosted fungal communities distinct between symptomatic and asymptomatic samples (Fig. 3A), with a greater shift of fungal communities in *P. notatum* than in *S. indicus*. The relative abundance of Basidiomycota was significantly greater in asymptomatic (*P. notatum* = 15.1% ± 15.1%, *S. indicus* = 22.0% ± 17.7%) than in symptomatic (*P. notatum* = 8.6% ± 9.5%, *S. indicus* = 6.7% ± 9.3%) leaves for both *P. notatum* ($P < 0.001$) and *S. indicus* ($P =$

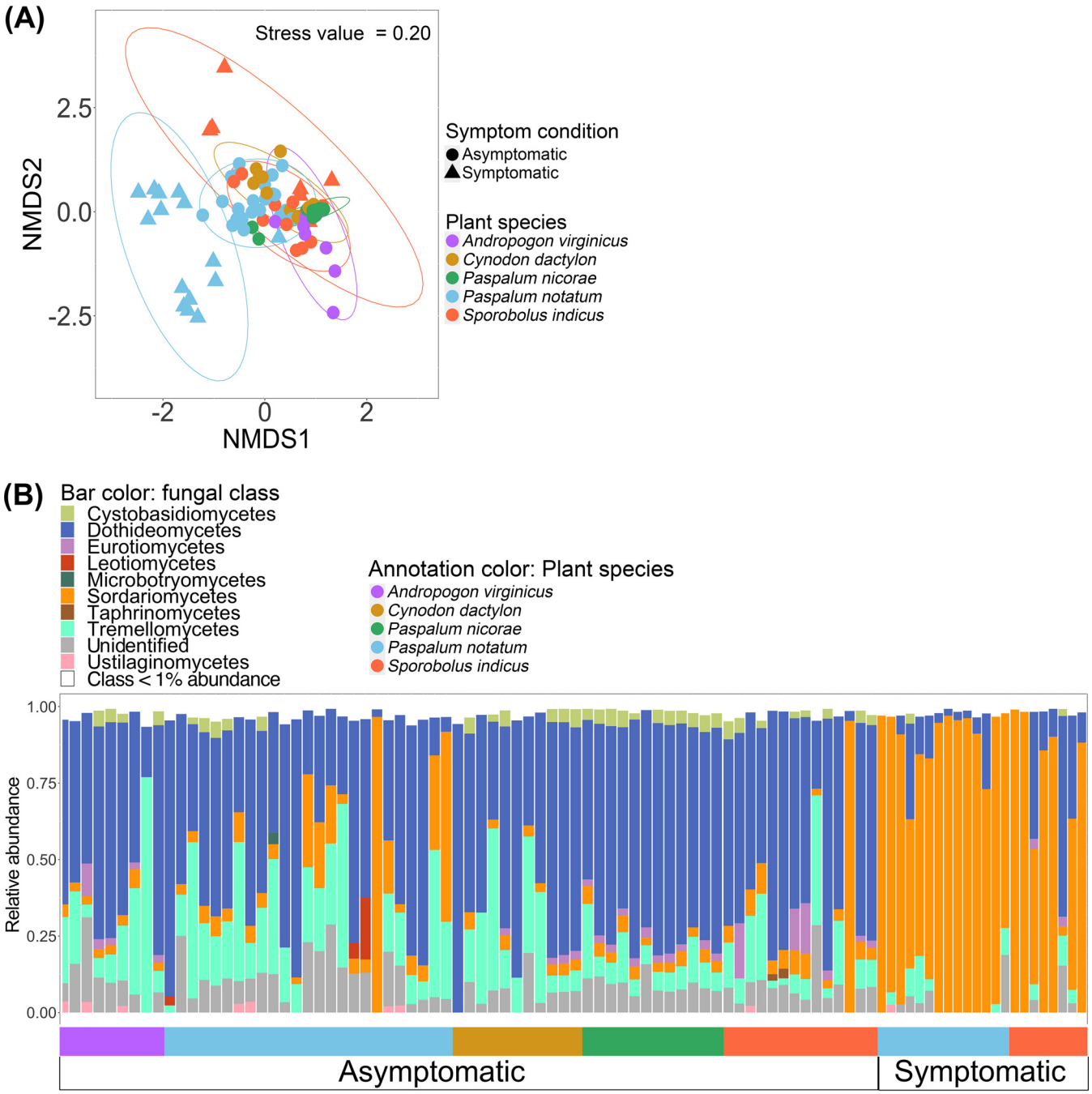

FIG 2 (A) Nonmetric multidimensional scaling (NMDS) plot of fungal beta-diversity (Bray-Curtis dissimilarity) of all grass samples. (B) Stack barplots of fungal community at class level for asymptomatic and symptomatic samples of the five forage grass species evaluated.

0.046) (Fig. 3B). Despite being relatively more abundant in symptomatic samples, the phylum Ascomycota dominated the grass-inhabiting fungal communities (Fig. 3B) regardless of the symptom condition (asymptomatic, *P. notatum* = 93.2% ± 9.3%, *S. indicus* = 91.3% ± 9.5%; symptomatic, *P. notatum* = 76.2% ± 15.1%, *S. indicus* = 77.9% ± 17.7%). Fungal richness (observed number of species and Chao1, *S. indicus*, $P = 0.002$; *P. notatum*, $P < 0.001$) and Shannon index (*S. indicus* and *P. notatum* both with *P* values of <0.001) were greater in asymptomatic leaves than in symptomatic leaves (Fig. 3C). Two ASVs, both assigned to *Myriogenospora*, were significantly more abundant (false-discovery rate [FDR] < 0.05) in symptomatic *P. notatum* (Fig. 3D). A *Clonostachys* ASV classified as *Clonostachys miodochialis* and a *Balansia* ASV were also

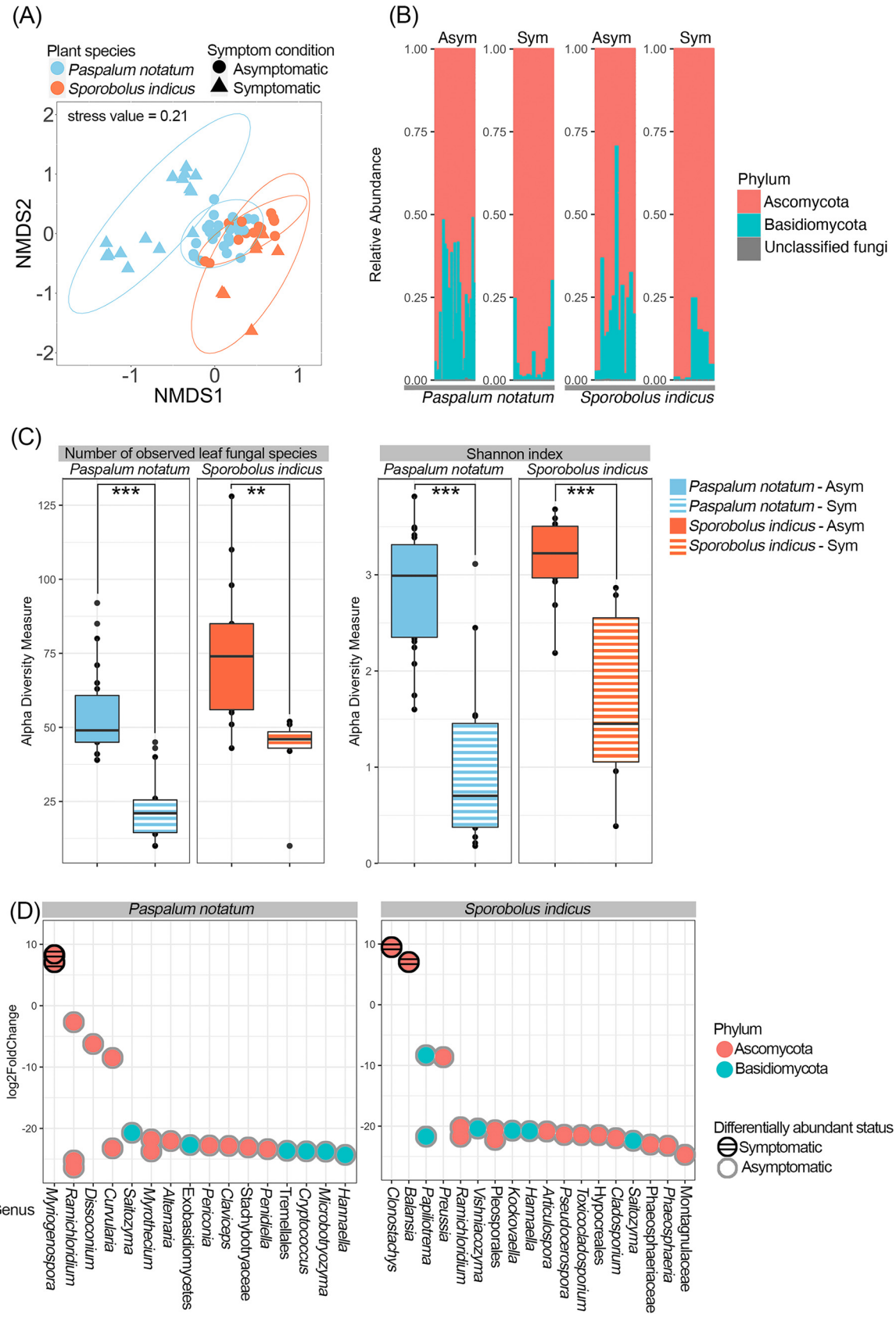

FIG 3 (A) Nonmetric multidimensional scaling (NMDS) plot of fungal beta-diversity (Bray-Curtis dissimilarity) of the symptomatic and asymptomatic *Paspalum notatum* and *Sporobolus indicus*. (B) Stack barplots of fungal phyla in asymptomatic and symptomatic *P. notatum* and

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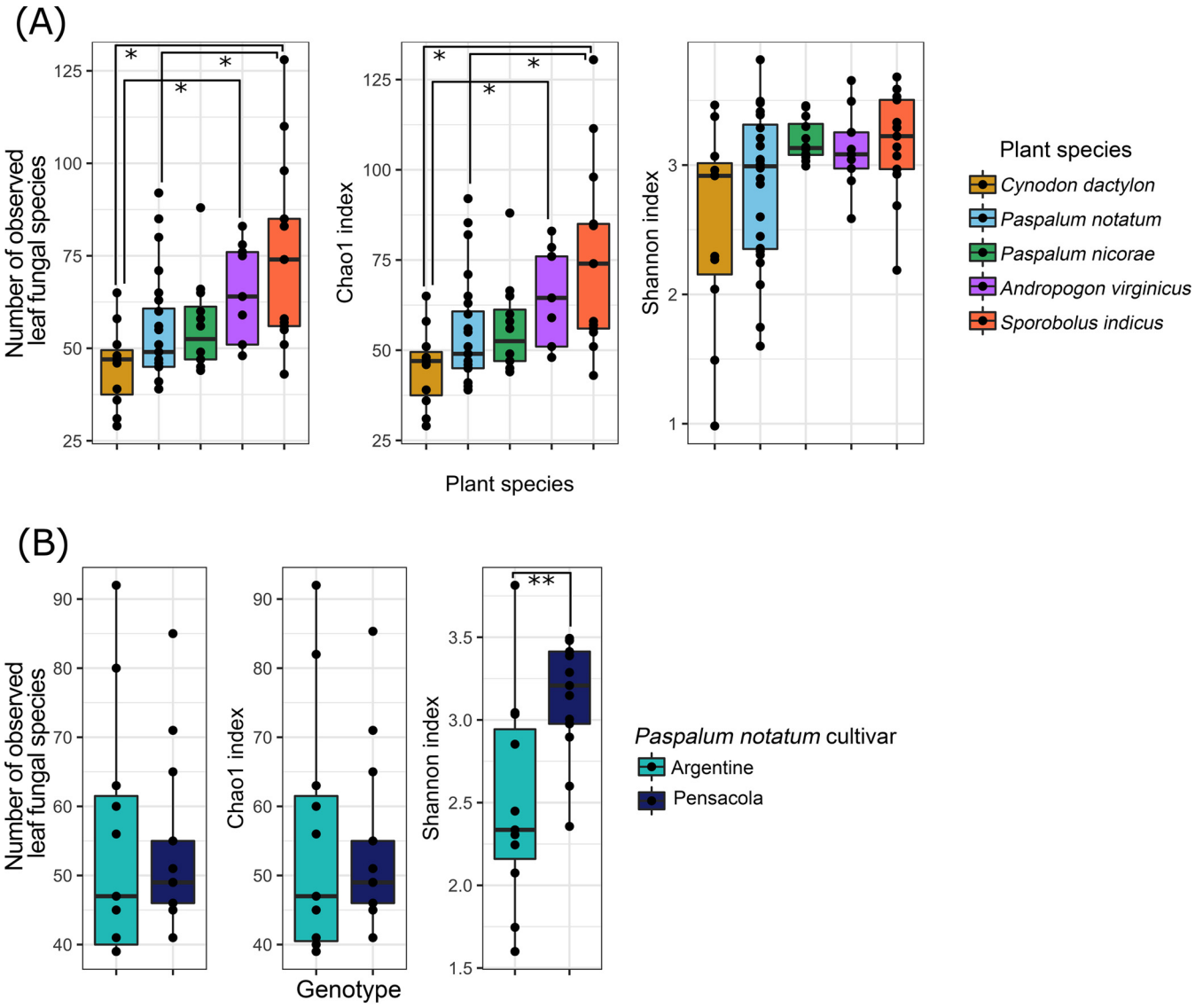

FIG 4 (A) Fungal alpha-diversity of asymptomatic grasses across plant species. (B) Fungal alpha-diversity of asymptomatic grasses of the two *Paspalum notatum* cultivars 'Argentine' and 'Pensacola'. *, $P < 0.05$; **, $P < 0.01$.

significantly more abundant in symptomatic *S. indicus* samples (Fig. 3D). A total of 19 ASVs spanning Ascomycota and Basidiomycota were more abundant in the asymptomatic samples of *P. notatum* and *S. indicus* (Fig. 3D).

Fungal diversity and composition in and on asymptomatic leaves across grass species, cultivar, and locality. We then focused on asymptomatic grass samples to assess factors impacting mycobiomes. We estimated the number of observed species, Chao1 index, and Shannon index of asymptomatic tissues of each plant species. Fungal diversity was not the same across a given plant species (number of observed species, $P = 0.002$; Chao1, $P = 0.002$, Shannon index, $P = 0.04$). Of all pairwise plant species comparisons, significant differences in fungal richness were observed in three pair-species comparisons (Fig. 4A). No significant differences in Shannon diversities of leaf fungi were found across all of the pair-plant species comparisons (Fig. 4A). Among the five examined grass species, *S. indicus* had the greatest

FIG 3 Legend (Continued)
S. indicus. Each bar corresponds to one sample. (C) Diversity measurements of asymptomatic versus symptomatic plants of *P. notatum* and *S. indicus*. (D) Significantly differentially abundant ASVs (Wald test, false-discovery rate [FDR] of <0.05) between symptomatic and asymptomatic *P. notatum* and *S. indicus*. Every circle corresponds to one ASV. ASV, amplicon sequence variant **, $P < 0.01$; ***, $P < 0.001$.

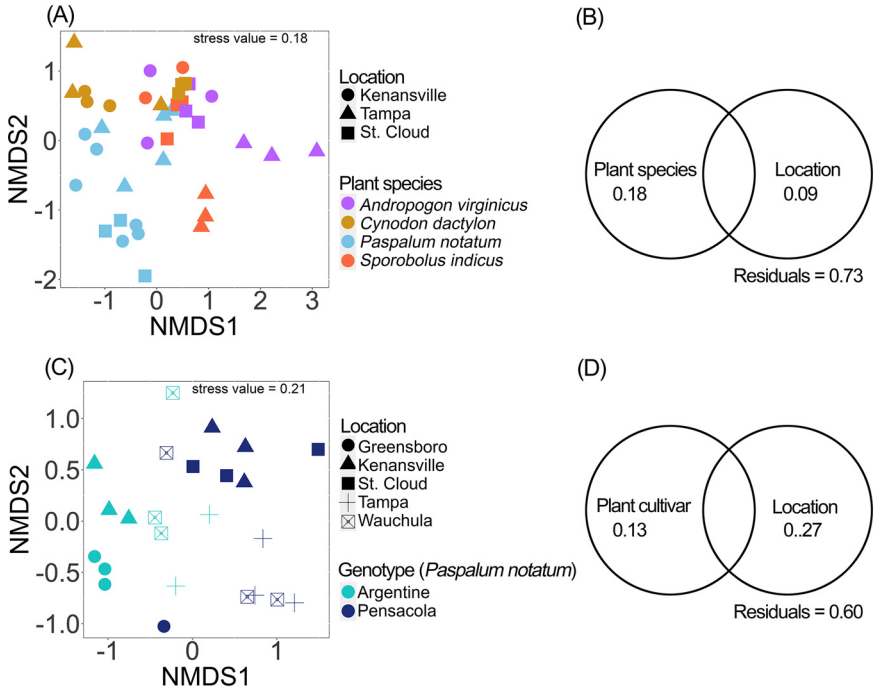

FIG 5 (A) Nonmetric multidimensional scaling (NMDS) plot of fungal beta-diversity (Bray-Curtis dissimilarity) of four asymptomatic grasses at three locations. (B) Variation partitioning of plant species and location effects on mycobiome composition. (C) Nonmetric multidimensional scaling (NMDS) plot of fungal beta-diversity (Bray-Curtis dissimilarity) of two *Paspalum notatum* cultivars. (D) Variation partitioning of *P. notatum* cultivar and location effects on mycobiome composition.

fungal diversity richness (Fig. 4A). This trend was observed across all sampling sites except for Kenansville, where the observed fungal species in *Andropogon virginicus* exceeded those in *S. indicus*.

The leaf mycobiomes of both *P. notatum* and *S. indicus* revealed similar richnesses and Shannon diversities across sampling sites ($P > 0.05$). The mycobiome of *P. notatum* was assessed for plant cultivar effect. While similar observed numbers of species and Chao1 measurements were detected for the two cultivars, the Shannon index suggested a significantly higher diversity of leaf fungi present in 'Pensacola' than in 'Argentine' (Fig. 4B).

To assess the plant identity and geography effects on mycobiome composition, we focused on three locations with four grass species sampled to avoid sampling bias. Plant species and location had an interactive effect on the beta-diversity of fungal communities living in and on grass leaves (permutational multivariate analysis of variance [PERMANOVA], plant species, location, and plant species-location had a *P* value of <0.01) (Fig. 5A). Therefore, these two factors were assessed separately. PERMANOVA suggested a significant difference for fungal beta-diversity among plant species (*P*, St. Cloud = 0.03, Kenansville = 0.01, and Tampa = 0.01) (Fig. 5A). Additionally, PERMANOVA suggested significant differences of fungal beta-diversity across locations for *P. notatum* and *S. indicus* ($P < 0.001$ for both plant species). Variance partitioning analysis revealed a slightly greater plant species effect (0.18) than location effect (0.09) (Fig. 5A and B). In comparison, the two *P. notatum* cultivars had significant cultivar, location, and cultivar-location effects (PERMANOVA, cultivar, location, and cultivar-location all with *P* values of <0.01). Variance partition analysis detected a greater impact of location effect (0.27) than cultivar effect (0.13) on mycobiome assembly (Fig. 5C and D).

Fungal indicators and core mycobiome of different plant species using asymptomatic grass samples. Asymptomatic leaf mycobiomes were investigated to find fungal members unique to certain grass species as "indicators" or present across different grasses as "cores" in the Florida pastures. Indicator ASVs are defined as ASVs found significantly more often with one plant species than with others. Among the four plant species, *S. indicus* had the highest number of indicator ASVs (60) followed by *A. virginicus* (28). *C. dactylon* and *P. notatum* only had 14 and 11 indicator ASVs, respectively. These ASVs were distributed across 48

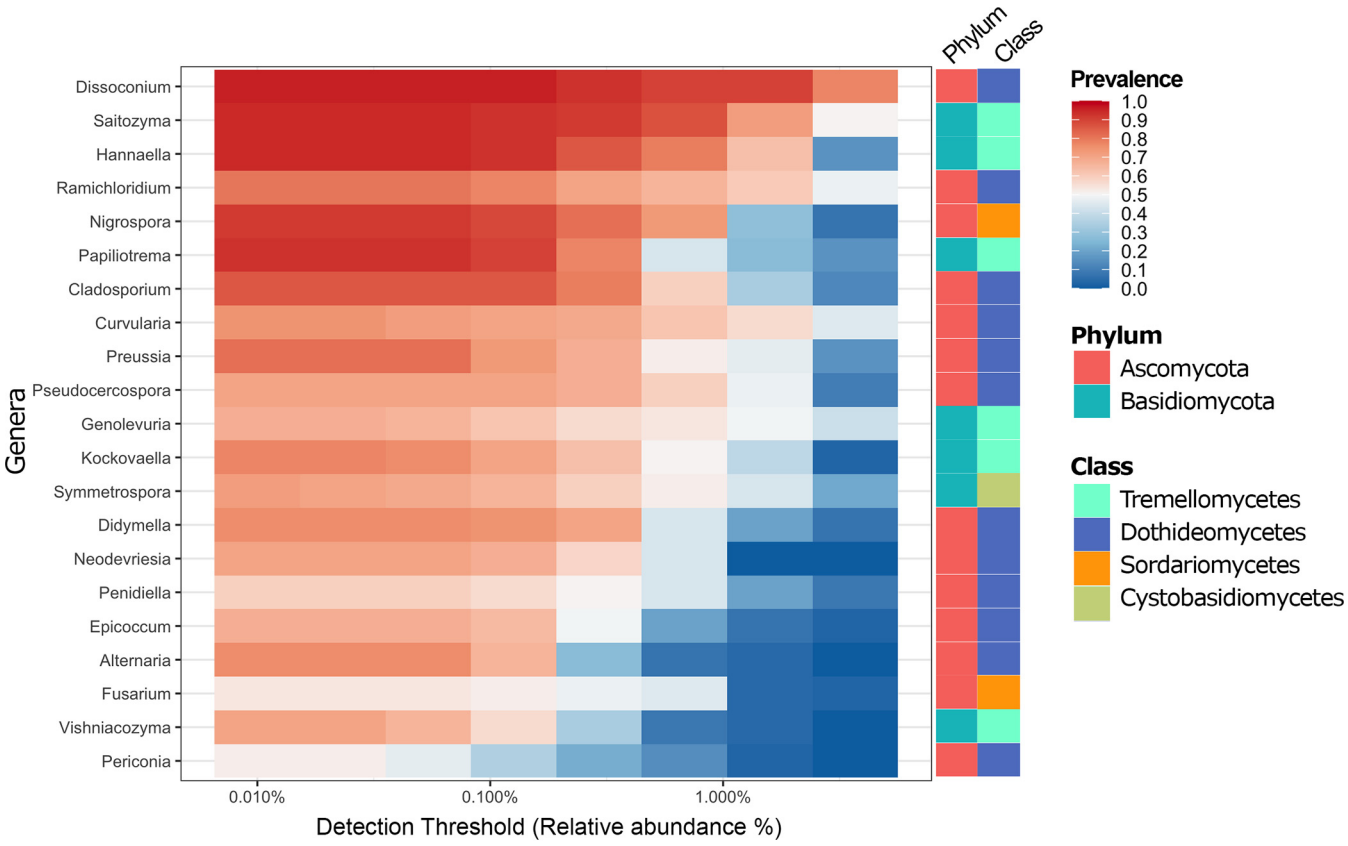

FIG 6 Core fungal genera (i.e., relative abundance of >0.01% in more than half of the samples) across all asymptomatic plant species.

genera (see Fig. S1 in the supplemental material). Only 33 were identified to include a single ASV as an indicator. Seven ASVs belonging to the genus *Ramichloridium* were identified as indicator ASVs in *S. indicus*, *P. notatum*, and *C. dactylon*.

To identify the core mycobiome shared across plant species, a genus with at least 0.01% relative abundance in over half of the asymptomatic samples was defined as a core genus of the mycobiome. Twenty-one genera were detected as being part of a core mycobiome within the asymptomatic leaf samples, with 12 being in the class Dothideomycetes and six in the Tremellomycetes, followed by two in the Sordariomycetes and one in the Cystobasidiomycetes (Fig. 6). All seven Basidiomycota fungal genera detected as core members across Florida pastures have yeast forms, highlighting the potential importance and ubiquity of yeast fungi in pasture grasses (Fig. 6).

Distribution of Clavicipitaceae fungi across plant species and locations. Because Clavicipitaceae are of interest in the grass mycobiome and could be major mycotoxin producers, we examined their relative abundance across symptomatic and asymptomatic leaves. A total of three genera (*Myriogenospora*, *Balansia*, and *Claviceps*) of the family Clavicipitaceae were detected from all grass samples (Fig. 7A), while symptomatic *P. notatum* and *S. indicus* had a greater abundance of *Myriogenospora* (78.7% ± 22%) and *Balansia* (32.8% ± 34.4%), respectively. These two genera were also present in asymptomatic samples (Fig. 7A). We further investigated the relative abundance of the three identified genera in asymptomatic grass samples for the two cultivars of *P. notatum*, 'Pensacola' and 'Argentine'. While similar relative abundances of *Balansia* and *Claviceps* were detected in the two cultivars, 'Pensacola' harbored a higher relative abundance of *Myriogenospora* than did 'Argentine' ($P < 0.05$, Fig. 7B).

Mycotoxin diversity and quantity in *Paspalum notatum*. Forty-five metabolites (see Table S1 in the supplemental material) were examined for the widely planted forage *P. notatum*. The goal was to evaluate if the epibiotic *M. atramentosa* is responsible for mycotoxin production. Six mycotoxins (alternariol methyl ether, beauvericin,

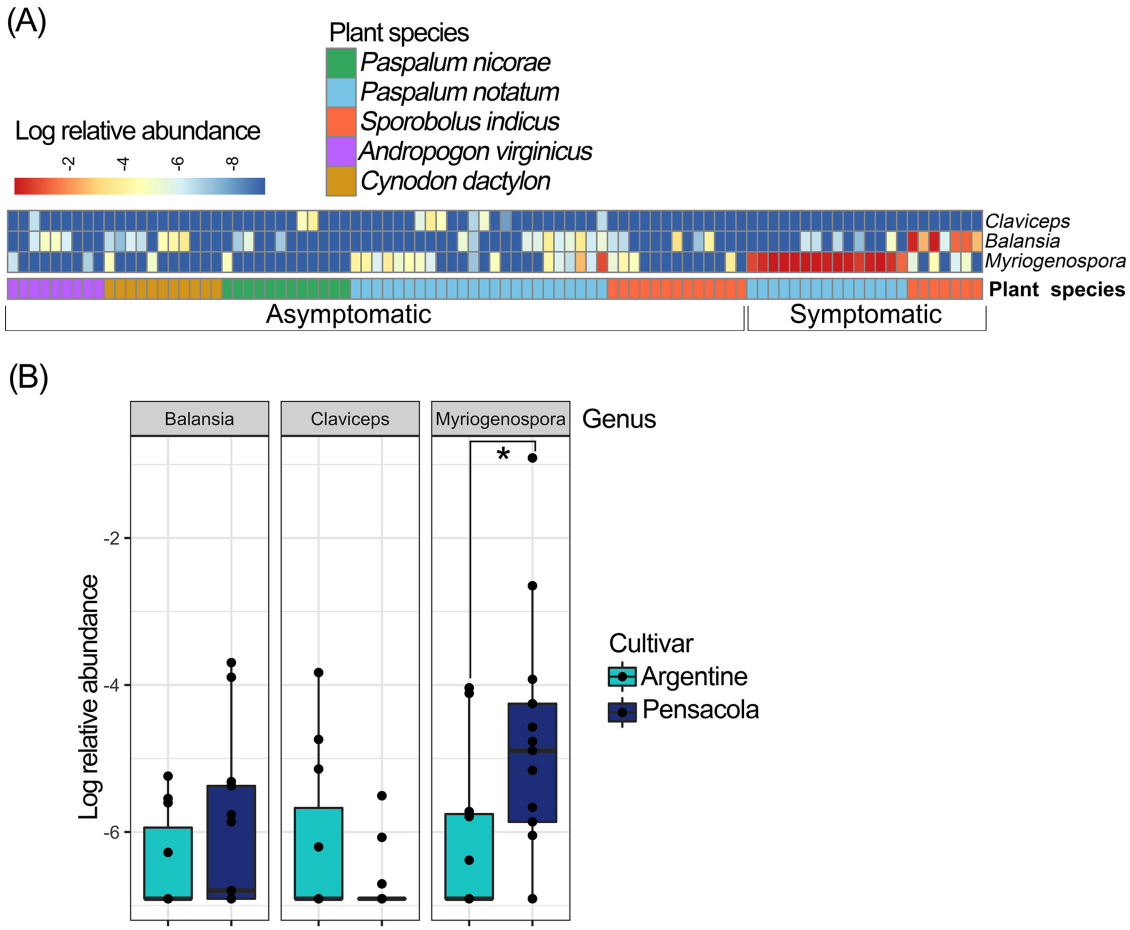

FIG 7 (A) Abundance of fungal genera of Clavicipitaceae across Florida pastures. (B) Clavicipitaceae of cultivars 'Argentine' and 'Pensacola' of the asymptomatic *Paspalum notatum*. *, $P < 0.05$.

dihydrolysergol, beta-zearalenone, emodin, fumonisin B1) were detected in at least one leaf sample of *P. notatum* (Fig. 8). Comparing these metabolites among mixed, symptomatic, and asymptomatic samples of *P. notatum*, emodin was significantly greater in symptomatic samples than in mixed or asymptomatic samples ($P = 0.01$). This suggests that the black fungal stromata associated with the symptomatic samples were associated with emodin production.

DISCUSSION

By using a culture-independent, high-throughput amplicon sequencing approach, we showed that the community of leaf fungi was structured by a combination of factors including plant species, cultivar, and sampling location, as well as the presence of fungal structures on leaves (symptomatic versus asymptomatic leaves). An unexpectedly high relative abundance of basidiomycetous yeasts was detected from the asymptomatic grasses, highlighting their overlooked ecological importance in warm-season grasses. The metabolite emodin was significantly more abundant in *P. notatum* with the epiphytic fungus *Myriogenospora atramentosa*. Our study underlines the complexity of the fungal community associated with Florida forages and begins to elucidate its ecological function, potential concern, and opportunity.

Fungal community assembly and diversity of warm-season grasses. Fungal communities of plants are often affected by complex factors. Similar to a previous study showing a more significant effect of grass host species than of geological factors like elevation (35) on the composition of the fungal community, our results suggest that leaf mycobiomes are likely manipulated by grass species and are less affected by

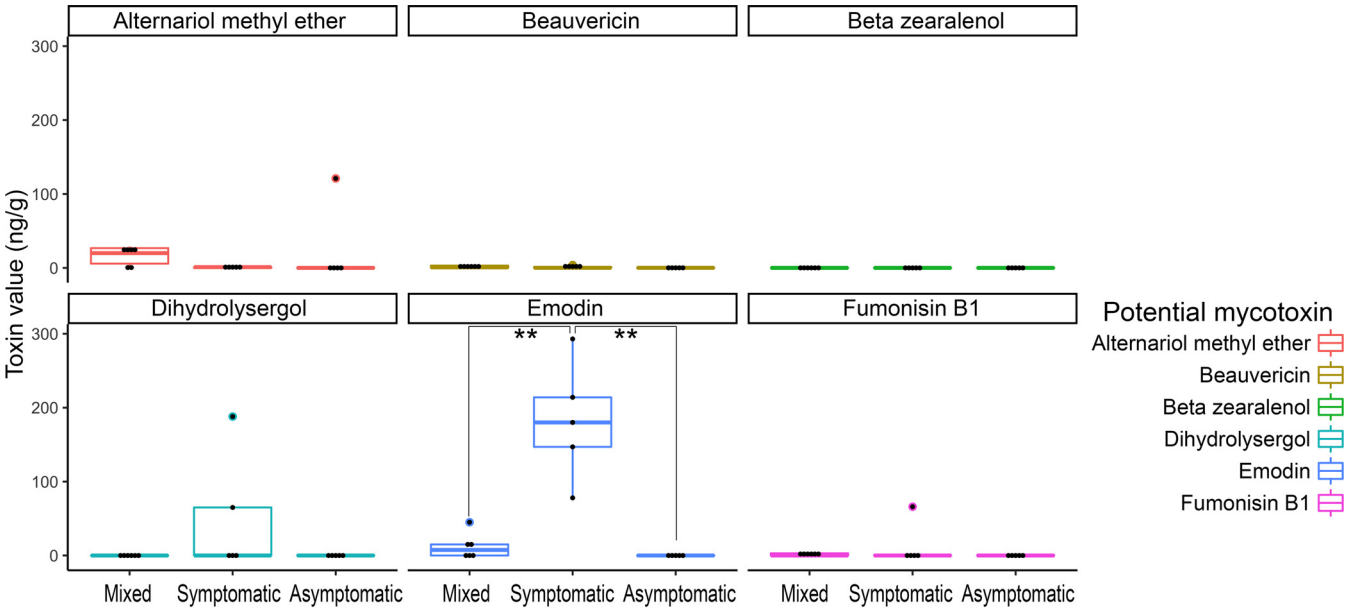

FIG 8 Six mycotoxins with nonzero values in at least one sample of *Paspalum notatum*. **, pairwise Wilcox test, false-discovery rate (FDR) of <0.01.

specific grass cultivars and regional-scale location. Studies of temperate grasses also revealed complex factors involved in shaping the foliar mycobiome. In prairie systems, seasonality, geographic separation, and host species played major roles in shaping the foliar grass mycobiome (36). In grassland farms, the mycobiome of different grass species responded distinctively to land management and within-field microclimate (37, 38). Of the five grass species investigated in this study, the two weedy species *S. indicus* and *A. virginicus* harbored significantly higher richness of leaf fungi than did the two forage grasses. Fungal richness may be determined by the outcome of plant-fungus interactions, especially for plant defense responses. Foliar endophytes have been shown to promote plant resistance to biological and environmental stress (39, 40) and to promote increased growth rates (41), leading to higher competitiveness with local plants. Future research can manipulate the fungal richness of these grasses and test for their competitiveness in warm-season pastures. In comparing the leaf mycobiomes between two *P. notatum* cultivars, despite no richness differences detected, 'Pensacola' *P. notatum* had a significantly higher Shannon diversity of leaf fungi, which measured both richness and evenness (Fig. 4B). Ploidy differences of 'Argentine' (tetraploid) and 'Pensacola' (diploid) (7) could lead to plant trait differentiation and may result in recruiting and retaining different groups and a diversity of mycobiomes through plant trait-fungal trait interactions (42).

Compositional differences of the fungal community in symptomatic versus asymptomatic samples were more significant in *P. notatum* than in *S. indicus* (Fig. 3A), suggesting that *M. atramentosa* and *B. epichloë* led divergent mycobiome responses. Interestingly, along with the black-stroma producer genus *Balansia*, an ASV of the genus *Clonostachys* also had higher abundance in symptomatic leaves. *Clonostachys* was previously reported as a hypermycoparasite on *Epichloë typhina* (43), a species that belongs to the same family as *Balansia*. *Clonostachys* has biocontrol features to deter phytopathogenic fungi and a high resistance to mycotoxins produced by *Fusarium* (44). The host-parasite association between *Clonostachys* and Clavicipitaceae has yet to be elucidated. Hypermycoparasites are often neglected in studies focusing on plant-associated fungi. Our finding highlights that hypermycoparasites should be considered for fungal metabolite accumulation and hold potential for bioprospecting fungal discovery.

The dominant fungal taxa living in/on warm-season grasses. Our results revealed high fungal diversity for those samples from apparently healthy (asymptomatic) grasses. Studies of grass-associated endophytic fungi, especially those detected by culture-based methods, often detected Ascomycota only (13, 45). Culture-independent methods recovered more

tremellomycetous (Basidiomycota) fungi (26, 46), likely due to their low rates of growth or inability to grow on artificial media. While fungi of the phylum Ascomycota dominated the community assembly here, a surprisingly high Basidiomycota biodiversity was detected (Fig. 2). Fungal members of Ascomycota and Basidiomycota probably interact in the phyllosphere. For example, Tremellomycetes (Basidiomycota) and Dothideomycetes (Ascomycota) have been reported to increase together in flower tissues during the ripening stage of wheat (46) and were detected as "hubs" of grass-mycobiome networks (31), suggesting possible cooperation of these two classes in the natural environment. Diverse reproduction strategies have been observed across different taxa of Tremellomycetes: some taxa produce complex fruiting bodies, some exist in the yeast form, and some are dimorphic (47). The core genera detected here (e.g., *Saitozyma* and *Hannaella*, Table S2, Fig. 6) prefer the yeast form. Although basidiomycetous yeasts are occasionally isolated from plants and soil (48), the roles this fungal group plays in fungal-plant interactions, soil biology, transmission, and ecology are largely unexplored. Some yeasts, including *Cryptococcus* of Tremellomycetes, have been proposed as agents for biocontrol against phytopathogens (49–51). Among dothideomycetous lineages, *Dissoconium* and *Ramichloridium* were the core genera detected in the asymptomatic leaves for all of the grass species sampled in this study (Fig. 6). Genomic analysis of one *Ramichloridium* species revealed its potential mechanism to be well adapted to extreme environments, including those with long periods of desiccation, low nutrients, and high solar intensity on the leaf surface (52). In addition to being the core genus, *Ramichloridium* had seven ASVs detected as indicators of three plant species (see Fig. S1 in the supplemental material), suggesting members of *Ramichloridium* had specific interactions with individual plant species. *Dissoconium* and *Ramichloridium* were both isolated from C4 grasses previously and demonstrated antifungal abilities (53, 54). Members of the tremellomycetous yeasts and Dothideomycetes were also identified as core in *Panicum virgatum* grown in a temperate climate (31). Together with our finding, tremellomycetous yeasts and Dothideomycetes are the core mycobiome of warm-season grasses regardless of the climate factor, highlighting their potential benefit to plants as fungal generalists. Future studies should be carried out to understand their ecology (e.g., trophic mode), physical association (e.g., epibiotic or endophytic), potential impact on plant host development, and fitness, as well as antagonistic effects on other organisms (54).

Culture-dependent and culture-independent methods can detect complementary microbial members of grass endophytes and epibionts (55, 56). For example, in *Paspalum*, the eurotiomycetous fungi such as *Penicillium* and *Aspergillus* were detected via culture-dependent methods (13), yet neither of these two genera was relatively abundant in the *P. notatum* samples investigated in this study using culture-independent methods. However, the culture-independent methods are generally thought to capture more comprehensive mycobiomes, especially those fungi that engage in interactions with plants or other microbial consortia (57).

Mycotoxins in *Paspalum notatum* and *Myriogenospora*. Plant-associated fungi can produce a chemically diverse variety of mycotoxins. These compounds can benefit the plant by making it less palatable, or even toxic, as a defense mechanism against grazing herbivores (58), thus allowing infected plants to thrive. Some of the best known mycotoxicoses in livestock are fescue foot and ryegrass staggers caused by clavicipitaceous fungi in cool-season grasses (59). Alkaloids, especially indole-diterpenes and ergot alkaloids, are the main types of toxins that cause the clinical signs seen in these syndromes (14). One ergot alkaloid precursor, dihydrolysergol, was detected in two out of the five symptomatic *P. notatum* samples (Fig. 8). Because the quantity of dihydrolysergol was not significantly different among symptom statuses, the black stroma (*M. atramentosa*) was unlikely to be the main source of dihydrolysergol production. If accidentally consumed by livestock, these toxins could raise health concerns, including average daily gain effects, reduced reproductive capacity, and hyperthermia (Table S3) (59–61). Such symptoms have recently been observed and were of concern in cattle operations in the southern part of the United States; however, the causal agents require future investigation (62). Albeit at a much lower level than on the symptomatic leaves, fungi of Clavicipitaceae were present across Florida pastures, even in asymptomatic plants (Fig. 7A).

This is unexpected for *Claviceps* and *Myriogenospora* as the former is an ovarian parasite and the latter is a leaf epibiont. According to an inoculation experiment, *Claviceps* can infect leaves, suggesting that a *Claviceps*-leaf association is possible (63). To our knowledge, *Myriogenospora* has not been confirmed microscopically inside the plant tissue as an endophyte. *Myriogenospora* reads of asymptomatic samples might be derived from early fungal infection without visible signs of fungi.

Although fungi of Clavicipitaceae such as *Balansia epichloë* that were detected in the samples of this study are well known for producing alkaloids, other fungi in the Dothideomycetes as well as Sordariomycetes, such as the core genera *Alternaria* and *Fusarium*, are also capable of producing other types of mycotoxins (Fig. 6) (17, 64). Toxicity symptoms could be triggered by changing environmental conditions such as fluctuation of temperature (65, 66). Previous research reported high zearalenone in *Paspalum notatum* (67), but similar concentrations were not recovered here (Fig. 8). Instead, we detected alternariol methyl ether in *P. notatum* (Fig. 8). Alternariol methyl ether was mostly produced by fungi of *Alternaria* (68), one of the core fungal genera examined here across Florida pastures. Finally, given the high consistency of emodin detected in grass samples with *Myriogenospora atramentosa* fruiting bodies (Fig. 8) and the fact that emodin can be produced by both fungi and plants (69), it is likely that *M. atramentosa* is responsible for producing or triggering the plant to produce emodin *in vivo* within *P. notatum*. Emodin is an anthraquinone also found in rhubarb which has been shown to have largely beneficial properties, including antimicrobial abilities, herbivore resistance, and properties of medicinal value (e.g., candidate as an antitumor drug) (69–71), highlighting the bioprospecting potential of this forage-associated mycotoxin. There is evidence that emodin can be hepatotoxic and nephrotoxic and lead to reproductive toxicity with high doses and chronic use (70), however, so caution must be exercised as a more complete picture surrounding the potential therapy/toxicity tradeoffs of this compound emerges. While previous research indicated seed heads from the cultivar 'Argentine' to be more susceptible than 'Pensacola' to ergot caused by *Claviceps paspali* (72, 73), we detected similar relative abundances of *Claviceps* in the leaves of both cultivars (Fig. 7B).

The fungal family Clavicipitaceae contains many plant-associated fungi capable of producing ergot alkaloids, especially in the Clavicipitaceae clade A (74) which includes *Verticillium*, *Balansia*, *Epichloë*, *Claviceps*, and *Myriogenospora*. Because clavicipitaceous fungi's main hosts are Poaceae, morning glories and sedges which are of essential use for humans in developing agricultural systems, these fungi and their mycotoxins have been widely studied (75). However, *Myriogenospora* represents an exception. Despite the wide host range of *Myriogenospora* on forage grasses and on crops such as sugar cane and lemongrass (19–21, 76), its capability of producing secondary metabolites including mycotoxins is unknown. In this study, ergot alkaloids were not detected in *Myriogenospora*-infected grass, suggesting a loss of function in the evolutionary history of the Clavicipitaceae clade A. *Atkinsonella hypoxylon* (75), an epibiotic fungus closely related to *Myriogenospora*, was predicted to produce ergot alkaloid according to its genome content. However, ergot alkaloid was undetectable in plant tissues infected by *A. hypoxylon*. The mycotoxin profile potentially produced in affected host plants needs to be more thoroughly studied by sampling *M. atramentosa in vivo* with different plant species or *in vitro* by culturing it in fungal medium as well as sampling across seasons. Other types of alkaloids, including indole-diterpenes, which cause livestock illness, should be investigated to eliminate food safety and animal health concerns of *Myriogenospora*-infected plants. Two different genotypes of *M. atramentosa*, confirmed with MiSeq and Sanger sequencing, were detected in our sampling (Fig. S2), agreeing with the work of Glenn et al. (19) that cryptic biodiversity is present within this fungal species. While many plant-associated clavicipitaceous fungi can transmit vertically with seeds, such as *Claviceps* and *Epichloë*, *M. atramentosa* is not known to be specifically vertically or horizontally transmitted (19). The seed heads were, however, occasionally found covered with *M. atramentosa* tissue (Fig. 1E to H). Whether the *M. atramentosa*-infected seeds remain viable is unknown and will require further investigation. Despite the many concerning aspects of clavicipitaceous fungi, they often provide a natural defense for plants against herbivore predation, holding promising applications in the realm of sustaining agriculture where insect resistance is a sought-after quality in crop systems (77).

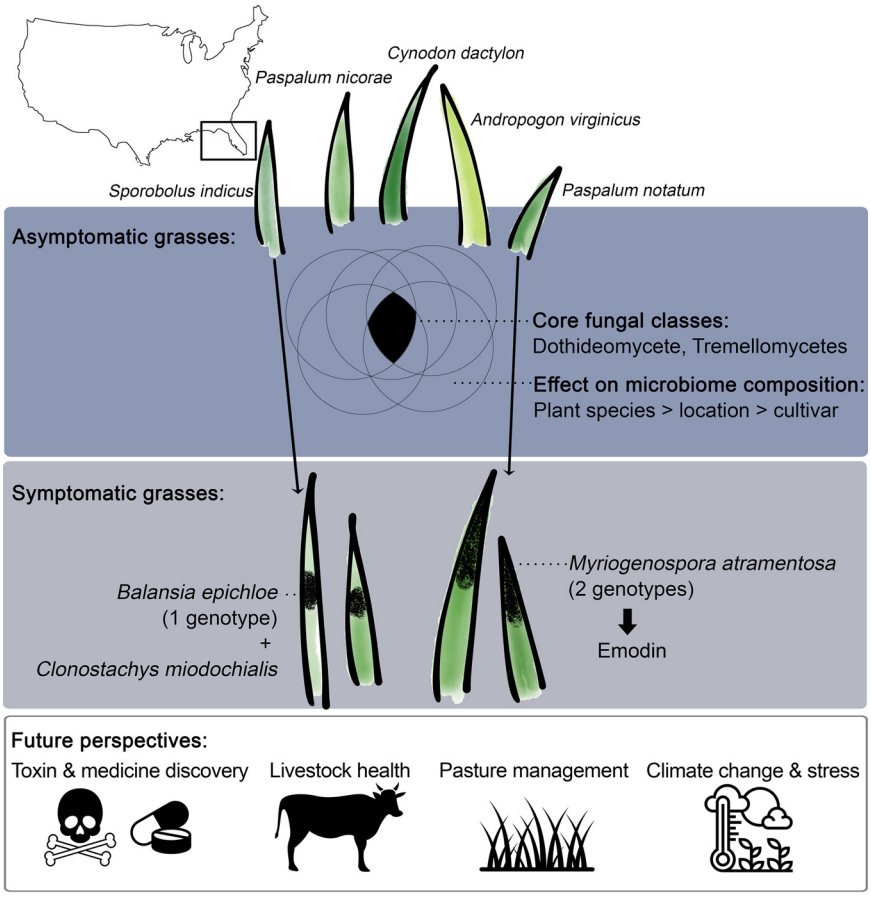

FIG 9 Schematic summary of warm-season grass leaf mycobiomes, mycotoxins, and future perspectives. Five perennial warm-season grasses were sampled. In the asymptomatic leaves, tremellomycetous yeasts and Dothideomycetes were revealed to be the core fungal classes across Florida pastures. The stroma-bearing (symptomatic) leaves of the forage grass *Paspalum notatum* were enriched by two *Myriogenospora atramentosa* genotypes. In comparison, the stroma-bearing leaves of the weedy grass *Sporobolus indicus* were enriched by *Balansia epichloë* and a mycoparasitic fungus, *Clonostachys* sp. *M. atramentosa* likely produced or triggered the production of emodin, an anthraquinone compound, in *P. notatum*. This study highlights the diverse leaf mycobiomes and potential production of secondary metabolites of warm-season grasses, holding potential concerns and opportunities. We call for interdisciplinary research that monitors animal health considering future climate change and pasture management strategies to better understand warm-season grass mycobiomes.

Conclusions. A holistic view of the foliar mycobiome of Florida warm-season grasses is illustrated in this study (Table S2, Fig. 9). We revealed that the weed *Sporobolus indicus* harbors the highest richness of fungi, posing potential concern about livestock grazing in pastures with cooccurring species of grass forage. Fungal assemblies are affected by both the host plant identity (i.e., species and cultivar) and the geographic location. Despite mycotoxins being a concern for livestock health, they also present a potential biocontrol tool for plants to combat insect predation and impacts from climate change as well as discovering drugs used to treat human diseases. We demonstrated that high-throughput amplicon sequencing is a powerful approach to delineate the mycobiome of healthy grasses. Additionally, unknown taxa (e.g., two *Myriogenospora atramentosa* genotypes) were corroborated, and overlooked fungal diversity (e.g., *Clonostachys miodochialis* accompanying *Balansia epichloë* stromata) was uncovered (Fig. 9). Significant knowledge gaps remain in understanding what mechanisms are involved in warm-season grasses and their interaction with leaf fungi, what environmental drivers can manipulate such interactions, and how these associations lead to secondary metabolite production and their subsequent impact on animal health. Future examination can incorporate host genetic differences, pasture management style, and other environmental factors such as weather data and soil nutrient level to better understand the complex factors shaping the mycobiome of warm-season grasses. We

advise that a multidisciplinary approach be taken in development of new research to be conducted on grass mycobiomes to further decipher plant-fungus interactions, determine their impacts on palatability and animal health, and elucidate the potential application of these fungal resources.

MATERIALS AND METHODS

Sample collection for fungal community assessment. We sampled five perennial warm-season grasses: *P. notatum*, *C. dactylon*, *P. nicorae*, *S. indicus*, and *A. virginicus*. *P. notatum*, *P. nicorae*, and *A. virginicus* belong to the subfamily Panicoideae while *S. indicus* and *C. dactylon* belong to Chloridoideae of the Poaceae family. The growth type of *P. notatum*, *P. nicorae*, and *C. dactylon* is rhizomatous in comparison to the bunch-type *S. indicus* and *A. virginicus*. Samples were collected from six pastures in Florida (Greensboro, St. Cloud, Kenansville, Tampa, Wauchula, and Chiefland; latitude and longitude range between 27°29′56″ to 30°32′33″N and 84°43′08″ to 80°53'13″W, respectively [Fig. 1]). Samples were collected in the summer of 2017 (between May and August) to avoid seasonal variation. All the collection sites have humid subtropical climates (78) but span four plant hardiness zones with average minimum winter temperatures ranging from 15°F to 35°F (79). Forage and weed species coinhabited all but the Chiefland site (Fig. 1A). All grass leaves sampled remained green without yellowing or signs of herbivory. Samples considered "asymptomatic" were leaves from plant individuals without any sign of fungal symptoms, while "symptomatic" samples were leaves with visible black stromata. The fungal stromata of symptomatic samples were included for all analysis. These symptomatic samples were collected from four and three out of six sampling sites for *P. notatum* and *S. indicus*, respectively (Fig. 1A to D). Three replicates were collected from each plant species at individual locations. Each replicate contained five leaf pieces. Samples were placed in sealed plastic bags, set in an ice-filled cooler, and transported back to the lab, where they were removed, placed into microcentrifuge tubes, and stored immediately in a low-temperature freezer (−80°C) until the DNA extraction step.

DNA extraction, library preparation, and sequencing. To extract DNA from leaf material, a cetyltrimethylammonium bromide (CTAB) protocol (80) was used with minor modifications. Specifically, 10 zirconia beads (2.0-mm diameter; BioSpec Products Inc., Bartlesville, OK, USA) and two stainless steel beads (2.3-mm diameter; BioSpec Products) were placed in 2-mL tubes, and samples were ground using a MiniG tissue homogenizer (SPEX SamplePrep, Metuchen, NJ, USA). Amplicon sequencing libraries were prepared with ITS1F and ITS2 primers (81, 82), targeting the ITS1 region of fungal nuclear ribosomal DNA (nrITS). To amplify the target region and to attach a barcode sequence for each sample, a three-step PCR approach was performed, as described previously (83). Briefly, the ITS region was amplified using standard primer sets (ITS1F, 5′CTTGGTCATTTAGAGGAAGTAA3′; ITS2, 5′GCTGCGTTCTTCATCGATGC3′) with 10 cycles (81, 82). The PCR product was then used as the DNA input for a second 10-cycle PCR with the same primer sets (ITS1F/ITS2) combined with Illumina adaptors. The third PCR then added a unique barcode to each sample, which enabled multiplexing. The PCR products generated from the third round of PCR were cleaned up with 1:1 (vol/vol) Agencourt AMPure XP beads prior to pooling and sequencing. The concentration and purity of the final PCR product were assessed by Nanodrop (ThermoFisher Scientific) and gel electrophoresis. The PCR products were pooled with equimolar ratios of DNA and sequenced by the Illumina MiSeq 250 pair-end platform at the Duke Genome Center of Biology (Durham, NC, USA). The raw reads were submitted to the National Center for Biotechnology Information (NCBI) Sequence Read Archive (BioProject identifier [ID], PRJNA733319). Whenever a Sanger sequencing verification step was involved, we used ITS1F and LR3 (84) primer sets and followed the work of Chen et al. (83) for PCR preparation and sequencing.

Bioinformatics and statistics. Reads generated with the Illumina MiSeq were demultiplexed, and primers were trimmed with the program Cutadapt (85). The paired-end reads were quality filtered and merged, followed by amplicon sequence variant (ASV) identification in DADA2 (86). The taxonomy was determined with the UNITE database (87) using the RDP naive Bayesian classifier algorithm (88) implemented in DADA2. Key taxa were checked twice by manually performing a BLAST search (BLASTN) with the sequences against NCBI GenBank. Data analyses were conducted in R unless otherwise noted (89). Alpha-diversity was calculated based on a data set rarefied to 9,805 reads per sample, and singletons were retained in this data set. For the remaining analyses, read count of each ASV was normalized to the proportion of all reads per sample (90). Fungal community assessment was conducted in Phyloseq (91). The R package ranacapa (92) was used to examine the species accumulation curve for sampling completeness. To visualize the community assemblies shaped by locality and plant identity, nonmetric multidimensional scaling (NMDS) analysis based on beta-diversity (Bray-Curtis dissimilarity) (93) was performed. The function ADONIS, which conducts permutational multivariate analysis of variance (PERMANOVA), was performed to assess community differentiation (93) with 999 permutations. Alpha-diversity was measured with observed species and Chao1 index for richness and the Shannon index, which considers both richness and evenness (93). Wilcoxon rank sum tests (for two treatments) or Kruskal-Wallis tests (for multiple treatments) were performed for data skewed from normal distribution; otherwise, Welch's *t* tests (for two treatments) and ANOVA (for multiple treatments) were conducted. A *post hoc* Tukey honestly significant difference (HSD) test was performed for pairwise comparisons. For multiple comparisons, *P* values were adjusted with false-discovery rate (FDR) correction.

To test if the relative abundance of certain ASVs was significantly more abundant in symptomatic or asymptomatic *P. notatum* and *S. indicus*, we performed pairwise comparisons with the Wald test using package DESeq2 (94). Members of core mycobiomes were detected at the genus level using the R package "microbiome" (95). A core member was defined by its relative abundance of >0.01% in more than half of the samples. Because not all plants were sampled at every site (e.g., only one plant species, *P. nicorae*, was sampled

at Chiefland), to understand how plant identity and geographic location contributed in shaping mycobiome composition, we focused on a sub-data set containing four plant species (*P. notatum*, *C. dactylon*, *S. indicus*, and *A. virginicus*) which were sampled at three locations (Kenansville, Tampa, and St. Cloud) to avoid sampling bias. Indicator species were identified by the indicspecies package (96). To test for plant cultivar effect on mycobiome assembly, two *P. notatum* cultivars ('Argentine' and 'Pensacola') were investigated. To determine the relative degree of host (i.e., at species or cultivar level)-versus-location effect, we performed variance partitioning with redundancy analysis (RDA), using the Varpart function in the Vegan package (93).

To reconstruct phylogeny, sequences were first aligned with MAFFT (97). The phylogeny was reconstructed in RAxML with a maximum likelihood algorithm (98).

Mycotoxin analysis. The Greensboro site (Fig. 1) was revisited in the summer of 2018 to collect *Paspalum notatum* samples for mycotoxin detection. Each sample contained at least 50 individual leaf pieces. We collected three sample types. In addition to the "asymptomatic" and "symptomatic" samples as defined above, "mixed" samples were collected without careful examination in the field. Each of the three sample types had 5 to 6 replicates. Samples were placed in an ice-filled cooler until return to the lab and then freeze-dried (FreeZone2.5; Labconco, MO, USA) on the same collection day.

Forty-five mycotoxins (see Table S1 in the supplemental material) were examined via multiple reaction monitoring of two transitions (quantitative and qualitative) per compound on an LC-MS/MS system. Each sample was ground to pass an 0.5-mm screen, then extracted in triplicate by adding 4 mL of a 79:20:1 (vol/vol/vol) acetonitrile-water-acetic acid extraction solution per gram of material, and then rotated in the dark for 90 min (99). Samples were centrifuged at 3,000 rpm for 5 min and then diluted 1:1 with 20:79:1 (vol/vol/vol) acetonitrile-water-acetic acid. Mycotoxins were detected on an AB/Sciex 3200 QTrap LC-MS/MS system (Applied Biosystems, Foster City, CA, USA) via electrospray ionization, with separation performed using a Perkin-Elmer (Waltham, MA, USA) series 200 high-pressure liquid chromatograph (HPLC) connected to a Gemini C_{18} column (150 by 4.6 mm, 5 μm; Phenomenex [Torrance, CA, USA]) with a 4- by 3-mm security guard cartridge with similar packing (99). Mobile phases consisted of 5 mM ammonium acetate and methanol-water-acetic acid in a ratio of 10:89:1 (vol/vol/vol) (A) or 97:2:1 (B) and were run in a gradient program at 1 mL/min. Ammonium acetate (HPLC grade) and methanol and acetic acid (LC-MS grade) were purchased from commercial sources. The 18-mΩ water was obtained from an Elga Ultra PureLab water system (Cary, NC, USA). The presence of a mycotoxin was confirmed when the signal was equal to or greater than a signal-to-noise (S/N) ratio of 3:1 (limit of detection [LOD]), and both quantitative and qualitative transitions were present. Mycotoxins were quantified for each sample against standard curves established using certified analytical standards purchased from commercial sources. To analyze mycotoxin content, the concentrations of different types of mycotoxins among the three sample types (symptomatic, asymptomatic, and mixed) of *P. notatum* were compared with the Kruskal-Wallis test followed by a pairwise Wilcox test.

Data availability. All sequences are available at NCBI GenBank. The raw reads were submitted to the Sequence Read Archive (BioProject identifier [ID], PRJNA733319). Sanger sequencing accession numbers are ON678271 to ON678272. The fungal specimen was deposited at the University of Florida Herbarium under no. FLAS-F-63886.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 1.1 MB.

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F.M., K.-H.C., C.M., and A.B. collected grass samples for molecular work and mycotoxin detection. K.-H.C. analyzed the data and led the writing of the manuscript. F.M. conducted library preparation toward sequencing. J.D. measured the mycotoxins. H.-L.L. designed and supervised the project. All authors contributed to the writing and editing of the manuscript.

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