SELBYANA
Tropical Botany and Conservation with an Emphasis on Epiphytes
Journal of MARIE SELBY BOTANICAL GARDENS

DIVERSE FUNGAL ENDOPHYTES IN THE LEAVES OF A WIDESPREAD BROMELIAD, *TILLANDSIA RECURVATA* (L.)L.

ROSS JOSEPH[1]*, BRAD OBERLE[2], JOEL THURMOND[2], AMY CLORE[2]

[1]Department of Microbiology and Cell Science, University of Florida 1355 Museum Dr, Gainesville, FL 32603, USA; E-mail: Josephr1@ufl.edu

[2]Division of Natural Sciences, New College of Florida 5800 Bay Shore Rd, Sarasota, FL 34243, USA

ABSTRACT

Fungal endophytes play important roles in vascular plants, especially for grass relatives and species in extreme habitats. We investigated the presence and diversity of endophytic fungi within healthy leaves of *Tillandsia recurvata* (Bromeliaceae), a widespread neotropical epiphyte in the plant order Poales. Microscopy confirmed the presence of fungal tissues *in situ* and subculturing yielded seven distinct fungal morphotypes. Standard barcode sequences for nuclear ribosomal DNA ITS1-5.8S-ITS2 (ITS) classified five of seven as Sordariomycetes and indicated cryptic diversity within five different isolates of the most prevalent morphotype. Phylogenetic analysis of partial ITS and β-tubulin (*TUB2*) sequences reinforced taxonomic classification and distance-based analyses. Overall, the results support previous evidence that epiphytic bromeliads host diverse fungal endophytes, including undescribed taxa, which collectively differ from the predominately clavicipitaceous fungal endophytes of grasses and their close relatives. Further research into the interactions between *T. recurvata* and its endophytic communities should help elucidate what role these endophytes might play within their hosts and within the larger context of ecosystems.

Key words: Community structure, Cryptic diversity, Defensive mutualism, Multi-locus phylogeny, Nutritional biology, Fungal biology

* Corresponding author

INTRODUCTION

Fungal endophytes are a highly diverse assemblage of microorganisms that inhabit the above- and belowground tissues of plants without causing apparent disease to their hosts during all or part of their life cycles (Hyde and Soytong 2008). Such host-fungal interactions appear to be ubiquitous, with an increasing number of studies reporting endophytes isolated from diverse geographic and environmental regions including tropical, subtropical and temperate forests, grasslands, and arctic tundra (Arnold and Lutzoni 2007; Rodriguez et al. 2009; Mugerwa, Saleeba, and McGee 2013). Within their hosts, endophytes may grow as a single dominant species, colonizing most or all of the plant's organs, or as rich communities with dozens or even hundreds of distinct organisms inhabiting a single organ (Clay 1988; Arnold et al. 2000). Clay (1988) suggested that these fungi may represent mutualistic symbionts in cool season grasses, acting to deter insects and larger herbivores through production of toxic secondary metabolites. Subsequent studies have shown that fungal endophytes may interact with a diverse range of plant hosts as mutualists, neutral inhabitants, latent pathogens and saprophytes (Carroll 1988; Porras-Alfaro and Bayman 2011). Additional positive effects of endophyte colonization, such as enhanced thermal, salt and drought tolerance, vegetative growth, and protection from pathogens, have also been uncovered (De Battista 1990; Arnold et al. 2003; Marquez et al. 2007; Kuldau and Bacon 2008). Furthermore, researchers have investigated endophyte communities in only a small fraction of plant species, so the probability of uncovering taxonomic novelty when surveying endophytes in previously unexplored plant species is extremely high (Strobel and Daisy 2003).

Epiphytic plants (epiphytes) represent one such group that remains understudied in terms of presence and diversity of their endophytic communities. Epiphytes are non-parasitic plants that grow on the surface of other plants, often trees or shrubs, throughout their entire life cycles and derive nutrients and water from air, moisture, rain, and surrounding debris (Brighigna et al. 1992; Benzing 2008). These plants account for approximately 10% of vascular plant species diversity worldwide and nearly 30% of vascular plant species in tropical and neotropical regions, where they are most abundant (Nieder, Prosperi and Michaloud 2001). Gentry and Dodson (1987) reported that 42 plant families containing as many as 15,540 species with epiphytic lifestyles occur in the neotropics, and the majority of these species reside in the families Orchidaceae and Bromeliaceae.

Characterizing fungal endophyte communities in epiphytic Bromeliaceae may help shed light on the role of fungal symbiosis in the development of epiphytic lifestyles, due to the unique nutritional biology and important phylogenetic position of these plants. Many epiphytic Bromeliaceae absorb mineral nutrients into their leaves through modified trichomes via atmospheric deposition or rainwater flow over their host (Benzing et al. 1976; Benzing 1980). Furthermore, Bromeliaceae includes the only epiphytic lineages in the order Poales, a hyperdiverse group that includes grasses and sedges. Both grasses and sedges host distinctive clavicipitaceous fungal endophytes, and cool-season grasses transmit systemic fungal infections vertically from parent to offspring through seeds (Clay 1990). Considering that Bromeliaceae, along with Typhaceae, form the basal lineage within the order, characterizing their fungal endophyte communities could shed light on the evolution of specialized fungal endophytes in the nutritional biology of this particularly diverse group of monocots.

In the present study, fungal endophytes from leaves of *Tillandsia recurvata* (L.) L., an epiphytic bromeliad widespread in the neotropics, were isolated and characterized by genetic sequencing, phylogenetic reconstruction, and microscopy. In contrast to the reported bacterial associations with *Tillandsia recurvata* (Brighigna et al. 1992), the communities and potential effects of fungal microbiomes within *Tillandsia* species are poorly understood. A previous study from Peru, comparing fungal endophyte communities in several species of *Tillandsia* to those of other plant species, found that epiphytic Bromeliaceae tended to have greater fungal operational taxonomic units (OTUs) from Xylariales, Sordariales and Pleosporales and fewer OTUs from Hypocreales, the order which includes the distinctive fungal endophytes of sedges and grasses (Unterseher et al. 2013). An undescribed Xylariaceae endophyte from the epiphyte *Tillandsia usneoides* in Florida produced diverse secondary metabolites with cytotoxic effects in human cells (Xu et al. 2015). While these studies show that fungal endophytes of *Tillandsia* are present, diverse and

metabolically active, the roles of fungal endophytes in plant adaptation to epiphytism and the evolution of specialized endophytic relationships within Poales remain unclear. In order to understand how the presence and diversity of fungal endophytes influence the extreme adaptations and ecological functions of these and other plant epiphytes, assessments of endophyte communities in other epiphytic *Tillandsia* from different regions are necessary.

The specific objective of this study was to advance knowledge of *Tillandsia* fungal endophyte associations by providing preliminary characterization of the fungal endophyte communities inhabiting *Tillandsia recurvata* (L.) in Florida. Here, we characterize 11 endophytes representative of morphotypes isolated from *T. recurvata* via tissue culture of surface-sterilized leaves. These endophytes were characterized through morphological analyses and the construction of a two-gene phylogeny. To our knowledge, this represents the first attempt to describe the fungal endophytes within *T. recurvata.*

Materials and Methods

Study Location

To begin sampling and characterizing fungal endophyte diversity in *T. recurvata*, the campus of New College of Florida was chosen as a study site. The campus spans 58 hectares, located in west Sarasota County on the shore of Sarasota Bay (27.3848°N, 82.5609°W). Sarasota County is a humid subtropical environment, with an annual average high temperature of 27.6°C and an average low of 17.8°C. Average annual precipitation in this region is 1,346.4 mm. The campus area was originally composed of coastal scrub and pine flatwood habitats, but was converted into a residential campus in the 1950's, and now consists largely of landscaped and restored habitats. A number of native and non-native tree genera can be found on the campus hosting robust populations of *T. recurvata.* Leaves of *T. recurvata* found growing on these abundantly available tree genera were collected and screened for fungal endophytes using culturing, morphological, and molecular methods as described below.

Fungal Isolation and Morphological Characterization

Tillandsia recurvata leaves were sampled from *Pinus*, *Quercus*, *Taxodium*, *Sabal*, and *Callistemon* host trees. For each host tree genus, four *T. recurvata* were collected and five healthy leaves collected per plant for a total of 20 *T. recurvata* leaves per host tree genus. From this pool of collected leaves, 10 were randomly selected from Pinus, Quercus, Sabal, and Callistemon samples, and 13 from Taxodium samples, for further processing and downstream experiments. An approximately 50 mm long segment was cut from each leaf and surface sterilized by rinsing in 90% (v/v) ethanol for 1 min, 70% (v/v) ethanol for 1 min, and 1.75% (v/v) sodium hypochlorite for 5 min followed by three rinses in autoclaved NANOpure™ water. The segments were then air dried in a UV-sterilized laminar flow hood on autoclaved filter paper. Once dry, a cutting approximately 10 mm in length was taken from the interior (not including previously cut edges) of the sterilized leaf segments and each resulting segment was plated onto 2% (w/v) malt extract agar (MEA), a standard mycological medium effective for culturing endophytes (Thirumalanadhuni et. al, 2018), in 90 x 15 mm Petri dishes. Two control plates were made along with each set of sample plates to test for incomplete surface sterilization. These controls consisted of one plate in which a sterilized leaf was imprinted into the medium and removed, and one plate in which several milliliters of autoclaved water that had been rinsed over a sterilized leaf was spread. Culture dishes were incubated for up to a month at room temperature in ambient light, and resulting fungal colonies were subcultured onto 2% MEA in 60 x 15 mm culture dishes to produce pure isolates.

Once pure isolates were obtained, they were organized into morphotype groups based on culture characteristics such as presence or absence of aerial hyphae, color, relative rate and shape of growth, and whether isolates were able to form spores in culture. Microscopic characteristics including hyphal septation, pigmentation, and spore shape were used to further divide cultures into morphotypes. Candidate isolates were then selected from each morphotype for downstream sample processing and cryopreservation. Herbarium specimens of isolates were deposited in the Forest Service Northern Research Station culture collection (CMFR). Microscopic visualization of *T. recurvata* tissues was also performed using Rose Bengal stain and a polarized light filter to visualize fungal hyphae *in situ.* Because endophytes had been cultured from all tree genera, they were assumed to be present in *T. recurvata* regardless of host tree species. Accordingly, *in situ* visualization was carried out on *Tillandsia* from a single host tree genus,

Callistemon. *Tillandsia recurvata* tissues collected from *Callistemon* were fixed in 4% formaldehyde and sectioned to a thickness of 2 µm using a vibratome before being stained. When stained tissues were viewed using this method, fungal tissue appeared red, while plant tissue appeared green (Bacon and White 1994).

DNA Isolation, Amplification, and Sequencing

DNA was extracted from 11 candidate isolates representing the diversity of morphotypes recovered, including one isolate each from the less prevalent morphotypes and five isolates from the most common morphotype (morphotype 2). Characterizing DNA from multiple isolates of morphotype 2 enabled an assessment of cryptic diversity within this prevalent type. PowerSoil™ and UltraClean™ Soil DNA Isolation kits (Qiagen, Netherlands, with the latter being the newer generation of the former) were used according to the 'experienced user protocol' in the manufacturer's instructions and DNA purity and concentration were assessed using a Thermo Scientific (MA) NanoDrop Lite™ Spectrophotometer. PCR was conducted using the fungal- specific primer pairs ITS1-F/ITS4 and Tub2Fd/Tub4Rd, which amplify two barcoding regions; (1) 600-800 bp of the internal transcribed spacers of nuclear ribosomal DNA ITS1-5.8S-ITS2 (ITS) (White et al. 1990; Gardes and Bruns 1993), and (2) a region (400-500 bp) near the 5′ end within the β-tubulin (*TUB2*) gene, respectively (Aveskamp et al. 2009). PCR mixes consisted of 5 µL 10x PCR Buffer (Invitrogen, CA), 4 µL dNTP mix (Takara, Japan), 0.25 µL *Taq* polymerase (Invitrogen, CA), 36.75 µL NANOpure™ water, 2 µL extracted fungal DNA, and 1 µL of both forward and reverse primer (100 picomole/µL). PCR conditions for ITS were as follows: 3 min at 95°C followed by 40 cycles of 50 sec at 94°C, 1 min at 45°C, and 1 min at 72°C, followed by a final 10 min incubation at 72°C. PCR conditions for *TUB2* were as follows: 5 min at 94°C followed by 40 cycles of 45 sec at 94°C, 30 sec at 52°C, 90 sec at 72°C, followed by a final 6 min incubation at 72°C. Amplification of fungal DNA was verified by electrophoresis on a 1.5% (w/v) agarose gel against an Invitrogen (CA) 1 kb Plus DNA Ladder. PCR products were then purified using a QIAquick® PCR Purification Kit (Qiagen, Netherlands) according to manufacturer's instructions and shipped to the University of Illinois Urbana Champaign Core DNA facility for Sanger sequencing using the two primer sets listed above, and an Applied Biosystems (CA) 3730xl automated sequencer with 50 cm capillary arrays.

Sequence Alignment and Phylogenetic Analysis

Contig generation, sequence alignment and annotation were completed using GENEIOUS 10.0.9 (http://www.geneious.com, New Zealand, Kearse et al. 2012). For each region, the first 30 base pairs for each trace file were excluded and ambiguous base calls were corrected manually. Each resulting consensus sequence was deposited in GenBank (SUPPLEMENTAL TABLE S1) and compared using BLAST to the NCBI collection for type specimens to reduce the chances of associating an isolate with an improperly identified fungal strain. The set of most similar sequences was incorporated into a preliminary alignment using the default settings for the Geneious alignment algorithm (Kearse et al. 2012). After checking the alignments, annotations from the GenBank files were transferred to the edited contigs and manually confirmed for *TUB2*.

Sequence data were used for two analyses. The first analysis constructed a phylogeny for all 11 cultured strains. Apparently deep phylogenetic divergences among strains complicated alignments for the internal transcribed spacers of ITS and the introns in *TUB2*. Consequently, these portions for each gene were excluded from the phylogenetic reconstruction. For the remaining 214 aligned bp of ITS representing the 3′ end of the 18S small subunit rRNA gene, the entire 5.8S rRNA gene and the 5′ end of the 28S large subunit rRNA gene, the best-fit nucleotide substitution model was identified using the small-sample size corrected Akaike Information Criterion in MEGA 7.0.21 (Kumar, Stecher, and Tamura 2015). The process was repeated for 224 aligned bp of exons from *TUB2*. To assess topological incongruences between regions, preliminary phylogenies for each region were reconstructed under maximum likelihood in MEGA. Because no hard topological conflicts occurred, both regions were concatenated and a phylogeny was estimated for the partitioned dataset in a Bayesian context using MR BAYES 3.2.6 (Ronquist and Huelsenbeck 2003). The priors were set to defaults and 200,000 Markov Chain Monte Carlo samples were drawn from the posterior distribution after discarding the first 20,000 samples as burn-in and retaining every 100 samples thereafter. Branch credibility was estimated using the

posterior frequency of the associated clades.
The second sequence-based analysis focused on the complete ITS sequence alignment. Based on the consensus ITS sequences, isolates were identified using the ribosomal database project (RDP) Bayesian Classifier with the Warcup V2 (2016) training set (Desphane et al. 2016; Shearin et al. 2018). In addition to the RDP classification, ITS consensus sequences were used to explore the molecular diversity within the most prevalent morphotype (morphotype 2). For these five strains, complete ITS sequences were realigned and the best-fit nucleotide substitution model was estimated using the methods described above. Based on this model, pairwise sequence distances were estimated in MEGA 7.0.21.

Functional Guild Classification

Functional guild classifications, host identity and geographic origins for isolates with similar sequences were determined in different ways depending on the marker and the sequence analysis method. For the most similar taxa based on the RDP classifier of ITS sequences, functional guild classifications were based on FUNGuild (Nguyen et al. 2016). For the most similar taxa based on top BLAST sequence similarity, functional guild classification was based on the Genbank accession "isolation method," "host" and/or "country" fields or additional data provided in the associated publication.

RESULTS

Healthy *Tillandsia recurvata* Leaves Harbor Endophytes

Rose Bengal staining revealed fungal hyphae growing in healthy *T. recurvata* leaf tissues. Tissues of *T. recurvata* stained green and contrasted with apparent fungal hyphae, which stained red

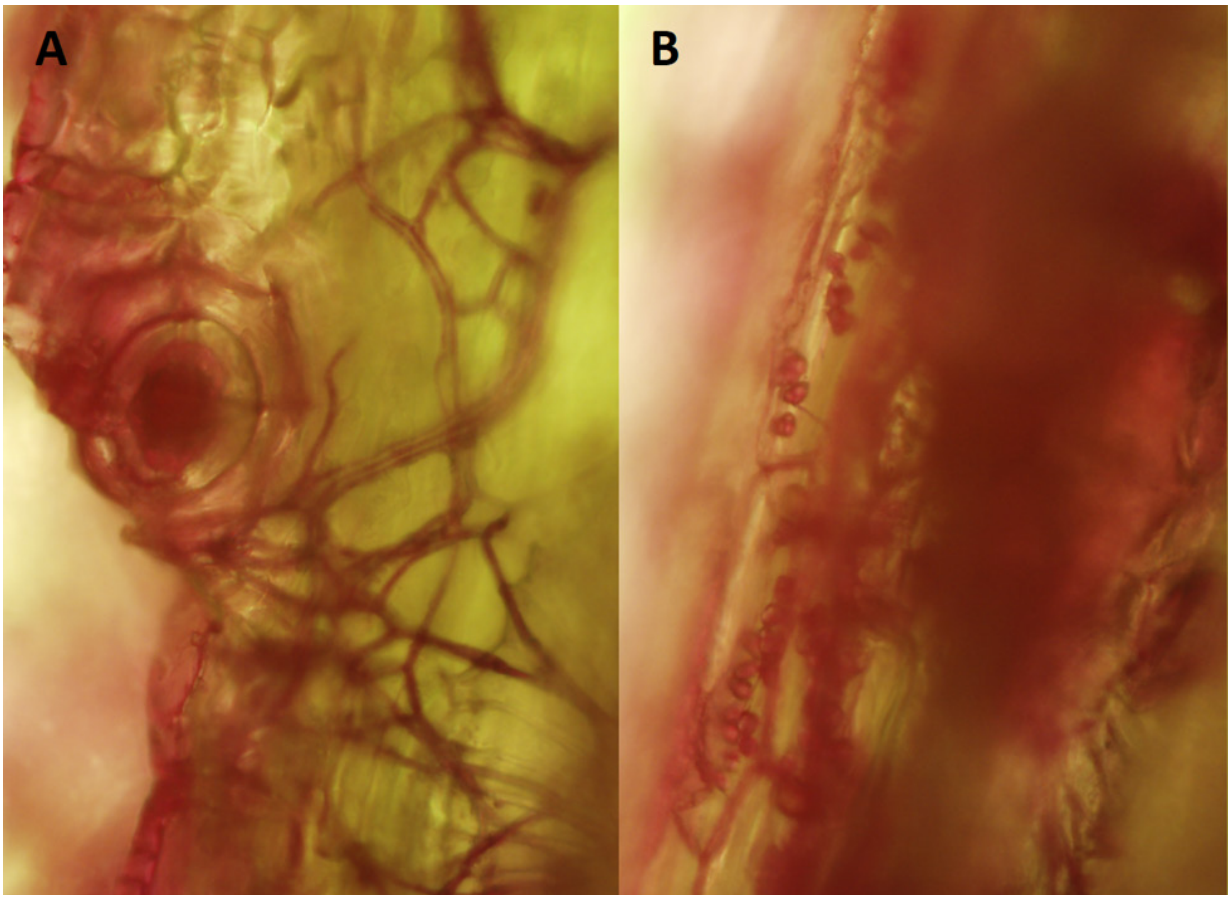

FIGURE 1. *In situ* (Rose Bengal) staining of fixed *T. recurvata* tissues displaying A) what appears to be fungal hyphae (red) growing within a stomate and surrounding tissue (green) of a *T. recurvata* leaf and B) fungal hyphae (red) presenting fruiting structures growing among parenchymal cells (green). Original magnification 400x.

(FIGURE 1). These fungal structures appeared in the intercellular space of the plant tissue throughout all 10 of the sections examined. Differences in cell morphology reinforced the interpretation of hyphal presence. *In situ* hyphae were thin and threadlike, with some septate and others aseptate, and clearly contrasted with leaf parenchyma and epidermal cells which were, by comparison, much thicker with distinctive primary cell walls.

Surface-sterilized leaves produced isolates consisting of multiple morphotypes, and little evidence for contamination (TABLE 1). Among 53 total leaves sampled, mycelia grew from 15, yielding 26 total fungal endophyte isolates. Plants collected from all five host tree genera yielded endophytes, but the number of isolates per host tree varied such that leaves from plants growing on *Callistemon* yielded 13, half of the total number of endophytes. Additionally, three cultures derived from Tillandsias growing on *Callistemon* displayed multiple morphotypes growing from a single leaf. Only one control plate developed mycelium, which originated on the medium and spread rapidly across the plate implying that it may have been from an external source. The infrequency of contamination in the controls suggests that our surface-sterilization technique was generally effective.

Endophyte Communities in *Tillandsia recurvata* Are Morphologically Diverse

Pure cultures displayed marked morphological diversity (FIGURES 2 and 3). Isolates were organized into morphotypes, groups sharing culture characteristics, to obtain an initial estimate of species diversity. Despite the potential for masking cryptic species diversity, this technique is useful for initial estimates of taxonomic richness by focusing broadly on distinct morphological features (Lacap et. al, 2003). Morphotypes were numbered 1-7, and in instances where multiple isolates were selected from a single group for sequencing, the isolates were further numbered by the order of their processing (e.g. 1.1, 1.2, 1.3, etc.). Descriptions of morphotypes are as follows.

Morphotype 1 was oily in appearance, black to brown or tan in color, with irregular margins and sparse white aerial hyphae in mature culture. Hyphae appeared pigmented, septate, displaying dimorphic sexual states in culture, including exophiala and cladophialophorous forms. Morphotype 2 was velvety in appearance, white with circular margins forming black and white stromata in mature culture. Hyphae appeared unpigmented, aseptate or sparsely septate, not forming spores in culture. Morphotype 3 was velvety in appearance, grey to olive green in color, with irregular margins forming concentric bands. Hyphae appeared pigmented, septate, not forming spores in culture. Morphotype 4 was thin, crust-like in appearance, white to tan in color with a circular margin, and a distinct ring separating younger and older tissue. Hyphae appeared unpigmented, aseptate or sparsely septate, branching, not forming spores in culture. Morphotype 5 was matted in appearance, white to green or brown in color, having circular margins with wispy aerial hyphae. Hyphae appeared

TABLE 1. *Tillandisa recurvata* sample collection, number, and characteristics of isolates recovered.[1]

# Plants sampled	Host Tree	# Leaves analyzed	# Isolates	Control 1	Control 2	Morphotypes recovered
4	*Taxodium*	13	2	Negative	Negative	1
4	*Pinus*	10	3	Negative	Negative	2,5
4	*Quercus*	10	5	Positive	Negative	2,3,4,5
4	*Sabal*	10	4	Negative	Negative	2,5,7
4	*Callistemon*	10	12	Negative	Negative	4,5,6,7

[1] Status of control plates (Control 1 being the plate containing only water that had been rinsed over the tissue and Control 2 being the plate into which the tissue was imprinted and then removed) refers to whether or not there was discernable growth, and morphotypes recovered correspond to those shown and described in FIGURE 2.

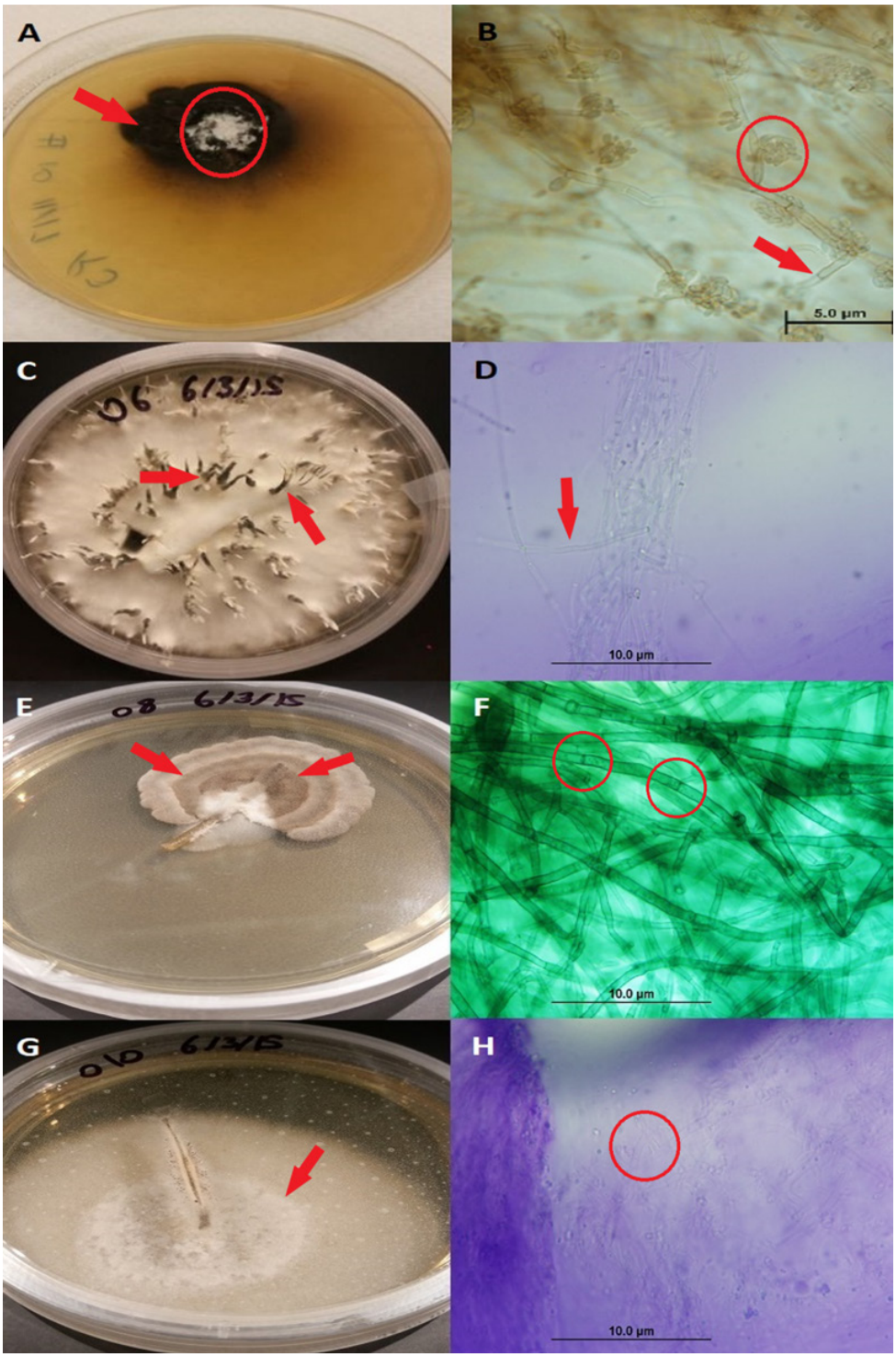

FIGURE 2. Morphotypes 1-4: Culture morphology and hyphal characteristics leading to morphotype designations. A) Morphotype 1: vegetative hyphae (arrow), aerial hyphae (circled). B) Morphotype 1: septate hyphae (arrow), exophiala state (circled) (cladophialophorous state not shown). C) Morphotype 2: stromata in mature culture (arrows). D) Morphotype 2: septate hyphae (arrow). E) Morphotype 3: mycelium with irregular margins forming concentric bands (arrows). F) Morphotype 3: septate hyphae (circled). G) Morphotype 4: distinct ring separating younger and older tissue (arrow). H) Morphotype 4: aseptate or sparsely septate branching hyphae (circled).

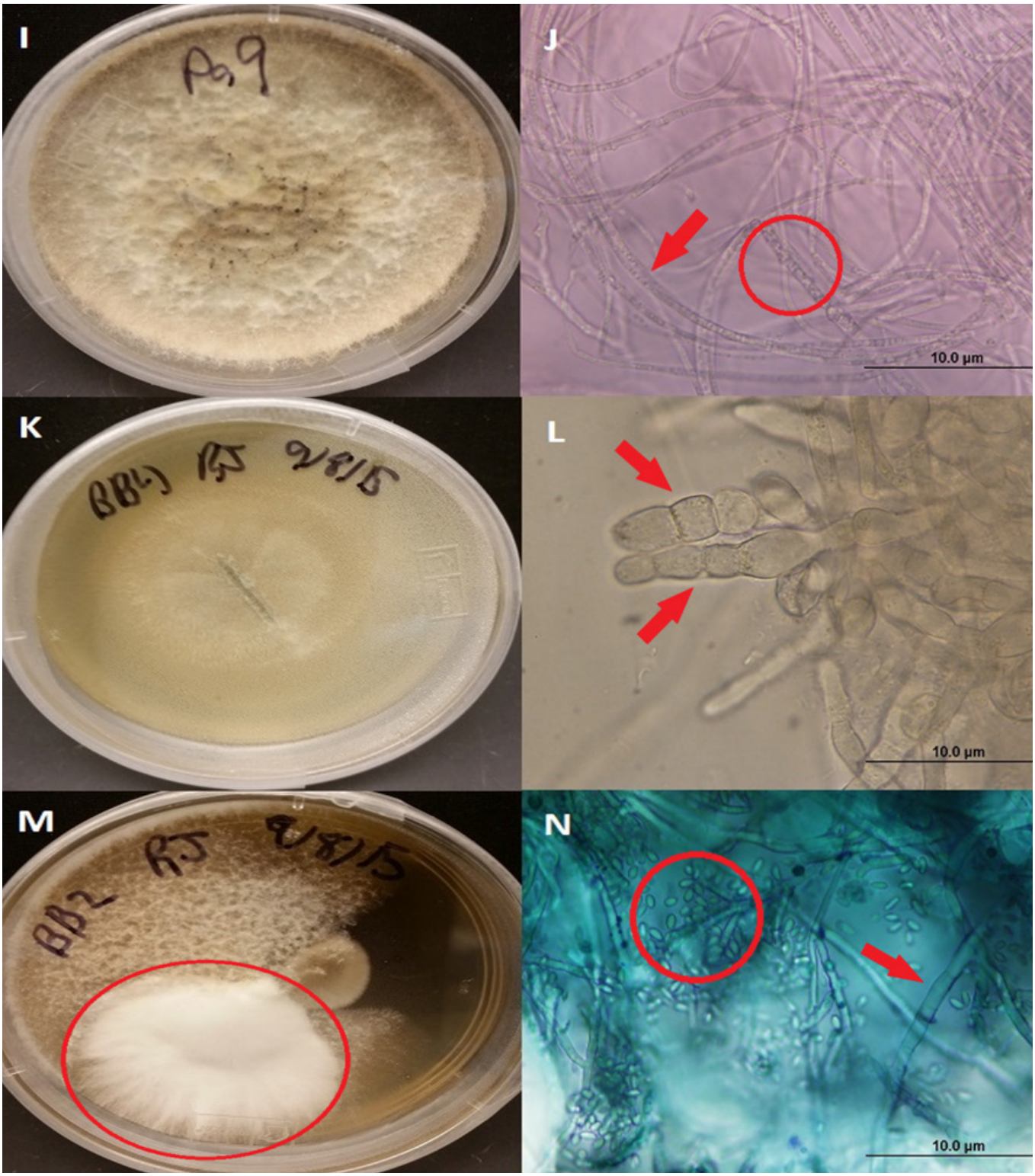

FIGURE 3. Morphotypes 5-7: Culture morphology and hyphal characteristics. I) Morphotype 5. J) Morphotype 5: septate, unbranching hyphae (arrow), green spherical structures on and in hyphae (circled). K) Morphotype 6. L) Morphotype 6: annelations present at hyphal tips (arrows). M) Morphotype 7 (circled). N) Morphotype 7: aseptate or sparsely septate hyphae (arrow), ovate or elliptical spores from trident-shaped fruiting structures (circled).

unpigmented, septate, and unbranching, containing many green spherical structures. Morphotype 6 was thin, crust-like, furrowed in appearance, tan in color with irregular margins. Hyphae appeared unpigmented, septate, with annelations present at hyphal tips. Morphotype 7 was velvety in appearance, white in color with irregular threadlike margins. Hyphae appeared unpigmented, aseptate or sparsely septate, and displayed ovate or elliptical spores from trident-shaped fruiting structures.

Morphotypes 2,4,5, and 7 were found in *T. recurvata* leaves collected from multiple host plants. The most prevalent morphotype (2) was represented by 5 isolates. Hyphae of this morphotype were generally aseptate and unpigmented. It is worth noting, however, that within this morphotype, isolates differed in their presentation of stromata, which varied in color and thickness (data not shown). In addition to the most prevalent morphotype, a number of endophytes recovered were only observed in a single culture, or in *T. recurvata* collected from a single host tree (TABLE 1). In total, 7 morphotypes were characterized and maintained during this study.

Isolated Strains Likely Represent Both Known and Novel Taxa

Isolated morphotypes varied in DNA sequences. The degree of correspondence between isolate sequences and existing sequences in the NCBI Nucleotide Collection varied by gene region and by isolate. Every ITS sequence had a maximum percent identity score greater than 96% and query coverage greater than 90% (SUPPLEMENTARY TABLE S1). Of the top matches, only one had a species-level taxonomic determination, *Preussia minima* (Sporomiaceae) for morphotype 6, with two more having genus-level determinations. RDP classification produced more detailed taxonomic

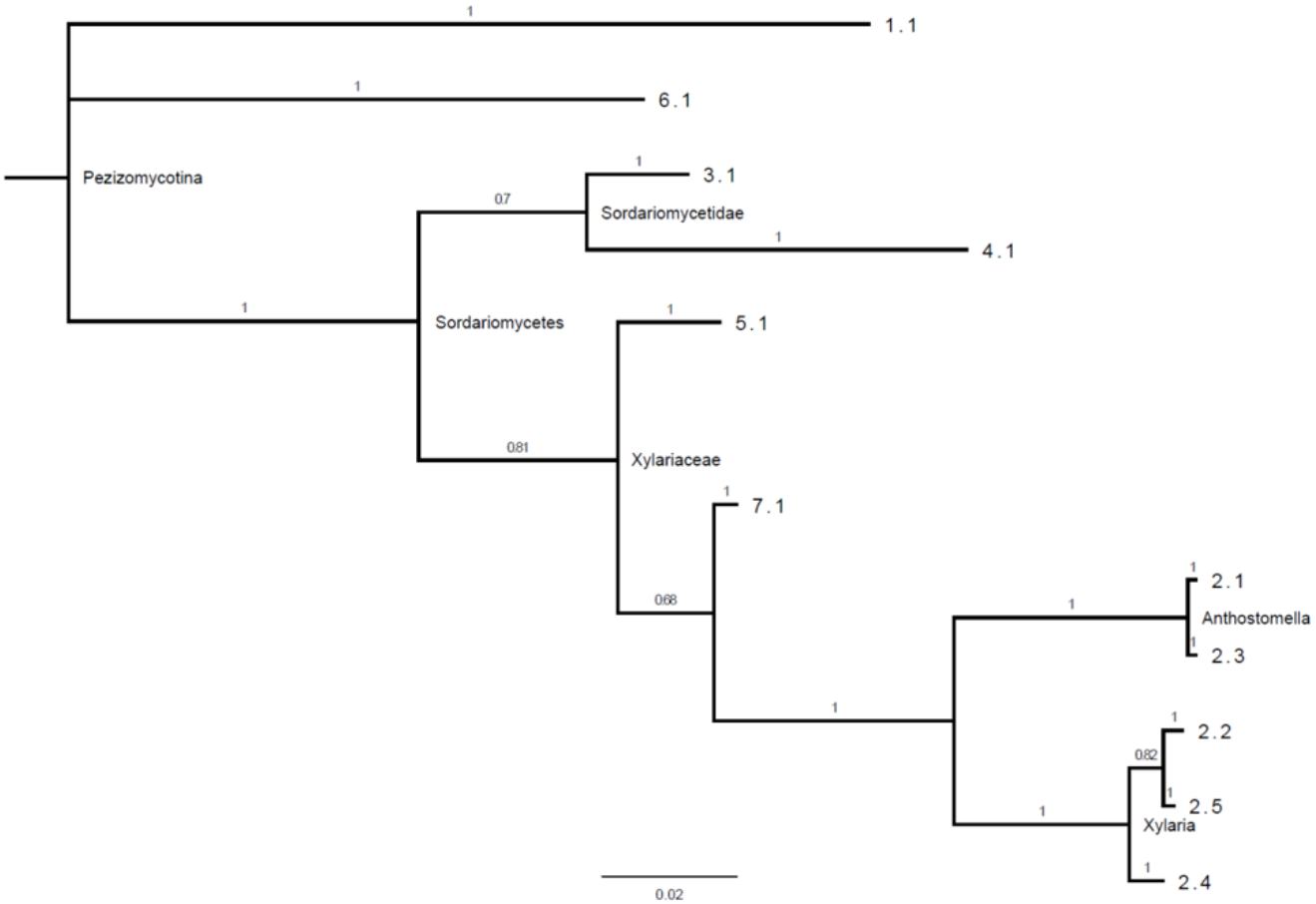

FIGURE 4. Phylogenetic reconstruction of candidate isolates based on concatenated sequences of *TUB2* and ITS partial gene regions. Node labels represent posterior probabilities. Branch lengths are in substitutions per site.

determinations with three isolates having more than 80% match to a taxon in the Warcup V2 training set (SUPPLEMENTARY TABLE S1). Besides consistent identification of morphotype 6 as *Preussia minima* (93%), the RDP classifier identified two isolates of morphotype 2 (2.1 and 2.3) as *Anthostomella eucalyptorum* (Xylariaceae: 84% and 81% respectively) and morphotype 7 as *Hypoxylon investiens* (Xylariaceae: 100%). One isolate, morphotype 3, had a very low RDP classification score, with the only taxon matching above 80% identity being the order Sordariales (93%). Maximum percent identity scores for BLAST searches of *TUB2* consensus sequences were lower than those for ITS, with a minimum value of 80% and the lowest query coverage of 50% (SUPPLEMENTAL TABLE S1). All of the top matching *TUB2* sequences had genus-level taxonomic determination. For every isolate, both ITS and *TUB2* sequences matched accessions with compatible higher taxonomic classifications.

Phylogenetic analysis of concatenated gene sequences recovered a topology that was consistent with other taxonomic assignment methods (FIGURE 4). Isolates 1 and 6, which shared their highest sequence identities with accessions assigned to Eurotiomycetes and Dothideomycetes respectively, formed part of a basal polytomy along with a strongly supported clade (posterior probability = 1) consisting of all other sequences. The latter clade shared their highest sequence identities with accessions assigned to Sordariomycetes. Within the putative Sordariomycetes, two isolates with ITS sequences that were highly similar to uncultured conifer-associated fungi formed a moderately supported clade that was sister to a clade of remaining isolates. Within the clade corresponding to Xylariales, two isolates had *TUB2* sequences that were highly similar to isolates identified as *Hypoxylon*. The remaining five isolates formed a strongly supported clade (posterior probability = 1) containing all isolates from the most frequently encountered morphotype. Three of these had pairwise ITS sequence distances less than 0.025 and shared their highest sequence identities with accessions assigned to the genus *Xylaria* (SUPPLEMENTAL TABLE S2). The other two had a pairwise ITS sequence distance of 0.013 and shared their highest sequences identities with accessions determined as unclassified Xylariaceae.

Among genetically similar isolates, the most commonly reported nutritional mode was symbiotroph (70%) followed by saprotroph (12%) (FIGURE 5). The specific functional guild associated with similar fungi varied depending on the marker and classification method, with foliar endophytes predominating (7/11) when identifying similar fungi based on RDP classification of ITS sequences, and lichenized fungi predominating (6/11) when identifying similar fungi based on BLAST scores for *TUB2* sequences. Among similar isolates identified using BLAST, the most common geographic locality was Florida (45%) followed by North Carolina and Europe (10% each). One isolate, 4.1 had an ITS sequence very similar to a species of Sordariomycete that had been isolated from *Tillandsia usneoides* in Florida. Another, 7.1, had an ITS sequence that was highly similar to an isolate that had been identified as being responsive to nitrogen addition in North Carolina soils (FIGURE 5).

DISCUSSION

In this study, we have shown that the neotropical epiphytic bromeliad, *T. recurvata*, contains fungal endophytes within its healthy tissues. *Tillandsia recurvata* leaves collected during this study were shown by culturing of surface-sterilized tissues and DNA sequencing methodologies to harbor diverse fungal endophytes, including likely members of *Exophiala, Hypoxylon, Preussia,* and *Xylaria*, as well as isolates with sequences that were most similar to unidentified fungi. Numerous distinct isolates were also noted to occur within a single leaf, suggesting that *T. recurvata* leaves may contain diverse communities of fungal endophytes. Furthermore, morphologically similar endophyte isolates were recovered from *T. recurvata* on diverse host trees, suggesting that certain fungal taxa may be common in tissues of this epiphyte.

Comparison of complete sequences to the NCBI nucleotide collection, RDP classification and phylogenetic reconstruction of concatenated expressed regions produced compatible hierarchical patterns of relationship among isolates. All but two samples occurred in a clade corresponding to Sordariomycetes and all samples from the same morphotype occurred in a clade corresponding to Xylariaceae. However, the majority of isolates could not be identified to species level with a high degree of confidence. This finding suggests that endophytes isolated during this study may represent undescribed species. For example, one isolate with sequences

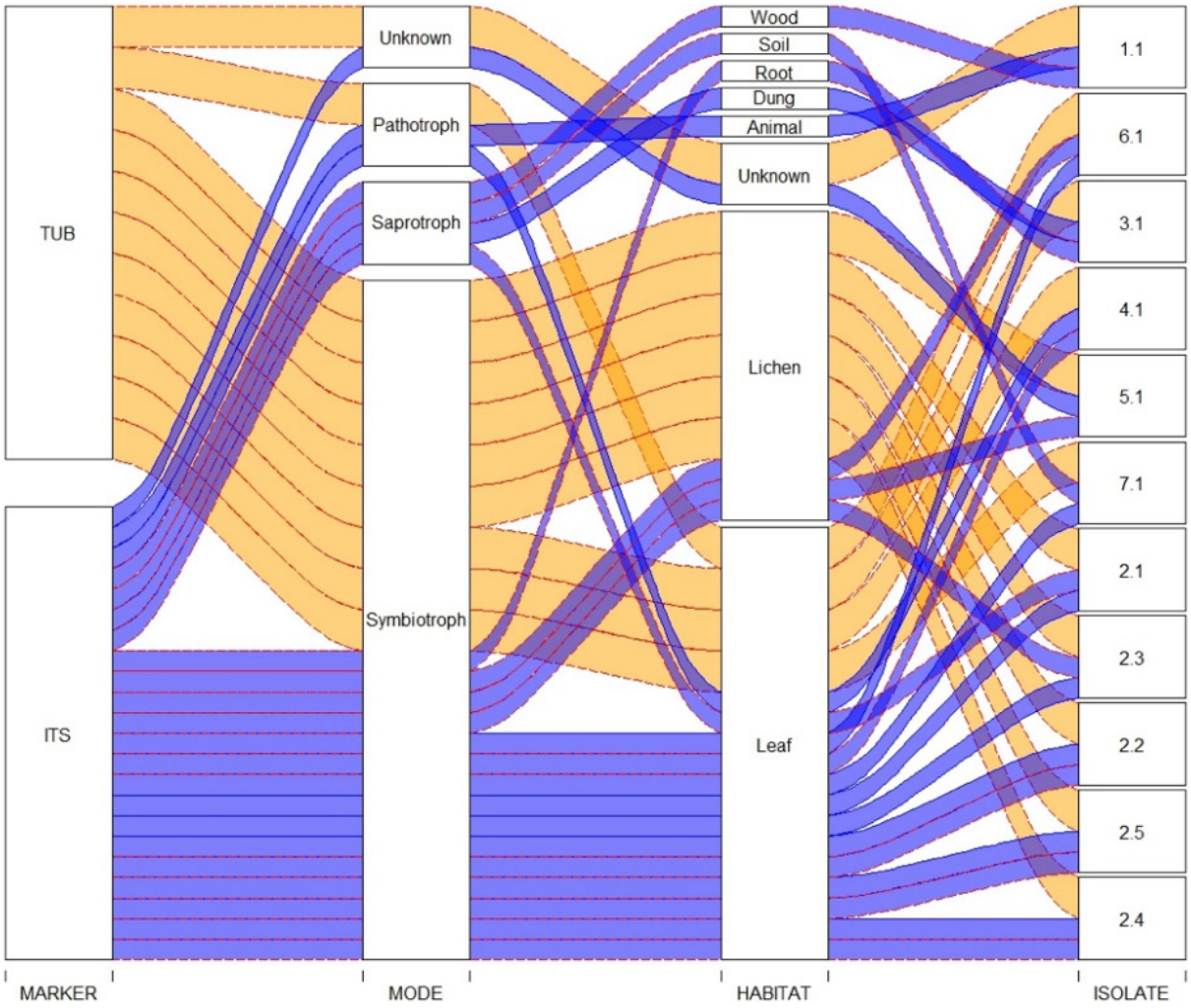

FIGURE 5. Nutritional modes and habitats assigned to isolates sequenced with either TUB (orange alluvia) or ITS (blue alluvia) markers and classified either by BLAST searches (dashed red borders) or by FUNGuild analysis (solid blue borders). Isolates are organized from top to bottom as they appear in the phylogeny (Figure 4).

similar to a described species of *Exophiala* from South Africa (Crous and Groenewald, 2011) would represent the first fungal endophyte from the class Eurotiomycetes discovered in a *Tillandsia*. Furthermore, the five isolates identified as the same morphotype belonged to two distinct clades, raising the possibility for cryptic diversity within morphologically similar cultured strains. While the methods employed in this study provide a valuable framework for understanding endophyte diversity in *T. recurvata*, future studies should address this issue of cryptic diversity by more extensive culturing and metagenomic approaches to achieve a more comprehensive view of the endophyte communities in this species.

Many of the endophytes recovered during this project are similar to endophytes isolated from other *Tillandsia* species in previous studies. Endophyte strains in these earlier studies were found to belong primarily to the classes Dothidiomycetes and Sordariomycetes, especially Xylariales and Sordariales, including a single *Xylaria spp.* isolate collected from *T. usneoides* in Florida and numerous isolates from three *Tillandsia* species in Peru (*T. usneoides, pupurea, and cacticola*) (Unterseher et al. 2013; Xu et al. 2015). It is possible that this commonality among endophytes cultured from numerous *Tillandsia* species in diverse geographic locations may suggest a level of specificity or dominance by some Sordariomycete fungi within

the tissues of *Tillandsia*. Endophyte studies of other families of epiphytes, such as Orchidaceae, have also revealed species of *Xylaria* and other Sordariomycetes; therefore, it is possible that these taxa may simply represent neutral, latently saprophytic, or endophytic species common in the environment, or in epiphytes as a whole (Yuan, Chen, and Yang 2009). Such ideas about endophyte community structure have been supported by recent studies such as that of Vincent et. al (2016), who demonstrated through extensive endophyte sampling from rainforest trees in Papua New Guinea that within a given species, relatively few generalist endophytes may dominate, accompanied by a large number of rare species.

While fungal endophyte communities in *T. recurvata* from Florida share features with communities of other *Tillandsias* and other epiphytes, they differ from communities described for more diverse terrestrial Poales. Perhaps unsurprisingly, none of the isolates had morphological or molecular characters typical of Clavicipitaceae, the family that has diversified in association with grasses and sedges (Clay 1990). Furthermore, a survey of several other species of *Tillandsia* in Peru (Unterseher et al. 2013) identified only a single fungal endophyte from the Hypocreales, the fungal order including Clavicipitaceae. These observations suggest either that the epiphytic habit is inhospitable to clavicipitaceous endophytes, or that the association between these fungi and plants occurred after the divergence between ancestors of Bromeliaceae and other Poales. More sampling from terrestrial bromeliads and other groups within the order is necessary to distinguish between these alternatives. It is also important to note that the sampling location from this study represents a disturbed habitat, and that sampling from more natural habitats may yield differing communities of endophytes. Further studies comparing these two habitat types could likely help determine the extent to which host tree habitat influences fungal communities associated with epiphytes, as has been elucidated for root-associated fungal endophytes in the epiphytic orchid, *Dendrobium sinense* (Wang et. al, 2017).

It is likely that only a fraction of the endophytes inhabiting a plant are capable of being cultured by current methods (Arnold 2007; Porras-Alfaro and Bayman 2009). Therefore, when attempting to estimate populations and community structures of endophytes within *T. recurvata*, it is essential to consider that the endophytes recovered through culturing in the present study likely do not represent the total diversity of fungal endophytes inhabiting the tissue. Testing different growth conditions and media may allow the identification of new endophytes in future studies, however culture-independent techniques such as environmental DNA sequencing should also be considered to achieve the greatest measure of species diversity. Arnold et al. (2007) demonstrated the complementarity of culture-based and environmental PCR (ePCR) techniques by reporting a number of Basidiomycete species recovered from ePCR, which were not seen in culture-dependent experiments, and a number of Sordariomycetes which were found by culturing, but not ePCR. With this in mind, assertions of dominant species based solely on culturing experiments may be premature.

Of the 11 candidate isolates recovered from tissues of *T. recurvata* during this study, five were identified with high degrees of confidence to four genera, while only one was confidently identified to the species level. Taxa with similar sequences had diverse nutritional modes, from endophytic and saprophytic to parasitic lifestyles. Isolates that were only identified to a class level by BLAST searching were all found to belong to the Sordariomycetes, which encompasses two of the known genera of isolates and is known to consist largely of parasitic and wood-decomposing fungi, though endophytes are also reported from this class (Arnold 2007). Analysis of all 11 isolates using either BLAST searching or the FUNGuild database to analyze ITS and *TUB2* markers revealed a range of nutritional modes including pathotroph, saprotroph, symbiotroph, and unknown nutritional modes, with symbiotrophs predominating. This may support the idea that many of these isolates may play a mutualist role in *T. recurvata*. Furthermore, the most prevalent habitat reported from fungi with similar ITS sequences was within leaves, while fungi with similar *TUB2* sequences had been predominately described as lichen forming. The most phylogenetically distinctive cultures in healthy *Tillandsia* leaves tended to have sequences similar to fungi encountered with relatively distinctive (e.g. animal pathogen) or incongruous (dung or root-associated) nutritional modes and habitats. In some cases where overall sequence identity was low, this pattern could reflect taxonomic novelty. In other cases where cultured fungi were highly similar to isolates

described from very different habitats, either these taxa may have ecological ranges that are very broad or the conditions in leaves of epiphytic *Tillandsia* species may select for fungi with unusual traits.

Due to the often generalist nature of the Sordariomycetes, it is difficult to speculate what beneficial roles, if any, these fungi may play within their hosts. It is possible that the candidate isolates are species that are largely neutral in a healthy plant, but may become pathogens or primary saprophytes under stress conditions, as has been suggested of fungi isolated from plants in previous studies (Carol 1988; Arnold and Lutzoni 2007; Damm et al. 2012). It is also possible that these fungi may form mutually beneficial associations with their plant hosts, potentially providing heightened resistance to biotic and abiotic environmental stressors, as has been described for other plant-endophyte associations (Kuldau and Bacon 2008). A candidate mutualist found growing as an endophyte in *T. recurvata* had an ITS sequence very similar to a fungus that was described as responsive to nitrogen addition in North Carolina soils (Hesse et. al, 2016). It is possible that endophytic fungi may be involved in epiphytic nutrient dynamics. However, further research would be necessary to elucidate the interactions between endophyte and plant host and to determine whether these interactions confer a direct benefit to the plant. One potential way to address this question could be by inoculating individual endophyte species into endophyte-free *T. recurvata* and assessing their effects on host fitness and stress response. *Tillandsia recurvata* is widespread across Florida, and often occurs in dry, hot, and high salinity environments and exhibits relatively low levels of defoliation by insect herbivory (Frank et al. 2004; Bernal et al. 2005; Larson et al. 2016). These features are often attributed to the epiphyte's elaborate trichomes and low nutrient density tissue, however the presence of beneficial fungal endophytes may provide an extra layer of defense to these plants.

ACKNOWLEDGEMENTS

We wish to thank Dr. Andrew Miller, Dr. David Benzing and Dr. Keith Clay for providing feedback on analyses and drafts and the New College of Florida Foundation for providing financial support. We also thank two anonymous reviewers and the editor for insightful comments on an earlier draft of the manuscript.

Arnold, A. E. (2007). Understanding the diversity of foliar endophytic fungi: Progress, challenges, and frontiers. Fungal Biology Reviews, 21: 51—66.

Arnold, A. E., Henk, D. A., Eells, R. L., Lutzoni, F., & Vilgalys, R. (2007). Diversity and phylogenetic affinities of foliar fungal endophytes in loblolly pine inferred by culturing and environmental PCR. Mycologia, 99: 185—206.

Arnold, A. E., & Lutzoni, F. (2007). Diversity and host range of foliar fungal endophytes: Are tropical leaves biodiversity hotspots? Ecology, 88: 541—549.

Arnold, A. E., Maynard, Z., Gilbert, G. S., Coley, P. D., & Kursar, T. A. (2000). Are tropical fungal endophytes hyperdiverse? Ecology Letters, 3: 267—274.

Arnold, A. E., Mejia, L. C., Kyllo, D., Rojas, E. I., Maynard, Z., Robbins, N., et al. (2003). Fungal endophytes limit pathogen damage in a tropical tree. Proceedings of the National Academy of Sciences of the United States of America, 100: 15649—15654.

Aveskamp, M. M., Verkley, G. J., de Gruyter, J., Murace, M. A., Perello, A., Woudenberg, J. H., et al. (2009). DNA phylogeny reveals polyphyly of Phoma section Peyronellaea and multiple taxonomic novelties. Mycologia, 101: 363—382.

Bacon, C. W., White, J. F. Jr. (1994). Stains, media, and procedures for analyzing endophytes. Biotechnology of Endophytic Fungi of Grasses. 47: 56.

Barbra C. Larson, J. Howard Frank, Martin B. Main, and Ginger M. Allen. (2016). Florida's native bromeliads. Retrieved 8/19, 2017, from http://edis.ifas.ufl.edu/uw205 UF IFAS Extension, CIR 1466: 1—10.

Bell, A. (1983). Dung fungi: an illustrated guide to coprophilous fungi in New Zealand. Victoria University Press. Wellington, New Zealand.

Benzing, D. H. (1980). The biology of the Bromeliads. Mad River Press. Eureka, California, USA.

Benzing, D. H. (2008). Vascular epiphytes: General biology and related biota. Cambridge University Press. New York City, New York, USA.

Benzing, D. H., Henderson, K., Kessel, B., & Sulak, J. (1976). The absorptive capacities of bromeliad trichomes. American Journal of Botany, 63: 1009—1014.

Bernal, R., Valverde, T., and Hernandez-Rosas, L. (2005). Habitat preference of the epiphyte Tillandsia recurvata (Bromeliaceae) in a semi-desert environment in Central Mexico. Botany, 83: 1238—1247.

Brighigna, L., Montaini, P., Favilli, F., & Trejo, A. C. (1992). Role of the nitrogen-fixing bacterial microflora in the epiphytism of Tillandsia (Bromeliaceae). American Journal of Botany, 79: 723—727.

Cai, L., Jeewon, R., & Hyde, K. D. (2005). Phylogenetic evaluation and taxonomic revision of Schizothecium based on ribosomal DNA and protein coding genes. Fungal Diversity. 19: 1—21.

Carroll, G. (1988). Fungal endophytes in stems and leaves: From latent pathogen to mutualistic symbiont. Ecology, 69: 2—9.

Christian, N., Sullivan, C., Visser, N. D., & Clay, K. (2016). Plant host and geographic location drive endophyte community composition in the face of perturbation. Microbial Ecology, 72: 621—632.

Clay, K. (1988). Fungal endophytes of grasses: A defensive mutualism between plants and fungi. Ecology, 69: 10—16.

Clay, K. (1990). Fungal endophytes of grasses. Annual Review of Ecology and Systematics, 21: 275—297.

Cortes-Tolalpa, L., Jiménez, D. J., de Lima Brossi, M. J., Salles, J. F., & van Elsas, J. D. (2016). Different inocula produce distinctive microbial consortia with similar lignocellulose degradation capacity. Applied Microbiology and Biotechnology, 100: 7713—7725.

Costa, I. P. M. W., Assuncao, M. M. C., Lima, T. E. F., Oliveira, R. J. V., & Cavalcanti, M. A. Q. (2012). Checklist of endophytic fungi from tropical regions. Mycotaxon, 119: 494.

Crous, P. W., and J. Z. Groenewald. "Why everlastings don't last." Persoonia: Molecular Phylogeny and Evolution of Fungi, 26: 70.

Damm, U., Cannon, P., Woudenberg, J., Johnston, P., Weir, B., Tan, Y., et al. (2012). The Colletotrichum boninense species complex. Studies in Mycology, 73: 1—36.

De Battista, J., Bouton, J., Bacon, C., & Siegel, M. (1990). Rhizome and herbage production of endophyte-removed tall fescue clones and populations. Agronomy Journal, 82: 651-654.

Deshpande, V., Wang, Q., Greenfield, P., Charleston, M., Porras-Alfaro, A., Kuske, C. R., ... & Tran-Dinh, N. (2016). Fungal identification using a Bayesian classifier and the Warcup training set of internal transcribed spacer sequences. Mycologia, 108: 1—5.

Frank, J. H., Sreenivasan, S., Benshoff, P. J., Deyrup, M. A., Edwards, G. B., Halbert, S. E., Hamon, AB., Lowman, M. D., Mockford, EL., Scheffrahn, R. H., Steck, G. J., Thomas, M. C., Walker, T. J., and Welbourn, W. C. (2004). Invertebrate animals extracted from native Tillandsia (Bromeliales: Bromeliaceae) in Sarasota county, Florida. Florida Entomologist, 87: 176—185.

Gardes, M., & Bruns, T. D. (1993). ITS primers with enhanced specificity for basidiomycetes—application to the identification of mycorrhizae and rusts. Molecular Ecology, 2: 113—118.

Gentry, A. H., & Dodson, C. (1987). Diversity and biogeography of neotropical vascular epiphytes. Annals of the Missouri Botanical Garden, 74: 205—233.

Hesse, C. N., Torres-Cruz, T. J., Tobias, T. B., Al-Matruk, M., Porras-Alfaro, A., & Kuske, C. R. (2016). Ribosomal RNA gene detection and targeted culture of novel nitrogen-responsive fungal taxa from temperate pine forest soil. Mycologia, 108: 1082—1090.

Hoffman, M. T., & Arnold, A. E. (2008). Geographic locality and host identity shape fungal endophyte communities in cupressaceous trees. Mycological Research, 112: 331—344.

Hyde, K., & Soytong, K. (2008). The fungal endophyte dilemma. Fungal Diversity, 33: 173.

Irinyi, L., Serena, C., Garcia-Hermoso, D., Arabatzis, M., Desnos-Ollivier, M., Vu, D., ... & da Cunha, K. C. (2015). International Society

of Human and Animal Mycology (ISHAM)-ITS reference DNA barcoding database—the quality controlled standard tool for routine identification of human and animal pathogenic fungi. Medical Mycology, 53: 313—337.

Kearse, M., Moir, R., Wilson, A., Stones-Havas, S., Cheung, M., Sturrock, S., Buxton, S., Cooper, A., Markowitz, S., Duran, C., Thierer, T., Ashton, B., Mentjies, P., & Drummond, A. (2012). Geneious Basic: an integrated and extendable desktop software platform for the organization and analysis of sequence data. Bioinformatics, 28: 1647—1649.

Kuhnert, E., Fournier, J., Peršoh, D., Luangsa-ard, J. J. D., & Stadler, M. (2014). New Hypoxylon species from Martinique and new evidence on the molecular phylogeny of Hypoxylon based on ITS rDNA and β-tubulin data. Fungal Diversity, 64: 181—203.

Kuldau, G., & Bacon, C. (2008). Clavicipitaceous endophytes: Their ability to enhance resistance of grasses to multiple stresses. Biological Control, 46: 57—71.

Kumar, S., Stecher, G., & Tamura, K. (2016). MEGA7: Molecular evolutionary genetics analysis version 7.0 for bigger datasets. Molecular Biology and Evolution, 33: 1870—1874.

Lacap, D. C., K. D. Hyde, and E. C. Y. Liew (2003). "An evaluation of the fungal 'morphotype' concept based on ribosomal DNA sequences." Fungal Diversity, 12: 53—66.

Marquez, L. M., Redman, R. S., Rodriguez, R. J., & Roossinck, M. J. (2007). A virus in a fungus in a plant: Three-way symbiosis required for thermal tolerance. Science, 315: 513—515.

Menkis, A., & Vasaitis, R. (2011). Fungi in roots of nursery grown Pinus sylvestris: ectomycorrhizal colonisation, genetic diversity and spatial distribution. Microbial Ecology, 61: 52—63.

Mugerwa, T. M., Saleeba, J., & McGee, P. (2013). A variety of melanised root-associated fungi from the Sydney basin form endophytic associations with Trifolium subterraneum. Fungal Ecology, 6: 70—82.

Nguyen, N. H., Song Z., Bates S. T., Branco S., Tedersoo L., Menke J., Schilling J. S., & Kennedy P. G. (2016) FUNGuild: an open annotation tool for parsing fungal community datasets by ecological guild. Fungal Ecology, 20: 241—248.

Nieder, J., Prosperí, J., & Michaloud, G. (2001). Epiphytes and their contribution to canopy diversity. Tropical forest canopies: Ecology and Management, 153: 51—63.

Porras-Alfaro, A., & Bayman, P. (2011). Hidden fungi, emergent properties: Endophytes and microbiomes. Annual Review of Phytopathology, 49: 291—315.

Rodriguez, R., White Jr, J., Arnold, A., & Redman, A. R. A. (2009). Fungal endophytes: Diversity and functional roles. New Phytologist, 182: 314—330.

Ronquist, F., & Huelsenbeck, J. P. (2003). MrBayes 3: Bayesian phylogenetic inference under mixed models. Bioinformatics, 19: 1572—1574.

Shearin, Z. R., Filipek, M., Desai, R., Bickford, W. A., Kowalski, K. P., & Clay, K. (2018). Fungal endophytes from seeds of invasive, non-native Phragmites australis and their potential role in germination and seedling growth. Plant and Soil, 422: 183—194.

Shimomoto, Y., Sato, T., Hojo, H., Morita, Y., Takeuchi, S., Mizumoto, H., ... & Hikichi, Y. (2011). Pathogenic and genetic variation among isolates of Corynespora cassiicola in Japan. Plant Pathology, 60: 253—260.

Strobel, G., & Daisy, B. (2003). Bioprospecting for microbial endophytes and their natural products. Microbiology and Molecular Biology Reviews: MMBR, 67: 491—502.

Thirumalanadhuni, V., and Umamaheswari D. P. (2018).Molecular Characterization and Endophytic Life Style Pattern of Cladosporium uredinicola Derived from Marine Brown Alga Dictyota dichotoma. Journal of Pure and Applied Microbiology, 12: 1661—1666.

Unterseher, M., Gazis, R., Chaverri, P., Guarniz, C. F. G., & Tenorio, D. H. Z. (2013). Endophytic fungi from Peruvian highland and lowland habitats form distinctive and host plant-specific assemblages. Biodiversity and Conservation, 22: 999—1016.

U'Ren, J. M., Miadlikowska, J., Zimmerman, N. B., Lutzoni, F., Stajich, J. E., & Arnold, A. E. (2016). Contributions of North American endophytes to the phylogeny, ecology, and taxonomy of Xylariaceae (Sordariomycetes, Ascomycota). Molecular Phylogenetics and Evolution, 98: 210—232.

Vincent, J., Weiblen, G., & May, G. (2016). Host associations and beta diversity of fungal endophyte communities in New Guinea rainforest trees. Molecular Ecology, 25: 825—841.

Wang, Xiaoming, et al. (2017). "Influence of host tree species on isolation and communities of mycorrhizal and endophytic fungi from roots of a tropical epiphytic orchid, Dendrobium sinense (Orchidaceae)." Mycorrhiza, 27: 709—718.

White, T. J., Bruns, T., Lee, S., & Taylor, J. (1990). Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. PCR Protocols: A Guide to Methods and Applications, 18: 315—322.

Xu, Y. M., Bashyal, B. P., Liu, M. X., Espinosa-Artiles, P., U'Ren, J. M., Arnold, A. E., et al. (2015). Cytotoxic cytochalasins and other metabolites from Xylariaceae sp. FL0390, a fungal endophyte of spanish moss. Natural Product Communications, 10: 1655—1658.

Yang, Y. L., Liu, Z. Y., Cai, L., Hyde, K. D., Yu, Z. N., & McKenzie, E. H. C. (2009). Colletotrichum anthracnose of Amaryllidaceae. Fungal Diversity, 39: 123—146.

Yuan, Z., Chen, Y., & Yang, Y. (2009). Diverse non-mycorrhizal fungal endophytes inhabiting an epiphytic, medicinal orchid (Dendrobium nobile): Estimation and characterization. World Journal of Microbiology and Biotechnology, 25: 295.