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Characterizing Culturable Bacterial Endophytes of Five Lycopodiaceae Species

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Abstract.—Microbial endophytes are integral factors in plant evolution, ecology, and physiology. However, the endophyte communities across all major plant lineages have yet to be characterized, and data are particularly scarce for lycophytes. Here we used a culture-based approach to survey the diversity of endophytic bacteria in five sympatric Lycopodiaceae species in central New York. The most notable endophyte isolated from this study is a bacterial species *Allobranchiibius huperziae*, which was only recently described from the roots of *Huperzia serrata* in China. The fact that the same endophyte was also found in our North American samples suggest a possible specific association with Lycopodiaceae species. The data and cultures from this study provide an important foundation for future metagenomic and functional studies to characterize better the diversity and significance of plant endophytes.

Key words.—*Allobranchiibius huperziae*, microbiome, symbiosis, 16S

Every macro-organism lives in close association with countless micro-organisms and plants are no exception. Some famed plant symbionts, such as arbuscular mycorrhizal fungi (AMF), are considered critical in the establishment of early land plants and their continued success (Heckman *et al.*, 2001; Taylor and Krings, 2005; Delaux *et al.*, 2012; Selosse *et al.*, 2015). Meanwhile, many plants harbor nitrogen-fixing bacteria, *e.g.*, *Rhizobium*, *Frankia*, and *Nostoc*, which not only influence plant growth, but also the surrounding environment (Santi, Bogusz, and Franche, 2013).

Yet, these well-known, specialized symbioses are not the only ones that impact plant growth and ecological interactions. In fact, communities of bacteria, archaea, fungi, and other eukaryotic microbes can live within healthy plant tissues (Berg *et al.*, 2016). These microbes, called "endophytes," make up communities that are often highly diverse, even within the same host species and geographic location (Higgins *et al.*, 2007; U'Ren *et al.*, 2010; Nelson and Shaw, 2019). This diversity is reflected in the various roles these endophytes fill within their host plant—they may be mutualistic, commensalistic, pathogenic, or latently saprotrophic, and may act to influence plant growth and interactions

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with other organisms, including pathogens (Porras-Alfaro and Bayman, 2011). Thus, plant-microbial interactions are undoubtedly underestimated but integral factors in shaping ecosystem structuring (Rudgers, Koslow, and Clay, 2003; Afkhami and Strauss, 2016; Aguilar-Trigueros and Rillig, 2016).

Regardless of their importance, microbial symbiotic communities have not been well characterized for all major lineages of land plants. Studies on the microbiomes of Lycopodiaceae are relatively scarce. Those that do exist focus mostly on fungal endophytes, largely employing light microscopy (Freeberg 1962; Duckett and Ligrone, 1992; Gemma, Koske, and Flynn, 1992; Schmid and Oberwinkler, 1993; Treu *et al.*, 1996; Zhi-Wei, 2000; Fernández, Messuti, and Fontenla, 2008; Kessler *et al.*, 2010a, b; Zubek *et al.*, 2010; Muthukmar and Prabha, 2013; Lehnert, Krug, and Kessler, 2017) and sometimes utilizing modern sequencing technologies (Winther and Friedman, 2008; Benucci *et al.*, 2020). Some studies have described culturable fungal endophytes of *Huperzia serrata* (Thunb.) Trevis., which can produce huperzine A, a bioactive cholinesterase inhibitor of pharmaceutical interest for treating Alzheimer's disease (Zhu *et al.*, 2010; Wang *et al.*, 2011; Le *et al.*, 2019).

Even fewer studies have explored the bacterial communities living in association with Lycopodiaceae species. Benucci *et al.*, (2020) utilized Illumina amplicon-sequencing technologies to characterize the bacterial communities of roots of nine Lycopodiaceae species (and one *Selaginella* species) in New Zealand. They identified 551 operational taxonomic units (OTUs) belonging to 28 classes of bacteria, with Proteobacteria, Acidobacteria, Actinobacteria, and Bacteriodetes being the most prominent. However, they did not sterilize the surface of the root material examined, thus there is no way of knowing which OTUs lived on the external surface of the roots and which are endophytes. Furthermore, Ghosh *et al.* (2016) explored the influence on plant growth of *Burkholderia* spp. living on the surface of *Lycopodium cernuum* L. rhizoids. Finally, Ai *et al.* (2017) described a novel genus and species of bacterial endophyte (*Allobranchiibius huperziae*) from *H. serrata*.

No study, to date, has specifically characterized the endophytic bacterial communities of Lycopodiaceae species. Could there be specialized symbioses between bacterial species and Lycopodiaceae species? Could these relationships impact plant growth, secondary chemical production, or interactions with other organisms (*e.g.*, fungal symbionts, herbivores, pathogens), and evolution? Our study aims to take a step towards answering these questions by characterizing and comparing the culturable endophytic bacteria in five Lycopodiaceae species found in central New York.

Materials and Methods

Plant collection location.—We collected *Huperzia lucidula* (Michx.) Trevis., *Spinulum annotinum* (L.) A. Haines, *Lycopodium clavatum* L., *Diphasiastrum digitatum* (Dill. ex A. Braun) Holub, and *Dendrolycopodium dendroideum* (Michx.) A. Haines from a sympatric population in Shindagin Hollow State Forest (GPS coordinates: 42.33707, -76.33905). The soil type of this location is

well-draining acidic silt-loam. Aerial and subterranean tissues from one plant of each species were collected once a week, for four consecutive weeks, in June 2019 (6/4, 6/10, 6/17, 6/24).

Plant sampling and endophyte isolation.—Intact plants were brought to the lab and refrigerated until they could be cleaned and processed (no more than 48 hours after collection). They were rinsed with deionized (DI) water to remove large debris. We divided plants into three categories of tissue: aerial, subterranean shoot, and subterranean root. Each tissue type was transferred to a sterile hood, rinsed again in sterile DI water to remove finer debris, and chopped into $2mm^2$ segments. Segments were surface sterilized by submerging them in 95% ethanol for 30 seconds, 10% bleach (with a few drops of 1% Tween 20) for two minutes, and 70% ethanol for two minutes (based on Arnold, 2002). Segments were allowed to air dry under sterile air on autoclaved filter paper. These protocols have been shown to eliminate epiphytic microbes (Schulz *et al.,* 1993; Arnold, 2002), but to ensure this, the exterior of one piece from each batch was rubbed on a lysogeny broth (LB) plate. If anything grew on the control plate, the entire batch was disregarded as contaminated. For each sample, ten segments of aerial tissue, five segments of subterranean shoot, and five segments of subterranean root were placed onto LB plates. Plates were sealed with parafilm, left at room temperature, and regularly checked for growth. Subcultures were made to separate different morphologies.

Culture identification.—Once pure cultures were obtained, we used a sterile pipet tip to transfer bacteria into a 0.2 mL tube for colony PCR with 27F (5′ AGAGTTTGATCMTGGCTCAG 3′; Lane *et al.*, 1991) and 1492Rl (5′ GGTTACCTTGTTACGACTT 3′; Turner *et al.*, 1999) primers, with the following recipe: 12.85 µL PCR water; 5 µL 5x GoTaq Flexi buffer; 2.5 µL dNTP's (1mM each); 2.5 µL BSA; 1µL 27F primer; 1µL 1492Rl primer; 0.15 µL GoTaq (Promega). The thermocycler program was set as: (1) 95°C for 4 minutes; (2) 95°C for 1 minute; (3) 60°C for 30 seconds; (4) 72°C for 1 minute 45 seconds; (5) repeat steps (2), (3), and (4) nine times, reducing step (3) by 1°C each time; (6) 95°C for 1 minute; (7) 50°C for 30 seconds; (8) 72°C for 1 minute 45 seconds; (9) repeat steps (6), (7), and (8) 17 times; (10) 72°C for five minutes 40 seconds. If colony PCR failed, DNA extractions were carried out based on Wilson (2001) and subject to the same PCR protocol as above. We cleaned all successful amplification products using an ExoSAP protocol (New England BioLabs) and submitted them to Eurofins for Sanger sequencing. Forward and reverse Sanger sequences were joined using Geneious Prime (Version 2019.2.1) and compared to the NCBI GenBank database using BLAST searches. Species names were assigned if sequences matched to only one species with at least 97% identity and full coverage. Unidentifiable taxa were grouped together if sequences were at least 97% similar (based on VSEARCH, Rognes *et al.,* 2016) and given an identifier based on higher level taxonomic ranks. The 16S rRNA sequences were deposited in GenBank and assigned accession numbers: MZ994597 through MZ994648.

Phylogenetic inference.—To support our taxonomic identifications, we created a phylogenetic tree with our 16S endophyte sequences and reference

TABLE 1. Number of cultures from each plant species and tissue type

	Aerial shoot	Subterranean shoot	Subterranean root	TOTAL
De. dendroideum	1	10	4	15
Di. digitatum	2	7	12	21
H. lucidula	29	20	5	54
L. clavatum	3	0	21	24
S. annotinum	10	14	19	43
TOTAL	45	51	61	157

16S sequences from GenBank. Sequence alignment and maximum-likelihood-based phylogenetic inference were done using PASTA (Mirarab *et al.*, 2015) with the default setting.

RESULTS

Of the 800 plates, 161 had bacterial growth (20.1% culture rate), of which 157 were identified (19.6% isolation rate; Table 1, Appendix 1). These 157 cultures belonged to 52 distinct OTUs (Table 2, Fig. 1) in 17 families. A total of 23 bacterial OTUs were isolated more than once. *Huperzia lucidula* had the greatest culture number and species richness, followed by *Spinulum annotinum*, *Lycopodium clavatum*, *Diphasiastrum digitatum*, and *Dendrolycopodium dendroideum*. Although aerial tissue had the fewest cultures, it had a higher richness than either subterranean shoots or roots, owing to the high richness in *H. lucidula* aerial tissue.

The phylogenetic trees (Figs. 2 and 3) generated overall support our taxonomic identifications. They also suggest that the two sequences which were only identifiable as bacteria ("Bacteria sp. 1" and "Bacteria sp. 2") belong in Bacillaceae, and are most closely related to *Lysinibacillus*.

DISCUSSION

Bacterial culturing rate and community composition.—In this study, 20.1% of the 800 surface sterilized plant segments had bacterial growth. No previous studies explored the culturable bacterial endophyte communities of Lycopodiaceae, and a canvassing of studies conducted on other plants

TABLE 2. Number of bacterial species identified from each plant species and tissue type

	Aerial shoot	Subterranean shoot	Subterranean root	TOTAL
De. dendroideum	1	7	4	9
Di. digitatum	2	5	8	12
H. lucidula	22	14	4	34
L. clavatum	2	0	14	15
S. annotinum	7	7	9	17
TOTAL	29	24	23	52

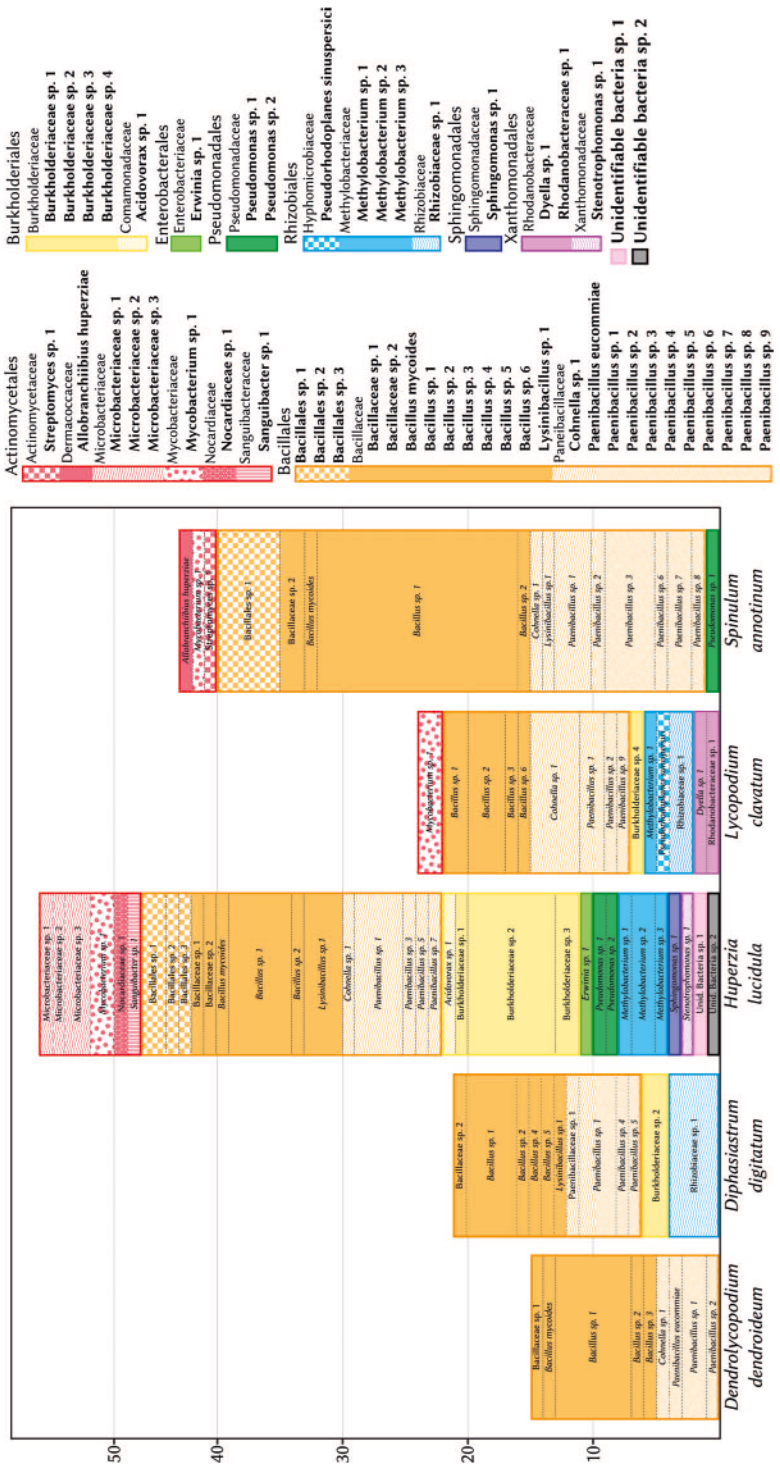

FIG. 1. Bacterial OTU's (bolded in legend) found in each plant species.

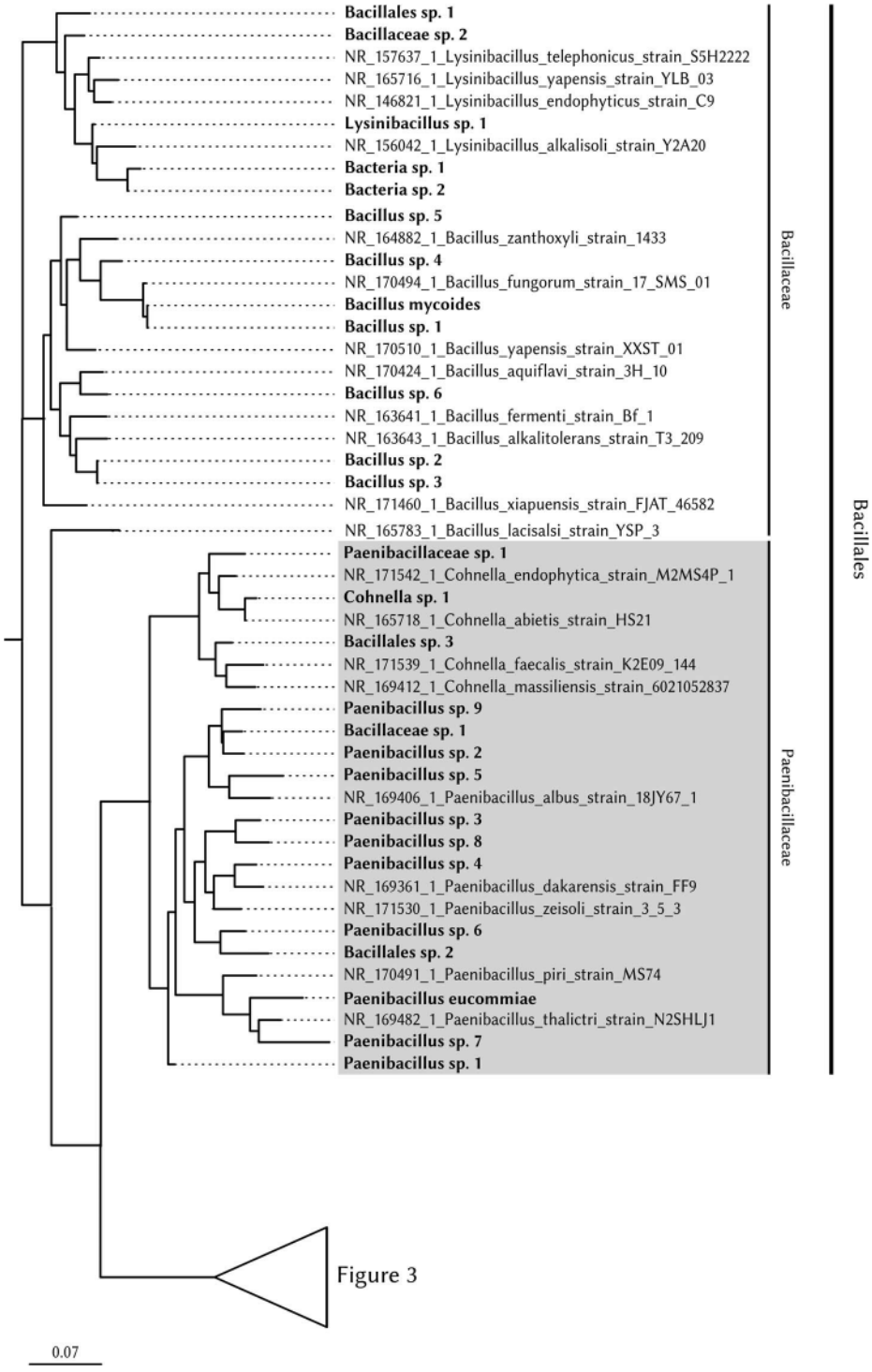

FIG. 2. Maximum likelihood phylogeny of the bacterial OTUs and confirmed reference sequences.

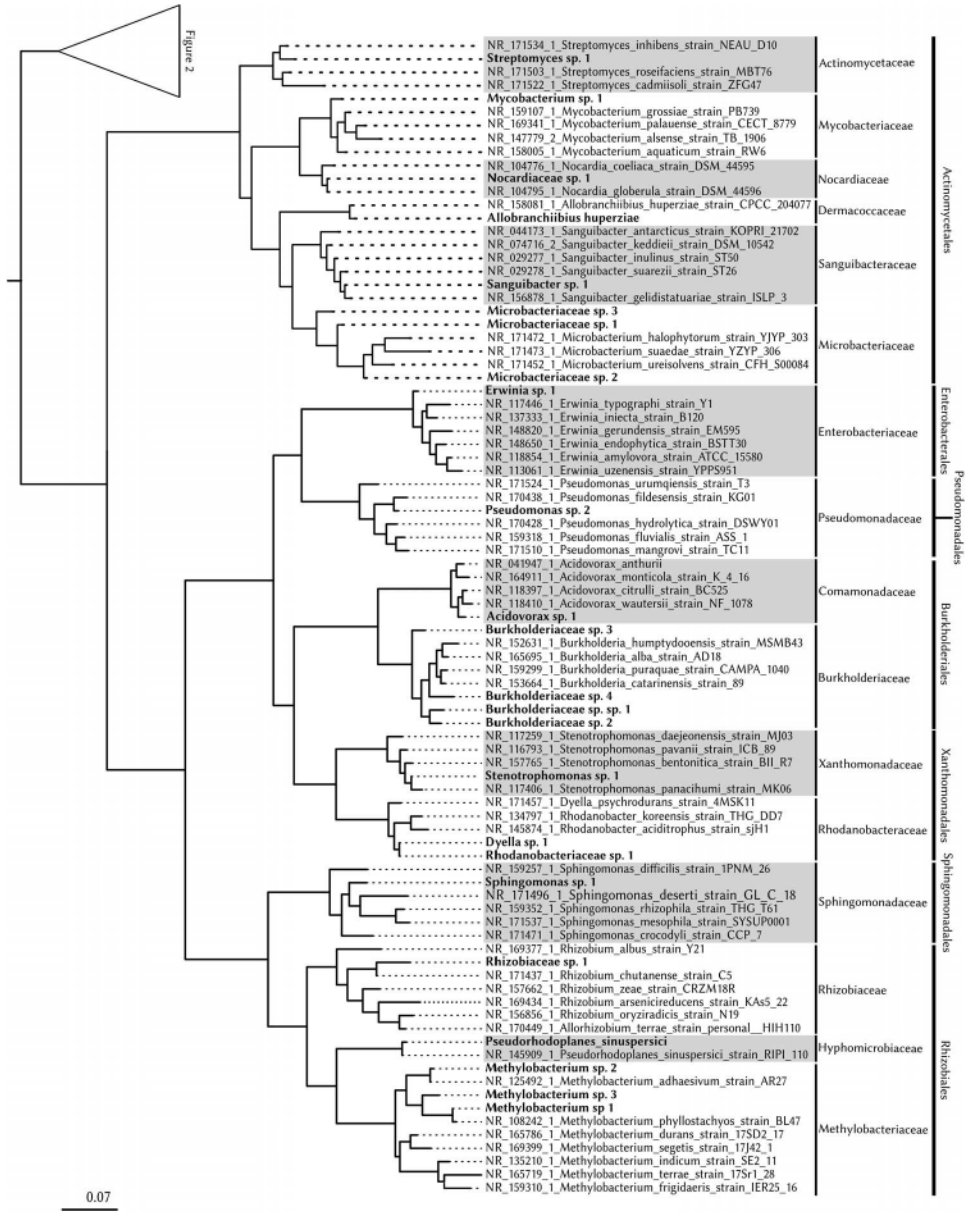

FIG. 3. Maximum likelihood phylogeny of the bacterial OTUs and confirmed reference sequences, *continued*.

revealed a distinct lack of reporting of bacterial culture rates. Thus, it is difficult to determine if 20.1% represents a relatively high or low culture rate.

Potential roles of isolated bacterial endophytes.—Bacterial endophytes can have a wide range of effects on their plant hosts, but specific functional studies

are needed to accurately describe the interactions between lycophytes and their endophytes. However, speculation can be made based on previously described interactions. For example, *Bacillus mycoides* may act to control pathogens in sugar beets (Bargabus *et al.,* 2002). Yet, for the many other unidentifiable *Bacillus spp.*, Bacillaceae *spp.*, and Bacillales *spp.* isolated in this study, it is impossible to hypothesize as to how they may impact their hosts. The same is true for the numerous unidentifiable *Paenibacillus spp.,* Paenibacillaceae, Microbacteriaceae, Nocardiaceae, and Rhodanobacteraceae.

Some *Pseudomonas spp.* promote plant growth via multiple mechanisms, including auxin production and increasing phosphate and nitrogen availability (Gnanamanickman, 2007; Oteino *et al.,* 2015). Similarly, there is evidence that some species of *Cohnella*, *Lysinibacillus,* and Burkholderiaceae promote plant growth either through phytohormone production or by increasing nutrient availability (Niang *et al.,* 2018; Gnanamanickman, 2007; Naureen *et al.,* 2017; Shabanamol *et al.,* 2017). *Sanguibacter spp.* may increase resistance to cadmium (Rajkumar, Ae, and Freitas., 2009). On the other hand, many *Acidovorax spp.* and *Erwinia spp.* are pathogens (Gnanamanickman, 2007).

Two genera isolated here are of particular interest as lycophyte symbionts: *Methylobacterium* and *Allobranchiibius*. Like some of the other bacterial taxa described above, *Methylobacterium spp.* may impact plant growth by producing phytohormones (like cytokinins and auxins) and increasing nitrogen availability (Holland, 1997; Kutschera, 2007). Additionally, *Methylobacterium spp.* have been hypothesized to be ubiquitous co-evolving symbionts across the land plant phylogeny (Holland, 1997). This hypothesis warrants further investigation. *Allobranchiibius* is represented by a single species, *A. huperziae,* isolated from *Huperzia serrata* roots in China (Ai *et al.*, 2017). We also found it in *S. annotinum* aerial tissue in this study, however its activity in the plant is unknown. Given that it has only been reported as a lycophyte endophyte, in two geographically disparate studies, further investigation should be conducted to determine if it is a lycophyte-specific symbiont and how it might be interacting with the plants.

Limitations of this study.—Any study on culturable endophyte communities inherently carries significant bias. First, microbial species abundances calculated from culturing have limited, if any, meaning. It is simply not possible to be sure that what is growing is representative of the communities that exist inside of a living plant. With our culturing methods, any obligate symbionts or obligate anaerobes would be missed entirely and fastidious bacteria may not have been able to grow. Thus, it is not possible to make confident conclusions regarding diversity indices and other statistical analyses. Second, the low number of cultures in this study did not allow for statistical analyses.

Future directions.—These culture data, while a valuable first step in characterizing microbial endophyte communities, need to be accompanied by a next-generation amplicon-sequencing dataset to characterize more thoroughly the complete endophyte community. We anticipate that the

results of an amplicon-seq study on Lycopodiaceae would yield a far greater number of OTU's, as well as more reliable species abundance data. The increase in statistical power inherent in amplicon-seq datasets, coupled with expanded sampling, would also allow for exploration of host and tissue specificity.

Furthermore, to fully characterize the microbiome of these plants, the unidentifiable OTUs from this study need to be fully described and given taxonomic assignment. Future large-scale functional assays on all OTUs are needed to determine how these endophytes interact with both their host plant and each other. These endophytes and their exudates may also possess utility in other applications, such as pest/pathogen management in agriculture or pharmaceutical development.

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APPENDIX 1.—Number of occurrences of each bacterial OTU in each plant tissue type

Bacteria	*De. dendroideum* aerial shoot	*De. dendroideum* subterranean root	*De. dendroideum* subterranean shoot
Acidovorax sp. *1*	0	0	0
Allobranchiibius huperziae	0	0	0
Bacillaceae sp. 1	0	0	1
Bacillaceae sp. 2	0	0	0
Bacillales sp. *1*	0	0	0
Bacillales sp. 2	0	0	0
Bacillales sp. 3	0	0	0
Bacillus mycoides	0	0	1
Bacillus sp. *1*	1	1	4
Bacillus sp. *2*	0	1	0
Bacillus sp. *3*	0	0	1
Bacillus sp. *4*	0	0	0
Bacillus sp. *5*	0	0	0
Bacillus sp. *6*	0	0	0
Burkholderiaceae sp. 1	0	0	0
Burkholderiaceae sp. 2	0	0	0
Burkholderiaceae sp. 3	0	0	0
Burkholderiaceae sp. 4	0	0	0
Cohnella sp. *1*	0	1	0
Dyella sp. *1*	0	0	0
Erwinia sp. *1*	0	0	0
Lysinibacillus sp. *1*	0	0	0
Methylobacterium sp. *1*	0	0	0
Methylobacterium sp. *2*	0	0	0
Methylobacterium sp. *3*	0	0	0
Microbacteriaceae sp. 1	0	0	0
Microbacteriaceae sp. 2	0	0	0
Microbacteriaceae sp. 3	0	0	0
Mycobacterium sp. *1*	0	0	0
Nocardiaceae sp. 1	0	0	0
Paenibacillaceae sp. 1	0	0	0
Paenibacillus eucommiae	0	0	1
Paenibacillus sp. *1*	0	1	1
Paenibacillus sp. *2*	0	0	1
Paenibacillus sp. *3*	0	0	0
Paenibacillus sp. *4*	0	0	0
Paenibacillus sp. *5*	0	0	0
Paenibacillus sp. *6*	0	0	0
Paenibacillus sp. *7*	0	0	0
Paenibacillus sp. *8*	0	0	0
Paenibacillus sp. *9*	0	0	0
Pseudomonas sp. *1*	0	0	0
Pseudomonas sp. *2*	0	0	0
Pseudorhodoplanes sinuspersici	0	0	0
Rhizobiaceae sp. 1	0	0	0
Rhodanobacteraceae sp. 1	0	0	0
Sanguibacter sp. *1*	0	0	0
Sphingomonas sp. *1*	0	0	0
Stenotrophomonas sp. *1*	0	0	0
Streptomyces sp. *1*	0	0	0
Unidentifiable Bacteria sp. 1	0	0	0
Unidentifiable Bacteria sp. 2	0	0	0

APPENDIX 1.—Extended.

Di. digitatum aerial shoot	*Di. digitatum* subterranean root	*Di. digitatum* subterranean shoot	*H. lucidula* aerial shoot	*H. lucidula* subterranean root	*H. lucidula* subterranean shoot
0	0	0	1	0	0
0	0	0	0	0	0
0	0	0	1	0	0
0	1	0	0	0	1
0	0	0	0	0	2
0	0	0	1	0	0
0	0	0	1	0	0
0	0	0	0	0	1
0	3	1	1	0	4
0	1	0	0	1	0
0	0	0	0	0	0
0	1	0	0	0	0
0	0	1	0	0	0
0	0	0	0	0	0
0	0	0	1	0	0
0	0	2	5	0	2
0	0	0	0	0	2
0	0	0	0	0	0
0	0	0	0	0	1
0	0	0	0	0	0
0	0	0	1	0	0
0	1	0	1	2	0
0	0	0	1	0	0
0	0	0	2	0	0
0	0	0	0	0	1
0	0	0	1	0	0
0	0	0	1	0	0
0	0	0	2	0	0
0	0	0	1	0	1
0	0	0	1	0	0
1	0	0	0	0	0
0	0	0	0	0	0
1	0	2	2	1	1
0	0	0	0	0	0
0	0	0	0	0	1
0	1	0	0	0	0
0	1	0	0	1	0
0	0	0	0	0	0
0	0	0	1	0	0
0	0	0	0	0	0
0	0	0	0	0	0
0	0	0	1	0	0
0	0	0	0	0	1
0	0	0	0	0	0
0	3	1	0	0	0
0	0	0	0	0	0
0	0	0	1	0	0
0	0	0	1	0	0
0	0	0	1	0	0
0	0	0	0	0	0
0	0	0	0	0	1
0	0	0	0	0	1

APPENDIX 1.—Extended.

L. clavatum aerial shoot	*L. clavatum* subterranean root	*L. clavatum* subterranean shoot	*S. annotinum* aerial shoot	*S. annotinum* subterranean root	*S. annotinum* subterranean shoot
0	0	0	0	0	0
0	0	0	1	0	0
0	0	0	0	0	0
0	0	0	2	0	0
0	0	0	0	3	2
0	0	0	0	0	0
0	0	0	0	0	0
0	0	0	0	1	0
1	1	0	2	9	5
0	3	0	1	0	0
0	1	0	0	0	0
0	0	0	0	0	0
0	0	0	0	0	0
0	1	0	0	0	0
0	0	0	0	0	0
0	0	0	0	0	0
0	0	0	0	0	0
0	1	0	0	0	0
0	4	0	1	0	0
0	1	0	0	0	0
0	0	0	0	0	0
0	0	0	0	1	0
1	0	0	0	0	0
0	0	0	0	0	0
0	0	0	0	0	0
0	0	0	0	0	0
0	0	0	0	0	0
0	0	0	0	0	0
0	2	0	0	0	1
0	0	0	0	0	0
0	0	0	0	0	0
0	0	0	0	0	0
0	2	0	2	1	0
0	1	0	0	1	0
0	0	0	0	1	3
0	0	0	0	0	0
0	0	0	0	0	0
0	0	0	1	0	0
0	0	0	0	1	1
0	0	0	0	1	0
0	1	0	0	0	0
0	0	0	0	0	1
0	0	0	0	0	0
0	1	0	0	0	0
1	1	0	0	0	0
0	1	0	0	0	0
0	0	0	0	0	0
0	0	0	0	0	0
0	0	0	0	0	0
0	0	0	0	0	1
0	0	0	0	0	0
0	0	0	0	0	0