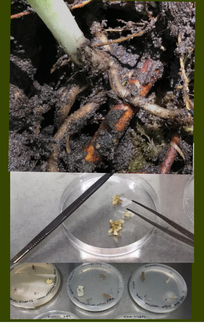

Endofungal bacteria isolated from mycorrhizal fungi in a North American terrestrial orchid

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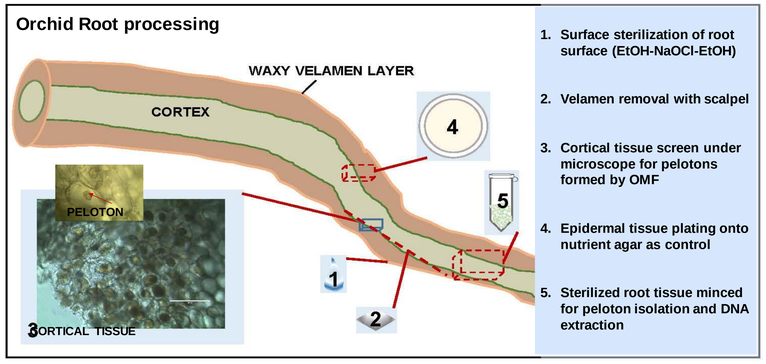
Introduction

Orchidaceae is among the largest and most diverse plant families consisting of approximately 30,000 orchid species worldwide with the majority of these being rare in nature because of their specialized ecological niches. To gain a mechanistic understanding of their complex ecological interactions, and to inform *in-situ* conservation, it is important to first discover and describe the diversity of organisms that orchids associate with. All orchids form obligate mycorrhizae with orchid mycorrhizal fungi (OMF), and accordingly, OMF diversity and interactions are commonly studied. However, endophytic bacteria are also known to enhance biomass and survival of orchid seedlings in vitro, suggesting another important symbiotic niche for orchids. Yet, orchid associated bacteria remain understudied, especially with respect to their role as mycorrhizal helper bacteria (MHB). We report here the first results of our investigation of culturable Endophytic Fungi (EF) and Endophytic Bacteria (EB), with specific focus on OMF and Endofungal Bacteria (EFB). We selected a North American terrestrial orchid, *Platanthera chapmanii*, for our studies, and sampled its roots across two years from two disjunct occurrences in Texas and Florida. Cultured fungal and bacterial isolates were identified by amplifying and sequencing the nrITS and 16S regions, respectively. We also ascertained the endosymbiotic status of bacteria within fungal hyphae by using scanning electron microscopy (SEM). To the best of our knowledge, **ours is the first report of isolation of endofungal bacteria from orchid mycorrhizal fungi**.

Methods



Symbionts targeted for culture-based isolation

Peloton-forming OMF and their EFB (EF and EB were simultaneously cultured)

Isolation Media

OMF and EF – Modified Melin-Norkrans (MMN) and Potato Dextrose Agar (PDA)
EB – Czapek Agar (CZ) and King's B Media (KB)

Endofungal bacterial isolation

PDA with Ampicillin with Streptomycin
Malt Extract Agar (MEA) with Cycloheximide

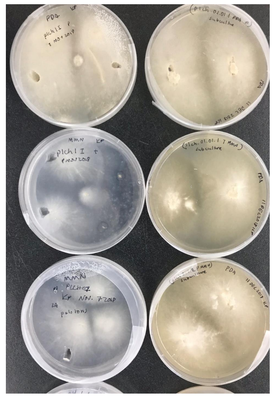
Visualization of hyphae

Scanning electron microscopy (SEM) of EF and OMF from above

DNA-based identification of fungi and bacteria

Amplification and sequencing of fungal nuclear ribosomal ITS region with primer pairs ITS1/ITS4OF and ITS1/ITS4-Tul

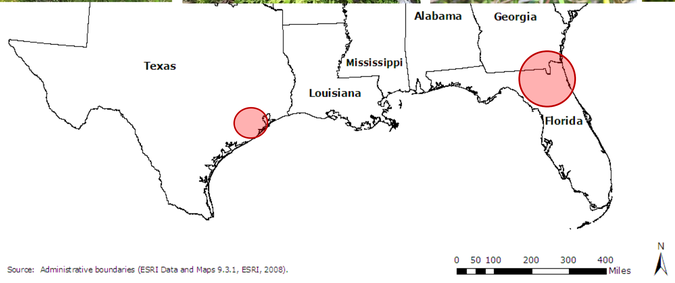
Amplification of the bacterial16S region with primer pairs 27F/1492R and 515F/916R



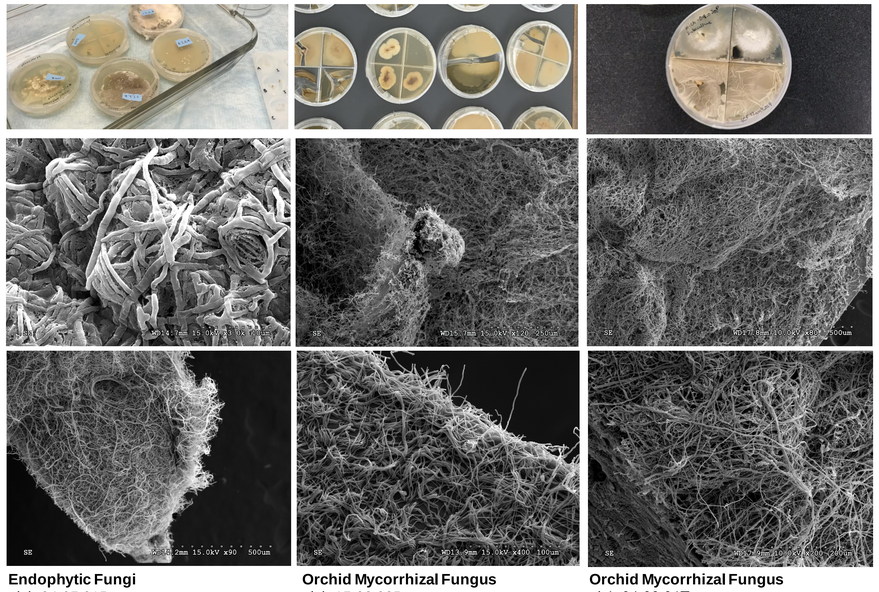
Research Questions

1. Are endofungal bacteria present and culturable from endophytic or orchid mycorrhizal fungi?

2. Do plants of *Platanthera chapmanii* from widely separated, disjunct populations host distinct orchid mycorrhizal fungal and / or endofungal bacterial communities?



SEM confirmation of EFB



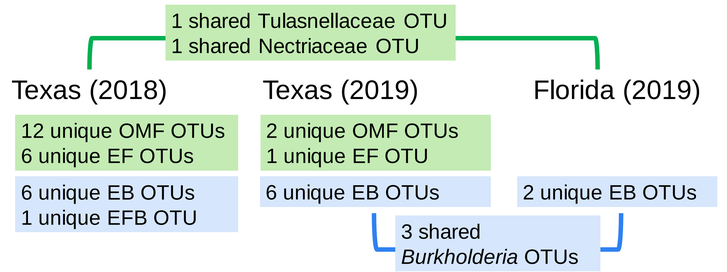
Endophytic Fungi
plch.04.05.01P
Fusarium coffeatum

Orchid Mycorrhizal Fungus
plch.15.02.02P
Uncultured Ceratobasidiaceae

Endofungal bacteria
[b.39.01(plch.15.02.01 on MEA)]
***Bacillus subtilis* strain 1-17**

Orchid Mycorrhizal Fungus
plch.04.03.01T
Uncultured Tulasnellaceae

Endofungal bacteria
[b.31.01(plch.04.03.01T on MEA)]
Bacillus subtilis



OMF -- Orchid Mycorrhizal Fungi; EF – Endophytic Fungi; EB -- Endophytic Bacteria;; EFB – Endofungal Bacteria; OTU – Operational Taxonomic Unit

Table 1. Diversity of EF, OMF, EB, and EFB OTUs recovered from *Platanthera chapmanii.* Sequences obtained from cultures were clustered at 97% similarity threshold into 23 fungal and 15 Bacterial OTUs.

Location	Year	Plant#	Fungal ID (number of OTUs)	Bacterial ID (number of OTUs)
Texas	2018	01	Tulasnellaceae (3)	
		02	Nectriaceae (1), Umbelopsidomycetes (1)	*Bacillus* sp. (1)
		04	Tulasnellaceae (6), Fungi (1)	*Bacillus* sp. (1)
		06		*Bacillus* sp. (2)
		07		*Burkholderia* sp. (1)
		08	Tulasnellaceae (1)	
		10	Tulasnellaceae (1)	
		11		*Bacillus* sp. (1)
		12	Fungi (1)	
		13	Ascomycota (1)	
		14	Nectriaceae (1), Hypocrealles (1)	
		15	Ceratobasidiaceae (1)	
	2019	03		*Burkholderia* sp. (1)
		04		*Bacillus* sp. (1)
		05		*Burkholderia* sp. (1), Bacteria (1)
		07	Umbelopsidomycetes (1)	*Burkholderia* sp. (1), Bacteria (1)
		10	Sodariomycetes (1)	*Burkholderia* sp. (1)
		11	Ceratobasidiaceae (1)	*Bacillus* sp. (1)
		12		
		14		*Paenibacillus* sp. (1)
		15	Ceratobasidiaceae (1)	
		16		*Burkholderia* sp. (1)
Florida	2019	01		*Bacillus* sp. (1), *Burkholderia* sp.(1)
		03		*Burkholderia* sp. (1)
		04		*Burkholderia* sp. (1)
		05		*Burkholderia* sp. (1)
		10		*Burkholderia* sp. (1)
		11		*Burkholderia* sp. (1)
		13	Tulasnellaceae (1)	*Burkholderia* sp. (1)
		14	Nectriaceae (1)	*Burkholderia* sp. (3)

Summary and Conclusions

- Our study is the first to isolate endofungal bacteria from orchid mycorrhizal fungi (OMF).
- In this ongoing work, we have thus far obtained 95 individual fungal cultures (EF and OMF combined), 105 individual EB cultures, and isolated and cultured 21 EFB. Of these, acceptable amplification and sequencing (>200 bp) is complete for 15 EF, 23 OMF, 25 EB, and 16 EFB.
- Altogether, we recovered 16 isolates of endofungal bacteria (EFB) from OMF (6) and EF (10). Taxonomically, the 16 isolates of EFB clustered into 1 *Burkholderia* OTU and 2 *Bacillus* OTUs. The 23 OMF isolates clustered into 12 Tulasnellaceae OTUs and 2 Ceratobasidiaceae OTUs.
- Further, the 15 EF isolates clustered into 3 unspecified fungal OTUs, 1 Hypocrealles OTU, 2 Nectriaceae OTUs, 1 Ascomycota OTU, 1 Umbelopsidomycetes OTU, and 1 Sodariomycetes OTU. The 25 EB isolates were clustered into 7 *Burkholderia* OTUs, 2 *Bacillus* OTUs, 1 *Paenibacillus* OTU, and 2 unspecified bacterial OTUs.
- Our research will ultimately steer an understanding of specialized, tripartite interactions of OMF, their endofungal bacterial symbionts, and orchids.

Acknowledgements

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