

Conservation of North America's native *Vanilla* (Orchidaceae) species: Population Structure,  
Isolation & Identification of Fungal Endophytes, and Mycobiome Metabarcoding

By

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**Dedication:**

“If there were ever a time to dare, to make a difference,  
to embark on something worth doing, it is now.”

– Unknown

“UNLESS someone like you cares a whole awful lot,  
nothing is going to get better. It’s not.” – The Lorax

“One raindrop raises the sea.”

– James Gurney

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**Universal Abstract:**

The orchid genus *Vanilla*, subfamily Vanilloideae, is distributed globally throughout the tropics with over 100 species. Six species inhabit southern Florida and only four are considered native: *Vanilla barbellata*, *V. dilloniana*, *V. mexicana*, and *V. phaeantha*. *Vanilla dilloniana* and *V. mexicana* are already considered locally extinct in Florida, while *V. barbellata* and *V. phaeantha*'s distribution is limited to protected areas. All four of these species are considered rare, threatened, and endangered in Florida. Their habitats face numerous environmental threats including loss of pollinators, seed dispersers, and habitat fragmentation, as well as anthropogenically driven threats such as habitat degradation and the effects of climate change like sea-level rise. Considering these escalating threats conservation efforts are needed now more than ever. Yet, little is known about their genetic structure, mycobionts, and mycobiont compositions. Through genotyping-by-sequencing (GBS) we determined genetic diversity, evidence of gene flow, and low heterozygosity of disjunct populations of sampled *Vanilla barbellata* populations. Additionally, we isolate and identify culturable endophytic fungi to ascertain their diversity and to acquire fungi with the potential to germinate orchid seeds. We were able to isolate and identify a wide variety of culturable fungi, from latent pathogen and non-mycorrhizal fungi to dark septate endophytes. Furthermore, with next-generation sequencing we explore and identify the composition and diversity of the mycobiomes of *Vanilla barbellata* and *V. phaeantha* and their different compartments. Counter to part of our initial hypotheses, we did not detect substantial differences of the mycobiome, between the two species, yet as we anticipated significant differences are evident between plant compartments. Data acquired in this study provide a foundational baseline for *Vanilla* conservation efforts that integrate their ecology, with a focus on the declining habitats of southern Florida. Accordingly, future

conservation efforts that consider the relocation of these *Vanilla* species via assisted migration or *ex situ* cultivation should consider the fungal community compositions found here. Adamantly and with the support of these findings, we recommend immediate conservation efforts focused on symbiotic seed germination and assisted migration of these imperiled species to habitats beyond reach of the threats of climate change.

**Chapter I: Genetic diversity in the endangered hemi-terrestrial orchid *Vanilla barbellata* (Orchidaceae) in south Florida (U.S.): implications for conservation.**

**Abstract:**

Genomics-based diversity analysis of natural populations is an indispensable tool for enabling informed, advanced, lineage-specific conservation strategies for threatened and endangered species, such as *Vanilla barbellata* (Orchidaceae). This native leafless orchid of south Florida faces several environmental threats including rising sea-levels as a result of climate change. A total of 115 individuals from two Floridian populations and three Caribbean populations were sampled. A *V. planifolia* draft genome was used to identify a filtered set of 12,825 single nucleotide polymorphism (SNP) markers generated via Genotyping-By-Sequencing (GBS). Principal Component Analysis (PCoA) and fastStructure provided resolution of GBS markers confirming distinct populations. Discriminant Principal Components (DAPC), Neighbor-Joining (NJ) tree, diversity statistics and the Fixation Index (Fst) were used to refine relationships among and within populations. Genomic diversity was identified among distinct populations. Floridian populations in the Everglades National Park and the Florida Keys are significantly different and exhibit low levels of heterozygosity. In contrast, populations of the Guánica State and Susúa State Forests of Puerto Rico have relatively high heterozygosity, but with no discernible gene flow with populations outside of Puerto Rico. Finally, the U.S. Virgin Islands population is characterized by an even higher level of heterozygosity, but no gene flow with other populations is evident. These results suggest that *ex situ* conservation of this species is

needed because of increased isolation and environmental change and will be best served by targeted botanical collection that captures the greatest level of existing diversity possible.

**Key Words:** Genotyping-By-Sequencing, next-generation sequencing, Orchidaceae, *Vanilla barbellata*, population genomics

## Introduction:

The Orchidaceae is the largest angiosperm family with approximately 20,000 to 30,000 species, many orchid species are among the most endangered plants on the planet (Dressler, 1993). They are highly vulnerable to habitat disturbances, reduction, and fragmentation, and the effects of climate change due to their complicated life histories and intimate interactions with specialized pollinators and fungi. Furthermore, due to centuries of over collecting for ornamental and traditional medicinal trades, all Orchidaceae species were listed on Appendix II of the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES), while many others, including entire genera such as *Paphiopedilum*, have been listed on Appendix I (CITES, 2021). National and State legislation provide further legal protection in several nations, but poaching has driven certain orchid species to the edge of extinction, even within nature reserves. As a result, significant resources have been devoted to efforts aiming to save native orchids from extinction. In 2010, the Smithsonian Institute and United States Botanical Garden collaborated to form the North American Orchid Conservation Center (NAOCC) (Whigham & Zettler, 2016). This organization's mission is to ensure the survival of native orchids throughout North America, and this represents the first international collaborative effort to identify strategies to conserve North American orchids. Re-introduction of the western prairie fringed orchid (*Platanthera praeclara*) in the upper Midwest, monitoring of the reproduction of smaller whorled pogonia (*Isotria medeoloides*) in northeastern states, and assisted reproduction and re-introduction programs like those focused on the ghost orchid in Florida (*Dendrophylax lindenii*), are just a few examples of long-term restoration efforts for iconic species in the United States (Bjugstad & Fortune, 1989; Whigham *et al.*, 2021; Zettler *et al.*, 2019).

In addition to ghost orchid, some of the most ambitious orchid conservation efforts in North America focus on species native and endemic to southern Florida. This area of the state is home to more than 100 species of native and introduced orchids, including six species of *Vanilla*. Large-scale projects managed by non-profit organizations like the Marie Selby Botanical Gardens and the Fairchild Tropical Botanic Garden strive to reintroduce orchids to environments where they once thrived through the Million Orchid Project (The Fairchild Tropical Botanic Garden, n.d.), while simultaneously educating the next-generation and local communities in conservation practices and native Floridian orchids. Despite many diverse species of North American orchids being studied and protected, many *Vanilla* are largely overlooked. Overshadowing by the neotropical *Vanilla planifolia*, the source of commercially produced *Vanilla* spice, is likely a partial cause of this.

The largest genus of the Vanilloideae subfamily is *Vanilla*, a pantropical genus of ca. 110 species of epiphytic, hemi-epiphytic, and terrestrial herbs (Soto-Arenas, 2003). Molecular research has supported *Vanilla* as an ancient lineage (>30 mya) and most basal subfamily of the Orchidaceae and their pantropical distribution is attributed to an ancient vicariance event prior to the breakup of Gondwana (Bouetard *et al.* 2010; Cameron 2000 & 2004; Givnish *et al.* 2015; Ramirez *et al.* 2007). More specifically, *Vanilla* are distributed from the New World tropics of southern Florida, Mexico, and the Antilles, to South America, to the African mainland and Madagascar, to tropical Asia including India and China to New Guinea and Southeast Asia (Karremans, 2020, Cameron, 2000). Vanilloideae are known for species having features atypical of most orchids such as fleshy aromatic, comparatively large-seeded fruits rather than dry capsules containing dust-like seeds, and their viney growth form growing terrestrially then hemi-epiphytically using aerial roots to climb trees and shrubs (Odoux & Grisoni, 2011; Correll *et al.*,

1950; Dressler, 1993; Soto-Arenas, 2003). More than half of all *Vanilla* species are endemic to tropical America and only a few New World species produce aromatic fruits containing vanillin (Flanagan & Mosquera-Espinosa, 2018). However, commercially cultivated *V. planifolia* only occur naturally in Mexico, Central America, and certain northern South American locations (Soto-Arenas & Dressler, 2010; Karremans, 2020).

In the late 1500s upon European invasion of the new world, *Vanilla* spice spread globally (Fouche, 1999). Modern commercially grown *Vanilla planifolia* crops are likely vegetatively propagated descendants from these original introductions resulting in limited genetic diversity and horticultural performance (Childers, 1948). Production was limited to Central America until approximately the 1850s when cultivation was expanded more globally. This was assisted by artificial pollination of *Vanilla*, discovered in 1838 by Charles Morren. Later in 1841, a more practical method of manual pollination was devised by Edmond Albius, who, at the time was a child slave, later freed but never compensated for his groundbreaking contribution to the *Vanilla* spice industry (Morren, 1838; Ecott, 2005).

Today, growers still rely primarily on clonal cultivation, and so manipulate a limited genetic diversity. This has led to an increasing interest in the industry of domestic *Vanilla* cultivation including expansion into other suitable environments and preservation of related species, like south Florida's four native species (Flanagan & Mosquera-Espinosa, 2018). The preservation of a crop's gene pool and genetic diversity through conservation of its wild relatives is necessary for the development, sustainability and continued use of a crop plant. Plant breeders need access to crop wild relatives, which provide a useful gene secondary gene-pool to maintain genetic diversity of crop plants (Brozynska *et al.*, 2016). *Vanilla barbellata* is one of four undomesticated, wild species native to south Florida (U.S.) and the Caribbean and which

constitute the secondary gene pool of the *Vanilla* crop (Flanagan & Mosquera-Espinosa, 2018). New diversity must be identified for commercial use to reduce production risks and detect novel genetics (Chambers *et al.*, 2021; Hu *et al.*, 2019).

*Vanilla barbellata* (“worm vine orchid”), is one of the leafless *Vanilla* that forms significant populations within the protected Everglades and throughout the Caribbean from the islands of the Bahamas, Cuba, the Dominican Republic, Florida, a few Leeward Islands, and Puerto Rico (Figure 1, 2). The ability of *V. barbellata* to grow in a variety of habitat types, especially drought prone habitats, makes it of unique concern and economic value for producing drought resistant crop plants (Flanagan & Mosquera-Espinosa, 2018; Koyyappurath *et al.*, 2016). *V. barbellata* faces numerous conservation risks in its native habitats including anthropogenic threats such as habitat loss, habitat fragmentation, and the effects of climate change like rising temperatures and sea-levels. These threats are not hypothetical; the melting of Greenland’s glaciers alone could cause the average sea level to rise by seven meters (23 ft; NASA, 2022). After only a three meter (>10ft) rise in sea level, most of the designated areas of conservation in south Florida and all current habitats of Florida’s *Vanilla* orchids will be underwater. In the past two decades, *Vanilla* populations throughout the Caribbean have significantly been affected by hurricane activity (i.e., Hurricane Maria, 2017 – Puerto Rico, Hurricanes Wilma, 2005 and Irma 2017 – Florida Keys), and in many cases, their current status is unknown (Govaerts *et al.*, 2018). As a result, this species has been reduced to very few populations scattered over vast distances, and the existing genetic diversity among and between these populations is not known. How *V. barbellata* are reproducing in the wild is unknown but vegetative (asexual/clonal) reproduction occurs naturally from stem cuttings and stems running on the ground. Reduced sexual reproduction is a result of environmental changes that could lead to populations that rely on

clonal reproductive (Barrett, 2015; Lin *et al.*, 2016). Clonal plants may be less effective in spreading into new habitat patches and respond to habitat change more slowly than non-clonal species, culminating in local extinction (Buckley and Freckleton 2010; Zobel *et al.*, 2010; Wiberg *et al.*, 2016). These scents may attract fragrance enticed neotropical male *Euglossine* bees or as a defense response (Lubinsky *et al.*, 2006; Kessler & Baldwin, 2002). Recently studies have demonstrated the potential evolutionary advantage of these fruit types via endozoochoric seed dispersal of Vanilloideae (Suetsugu *et al.*, 2015; Pansarin 2021).

Long term conservation depends on genetic diversity because it influences a population's ability to adapt to a changing environment, but rare and endangered plant populations frequently have low levels of genetic diversity (Reed, 2003; Frankham, 1996; Leimu *et al.*, 2006) and establishing which populations have the highest conservation value is no simple task. Allelic richness is a basic indicator of genetic diversity that is frequently employed when selecting populations to target for conservation efforts and are identified using molecular marker-based research methods (Petit *et al.*, 1998; Vinceti *et al.*, 2013). However, there has recently been an increase in studies of *Vanilla* orchid genetic diversity. *V. barbellata* is understudied, and its current habitat range in south Florida is at risk from the effects of climate change, rising sea levels, hurricanes, development, and other threats (Chambers *et al.*, 2021; Hu *et al.*, 2019). *Vanilla barbellata* deserves attention and conservation, if not for its intrinsic value, then for its economic value and potential to provide genetic variability to commercial propagation and production of *V. planifolia*. Further, *V. barbellata* has a relatively unique leafless growth habit amongst *Vanilla* orchids that has arisen three separate times in the *Vanilla* clade. This habit was developed through its evolutionary history that involves not one but two trans-oceanic dispersals first to Africa then back to Mesoamerica (Bouetard *et al.*, 2010; Cameron & Bolin, 2010).

Considering the desire to maintain native populations for future generations, the objective of this study is to investigate existing genetic diversity of *V. barbellata* by quantifying that genetic variation using next-generation Genotyping-by-Sequencing (GBS) of individuals within and among populations in southern Florida and other Caribbean locations. To achieve this objective, we aim to investigate: 1) what genetic diversity exists in southern Florida and how that genetic diversity compares to other *V. barbellata* populations in the Caribbean, 2) whether there is there evidence of gene flow between highly fragmented populations in Florida, and 3) whether there is there evidence of inbreeding via limited outcrossing or small population size, and to what extent. Based on observations from local experts we anticipate that asexual or clonal reproduction is the dominant form of reproduction for *V. barbellata* in southern Florida, perhaps due to low pollination rates, infrequent flowering, fragmented habitats, and small populations causing population bottlenecks. Prevalence of asexual reproduction would result in little to no gene flow, high genetic drift, and fixed, low heterozygosity, which we expect to be reflected in genetic diversity analysis.

### **Methods:**

Due to the endangered and threatened status of *Vanilla barbellata*, permits are required for their collection. Plants included in this study were mostly collected from protected areas, and sampling of a 1–2 cm<sup>2</sup> tissue segment was done with consent of local nature conservation authorities. Individual plants at a site were only sampled if that sampling was clearly not detrimental to the plant's survival.

Collections in south Florida were made in the Everglades National Park (NP) and the Florida Keys on lands protected and maintained by Florida Fish and Wildlife Conservation

Commission. In total, 115 individuals were included in this study from five wild sites and plants in three botanic collections. Precise locations cannot be disclosed for some due to permitting restrictions instituted to protect endangered species (Table 1).

Depending on a population's size, approximately 10 to 30 tissue samples (1-10 g tissue or 1–2 cm<sup>2</sup>) per population were collected between 2016 and 2018. Considering the endangered and threatened status of *V. barbellata* the ideal minimum of samples per population was not possible, as some collection sites have very few plants present. Furthermore, only distinctly separate individuals, those not growing on top of each other, were sampled. A collected sample was immediately placed in silica gel for drying. If necessary, due to the succulent nature of the plants, collected samples were cut into smaller pieces to ensure drying and to prevent DNA degradation. Once dehydrated all tissue samples were preserved and stored at -80°C. Tissue from genus *Vanilla* is both fibrous and mucilaginous increasing difficulty of grinding tissue for DNA extraction. We ground tissue samples for 1.5 minutes at 25 Hz. in a TissueLyser (Qiagen) along with two to three 4.5mm aluminum pellets and buffer from Promega Extraction Kit in preparation for DNA extraction. DNA extraction was completed using Promega Maxwell® 16 Tissue DNA Purification Kit and Promega Maxwell AS2000 for automated nucleic acid extraction in the Cameron Lab at UW Madison. Gel electrophoresis and DNA Quantification using TECAN Infinite M Plex plate reader for fluorescent readings of DNA using Invitrogen Qubit™ dsDNA HS Assay kit by Thermo Fisher Scientific.

Library preparation and sequencing for genotyping-by-sequencing (GBS) was performed at the Biotechnology Center at the University of Wisconsin – Madison using an Illumina NovaSeq6000 2x150bp, 250 million reads per sample and size selection with dual restriction

enzymes PstI and MspI. The reference genome of *Vanilla planifolia* ‘Daphna’ from National Center for Biotechnology Information (NCBI) was used to facilitate read mapping and Single Nucleotide Polymorphisms (SNP) calling (Hasing *et al.*, 2020). This method multiplexes samples on the high-throughput Illumina HiSeq 2000 platform while retaining adequate sequencing depth (Elshire *et al.*, 2011). Genetic markers from GBS are highly effective in phylogeographic and population structure analyses that include non-model organisms (Andrews *et al.*, 2016; Emerson *et al.*, 2010; Davey *et al.*, 2011; Deschamps *et al.*, 2012; Sonah *et al.*, 2013).

STACKS v2.6 GBS pipeline was used to call SNPs from the GBS library using a reference draft of *V. planifolia* ‘Daphna’ genome (Catchen *et al.*, 2011; Hasing *et al.*, 2020). Process\_radtags examined raw reads and checked for intact barcodes. It then demultiplexed data and if an error existed in the barcode, it was corrected. Secondly, it slides a window down the length of reads and checks average quality score within the window. If it scores below 90% probability of being correct, then the read is discarded. The *de novo* assembly protocol contained three phases. First, the ustacks program generates “stacks” inside individuals. Second, cstacks builds loci and catalog samples and GBS tag alignment via BWA MEM algorithm seeded alignments with maximal exact matches (Langmead *et al.*, 2012). Finally, gstacks finds alleles and single-nucleotide polymorphisms for each sample against the catalog. This was followed by using the populations module to produce datasets for downstream population analyses. Quality filtering of SNPs was performed primarily using built-in functions in VCFtools (Danecek *et al.*, 2011). Minor allele frequency (MAF) was set to 0.05 and minimum locus coverage was set to retain SNPs that are covered in >80% of individuals with >10x average SNP coverage to generate high-quality filtered SNPs (97 accessions). PLINK (Prucell *et al.*, 2017) pruned the

SNP dataset to reduce linkage disequilibrium (LD) within the dataset to lower than a threshold ( $r^2 \leq 0.5$ ) site call, MAF rate,  $r^2$  (LD), and site heterozygosity.

An analysis of molecular variance (AMOVA) of SNP data was done using the R package “poppr” (Kamvar *et al.*, 2014). Significance of the AMOVA and Phi statistical analyses were tested using 999 permutations with the R package “ade4” (Chessel *et al.*, 2004).

Population stratification was inferred using fastStructure. Prior to analysis, SNPs with missing data and with a MAF < 0.05 were removed. K (number of populations) values, ranging from 1 to 10, were tested, and the most probable number of populations was chosen running the Python script provided for multiple choices of K. Admixture proportions of each genotype, estimated by fastStructure, were visualized using the provided Python script to construct DISTRUCT plots (Raj *et al.*, 2014; Rosenberg, 2004).

Principal Component Analysis (PCoA) & Discriminant Principal Components (DAPC) plot of 115 *Vanilla* accessions for PC1/PC2 showing multiple distinct clusters was conducted using the R packages ‘poppr’ and ‘ggplot2’ (Kamvar *et al.*, 2014; Wickman, 2016). The filtered VCF file of the complete genotype matrix was used to construct a neighbor joining (NJ) tree with 100 bootstrap replicates to assess branch support, using the R packages ‘vcfR’ and ‘ape’ (Knaus & Grünwald, 2017; Paradis & Schliep, 2019).

## **Results:**

Sequencing of GBS libraries resulted in 892,018,743 total single reads from 115 accessions. Cleaning and demultiplexing followed, resulting in good, barcoded reads, which are sequence reads with a phred score of 10 (Ewing *et al.*, 1998; Ewing & Green, 1998). This means it is considered a perfect match to one of the barcodes used in sequencing and deemed acceptable

by process\_radtags of STACKS (Catchen *et al.*, 2011). If the score dropped below 90% (a phred score of 10) the read was discarded. Demultiplexing resulted in 735,135,762 reads. The *de novo* pipeline reconstructed 64,885 loci, 52,060 of which did not pass the populations constraints and were removed (sites per loci; mean=69.5, stdev. =40.8, min=1.6, & max=176.1). The remaining 12,825 loci composed 830,656 variable sites. Reads of these variable sites were then aligned to the *Vanilla planifolia* ‘Daphna’ reference genome (Hasing *et al.*, 2020) and filtered out based on minimum minor allele frequency (MAF) >0.05 resulting in 28,589 variant sites and a total of 6,751 high-quality filtered SNPs. 26.14% of SNPs reached 0x-2x coverage, 12.51% of SNPs reached 3x-10x coverage and coverage is descending as expected (Figure 4). The highest average SNP coverage existed near 0x, however the second highest SNP coverage existed between 40-60x average SNP coverage (Figure 5). The genome-wide observed heterozygosity over all populations was very low ( $H_o=0.05$  to  $0.09$ ) suggesting frequent inbreeding in the wild. Among the populations, the Virgin Islands NP demonstrated the highest heterozygosity at 0.09054.

The first, second, and third coordinates of the Principal Component Analysis (PCoA) of *V. barbellata* accessions explain 71.5% of the present genetic variability (Figure 6). A PCoA plot of 115 *Vanilla* accessions of the first and second coordinates show multiple distinct clusters. Notably tight clusters of individuals are formed by individuals from the Virgin Islands NP, Florida Keys, and the Florida Everglades NP & Keys. Additionally, slightly overlapping clusters are formed for Guánica SF and Susúa SF individuals from Puerto Rico (Figure 6A). Discriminant Principal Components (DAPC) analysis demonstrates the same clustering patterns (Figure 6B). The neighbor joining tree exhibits a distinct Virgin Islands NP clade, a Florida clade that is a combined Everglades NP clade and Florida Keys sister clade, and a Guánica SF/Susúa

SF clade with Virgin Island NP clade embedded within it. A distinct Guánica SF clade is sister to all other clades (Figure 7).

FastStructure analysis was conducted to investigate relationships among accessions. These results indicate that the optimal number of clusters to designate the data is  $K=4$  populations. The  $K=4$  admixture plot distinguishes four different populations: Florida Everglades NP (orange), Florida Keys (pink), Puerto Rico (Guánica SF/ Susúa SF) (blue), and the U.S. Virgin Islands NP (green). However, the  $K=5$  admixture plot distinguishes five different populations: Florida Keys (pink), Florida Everglades NP (orange), Guánica SF/Susúa SF (PR)-1 (light blue), Guánica SF/Susúa SF (PR)-2 (royal blue), and the U.S. Virgin Island NP (green). These results mirror the phylogenetic analyses and further confirmed clustering results where collections sites separated into subgroups. (Figure 8).

In our comparisons of population samples by a pairwise difference distance method with 110 permutations, the highest differentiation is notable between the Florida Keys and the Virgin Islands NP (FK/VI=0.54765), the second greatest difference is shown between the Florida Everglades NP and the Virgin Islands NP (FE/VI=0.50585), while the lowest is seen between Susúa SF and Guánica SF Puerto Rico (SPR/GPR=0.07740). Overall, limited genetic differentiation is demonstrated and supported by average  $F_{st}$  value of 0.30652.

## **Discussion:**

Conservation efforts for the endangered *V. barbellata*, including collection, propagation, and reintroduction, can be aided by diversity analysis, with the emphasis of such efforts intending to maintain as much genetic diversity as possible. The ability of GBS to inform conservation practices and identify needs in *Vanilla* has been previously acknowledged (Hu *et*

al., 2019). Collaborative efforts will be necessary to expand *Vanilla* sampling, conservation, and genomics to support *Vanilla* plant improvement and prevent extinction of wild relatives in the genus. The primary objective of this study is to identify existing genetic diversity of Floridian *V. barbellata* and how it compares to Caribbean populations. We also survey the population for the occurrence of heterozygosity via limited outcrossing or small population size.

**Virgin Islands National Park, St. John** - The Virgin Islands NP population of *V. barbellata* was the largest, most sampled, and geographically isolated of this study, exclusively inhabiting rocky coastal lowland hammocks (Figure 2A). It harbored the highest observed heterozygosity ( $H_o = 0.089$ ), lacks inbreeding depression ( $F_{is} = -0.816$ ), and deviates significantly from Hardy-Weinberg Equilibrium (Table 2). This population is the most genetically distant from all other populations according to our analysis of pairwise  $F_{st}$ , supporting our assertion that  $F_{st}$  is correlated to geographic distance in *V. barbellata*. Pairwise  $F_{st}$  values between the Virgin Islands NP and relatively nearby Puerto Rican populations of Guánica SF (0.337) and Susúa SF (0.392) are much lower than those of the more distant Floridian populations of the Florida Keys (0.548) and the Florida Everglades NP (0.506) (Table 4). The closer relationship of the VINP population to the two Puerto Rican populations is evident from neighbor joining tree in which the Puerto Rican clade is sister to the Virgin Islands NP clade (Figure 7). Moreover, the outcome of our fastStructure admixture plots reveal a small level of gene flow occurring between these populations (Figure 8), demonstrating something important here. The genetic distance from other populations is further demonstrated by the PCoA & DAPC analyses, where all members of the Virgin Islands NP population form a tight cluster entirely separate from all other populations (Figure 6A, 6B). The conclusions of these analyses ultimately

point to its significant genetic value and the necessity of conserving this population, comprising definitive genetic distance and high heterozygosity ( $H_o=0.089$ ).

**Puerto Rico, State Forests** – There are two populations of *V. barbellata* in Puerto Rico, one in Guánica SF and one in Susúa SF. The habitat in Guánica SF's is rocky coastal lowland hammock, similar to that of the Virgin Islands NP's population. Conversely, the Susúa SF habitat is uniquely located at a higher elevation and inland montane forest with a more open understory than any other population of *V. barbellata* we studied. Despite these comparatively different environments both populations seem to be thriving. Guánica SF exhibited quite low heterozygosity ( $H_o = 0.045$ ) and a low level of inbreeding depression ( $F_{is} = 0.043$ ) amongst the five *V. barbellata* populations. Susúa SF similarly demonstrated low heterozygosity ( $H_o = 0.069$ ) and slightly higher inbreeding depression ( $F_{is} = -0.078$ ) (Table 2). These Puerto Rican populations demonstrate the lowest genetic distance between each other with pairwise  $F_{st}=0.077$ . Pairwise  $F_{st}$  values between Puerto Rican populations and adjacent populations are nearly equally genetically distant ranging only between 0.329 to 0.392 (Table 4). Individuals from both populations are intermingled forming a single clade with significant bootstrap values in the neighbor joining tree (Figure 7). Furthermore, PCoA & DAPC analysis also show significant overlap between the two and our admixture plot of  $K=5$  reaffirms the presence of gene flow (Figure 6 A&B, Figure 8). We consider two possible explanations for this occurrence. First and more likely, a translocation event that transferred an individual or part of an individual of Susúa SF ancestry to Guánica SF location. Sexual reproduction then occurred and resulted in individuals of combined ancestral lineages. This appears more probable because more than one sampled individual of Guánica SF is entirely composed of the apparent Susúa SF ancestry. While Susúa SF individuals do not demonstrate this but do express individuals with a combination

Susúa SF and Guánica SF ancestry suggesting sexual reproduction (Figure 8). Our second explanation is much less likely although still possible. Local pollinators could traverse that distance and successfully pollinate another flowering *V. barbellata*. In either case, plants that are combinations of the two ancestries are present and because of this both populations are contain higher heterozygosity than others. The apparent gene flow between these two populations distinguishes them among all others in this study. The potential sexual reproduction occurring between them deserves further examination for conservation and breeding efforts. The genetic diversity that is unique to this population also warrants the attention of conservationists.

**South Florida** - The Everglades National Park and the Florida Keys are home to *V. barbellata* as well, quite possibly the last remaining populations in the State of Florida. The Florida Everglades NP is a 1.5-million-acre wetlands preserve in the southern most portion of the state. This population's environment is a thick wetland hammock with limestone covered by shallow soils and dominated by native cycads, pines, grasses, cabbage palm, and poisonwood trees. This population is more inland than any other population yet remains just above sea-level. The nearest *V. barbellata* population is approximately 112 km. away, likely significantly restricting gene flow. *V. barbellata* of the Florida Keys occupies areas with sandier soil types and similar vegetation with the addition of mangroves (Figure 2B). These two Floridian populations are sister to each other and together they are sister to all other populations of this study (Figure 7). The population differentiation between them is the lowest of all groups included ( $F_{st}=0.162$ ) and they form tight overlapping clusters in the PCoA (Table 4 & Figure 6A), yet they maintain a significant difference from each other in both K=4 and K=5 admixture plots (Figure 8). *V. barbellata* of the Everglades NP was flowering at the time of collection and had produced and mature fleshy fruits. In the hopes of including them in seed germination

studies we collected these fruits which later proved to contain embryo-less seeds. Despite the inviable seeds of this plant, the inbreeding coefficient is low ( $F_{is} = -0.014$ ), suggesting perhaps sexual reproduction has some success here. This is not the case for the Florida Keys though, where  $F_{is} = 0.291$  and observed homozygosity is  $H_o = 0.961$  (Table 2). These high values are greater than all other populations, implying a concerning and significant amount of inbreeding. The low heterozygosity of the Florida Keys and the isolating distance of the Everglades NP are causes of great concern that illustrate the need for further concentrated conservation efforts.

**Implications for Conservation & Recommendations** - The patterns of population structure demonstrated in our study reveal important conservation implications. Above all, the most immediate threats to this species are the effects of climate change, in particular rising sea levels. These findings are essential as decisions of population priority in conservation efforts are made and for development of conservation management strategies for this rare species. Susúa SF of Puerto Rico is the only location included in this study in which the population is not coastal or near sea level. The remainder of the populations' habitats are near sea-level and coastal, making them particularly vulnerable. According to NASA (2022), these lowland habitats will vanish with a rise in sea-level of merely one-meter. In conjunction with hurricane activity and direct anthropogenic disturbances this species may very well become extirpated or locally extinct in Florida, just as *V. dilloniana* and *V. mexicana* have been.

Management plans should involve *ex situ* conservation efforts with a primary goal of promoting assisted sexual reproduction to create viable seeds and offspring with combined genotypes between populations. These seeds would be important for seed germination studies and germplasm/seed preservation. We suggest two simultaneous large-scale integrated *ex situ* conservation actions for all populations. First, we suggest assisted migration, establishing one or

more new genetically diverse population in strategic locations by transplanting clonal cuttings from each population. Such locations should consider their ecological contexts and should be located as close to existing populations as possible. Additionally, this should be beyond the reach of rising sea levels and semi-protected from hurricanes, while remaining easily accessible by conservationists for continued observation and monitoring. In this scenario, efforts may be ongoing depending on available pollinators and seed dispersers. Further efforts should concentrate on understanding causes of *V. barbellata*'s rarity and decline to provide better understanding for ongoing conservation. The collection of representative individuals from all populations which will ensure preservation of genetic resources is an essential start for conservation. An *ex situ* breeding program should be established with the aforementioned living collection to increase genetic diversity of the collection, stymie the effects of inbreeding depression, and bolster population sizes.

This study revealed the low genetic diversity of *V. barbellata* in both Floridian and Caribbean populations. The overall low genetic diversity, significant genetic differentiation in populations, and declining populations of *V. barbellata* are consequences of limited gene flow, habitat fragmentation, and geographic isolation. The low heterozygosity suggests that populations have been declining and the decline is escalating. Extinction of any population may lead to a considerable loss of existing genetic variation given the significant genetic differentiation among populations. These populations are particularly vulnerable and at imminent risk of extinction in the wild and need to be brought into cultivation. This study supports an urgency for *ex situ* conservation and management efforts.

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**Tables:**

Samples Collected of <i>V. barbellata</i> for Population Structure Analysis	
Locality	N
Guánica State Forest, Puerto Rico	19
Susúa State Forest, Puerto Rico	13
Florida Keys, FL	22
Everglades National Park, FL	25
Virgin Islands National Park, U.S.V.I.	29
Marie Selby Botanical Garden & Fairchild Tropical Botanic Garden, FL	7
<b>TOTAL</b>	<b>115</b>

**Table 1:** Samples collected of *Vanilla barbellata* and collection sites for population structure analysis.

<i>Population Sites</i>	<b>n</b>	<b>Private alleles</b>	<b>Obs. Het. (Ho)</b>	<b>Obs. Hom.</b>	<b>Exp. Het. (He)</b>	<b>Exp. Hom.</b>	<b>Pi</b>	<b>Fis</b>
<i>Marie Selby Botanical Garden &amp; Fairchild Tropical Botanic Garden, FL</i>	6	2684	0.066	0.934	0.081	0.919	0.088	0.185
<i>Florida Keys</i>	18	3837	0.039	0.961	0.055	0.945	0.057	0.291
<i>Florida Everglades NP</i>	23	7798	0.071	0.929	0.07	0.93	0.071	- 0.014
<i>Susúa SF (PR)</i>	17	2138	0.069	0.931	0.064	0.936	0.066	- 0.078
<i>Guánica SF (PR)</i>	10	285	0.045	0.955	0.047	0.953	0.05	0.043
<i>Virgin Islands NP</i>	28	1079	0.089	0.911	0.049	0.951	0.05	- 0.816

**Table 2:** Summative diversity statistics from STACKs populations module. Fis was averaged across all loci. Significant evidence of high inbreeding in south Floridian populations.

<b>Global AMOVA results as a weighted average over 1219 loci:</b>			
Source of Variation	Sum of squares	Variance components	Total Variance
Among all Populations	9293.532	55.786	30.652
Within Populations	23695.464	126.210	69.348
Total	32988.996	181.997	
Average F-Statistics over all loci Fixation Indices		F <sub>ST</sub> : 0.307	

**Table 3:** Global AMOVA results as weighted average over 1219 loci of *Vanilla barbellata* populations: Virgin Islands NP, Florida Keys, Florida Everglades NP, Susúa SF of Puerto Rico and Guánica SF of Puerto Rico. The majority of variation was found within populations (69%), rather than among populations (30%). Overall, limited genetic differentiation is demonstrated and supported by an average Fst value of 0.307.

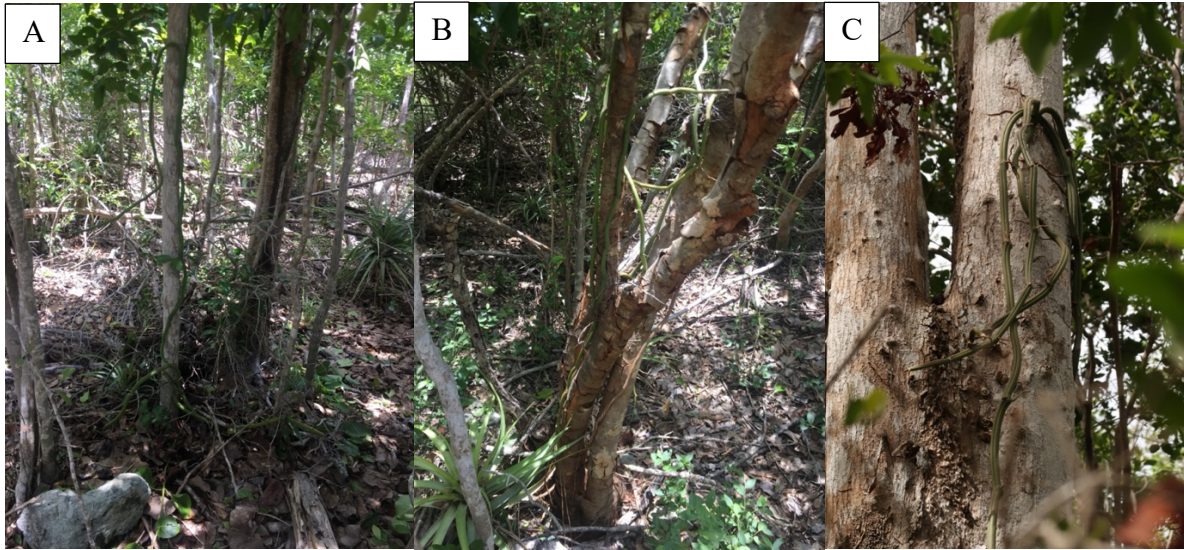
<b>Population pairwise Fst</b>					
	<b>FL Keys</b>	<b>FL Everglades NP</b>	<b>Susúa SF (PR)</b>	<b>Guánica SF (PR)</b>	<b>US Virgin Islands NP</b>
<b>FL Keys</b>	-				
<b>FL Everglades NP</b>	0.162	-			
<b>Susúa SF (PR)</b>	0.356	0.336	-		
<b>Guánica SF (PR)</b>	0.367	0.329	0.077	-	
<b>US Virgin Islands NP</b>	0.548	0.506	0.392	0.337	-
<b>p&lt;0.0001</b>					

**Table 4:** Comparisons of pairs of population samples by pairwise difference distance method with 110 permutations. Population pairwise Fst values are show below the diagonal. Highest differentiation was notable between the Florida Keys and the Virgin Islands NP, while the lowest was seen between Susúa SF and Guánica SF Puerto Rico.

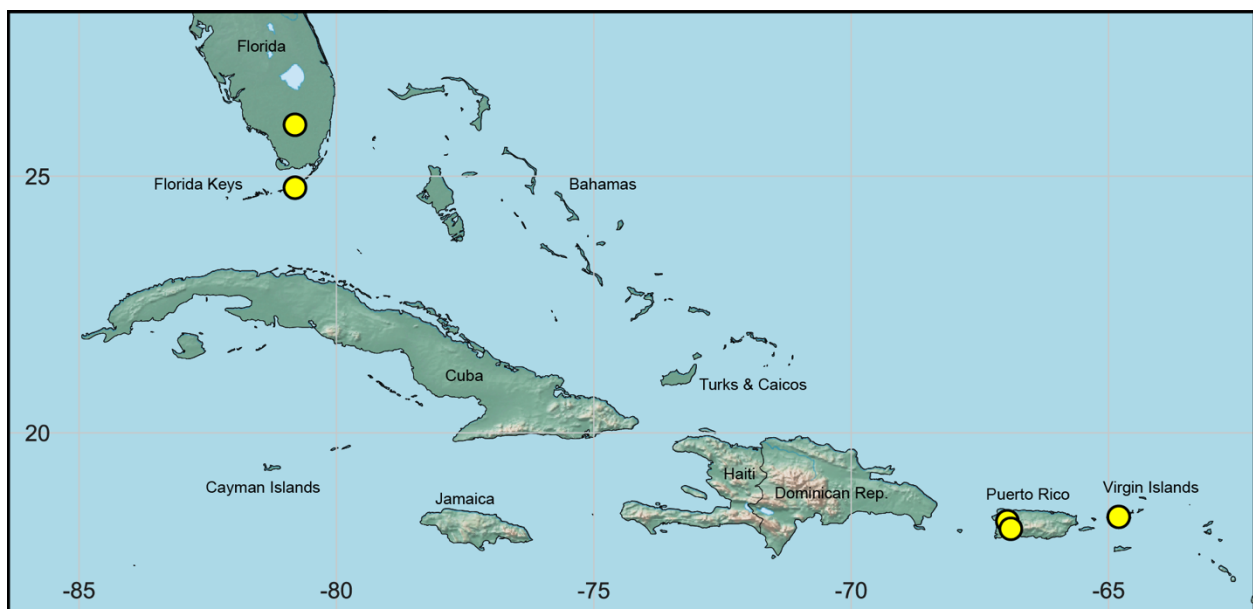
**Figures:**

**Figure 1:** North America's native *Vanilla* species in south Florida ([regionalconservation.org](http://regionalconservation.org)).

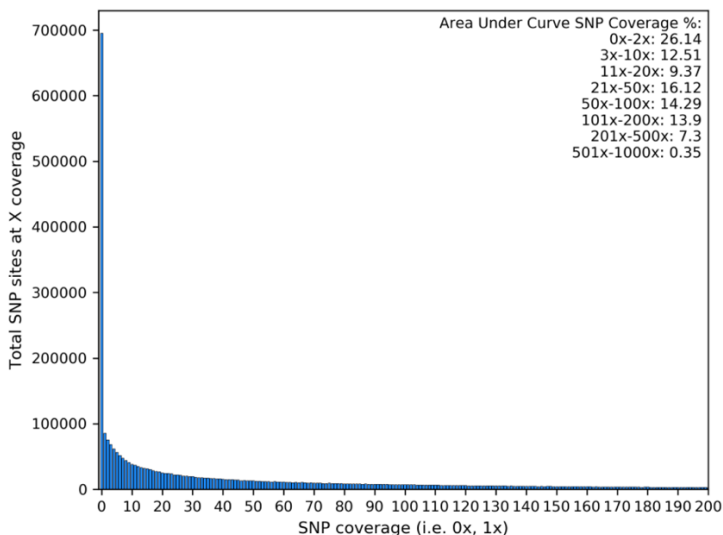
From the left to right: *V. dilloniana*, *V. barbellata*, *V. phaeantha*, and *V. mexicana*. These images are from The Institute for Regional Conservation's Floristic Inventory of South Florida.



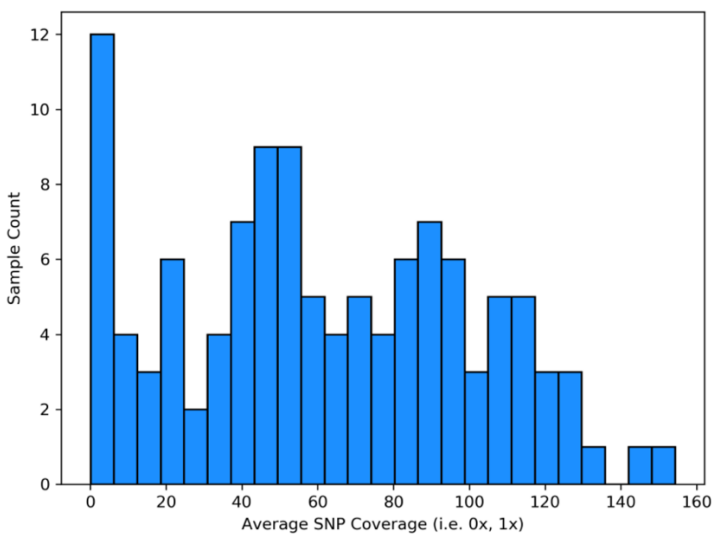
**Figure 2:** *Vanilla barbellata* in its natural habitats from select populations of our analyses. A: US Virgin Islands National Park – coastal thick hammock habitat. B: Florida Keys- coastal thick hammock with slightly sandier soil. C: Puerto Rico, Susúa State Forest – higher elevation (not sea-level) montane forest with rocky soil



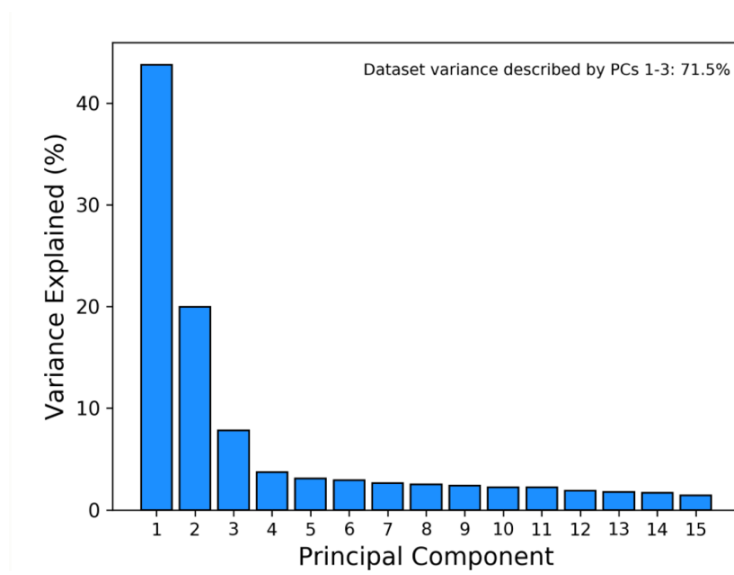
**Figure 3:** Map of *Vanilla barbellata* collection sites.



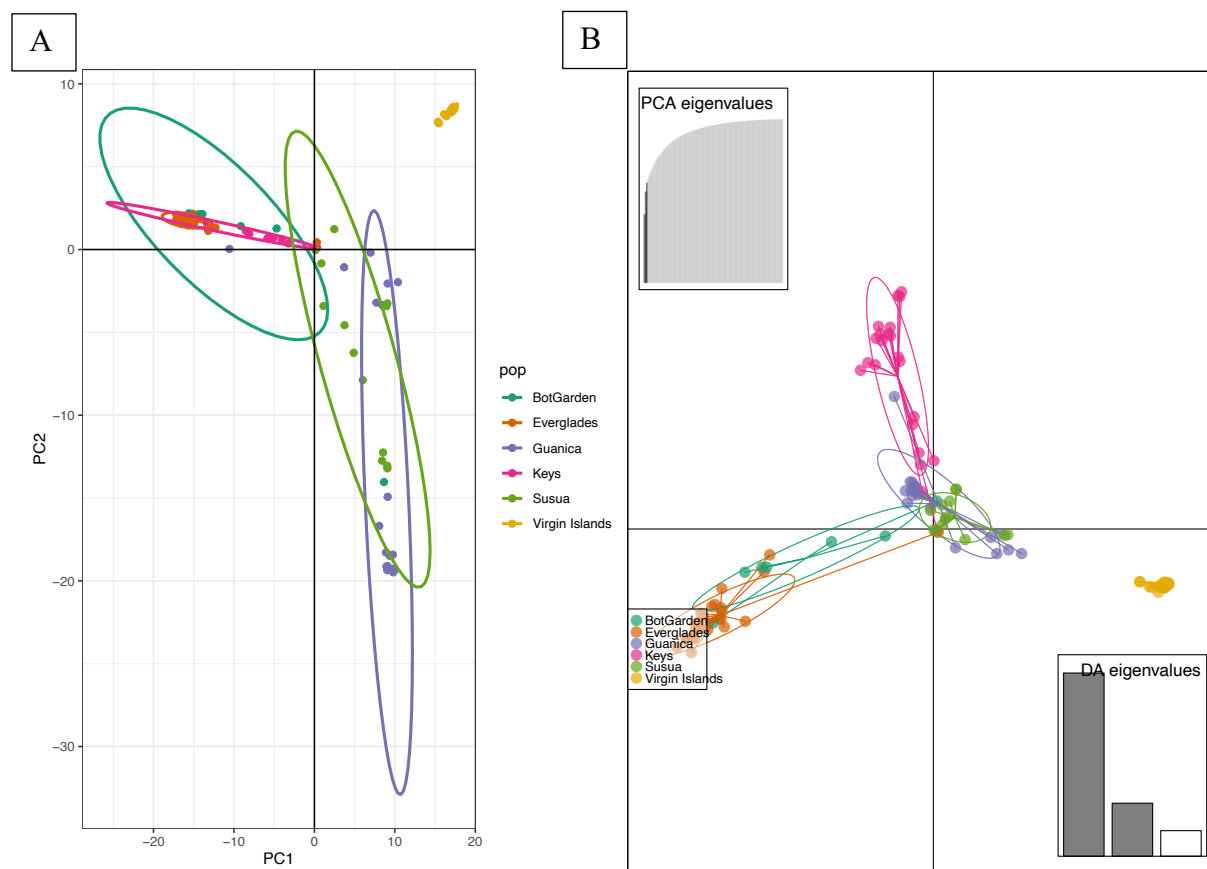
**Figure 4:** Total SNP sites at x coverage. 26.14% of SNPs reached 0x-2x coverage, 12.51% of SNPs reached 3x-10x coverage.



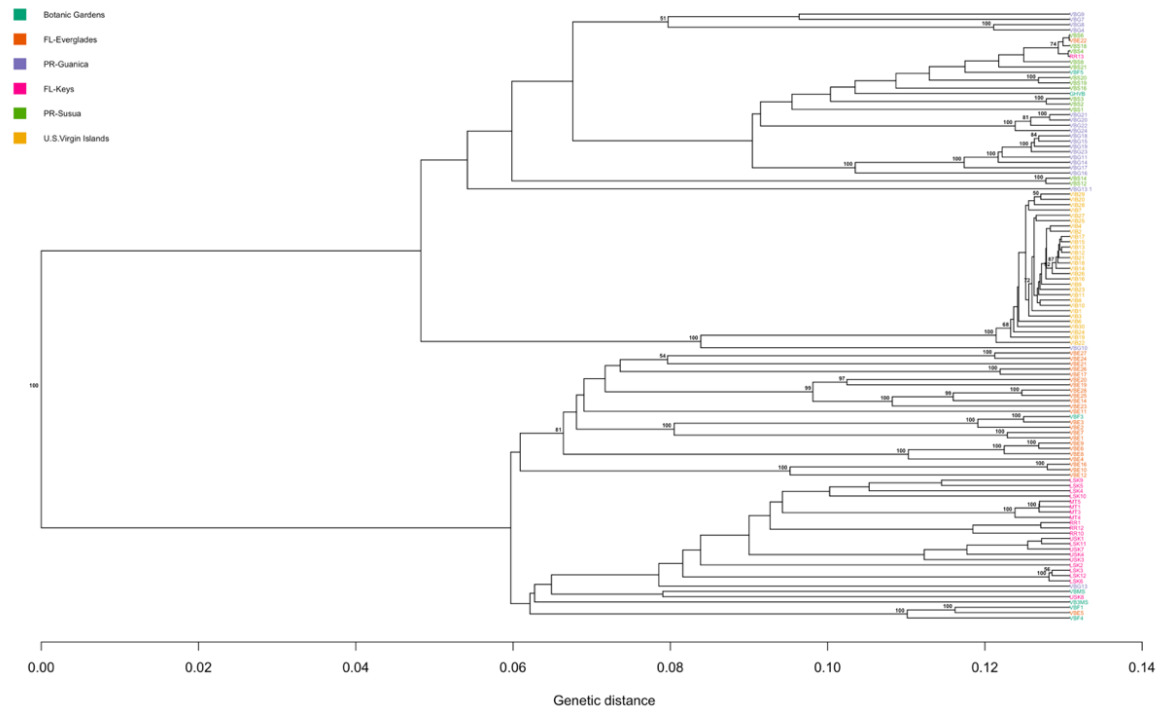
**Figure 5:** Average SNP coverage vs sample counts. The highest average SNP coverage existed near 0x, however the second highest SNP coverage existed between 40-60x average SNP coverage.



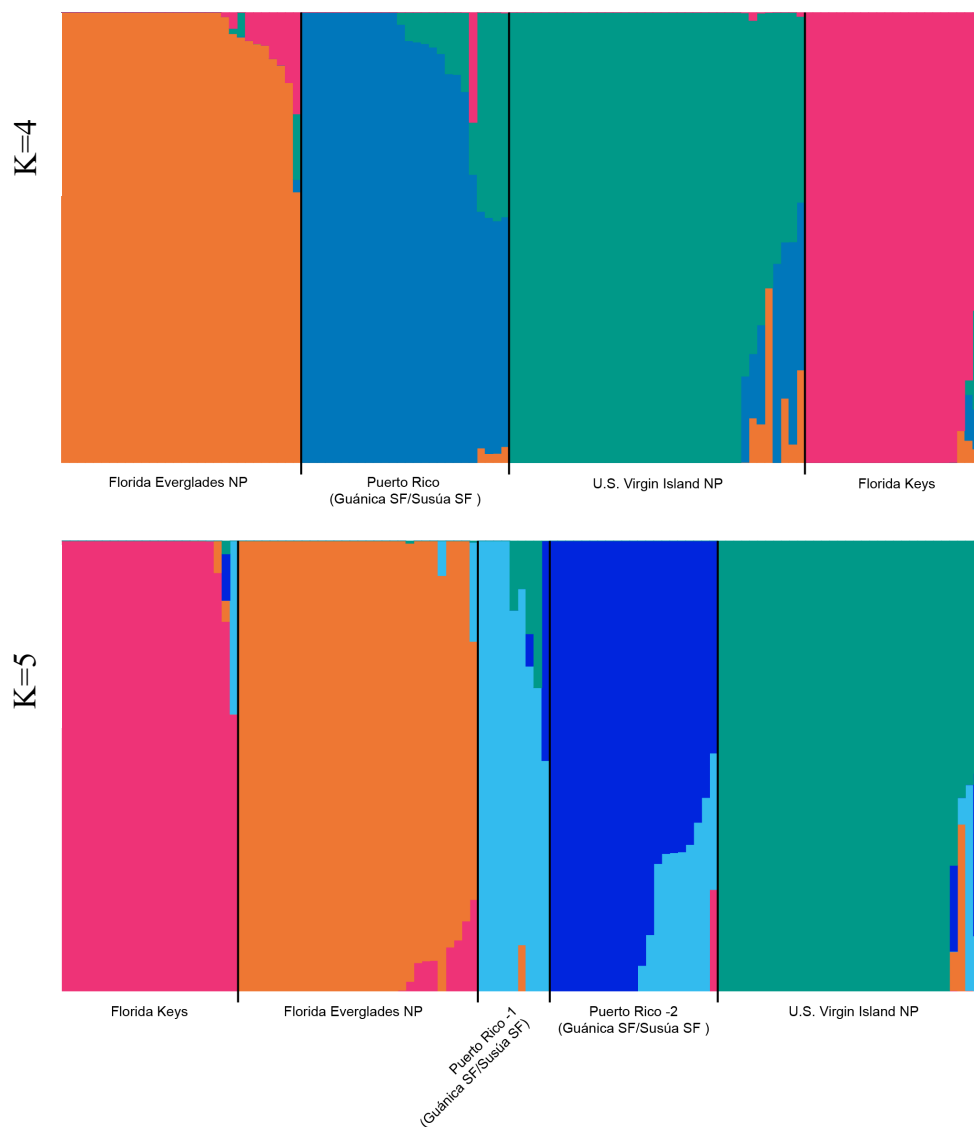
**Figure 6:** Variance explained (%) by principal components where 71.5% of variance comprise the first, second, and third components.



**Figure 6:** A) Principal Component Analysis plot of 115 vanilla accessions for PC1/PC2 showing multiple distinct clusters, notably tight clusters of individuals from the Virgin Islands NP, Florida Keys, and the Florida Everglades NP, and additional slightly overlapping clusters for Guánica SF and Susúa SF. B) Discriminant Principal Components (DAPC) demonstrating the same clustering patterns.



**Figure 7:** The Neighbor Joining (NJ) tree built with the complete genotyping matrix. This tree demonstrates distinct Virgin Island NP clade, Florida clade that is a combined Everglades NP clade and Florida Keys sister clade, and a Guánica SF/ Susúa SF clade with Virgin Islands NP clade embedded within it. A distinct Guánica SF clade is sister to Virgin Islands NP clade.



**Figure 8:** DISTRUCT admixture plots produced from fastStructure output. Model complexity that maximizes marginal likelihood = 4. Model components used to explain structure in data = 5. Optimal K is between K=4 and K=5. The K=4 admixture plot distinguishes four different populations: Florida Everglades NP (orange), Florida Keys (Pink), Puerto Rico (Guánica SF/Susúa SF) (Blue), and the U.S. Virgin Islands NP (green). The K=5 admixture plot distinguishes five different populations: Florida Keys (pink), Florida Everglades NP (orange), Guánica SF/Susúa SF (PR)-1 (light blue), Guánica SF/Susúa SF (PR)-2 (royal blue), and the U.S. Virgin Islands NP (green).

**Chapter II: Isolation and identification of fungal endophytes from roots of four *Vanilla* species (Orchidaceae) native to south Florida.**

**Abstract:**

Orchids have evolved obligate associations with mycorrhizae, and so a fundamental understanding of mycorrhizae is required for studying orchid ecology and implementing conservation plans. Within the United States *Vanilla barbellata*, *V. phaeantha*, *V. dilloniana*, and *V. mexicana* (Orchidaceae) are native to the state of Florida, but are considered rare, threatened, and/or endangered. In this study we identified and calculated diversity indices of culturable endophytic fungi associated with these four *Vanilla* species, with a greater level of sampling from among three populations of *V. barbellata*. In total 166 endophytic fungal isolates were obtained and identified using ITS sequencing, revealing a wide variety of mycorrhizae including latent pathogens and non-mycorrhizal fungi such *Fusarium* spp., dark-septate endophytes of the genus *Periconia*, and fungi of the genus *Tulasnella* known to be orchid mycorrhizae. In general, differences among species were minimal aside from those that are the probable result of sampling error. In contrast, we observed significant differences among *V. barbellata* population sites and detected the presence of multiple dominant fungal taxa. Our results revealed a high diversity of fungal partners that should be considered in any relocation efforts of the species in response to their dwindling threatened habitats. Fungal isolates from this study or related to those of our findings may also be an important to future conservation efforts of these orchids that will rely on *ex situ* seed germination and re-introduction.

**Key Words:** *Vanilla*, ITS sequencing, Orchidaceae, Orchid mycorrhiza, Conservation

**Introduction:**

Many orchids require specialized habitats, some have unique pollinators, and all form symbiotic relationships with mycorrhizal fungi that are crucial for orchid seed germination, seedling survival, and even contribute to survival of adult orchids. Orchid species can depend on different genera of fungi to form a symbiotic relationship and even change their symbionts throughout their lifespan (Zettler *et al.*, 2007). Orchid seeds are unique in structure compared to most other angiosperms and require a fungal symbiont to germinate. Their seeds are microscopic, not provisioned with endosperm and their seed coat is comprised of a thin, single layer of cells around an undifferentiated embryo. In more typical angiosperm seeds, an endosperm is present to provide the developing embryo with nutrients such as starches and proteins (Dressler, 1990; Rasmussen 1995, 2002). For this reason, orchid seeds depend on mycorrhizal fungi and are mycoheterotrophic, meaning they obtain nutrients from fungi (Currah *et al.*, 1997b, Rasmussen 2002, Taylor *et al.*, 2002). Due to their dependence on these numerous complex interactions, orchids are sensitive to environmental degradation and will quickly disappear if habitats begin to change, allowing them to serve as indicator species (Akhalkatsi *et al.*, 2014).

A mycobiont relationship is characteristic of the Orchidaceae and germinating orchid seeds initially consume nutrients through fungi only. The physiological impact of mycorrhizae is often uncertain, but it is known that they often contribute basic nutrients utilized by orchids (Rasmussen *et al.*, 2015). The absence of an endosperm in orchid seeds makes a relationship with mycorrhizal fungi all the more critical for orchid seed germination and seedling survival because orchids rely entirely on fungi for energy and nutrition in this phase of growth and development (Zettler *et al.*, 2007; Taylor & McCormick, 2008; Waterman *et al.*, 2008). Seedling

recruitment of any plant population is essential for its sustainability (Rasmussen *et al.*, 2015). Symbiotic germination of *Vanilla* orchid seeds is a necessity for their conservation and sustainability, but successful studies germinating *Vanilla* seeds have been limited to few species. Modern studies have broadened the scope of investigation into these mycorrhizae to include those not traditionally thought to promote seed germination. Mycorrhizae, including non-orchid mycorrhizal fungi (non-OMF) such as latent pathogens, dark septate endophytes (DSE), and more, have seen a surge of interest.

Vanilloideae are unique among orchids because of their viny growth form, first growing terrestrially and subsequently hemi-epiphytically by using aerial roots to climb trees and shrubs (Figure 2) (Odoux & Grisoni, 2011; Correll *et al.*, 1950). Moreover, this unique group of orchids has a much larger seed and scleral seed coat that is produced in a fleshy fruit, unlike the majority of extant orchids which produce small, dust-like seeds in a capsule (Cameron & Chase, 1998; Zettler *et al.*, 2013). These seeds, like all orchid seeds, require a fungal symbiont for germination (Rasmussen, 2002). The orchid embryo becomes actively mycoheterotrophic, deriving its necessary carbon, nitrogen, and other nutrients from the fungal symbiont. In many cases, the dependency and specificity of an orchid's mycorrhizae differ by species (Smith & Read, 2008). Leafless orchids are likely to rely on their fungal symbiont more than those with leaves, which has been witnessed in nature previously in the genus *Erythrorchis*, a leafless member of the Vanilloideae (Ogura-Tsujita, 2018). *Vanilla barbellata* and *V. dilloniana* are two such leafless examples that are native to Florida. The leafless habit of *Vanilla* is thought to have established itself after the transoceanic dispersal of the genus to the continent of Africa. The genus then speciated with leaflessness on the African continent prior to a secondary transoceanic dispersion

back to the Caribbean where the genus then further speciated with the leafless habit (Bouetard *et al.*, 2010).

Six *Vanilla* species inhabit the state of Florida. *Vanilla planifolia* Rchb.f. and *V. pompona* are originally Mexican species presumed to have escaped from cultivation or were otherwise anthropogenically introduced into their non-native environment. *Vanilla barbellata* is one of the leafless *Vanilla* that forms significant populations within the protected Everglades (pers. comm. Kenneth Cameron). *Vanilla barbellata* is distributed on the islands of the Bahamas, Cuba, the Dominican Republic, Florida, a few Leeward Islands (Lesser Antilles), and Puerto Rico. *Vanilla phaeantha* Rchb.f. is a rare species in Florida, restricted to only two sites in the state. These sites include the Fakahatchee Strand Preserve State Park, a highly protected area of twenty miles of linear swamp forest where they are thriving, and the Florida Everglades. *Vanilla phaeantha* is also distributed throughout the Bahamas, Costa Rica, Cuba, El Salvador, Florida, Jamaica, Mexico, Panama, Trinidad-Tobago, and Windward Islands (Greater Antilles) (Wegier, 2018). The conservation status of *V. dilloniana* and *V. mexicana* are far more dire. *Vanilla dilloniana* Correll is now presumed to be entirely extirpated from the state of Florida. Although cuttings from the last wild plants may be available in private or botanical collections, there is no effort underway to re-introduce plants into their native Floridian range. Their range previously included Cuba, the Dominican Republic, Haiti, Florida, Leeward Islands, and Puerto Rico. Lastly, although *V. mexicana* was assumed to be extirpated from North America after Hurricane Andrew (1992), at least two populations of juvenile and adult plants were recently found on private property north of the Everglades. *Vanilla mexicana* maintains its rare status in Florida and its locations have not been made public. The previous range of *Vanilla mexicana* included Cuba, the Dominican Republic, Haiti, Florida, Jamaica, Leeward and Windward Islands, Puerto

Rico, Trinidad-Tobago, and Venezuela. Repeated attempts at cultivating this species have failed, and it is hypothesized that they can only be propagated from seed in combination with the appropriate mycorrhizal fungi. Seed germination facilitated by OMF is an essential tool for orchid conservation and is a necessity for future conservation efforts of endangered orchid species, including Floridian *Vanilla*.

This study used molecular methods to investigate and compare biodiversity of endophytic and mycorrhizal fungi associated with roots of south Floridian *Vanilla* populations that are endemic and threatened. In this study, only cultivable fungi were isolated and investigated for this purpose. Molecular analysis of fungi associated with these *Vanilla* provides new and valuable information on the biology of these species that could be of great value for its conservation. To compare endophytic fungi of these four *Vanilla* species, we generated and analyzed datasets of fungi we cultured from roots of each species. Information on the interactions between the studied orchid species and fungi may contribute to the knowledge of factors governing *Vanilla* distribution and population dynamics.

### **Methods:**

Considering the threatened and endangered status and general sensitivity of these species, it was important to limit the number of roots sampled from any single plant or population in an effort minimize damage to plants. Thus, only 3-5 root samples were collected from any given population. Root samples of approximately 5-10cm in length were collected. Photographs of voucher specimens are available at the Wisconsin State Herbarium, at the University of Wisconsin-Madison, Botany Department (<https://herbarium.wisc.edu/>). Upon collection, roots were placed in a sterile plastic bag with a moistened paper towel to prevent drying and placed on

ice. Within 12 hours of collection these bags were refrigerated at 4°C. *Ex situ* plants from the Marie Selby Botanical Garden included *V. dilloniana*, and *V. barbellata*, where one root was sampled from each. *In situ* plant samples included the following: three roots of *V. phaeantha* from the Everglades, three *V. mexicana* roots from private property, nine *V. barbellata* roots from the Florida Keys (Ramrod Key, Sugarloaf Key, and Middle Torch Key) and three more from the Everglades (Table 1).

Within one week of collection, mycorrhizae were isolated using standard protocols applied to epiphytic orchids (Zettler *et al.*, 2013; Yokoya *et al.*, 2015). Collected roots were washed gently in soapy water (Dawn® soap), and surface-sterilized by agitating for one minute with a sterilization solution composed of 5ml 5.25% NaOCl un-concentrated bleach (Clorox® Company, Oakland Ca.), 5ml 70% ethanol, and 90ml sterile deionized water. Roots were then triple rinsed with sterilized water, one minute per rinse (Zettler & Piskin, 2011). With a flame sterilized pair of forceps and a scalpel, the roots were cut into 1 cm length segments and individually macerated into Petri dishes with approximately 1 ml sterile water to release fungal pelotons. Once macerated, Fungal Isolation Media (FIM) was poured into Petri dishes over macerated root tissue (Hollick, 2009). FIM containing petri dishes were left to incubate at room temperature (~21°C) for three days (Clements & Ellyard, 1979). Under a compound microscope at 40x magnification fungal pelotons and mycelia protruding from plant tissues were first located and then isolated. After visible hyphae appeared around root segments, hyphal tips and freed pelotons were transferred into a Petri dish containing potato dextrose agar (PDA) medium and sub-cultured several times to obtain pure cultures. Each isolation was placed on PDA medium, and incubated at room temperature, 21°C, following Yu *et al.* (2009).

Genomic DNA was extracted using a modified method for a DNeasy Plant Kit (Qiagen Company©) incorporating the use of a tissue lyser in the first step of extraction. The tissue sample along with AP1 solution and 2-3, 4.5mm aluminum pellets were agitated in the tissue lyser for 1 min. DNA extraction was confirmed by gel electrophoresis. After extracting DNA from fungal samples, amplification reactions of 20µl were conducted using ITS primers (White *et al.*, 1990; Taylor & McCormick, 2008) selected based on the findings of White *et al.*, 1990 and purchased from Integrated DNA Technologies. The temperature of melting was calculated for each.

ITS1 (5'-TCCGTAGGTGAACCTGCGG-3')  $T_m = 66.8^\circ\text{C}$

ITS4 (5'- TCCTCCGCTTATTGATATGC -3')  $T_m = 60.4^\circ\text{C}$

ITS1 forward primer amplifies from the ITS region between the SSU (18S) RNA region and 5.8S RNA region and the ITS4 reverse primer amplifies from the ITS region between the LSU (25-28S) RNA region and 5.8S RNA region (White *et al.*, 1990; Vilgalys, 2015). Although ITS1 and ITS4 had annealing temperatures of 66.8°C and 60.4°C, respectively, these temperatures were attempted in PCR amplification but were unsuccessful. Instead, a much lower temperature of 54°C was attempted, following White *et al.*, (1990) and DNA amplification was successful. PCR was conducted under the following conditions: 2 min for initial denaturation at 94°C, before thermocycling 35 cycles with 30s denaturation at 94°C, 40s annealing at 54°C, and 1 minute elongation at 72°C, and the last cycle a final extension for 10 min. at 72°C (White *et al.*, 1990; Taylor & McCormick, 2008; Zettler *et al.*, 2013; Yokoya *et al.*, 2015). PCR products were detected by gel electrophoresis and DNA Quantification using TECAN Infinite M Plex plate reader for fluorescent readings of DNA using Invitrogen Qubit™ dsDNA HS Assay kit by thermo Fisher Scientific.

Crude ITS1/ITS4 amplification products were used for Sanger sequencing at Functional Biosciences™, DNA Sequencing Services (<https://functionalbio.com/>). Fungal DNA sequences were quality checked, trimmed, and aligned producing consensus sequences using Geneious Prime 2019.0.3 (<https://www.geneious.com>) and a National Center for Biotechnology Information (NCBI) BLAST™ (blastn) search was performed on GenBank to identify possible matches to fungal sequences (Altshul, 1997, Taylor & McCormick, 2008).

Relative abundance and frequency of endophytic fungi isolated from the orchid roots collected from the same site were determined. Fungal diversity was compared among orchid species and among sampling sites of *V. barbellata*. Analyses were performed using the ‘vegan’ package in R Studio. We calculated the following diversity indices; Fischers’ Alpha, Chao’s Beta Estimator, Whittaker’s Beta Diversity, Shannon-Wiener Index, Simpson’s Index and Inverse Simpson’s Index, as well as Pielou’s Evenness. Additionally, one-way ANOVAs were calculated using Microsoft Excel.

## **Results:**

In this study, we identify culturable endophytic fungal associates of four species of *Vanilla* (*V. barbellata*, *V. phaeantha*, *V. dilloniana*, and *V. mexicana*), as well as different communities of *V. barbellata* (Florida Keys, Florida Everglades, and Floridian Botanic Gardens) using ITS sequences. In total, 166 endophytic fungal isolates belonging to 44 different genera were identified (Tables 2, 3). All four *Vanilla* demonstrate greater fungal colonization in terrestrial achlorophyllous root segments than chlorophyllous aerial root segments.

In total 123 fungal isolations from roots of *V. barbellata* were successfully grown in culture; 53 isolates from 9 root segments were collected from the Florida Keys (Ramrod Key,

Sugarloaf Key, and Middle Torch Key), 62 isolates from three roots segments from the Everglades National Park, and eight isolates from one root from the Marie Selby Botanic Garden. *Fusarium* sp. were the most abundant in isolation from both the Florida Keys and Everglades samples (none were isolated from the Marie Selby Garden). The second most abundant isolate made from the Florida Keys was *Chaetomium* sp. and from the Florida Everglades the second most abundant isolate was *Aspergillus* sp. (Table 3).

Overall, 83% of endophytic fungal isolates may be ascribed to the classification of Ascomycetes. Roughly 16% of mycorrhizae were classified as Basidiomycetes and less than 1% as Zygomycetes (Table 3). From the roots samples of *V. mexicana*, 11 fungal isolates were successfully grown in culture. This paucity of isolates may be attributable to freezer burn incurred during transport from the field to the laboratory. These isolates were identified to seven different genera, the most common of which was *Pestalotiopsis* sp. *Vanilla phaeantha* roots yielded 18 successful isolations of 13 different genera. Most notably, a fungal pathogen well known for its effects on agriculturally grown *Vanilla*, *Fusarium* sp. was isolated most frequently. 18 fungal isolates from eight distinct genera, were successfully grown in culture from root segments of *V. dilloniana* collected from the Marie Selby Garden. Three of the isolates were identified as *Tulasnella*, a genus well known for its potential to germinate orchid seeds and another three isolates were identified as *Fusarium* sp. The genus *Fusarium* is commonly known for its potential as an orchid pathogen (Table 3).

Fungal isolates made from *V. barbellata* roots collected from three different locations are significantly different in a single-factor ANOVA ( $n = 44$ ,  $F = 7.09$ ,  $P\text{-value} = 0.0012$ ) (Table 4). Fischer's alpha diversity demonstrates that the highest richness and diversity of fungi among *V. barbellata* sites is in the Florida Keys. Evenness ( $J'$ ) suggests the existence of dominant fungal

species in *V. barbellata* from the Florida Keys, as made evident by the high collection of *Fusarium* sp. isolates.

In general, beta diversity for sample sites of *V. barbellata* is lowest between the Florida Keys and the Florida Everglades, and highest between either *in situ* location compared to the *ex situ* location (Marie Selby Garden). The same difference is demonstrated by Shannon-Wiener and Simpson indices which also correspond with species richness (Table 5 & Figure 1).

Fungal isolates made from roots of four different *Vanilla* species are significantly different in a single-factor ANOVA ( $n = 44$ ,  $F = 16.97$ ,  $P\text{-value} = 1.05e-9$ ) (Table 6). Analysis of alpha diversity by species indicates that the highest richness and diversity of fungal species is present in *V. phaeantha*. Beta diversity by *Vanilla* species is greatest between *V. barbellata* and *V. phaeantha*, this may be an artifact of collection as both came from the Florida Everglades. Overall, Shannon-Wiener and Simpson diversity indices between species are dissimilar but do correspond to species richness (Table 7, Figure 2).

Both between *Vanilla* species isolations and between *V. barbellata* site isolations (Figures 3, 4) Nectriaceae was recovered as the most abundant fungal family (26% and 32% respectively). Sporomiaceae, Chaetomiaceae, Aspergillaceae, and Psathyrellaceae are also recovered as equally secondarily present in both. Additionally, Nectriaceae is significantly abundant in all four *Vanilla* species (Figure 5).

## **Discussion:**

Orchids are the largest plant family and native to all continents, except Antarctica, meaning they play a role in many diverse ecosystems. Orchids require mycorrhizal fungi at different stages of their life, including germination, to obtain carbon. This dependency on

mycorrhizal fungi makes collection and identification of these mycorrhizae essential for future conservation efforts utilizing the same fungi in an attempt to germinate rare and endangered *Vanilla* (McCormick *et al.*, 2004).

**Fungal Ecology** - Saprotrophic or soil fungi infect orchid root tissue penetrating through cell walls of the orchid where they then form pelotons within the cells. These pelotons are characteristic intracellular hyphal coils, which are then digested by the plant cells (Yoder *et al.*, 2000). The fungus infecting the plant is restricted only to the cortical cells, providing the plant with critical nutrients for survival (Waterman *et al.*, 2008). These pelotons are integrated into the root and allow nutrients to be transferred from the mycorrhizal fungi to orchids. Yet, the fungi receive little to no nutrients from the orchid, casting doubt on the nature of this interaction. Adult orchids have pelotons integrated into their roots, and they can even be found in their leaves (Bayman *et al.*, 2002; Phillips *et al.*, 2011, Porras-Alfero & Bayman, 2007; Whigham & Zettler, 2016). Many orchid-associated mycorrhizal fungi are still unknown. Furthermore, at different phases in their life cycles, different orchids demonstrate different mycorrhizal partnerships and in different combinations (Zettler *et al.*, 2007). These preferences could be influenced not just by individual variances from orchid to orchid, but also by differences habitat to habitat (Hollick, 2004; Otero *et al.*, 2004; Phillips *et al.*, 2011; Shefferson *et al.*, 2005; Zettler *et al.*, 2007). The fungi that are most typically associated with orchids are *Rhizoctonia*; (anamorph/telomorph) *Opadorhiza/Sebacina*, *Epulorhiza/Tulasnella*, and *Ceratorhiza/Ceratobasidium* (Rasmussen *et al.*, 2015; Wells, 1994; Taylor & McCormick, 2008).

Naturally, not all fungal partnerships are beneficial to orchids. Latent pathogens and non-mycorrhizal fungi are presumably present and simply asymptomatic. *Vanilla* crops have shown a rather severe susceptibility to certain pathogenic *Fusarium* species (Pinaría *et al.*, 2010;

Gamboa-Gaitan, 2013). The monoculture of *Vanilla* crops created by current agricultural practices necessitate seed germination of *Vanilla* species is even more essential (Cameron 2011; Koyyappurath *et al.*, 2016; Hernandez-Hernandez, 2018). Conversely, researchers have found that certain asymptomatic latent pathogens, like *Fusarium*, may have the capacity to promote orchid seed germination, even assisting to the point of rupturing the testa. More recently *Trichoderma*, *Fusarium*, and *Clonostachys* have been shown to exhibit germinative potential on seeds of *Pogoniopsis schenckii* (Vanilloideae), causing a rupture of the external tegument (Sisti *et al.*, 2019).

***Vanilla mexicana*** - *Vanilla mexicana* belongs to the New World membranaceous-leaved clade sister to the rest of the *Vanilla* genus (Bourtard *et al.*, 2010). The Floridian population of this species that was sampled for this study is private property and a well-kept secret, as it is now the only known location of the species in the state. The severe decline of this species may be in large part due to its inability to be clonally cultivated. This may also suggest a strong dependency on mycorrhizal partnerships as seed germination seems to be its only mode of reproduction. Although samples were exposed to freezer burn during transportation from the field to the laboratory, *Pestalotiopsis* sp. were identified four times. *Pestalotiopsis* sp. are known as non-mycorrhizal endophytes that do have the ability to transfer nutrients such as carbon and nitrogen to host plants (Table 3; Figures 3, 5).

***Vanilla phaeantha*** - *Vanilla phaeantha* is closely related to the cultivated crop plant *V. planifolia* and is part of the American fragrant clade of *Vanilla* (Bouetard *et al.*, 2010). This neotropical fragrant leafy species shares many similarities with the crop plant *Vanilla*, often including habitats like those in Florida and susceptibility to similar diseases such as fusariosis caused by *Fusarium*. *Fusarium* was also the most frequently identified among fungal isolates of

this species. Additionally, *Preussia* sp., a common soil fungus, and *Periconia* sp., a dark septate endophyte (DSE), were isolated from *V. phaeantha*. These as well as *Coprinellus* sp., *Gymnopilus* sp., *Phomopsis* sp., *Paecilomyces* sp., *Diaporthe* sp., and others were isolated in other species of *Vanilla* included in this study (Table 3; Figures 3, 5).

***Vanilla dilloniana* & *V. barbellata*** - *Vanilla dilloniana* and *V. barbellata* are both members of the Old World (African & Asian) and Caribbean clade. Their common ancestor experienced transoceanic dispersions, the first of which was to Africa from the Caribbean where the leafless habitat was developed. This leafless African ancestor experienced the secondary dispersal back to the Caribbean where it further speciated with the leafless habit (Bouetard *et al.*, 2010). It is often hypothesized that leafless members of the Orchidaceae require a stronger partnership with mycorrhizal fungi. *Tulasnella* sp. was identified from *V. dilloniana* isolates on three occasions and are commonly known to be beneficial to orchid seed germination and growth. *Fusarium* sp. were equally present in fungal isolates from the species. *V. barbellata* included in this study demonstrated a high frequency of *Fusarium* sp. among isolations. Additionally, *Coprinellus* (Psathyrellaceae), *Aspergillus* (Aspergillaceae), *Chaetomium* (Chaetomiaceae), and *Preussia* sp. (Sporomiaceae) were all frequently isolated as well, all of which are known as either latent-pathogens or non-mycorrhizal endophytes. Many differences are evident between *V. barbellata* mycorrhizae isolated from the Florida Keys and the Everglades National Park, some appearing at one site and not the other. However, in both cases the mycorrhizae that are most abundant are the mycorrhizae they have in common (Table 3; Figures 3, 5).

**Diversity of Fungal Communities** - The diversity of fungal communities differs among orchid species and between locations of *V. barbellata*. The Shannon-Wiener index values

obtained from empirical data range from 1.9 to 3.1 between species and 1.9 and 2.9 between locations of *V. barbellata* (Tables 5, 7; Figures 1, 2). These values are similar to or slightly higher than those of similar studies (Stark *et al.*, 2009; Tao *et al.*, 2008; Oliveira *et al.*, 2014; Beltran-Nambo *et al.*, 2018). Beta diversity results between *Vanilla* species and *V. barbellata* sites, is related to mycobiont richness and evenness. Based on the Simpson diversity index values, all species and communities indicate the presence of dominant species. Diversity is highest in the Florida Keys (Table 6 & Figure 1). Although this may be an artifact of this study's collection scheme as more individuals were available for sampling in the Florida Keys, this may also indicate that associations with a diverse fungal community may influence the persistence and vulnerability of orchids. Thus, the lower values may explain the decline or diminished number of individuals in natural populations likely due to habitat deterioration (Table 2; Figures 4, 5).

Most notably, only three isolates of the total 168 were identified as known orchid mycorrhizal fungi belonging to the genus *Tulasnella*. *Tulasnella calospora* is a recognized orchid mycorrhizal fungus and could be potentially useful for seed germination. Isolates also include numerous non-mycorrhizal endophytes, saprobes, latent pathogens such as the genus *Fusarium*, and dark-septate endophytes. Recently, there has been a growing interest in dark septate fungi like *Periconia* sp. and their potential role in seed germination, including orchid seeds, although, the majority of the fungi are mutualists with the potential to be pathogens. *Fusarium* sp. were identified numerous times from all *Vanilla* species and sites sampled. *Fusarium* is known as the cause for fusariosis or wilt disease, a common issue in *Vanilla* crops.

**Latent Pathogens & Non-mycorrhizal Fungi** - Symptoms of fusariosis include, but are not limited, to plant tissue yellowing, necrosis, stunted growth, xylem tissue turning brown;

fusariosis ultimately leads to plant death. *Fusarium* is considered a ubiquitous fungus and is often asymptomatic, as in this case. Latent pathogenic endophytic fungi also deserve additional study. *Colletotrichum* sp. were also isolated and identified, along with the latent pathogens *Aspergillus* sp., *Paecilomyces* so., and *Chaetomia* sp. Contemporary work has demonstrated that *Clonostachys* sp. (a saprobe) and *Trichoderma* sp. (a non-mycorrhizal ectomycorrhiza) have in fact shown some promising potential for symbiotic seed germination (Sisi *et al.*, 2019). Additional non-mycorrhizal endophytes that were identified include *Xylaria* sp., *Pestalotiopsis* sp., *Phomopsis* sp., *Diaporthe* sp., and *Cladosporium* sp. Orchids are critically dependent on fungi and identifying mycorrhizal preferences is key to their conservation (McCormick *et al.*, 2004). Although non-mycorrhizal endophytes are not providing the plant with essential carbon, nitrogen, or water, it has been reported that some species like *Xylaria* sp. may be contributing antibacterial compounds (Sisi *et al.*, 2019).

**Dark Septate Endophytes** - Interest in dark septate fungi and their potential role in seed germination, including orchid seeds, has been growing. Current research on orchid seeds with dark septate endophytes as mycorrhizae is exclusively restricted to use on terrestrial orchids, such as *Dactylorhiza praetermissa* (Zimmerman & Peterson, 2007) and *Aa achalensis* (Sebastián *et al.*, 2014). As a hemi-terrestrial genus, *Vanilla* is an interesting option for future seed germination experiments using dark septate endophytes.

***Tulasnella*, A Well-known Orchid Mycorrhizal Fungi** - Three isolates were identified as known orchid mycorrhizal fungi belonging to the genus *Tulasnella* and have the potential to be useful for seed germination. Different *Tulasnella* species have been identified as mycorrhizae in members of the *Vanilla* genus in Puerto Rico and Colombia (Porras-Alfero & Bayman, 2007; Alomía *et al.*, 2017). However, there has been limited success utilizing it in in germination

experiments for the *Vanilla* genus. Other orchid genera have had great success with *Tulasnella* inoculum for symbiotic seed germination including conservation efforts of Floridian orchids (Zettler *et al.*, 2011). Additionally, *Tulasnella* sp. has shown great value in seed germination and conservation efforts with leafless Floridian orchids such as *Dendrophylax lindenii* and *D. porrectus* (Mujica *et al.*, 2018). Notably, the closest sequence match for the three *Tulasnella* sp. isolates from *V. dilloniana* is to that of another orchid based study conducted on Reunion Island's tropical epiphytes that included *Vanilla* as an outgroup (Martos *et al.*, 2012).

**Future Work** - In summary, fungal communities of the four *Vanilla* species analyzed showed high diversity. Basidiomycete fungi in this study have probable mycorrhizal fungi functions, as the Ascomycetes found included fungi that are potentially endophytic. Additionally, these native fungal communities should be considered in any translocation of plants and reintroduction of plants produced from symbiotic seed germination. This study adds to the growing body of literature that considers the ecology of *Vanilla* that is contributing to conservation efforts for the genus. Identities of the mycorrhizae of this study may inform future seed germination research. In the case of *V. barbellata*, flowers in the U.S. Virgin Islands, Puerto Rico or the State of Florida should be hand pollinated and monitored. For additional confidence in pollination efforts, seed viability tests with the new technique using tetrazolium chloride according to the procedures of Alomía *et al.*, 2017 are recommended. We suggest a seed sterilization and scarification technique utilizing vacuum filtration with the addition of sulfuric acid (H<sub>2</sub>SO<sub>4</sub>) and seed coat degrading enzymes (pepsin) to mimic small animal digestion (Batty 2001 a; b; Dixon *et al.*, 2003, Suetsugu *et al.*, 2015; pers. comm. Giovanni Giraldo-Caro, D. Whigham, Kingsley Dixon, & Lawrence Zettler). Recent evidence suggests that zoochory is necessary for seed scarification and dispersal and the use of sulfuric acid and seed coat degrading

enzymes effectively mimic digestion need to break down the scleral coat of *Vanilla* seeds. The presence of such dispersals appear absent in Floridian and possibly Caribbean environments but present in Central and South America. This technique could replicate natural scarification of small vertebrate digestion, thinning the sclerified seed coat of *Vanilla* seed allowing for mycorrhizal infection. Given that *Vanilla* is a threatened taxon, included in the IUCN Redlist and the Endangered Species Act lists, a better understanding of its ecology may be of critical importance for long-term conservation of these delicate and economically important orchids. Molecular analysis of fungi associated with Floridian *Vanilla*, performed for the first time in this work, provides valuable information on the biology of this threatened orchid species that could be of great value for its conservation. Additionally, future use of mycorrhizae identified here may inform subsequent seed germination studies. Non-OMF endophytes are frequently disregarded, however there has recently been a resurgence of interest in non-mycorrhizal endophytic fungus and the nature of their connection with orchids when they are latent pathogens. Information gained from this study will contribute to the efforts of NAOCC, IUCN, and Florida State Conservation efforts

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**Tables:****Table 1:** Root Samples Collected for Fungal Isolations from each *Vanilla* species and the collection site.

*Samples Collected for Fungal Isolation*

<i>Vanilla Species</i>	Location	Number Collected	Fungal Isolates	Mean
<i>V. barbellata</i>	Selby Garden	1	8	0.048
	Florida Keys	9	53	0.315
	Everglades	3	62	0.369
<i>V. phaeantha</i>	Everglades	3	18	0.107
<i>V. mexicana</i>	Lake, FL	3	11	0.065
<i>V. dilloniana</i>	Selby Garden	1	16	0.095

**Table 2:** Fungal genera identified in axenic cultures from three different sites of *Vanilla barbellata* populations (Florida Keys, the Everglades National Park, and the Marie Selby Garden & Fairchild Topical Botanic Gardens) and number of fungal strains obtained from each.

Division	Family	Genera (sp)	NCBI Accession #	<i>V. barbellata</i> Total				
				Fl Keys	Everglades	Botanic Gardens	Total	
<i>Ascomycota</i>	Aspergillaceae	<i>Aspergillus spelaesus</i>	MH911347, MH911348	2	8	0	10	
		<i>Penicillium</i> sp.	GU055994, MT582767	2	0	0	2	
	Bionectriaceae	<i>Clonostachys</i> sp.	MW765066	1	2	0	3	
	Chaetomiaceae	<i>Chaetomium</i> sp.	KJ767122, LC133884, MT035919, MN547398	5	3	1	9	
			<i>Humicola</i> sp.	MN006389	0	1	0	1
	Cordycipitaceae	<i>Beauveria</i> sp.	MW077719, MH185848	0	2	0	2	
	Dermateaceae	<i>Trichosporiella</i> sp.	DQ069049, MH512968	0	1	0	1	
	Diaporthaceae	<i>Diaporthe</i> sp.	KY413768, AB899788, MW767084, MT158487, MH044708	1	1	1	3	
	Didymellaceae		<i>Epicoccum</i> sp.	KT588465	1	0	0	1
	Glomerellaceae	<i>Colletotrichum</i> sp.	MW045583, FJ224109	0	3	2	5	
	Nectriaceae	Nectriaceae sp. <i>Fusarium</i> sp.	MK116450 MT529795, KU535783, LC13762, MW497617, KY910845, MW789355, MT299291, LT746254, MK583509, LT841264, LT841265, MH486974, MH707085, MN759070	1 11	0 9	0 0	1 20	
			<i>Nectria</i> sp.	AB237663, MH748176, MK116450, MH748176	3	2	0	5
			<i>Volutella</i> sp.	AJ301967	1	0	0	1

		<i>Xenoacremonium</i> sp.	MN701717, KM231835, KM231834	1	4	1	6
	Periconiaceae	<i>Periconia</i> sp.	MW442967, MW826181, KX394553	0	1	0	1
	Pleurostomataceae	<i>Pleurostoma</i> sp.	MK268142, MK267789	0	1	1	2
		<i>Pleurostomophora</i> sp.	MK647985	0	3	0	3
	Pseudeurotiaceae	<i>Geomyces</i> sp.	HQ115661, KF212290	1	0	0	1
		<i>Pseudogymnoascus</i> sp.	MH865212	1	0	0	1
	Schizoparmaceae	<i>Coniella</i> sp.	NR_111706	1	0	0	1
	Sordariaceae	<i>Asordaria</i> sp.	AY681174	1	0	0	1
	Sporocadaceae	<i>Pestalotiopsis</i> sp.	KY609340, MK120574, LC387821, JN222976, MK278904, KP747709	2	1	0	3
	Sporormiaceae	<i>Preussia</i> sp.	KY124642, AB470820, KP101211, MN341256	4	6	0	10
	Thermoascaceae	<i>Paecilomyces</i> sp.	OK210352, MG827159	0	2	0	2
	Togniniaceae	<i>Phaeoacremonium</i> sp.	MT022468	1	0	0	1
	Trichocomaceae	<i>Talaromyces</i> sp.	KP235606, MK139987	0	4	0	4
	Unclassified Hypocreales	Unclassified Hypocreales sp.	LC314677, GU017510, MZ666365	1	0	1	2
	Valsariaceae	<i>Myrmaecium</i> sp.	KP687884	1	0	0	1
<i>Basidiomycota</i>	Cortinariaceae	<i>Gymnopilus</i> sp.	MT237518, KT368682, AF501553	1	1	0	2
	Irpicaceae	<i>Irpex</i> sp.	KUC8508, MN856293	2	0	0	2
	Phanerochaetaceae	<i>Bjerkandera</i> sp.	KC176315	1	0	0	1
	Polyporaceae	<i>Trametes</i> sp.	KM822753, GU738022	1	4	0	5
	Psathyrellaceae	<i>Coprinellus</i> sp.	MN622748, LC612525, MT507812, OK210350, AB470820, MK087751, OK178929	4	3	1	8
<i>Mucor - Fungi incertae sedis</i>	<i>Mucor - Fungi incertae sedis</i>	<i>Mucor</i> sp.	NR_111683	1	0	0	1
	Umbelopsidaceae	<i>Umbelopsis</i> sp.	KP172537	1	0	0	1
		Total		53	62	8	123

**Table 3:** Fungal genera identified in axenic cultures from four *Vanilla* species studied and number of fungal strains obtained from each.

<i>Division</i>	<i>Family</i>	<i>Genera (sp)</i>	<i>NCBI Accession #</i>	<i>V. barbellata</i>	<i>V. phaeantha</i>	<i>V. dilloniana</i>	<i>V. mexicana</i>	<i>Total</i>	
<i>Ascomycota</i>	Aspergillaceae	<i>Aspergillus spelaeus</i>	MH911347, MH911348	10	0	0	0	10	
		<i>Penicillium</i> sp.	GU055994, MT582767	2	0	0	0	2	
	Bionectriaceae	<i>Clonostachys</i> sp.	MW765066	3	0	0	0	3	
	Chaetomiaceae	<i>Chaetomium</i> sp.	KJ767122, LC133884, MT035919, MN547398	9	0	0	0	9	
		<i>Humicola</i> sp.	MN006389	1	1	0	0	2	
	Cordycipitaceae	<i>Beauveria</i> sp.	MW077719, MH185848	2	0	0	0	2	
	Debaryomycetaceae	<i>Meyerozyma</i> sp.	MW710001	0	0	1	0	1	
	Dermateaceae	<i>Trichosporia</i> sp.	DQ069049, MH512968	1	1	0	0	2	
	Diaporthaceae	<i>Diaporthe</i> sp.	KY413768, AB899788, MW767084, MT158487, MH044708	3	1	0	2	6	
			KT588465	1	0	0	0	1	
	Didymellaceae	<i>Epicoccum</i> sp.	KT588465	1	0	0	0	1	
	Glomerellaceae	<i>Colletotrichum</i> sp.	MW045583, FJ224109	5	0	0	0	5	
	Leotiomycetes incertae sedis	<i>Scytalidium</i> sp.	MF992675	0	0	1	0	1	
	Nectriaceae	Nectriaceae sp.	<i>Fusarium</i> sp.	MK116450	1	0	0	0	1
			MT529795, KU535783, LC13762, MW497617, KY910845, MW789355, MT299291, LT746254, MK583509, LT841264, LT841265, MH486974, MH707085, MN759070	20	4	3	0	27	
		<i>Nectria</i> sp.	AB237663, MH748176,	5	0	0	1	6	

<i>Basidiomycota</i>		MK116450, MH748176 KY554751	0	1	0	0	1	
		<i>Neocosmopora</i> sp.						
		<i>Volutella</i> sp.	AJ301967	1	0	0	0	1
		<i>Xenoacremonium</i> sp.	MN701717, KM231835, KM231834	6	0	0	1	7
	Periconiaceae	<i>Perconia</i> sp.	MW442967, MW826181, KX394553	1	2	0	0	3
	Pleurostomataceae	<i>Pleurostoma</i> sp.	MK268142, MK267789	2	0	0	0	2
		<i>Pleurostomophora</i> sp.	MK647985	3	1	0	0	4
	Pseudeurotiaceae	<i>Geomyces</i> sp.	HQ115661, KF212290	1	0	0	1	2
		<i>Pseudogymnoascus</i> sp.	MH865212	1	0	0	0	1
	Schizoparmaceae	<i>Coniella</i> sp.	NR_111706	1	0	0	0	1
	Sordariaceae	<i>Asordaria</i> sp.	AY681174	1	0	0	0	1
	Sporocadaceae	<i>Monochaetia</i> sp.	JN222972	0	0	0	1	1
		<i>Pestalotiopsis</i> sp.	KY609340, MK120574, LC387821, JN222976, MK278904, KP747709	3	0	0	4	7
	Sporormiaceae	<i>Preussia</i> sp.	KY124642, AB470820, KP101211, MN341256	10	2	0	0	12
	Thermoascaceae	<i>Paecilomyces</i> sp.	OK210352, MG827159	2	1	0	0	3
	Togniniaceae	<i>Phaeoacromonium</i> sp.	MT022468	1	0	0	0	1
	Trichocomaceae	<i>Talaromyces</i> sp.	KP235606, MK139987	4	0	0	0	4
	Unclassified Hypocreales	Unclassified Hypocreales sp.	LC314677, GU017510, MZ666365	2	1	0	0	3
	Valsaceae	<i>Phomopsis</i> sp.	GU066650, KF032029, LT560085	0	1	1	1	3
	Valsariaceae	<i>Myrmaecium</i> sp.	KP687884	1	0	0	0	1
Xylariaceae	<i>Xylaria</i> sp.	JQ760825, JQ760694	0	0	2	0	2	
Cortinariaceae	<i>Gymnopilus</i> sp.	MT237518, KT368682, AF501553	2	1	2	0	5	
Irpicaceae	<i>Irpex</i> sp.	KUC8508, MN856293	2	0	0	0	2	

<i>Mucor - Fungi incertae sedis</i>	Phanerochaetaceae	<i>Bjerkandera</i> sp.	KC176315	1	0	0	0	1
	Polyporaceae	<i>Trametes</i> sp.	KM822753, GU738022	5	0	0	0	5
	Psathyrellaceae	<i>Coprinellus</i> sp.	MN622748, LC612525, MT507812, OK210350, AB470820, MK087751, OK178929	8	1	1	0	10
	Tulasnellaceae	<i>Tulasnella</i> sp.	JF691405	0	0	3	0	3
	<i>Mucor - Fungi incertae sedis</i>	<i>Mucor</i> sp.	NR_111683	1	0	0	0	1
	Umbelopsida ceae	<i>Umbelopsis</i> sp.	KP172537	1	0	0	0	1
		Total		123	18	16	11	166

**Table 4:** ANOVA Single Factor - *Vanilla barbellata* by collection site exhibiting significant difference exists between the Florida Everglades and the Florida Keys.

SUMMARY						
Groups	Count	Sum	Average	Variance		
Florida Keys	44	53	1.205	3.655		
Everglades	44	62	1.476	4.792		
Bot. Gardens	44	8	0.182	0.199		
ANOVA						
Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	40.442	2	20.221	7.091	0.001	3.068
Within Groups	362.181	127	2.852			
Total	402.623	129				

**Table 5:** Diversity Indices of fungal endophytes by collection site of *Vanilla barbellata*.

Shannon-Wiener and Simpson Indices exhibit similarities between the Florida Everglades and Florida Keys. Beta Diversity demonstrates no significant differences between the Florida Everglades and the Florida Keys.

Collection Sites	Species Richness	Pielou's Evenness (J')	Fischer Alpha	Shannon-Wiener (H)	Simpson Index
Florida Keys	27	0.893	22.035	2.945	0.921
Florida Everglades	21	0.918	11.175	2.796	0.925
Marie Selby Garden	7	0.98	26.782	1.906	0.844
Overall	26	0.876	17.128	3.14	0.937
Beta Diversity (Whittaker's)	Florida Keys	Florida Everglades			
Florida Everglades	0.5	-			
Marie Selby Garden	0.706	0.571			

**Table 6:** ANOVA Single Factor - *Vanilla* species exhibiting significant difference exists

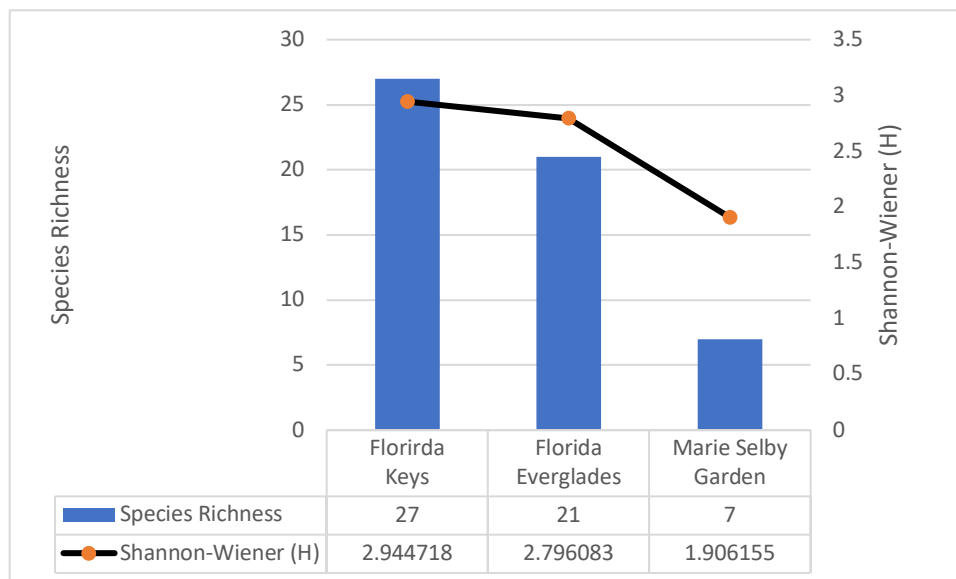
between each. *Vanilla* sp with a smaller sample size also show the same differences.

SUMMARY						
Groups	Count	Sum	Average	Variance		
<i>V. phaeantha</i>	44	18	0.409	0.619		
<i>V. dilloniana</i>	44	14	0.318	0.594		
<i>V. mexicana</i>	44	11	0.25	0.517		
<i>V. barbellata</i>	44	123	2.795	14.12		
ANOVA						
Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	201.841	3	67.280	16.978	1.05E-09	2.657
Within Groups	681.591	172	3.963			
Total	883.432	175				

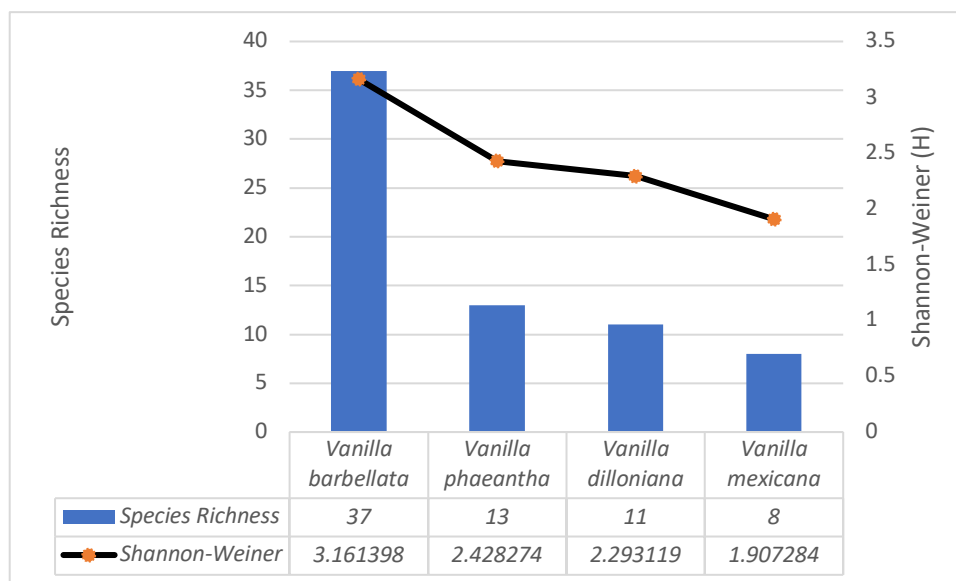
**Table 7:** Diversity Indices of fungal communities by *Vanilla* species. The Shannon-Wiener and Simpson indices correlate with each other and Alpha diversity.

Species	Species Richness	Pielou's Evenness (J')	Fischer's Alpha	Shannon-Wiener	Simpson Index
<i>Vanilla barbellata</i>	37	0.876	17.851	3.161	0.938
<i>Vanilla phaeantha</i>	13	0.947	21.001	2.428	0.895
<i>Vanilla dilloniana</i>	11	0.956	12.014	2.293	0.889
<i>Vanilla mexicana</i>	8	0.917	10.489	1.907	0.819
Total	44	0.907	19.108	3.433	0.956
Beta Diversity (Whittaker's)	<i>Vanilla barbellata</i>	<i>Vanilla phaeantha</i>	<i>Vanilla dilloniana</i>		
<i>V. phaeantha</i>	0.56	-	-		
<i>V. dilloniana</i>	0.667	0.75	-		
<i>V. mexicana</i>	0.778	0.905	0.895		

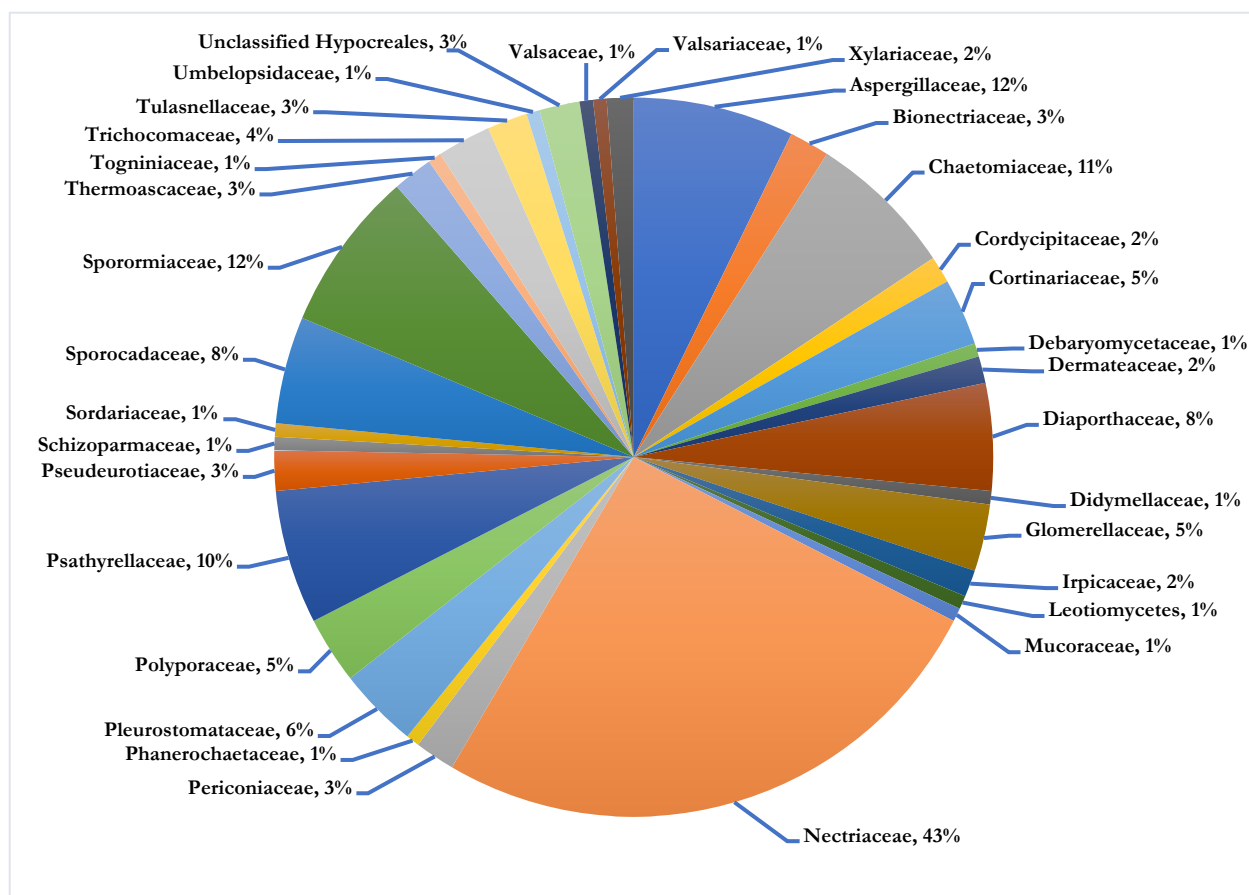
### Figures:



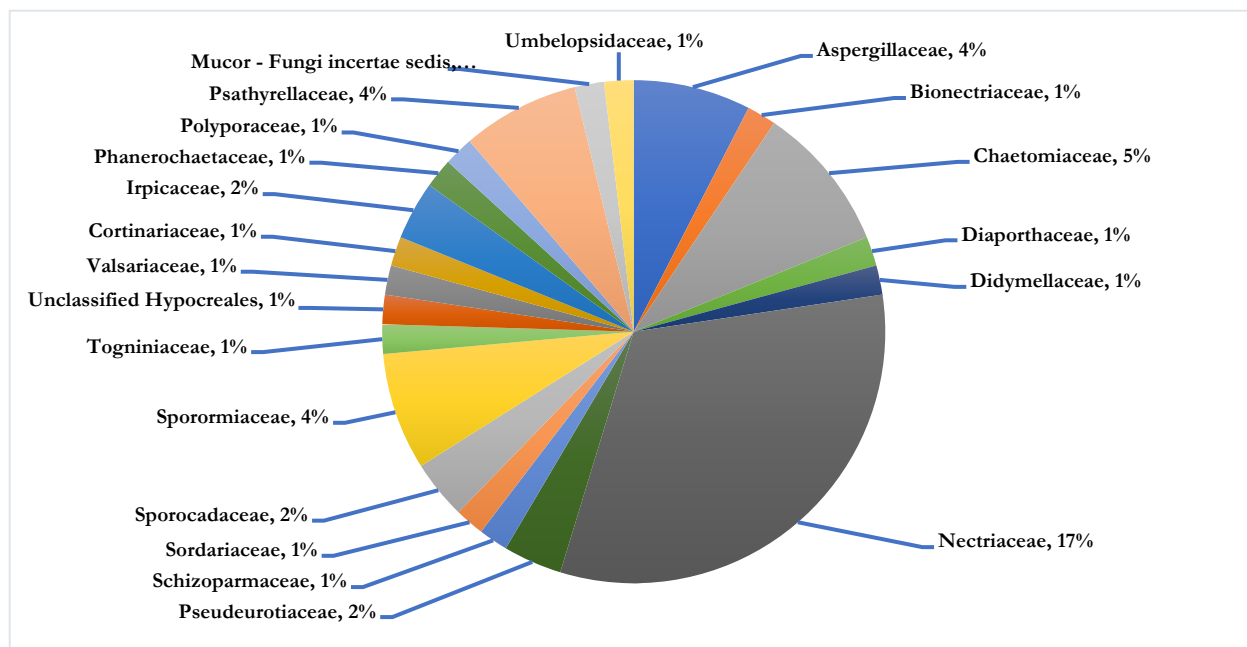
**Figure 1.** Graph of richness and fungal diversity (Shannon-Wiener) among sites of *Vanilla barbellata*. Shannon-Wiener index correlates with species richness.



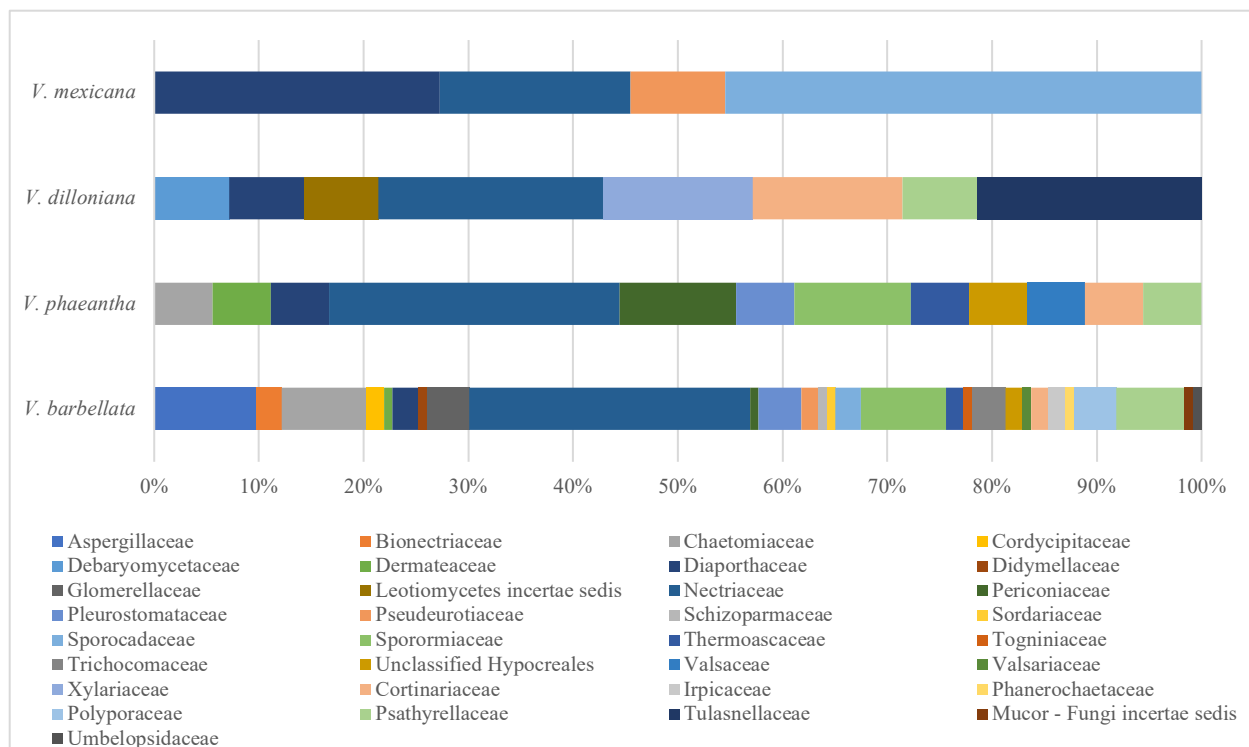
**Figure 2.** Graph of richness and fungal diversity (Shannon-Wiener) among four species of *Vanilla*. Shannon-Wiener index correlates with species richness.



**Figure 3.** Frequency distribution of the number of endophytic isolates identified to family from all four *Vanilla* species included in this study (*Vanilla mexicana*, *V. barbellata*, *V. dilloniana*, & *V. phaeantha*).



**Figure 4.** Frequency distribution of the number of endophytic isolates identified to family with the *Vanilla barbellata* from differing sites.



**Figure 5.** Relative abundance of orchid mycorrhizal families associating with the four *Vanilla* species native to south Florida. Relative abundance was calculated as the proportion of fungal isolates identified to each fungal family from each *Vanilla* species.

**Chapter III: Organographic Meta-Barcoding of Mycobiomes Comparing Leafless *Vanilla barbellata* and Leafy *Vanilla phaeantha* (Orchidaceae).**

**Abstract:**

Only a few genera of Orchidaceae subfamily Vanilloideae exhibit a climbing growth habit otherwise absent from this most species-rich family of flowering plants. Among these are several leafless genera, some of which are achlorophyllous mycoheterotrophs, but also a handful of photosynthetic vines within the genus *Vanilla*. Leafless orchids, such as *V. barbellata*, have been hypothesized perhaps to have greater dependence on mycorrhizal fungi than their leafy counterparts, such as *V. phaeantha*. Although mycorrhizal fungi play a fundamental role in the life cycle of orchids, the full diversity and composition of orchid mycobionts is poorly documented. Meta-barcoding using Illumina amplicon sequencing can reveal distributions of operational taxonomic units (OTUs) characterizing fungal community profiles. We analyzed mycobiome composition and diversity between the leafless *V. barbellata* and leafy *V. phaeantha* native to south Florida and compared plant compartments within and among these two species from different clades. Despite their phylogenetic distance and the distinctive leaflessness of *V. barbellata*, both species shared most mycobiont taxa in all analyzed compartments and habitats. Yet, plant organs demonstrated significant differences, suggesting the plant host influences fungal communities and that fungi establish specific interactions with their plant host. Our results suggest that the mycobiont associations of these *Vanilla* species are not species specific. These similarities would permit the ability to undertake conservation efforts for multiple *Vanilla*

species in aggregate. Data acquired in this study provides a foundational baseline for the conservation efforts for the *Vanilla* genus that more fully integrates their fungal ecology.

**Key Words:** Orchidaceae, *Vanilla barbellata*, *Vanilla phaeantha*, Mycobiome, Meta-barcoding

**Introduction:**

The pantropical genus *Vanilla* is the largest of the Orchidaceae subfamily Vanilloideae consisting of 118 species (Cameron, 2000; Soto-Arenas & Cameron, 2003). Southern Florida is home to six *Vanilla* species, two of which are *Vanilla barbellata* and *Vanilla phaeantha*. *V. barbellata* is a leafless *Vanilla* that grows in the Bahamas, Cuba, the Dominican Republic, Florida, a few Leeward Islands, and Puerto Rico. *V. phaeantha* Rchb.f. is a rare species in Florida with only two known locations; the Fakahatchee Strand Preserve State Park, and the Florida Everglades, but with an accepted range of the Bahamas, Costa Rica, Cuba, El Salvador, Florida, Jamaica, Mexico, Panama, Trinidad-Tobago, and the Windward Islands (Wegier, 2018).

Vanilloideae exhibit a viny growth type, which is unique among orchids, growing terrestrially first, and subsequently hemi-epiphytically by climbing trees and shrubs with aerial roots (Soto-Arenas & Cameron, 2003; Odoux & Grisoni, 2011; Correll *et al.*, 1950). They are known for species having atypical features of most orchids having a much larger seed and scleral seed coat and fleshy aromatic fruits rather than dry capsules containing microscopic dust-like seeds (Dressler, 1993; Cameron & Soto-Arenas, 2003; Cameron & Chase, 1998; Zettler *et al.*, 2013). These seeds require a fungal symbiont for germination, like most orchids (Rasmussen, 2002). The orchid embryo becomes actively mycoheterotrophic, relying on the fungal symbiont. An orchid's mycorrhizae vary by species in several circumstances deriving its necessary carbon, nitrogen, and other nutrients from the fungal symbiont (Smith & Read, 2008).

Orchids can form symbiotic relationships with a variety of fungal genera and can change symbionts throughout their lives (Zettler *et al.*, 2007; Taylor & McCormick, 2008; Waterman *et al.*, 2008). Seedling recruitment is crucial to long-term viability of any plant community (Rasmussen *et al.*, 2015). Some even more atypical members of *Vanilla* are leafless. Two such

leafless examples that are native to Florida are *V. barbellata* and *V. dilloniana*. The leafless habit of *Vanilla* is thought to have established itself after the transoceanic dispersion of the genus to the continent of Africa. The genus then speciated and developed this habit on the African continent prior to a secondary transoceanic dispersal back to the Caribbean where the genus then further speciated with the leafless habit (Bouetard *et al.*, 2010). It is thought that without the additional surface area of leaves to conduct photosynthesis these orchids are likely to rely more on their fungal symbiont for nutrients than those with leaves, as demonstrated recently in the genus *Erythrorchis*, a leafless member of the Vanilloideae (Ogura-Tsujita, 2018).

The mycobiome of a plant consists of resident fungi from a particular environment, including the plant body or a portion of it. Within the mycobiome, mycobionts have a broad range of roles from pathogen to symbiont. Ascertaining how these communities assemble to effectively predict or modify their intended activities requires determining the diversity and composition of these component fungal communities. Some fungal constituents of the mycobiome increase water and nutrient uptake. They may also play pathogenic roles, like *Fusarium*, which can cause root rot or fusariosis. Endophytic fungi are distributed inside the host plant's living tissue, often causing no obvious damage (Hardoim *et al.*, 2015). Plant development, pathogen defense, and abiotic stress response are only a few of the crucial processes facilitated by their complex relations inside the host plant (Rodriguez *et al.*, 2009; Khare *et al.*, 2018). The agricultural and industrial potential of these interactions has piqued interest in these endophytes in recent years. It is first necessary to identify those fungal taxa forming and influencing the existing fungal community to determine if their management for conservation or agricultural purposes is possible. *Vanilla planifolia* is primarily cultivated for manufacturing of *Vanilla* spice. Clonal propagation practices have created monocultures, which

could lead to *Vanilla* crop failure. Monoculture crops are more prone to common infections from viruses and *Fusarium* pathogenicity (*Fusarium* rot), jeopardizing global commercial cultivation efforts (Cameron 2011; Koyyappurath *et al.*, 2016; Hernandez-Hernandez, 2018). Although *F. oxysporum* may be present in wild or cultivated plants, the plants will not necessarily show signs or symptoms of infection (Pinaría *et al.*, 2010; Gamboa-Gaitan, 2013). Rather, some *F. oxysporum* strains can act mutualistically, inhibiting pathogenic strains while also providing benefits to their host (Nel *et al.*, 2006). This suggests at least some *Fusarium* species have a mycorrhizal role, and probably a dual pathogen-endophyte role in *Vanilla* that is likely partially dependent on the microbiome context and overall plant health (Carbajal-Valenzuela *et al.*, 2021).

It is additionally necessary to identify fungal endophyte communities of complex orchid species, such as *V. barbellata*, to inform conservation efforts. A lack of data concerning seed germination and required soil-fungal interactions, unique pollinators, and specific habitat requirements all hinder orchid conservation efforts. Orchids are often the first to be lost during ecosystem degradation or declines, and are indicator species or early warning signs that conservation efforts are necessary to preserve a system (Noss, 1990). South Floridian *Vanilla* face additional natural threats such as wildfires and flooding as well as anthropogenically driven threats such as habitat loss, degradation, and fragmentation. Furthermore, the future of these habitats is under clear threat from the effects of climate change, particularly rising sea levels and hurricane activity, according to the National Aeronautics and Space Administration (NASA). This adds extra urgency for the need to understand the endophytes of *V. barbellata* and *V. phaeantha*. Understanding the necessary mycobionts and their dynamics for successful cultivation of these *Vanilla* requires identifying fungal endophytes and currently are unknown. *V. barbellata* and *V. barbellata* deserve both attention and conservation, if not for their intrinsic

value, then for their economic value.

Mycobiome meta-barcoding is a relatively new research frontier, and this study of *V. barbellata* and *V. phaeantha* mycobiomes will be the first of its kind, elaborating on the full spectrum of symbiont relations. *V. barbellata* and *V. phaeantha* are two species that require conservation consideration and are not agriculturally cultivated species like *V. planifolia*. However, findings of this study are transferrable to the agriculturally cultivated *V. planifolia*, which has similar habitat requirements and lifestyles. The viny habit is characteristic of all *Vanilla*, having both terrestrial roots planted in the soil and aerial roots clinging to trees, which are used to reach the canopy. The presence of both root types on the same plant presents a unique additional dimension to this analysis rarely found in mycobiome studies. This study will focus primarily on fungal associates with the following goals: 1) sequencing DNA to identify endophytic fungi, 2) producing a more comprehensive analysis of fungal communities from terrestrial roots, aerial climbing roots, and (or photosynthetic organs) leaves of *Vanilla* plants, and 3) investigate mycobiome differences between individual plants. We expect there will be a difference in the mycobiont community of each plant compartment, not necessarily plant to plant and in mycobionts of individual plants based on the function of that plant compartment. In addition, we anticipate there will be a significant difference between ectomycorrhizal fungi associated with leaved *V. phaeantha* and leafless *V. barbellata* which likely relies on a more substantial symbiosis.

### **Methods:**

Due to the endangered and threatened status of *V. barbellata*, permits are required for their collection. Plants included in this study were mostly collected from protected areas, and

sampling of a 1–2 cm<sup>2</sup> tissue segment was done with consent of the local nature conservation authority and in the presence of a representative person. Individual plants present at a site were only sampled if that sampling was not clearly detrimental to the plant's survival.

Collection of *V. barbellata* and *V. phaeantha* samples took place in the summer of 2018 (July and August) from natural populations in the Florida Keys, Virgin Islands, and Puerto Rico (Figure 2). *V. barbellata* of the Florida Keys were located on lands protected and maintained by the Florida Fish and Wildlife Conservation Commission, this included three individuals from three different adjacent islands (Keys). These *V. barbellata* occupy thick wetland hammocks with limestone covered by shallow sandy soils and dominated by native cycads, pines, grasses, cabbage palm, poisonwood trees and mangroves. They remain just above sea-level and are adjacent to the island's coast (Figure 1B). Three *V. barbellata* were sampled from the U.S. Virgin Islands National Park (NP), the largest population of the species included in this study (Figure 1A). In Puerto Rico, three *V. barbellata* were sampled, from two sites Susúa State Forest (SF) and Guánica State Forest (SF). The Guánica SF habitat is a rocky coastal lowland hammock, quite similar to that of the Virgin Islands NP's population. The Susúa SF habitat is uniquely located at a higher elevation and inland montane forest, the understory is less dense compared to the thickets of the other habitats (Figure 1C). Despite these comparatively different environments both populations seem to be thriving. To achieve the goal of this study, it was imperative to include a *V. phaeantha* population that grows sympatrically or adjacently to *V. barbellata* under natural conditions. With this in mind, three *V. phaeantha* of Maricao State Forest (SF) were sampled. The Maricao SF is located just north of Susúa SF & Guánica SF. Maricao SF is a verdant montane forest with rocky soils (Figure 1D). Precise locations cannot be disclosed due to permitting restrictions instituted to protect endangered species (Table 1).

Eight samples per plant were collected to analyze four different communities (Figure 6): One leaf or photosynthetic stem for photosynthetic endosphere and phyllosphere, aerial root tissue for aerial root endosphere and aerial rhizosphere, terrestrial root tissue for terrestrial root endosphere and terrestrial rhizosphere, and a bulk soil gathered one meter away of selected plants. All samples were stored at 4 °C in RNAlater tissue storage reagent (Sigma-Aldrich®) until processing.

Leaf and root samples were washed under a flow hood with phyllosphere removal buffer (6.75 g  $\text{KH}_2\text{PO}_4$ , 8.75 g  $\text{K}_2\text{HPO}_4$ , and 1 mL Triton X-100 per liter) and a 0.9 percent NaCl (unconcentrated bleach; Clorox® Company, Oakland Ca.) solution, respectively, to extract microorganisms from the phyllosphere and rhizosphere. The microbial pellet was collected by centrifugation from these solutions and kept at -80 °F prior to DNA extraction. Tissues were disinfected with sterilization solutions of 70% ethanol and 5% NaCl, and triple rinsed with sterile distilled water for two minutes each. Surface sterilization was evaluated by testing 200  $\mu\text{l}$  of the third rinse streaked onto nutrient agar plates and observed for several days of incubation at 21°C. Surface sterilization was considered successful when no microbial growth occurred, and surface sterilization was conducted a second time if any growth was observed. The surface sterilized leaf and root tissues comprised the endophytic samples. All samples were stored at -80 °F prior to DNA extraction. (Lottmann *et al.* 2010; Desgarenes, 2014; Franseca-García *et al.*, 2016; Coleman-Derr *et al.* 2016). Tissue from genus *Vanilla* is both fibrous and mucilaginous increasing difficulty of grinding tissue for DNA extraction. Tissue samples were ground for 1.5 minutes at 25 Hz. in a TissueLyser (Qiagen) along with 2-3, 4.5mm aluminum pellets and CTAB buffer in preparation for DNA extraction. DNA was extracted following three different protocols depending on sample type as previously described by Lottmann *et al.* 2010 (Desgarenes, 2014;

Franseca-García *et al.*, 2016; Coleman-Derr *et al.* 2016). Total DNA was extracted from root tissues with the Soltis Lab CTAB DNA Extraction Protocol (Doyle & Doyle, 1987; Cullings 1992). DNA from bulk soil samples were extracted, cleaned, and concentrated using Power Soil DNA Isolation Kit, according to the manufacturer's protocol. DNA from terrestrial rhizospheres and phyllospheres were extracted, cleaned, and concentrated with Ultra Clean® Water DNA Kit (MO BIO Laboratories, Carlsbad, CA), according to the manufacturer's protocol. 1% Gel electrophoresis and DNA quantification using TECAN Infinite M Plex plate reader for fluorescent readings of DNA using Invitrogen Qubit™ dsDNA HS Assay kit by Thermo Fisher Scientific, confirmed extraction integrity.

Purified genomic DNA was submitted to the University of Wisconsin-Madison Biotechnology Center for construction and sequencing of ITS libraries. DNA concentration was verified fluorometrically using Quant-iT™ PicoGreen® dsDNA Assay Kit (ThermoFisher Scientific, Waltham, MA, U.S.A.). Sample preparation was process similarly to the process described in Illumina's 16s Metagenomic Sequencing Library Preparation Protocol, Part # 15044223 Rev. B (Illumina Inc., San Diego, California, U.S.A.) with the following modifications. The ITS region was amplified with fusion primers forward primer:

5'- ACACTCTTCCCTACACGACGCTCTTCCGATCTCTTGGTCATTTAGAGGAAGTAA-  
3'

reverse primer: 5'-

GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTTCCCTCCGCTTATTGATATGC-3'.

Region specific primers were previously described (ITS1-F Gardes and Bruns, 1993; ITS4

White *et al.*, 1990) and were modified to add Illumina adapter overhang nucleotide sequences to region-specific sequences. Following initial amplification, reactions were cleaned using a 0.7x volume of AxyPrep Mag PCR clean-up beads (Axygen Biosciences, Union City, CA). Using the initial amplification products as template, a second PCR was performed with primers that contain Illumina dual indexes and Sequencing adapters where bracketed sequences are equivalent to the Illumina Dual Index adapters D501-D508 and D701-D712, N716, N718-N724, N726-N729.

Forward primer: 5'-

AATGATACGGCGACCACCGAGATCTACAC[55555555]ACACTCTTCCCTACACGACGCTCTTCCGATCT-3',

Reverse Primer: 5'-

CAAGCAGAAGACGGCATAACGAGAT[77777777]GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT -3',

Following PCR, reactions were cleaned using a 0.7x volume of AxyPrep Mag PCR clean-up beads (Axygen Biosciences). Quality and quantity of finished libraries were assessed using an Agilent DNA 1000 kit (Agilent Technologies, Santa Clara, CA) and Qubit® dsDNA HS Assay Kit (ThermoFisher Scientific), respectively. Libraries were pooled in an equimolar fashion and appropriately diluted prior to sequencing. Paired end, 300 bp sequencing was performed using Illumina MiSeq Sequencer and a MiSeq 600 bp (v3) sequencing cartridge. Images were analyzed using the standard Illumina Pipeline, version 1.8.2.

Microbiome Analysis was performed by the University of Wisconsin-Madison Biotechnology Center using Quantitative Insights Into Microbial Ecology (QIIME2) version 2

(Bolyen *et al.*, 2019; Caporaso *et al.*, 2010). Illumina sequencing reads were denoised and quality filtered using the denoising program DADA2 (Callahan *et al.*, 2016). This trimmed low quality bases, filtered out noisy sequences, corrected errors in marginal sequences, removed chimeric sequences and singletons, and then dereplicated those sequences. The resultant dereplication produced the operational taxonomic units (OTUs) (Bai *et al.*, 2015; Laforest-Lapointe, 2017). Sequence variants were aligned and masked using Mafft. Taxonomy was assigned using Bayesian classifiers based on a pretrained Unite database curated to the ITS amplicon region for further downstream analysis. Low frequency reads (<0.01%) were filtered and excluded. Alpha diversity across various treatments was compared using the Kruskal Wallis test. Alpha rarefaction curves utilizing Shannon, Simpson, and observed were constructed for all samples with a rarefaction upper limit of median depth/sample count. If there were insufficient reads in a sample, it was discarded from further analysis. On ASV data leveled according to the lowest sample depth, beta diversity was computed, and ordination plots were constructed using Bray-Curtis and Jaccard (Non-Phylogenetic), Weighted and unweighted Unifrac (Phylogenetic). Venn diagrams were plotted with the function 'venn.diagram' using the package 'VennDiagram' (Chen & Boutros, 2011). Mean values and standard deviation and further statistical analyses of each experiment were calculated using R packages 'Phyloseq', 'MicrobiotaProcess', 'microbiome', and 'microbiomeutilities' (McMurdie & Holmes, 2013; Xu & Yu, 2021; Lahti & Shetty, 2012-2019 & 2018). Distances were calculated using the 'vegdist' function of the package 'vegan' for Bray-Curtis. Nonmetric multidimensional scaling (NMDS) was performed using 'vegan' packages (Oksanen *et al.*, 2017). Diversity statistics were calculated using Qiime2. ANOVAs were performed using Microsoft Excel with average relative abundances and relative frequencies of each OTU in each community across the two *Vanilla* species.

**Results:**

We analyzed fungal communities associated with six sample types were obtained from two *Vanilla* (*V. barbellata* and *V. phaeantha*) species. Through Illumina MiSeq platform we obtained 13,627,090 total high-quality reads which resulted in 3,232 operational taxonomic units (OTUs) after applying technical reproducibility thresholds for fungal data sets. Due to human error and/or low quality of sequences 35 of the original 133 samples met these necessary and stringent quality checks and filters. The greatest differences between fungal community members are evident between aerial root endospheres and terrestrial root endospheres or exospheres. Additionally, a difference was observed between *V. barbellata* and *V. phaeantha* associated fungi and their abundance. However, we attribute this difference to sampling disparities as significantly more *V. barbellata* were sampled in this study (Figure 3: A&B).

The Shannon Index ( $H'$ ) between plant compartments of the two species is generally higher within *V. barbellata* than within *V. phaeantha*, with the exception of aerial root endospheres. Additionally, Simpson Index for all *V. phaeantha* plant organs demonstrate relatively low diversity (Table 2). The analysis of variance (ANOVA) between *Vanilla* species plant organs demonstrates significant variance within groups and a significantly lower variance between groups (Table 3A). Similar variance is exhibited between *Vanilla* species (Table 3B)

Levels of fungal diversity differ among compartments and between the two species. In general, alpha diversity as measured by the Shannon ( $H'$ ) index is lower in endospheric samples as compared to exospheric samples. Alpha diversity of fungal communities are higher in rhizosphere soil than in bulk soil across both species, suggesting that root exudates or other plant-associated factors may be responsible for increasing local microbial diversity. Remarkably, alpha diversity associated with aerial rhizosphere of both species vary greatly, possibly

suggesting intermittent fungal colonization of aerial roots. Furthermore, these levels were even lower in the phyllosphere, suggesting environmental conditions for aboveground plant surfaces are less hospitable than underground or velamen-covered surfaces (Figure 4).

While the relative abundance profile of *V. barbellata* seems similar to *V. phaeantha*, community composition differs substantially between the rhizosphere, terrestrial root endosphere, and aerial root endosphere (Figure 5: A&B). Additionally, relative abundance profiles between fungal communities are more similar within *Vanilla* species for the terrestrial root endosphere and terrestrial rhizosphere sample types. In contrast, sample types differ between each other by several taxonomic orders that exhibit increases or decreases in abundance across terrestrial rhizosphere, terrestrial root endosphere, and aerial root endosphere samples. Fungal communities across all six sample types were dominated by Ascomycota (average 51.7% of total relative abundance), while Basidiomycota represented a smaller portion of communities (34.4%). Strikingly, very few members of the known arbuscular mycorrhizal fungi (AMF) phylum Glomeromycota (3.13%) were detected. The fungal orders Sebaciniales and Cantharellales which are known to encompass fungi considered orchid mycorrhizae were only found in terrestrial rhizospheres and some terrestrial root endospheres.

The most frequent genera identified by OTUs included fungi known to live in association with many orchids as potentially useful for seed germination or as known pathogens. This includes genera such as *Fusarium* (Hypocreales) which is found in a much higher abundance in *V. phaeantha* than *V. barbellata*. Known endophytes such as *Colletotrichum* (Glomerellales), *Cladosporium* (Capnodiales), *Coprinellus* (Agaricales), *Diaporthe* (Diaporthales), and *Pestalotiopsis* (Xylariales) are found in a higher abundance for few samples (Figure 6).

The NMDS plot reveals that aerial root endosphere samples exhibit the greatest between-sample variation. Conversely, bulk soil, terrestrial root endosphere, and terrestrial rhizosphere samples cluster more closely together (Figure 7).

### **Discussion:**

This study is the first of its kind to identify the components and diversity of fungal taxa composing the identifiable mycobiome of an endangered orchid species, moreover endangered species of the *Vanilla* genus. We aimed to determine what fungi compose the mycobiome of *V. barbellata* and *V. phaeantha*? How do mycobiomes of *V. barbellata* and *V. phaeantha* differ from 1) plant compartment to plant compartment of the same species, 2) plant compartment to plant compartment between species, and 3) overall composition between species?

The taxonomic composition of fungal communities of the endangered *Vanilla* species, *V. barbellata* and *V. phaeantha*, have not been studied to identify overall trends until now. Understanding of variability and components of these communities can be key to informing conservation efforts both *in situ* and *ex situ*. The present study not only investigates fungal diversity but also makes comparisons between the leafy habit of *V. phaeantha* to the leafless habit of *V. barbellata*, as well as between plant compartments i.e., aerial root endosphere, terrestrial rhizosphere, and terrestrial root endosphere.

To our knowledge there has been limited microbiome metabarcoding research focused on members of the Orchidaceae. Indeed, the predominant orchid-fungal research style focuses on identification of culturable mycorrhizal fungi for their use in seed germination trials in the interest of increasing populations of endangered or rare orchids. The rare and endangered leafless Floridian natives *Dendrophylax lindenii* and *D. porrectus* are clear examples of this, where

fungus isolates were acquired from wild relatives and then used for seed germination studies (Zettler *et al.*, 2019; Mujica *et al.*, 2018). This type of research is pivotal as seedling survival is significantly increased when reintroduced to natural environments after being cultivated *in situ* with symbiotic fungi (Rasmussen *et al.*, 2015). Mycorrhizal fungi that promote seed germination are thought to persist in this partnership with the orchid through adulthood, making identification of these mycorrhizal fungi all the more essential for conservation efforts. Furthermore, even less orchid focused microbiome research is specifically dedicated to the *Vanilla* genus. As with the majority of *Vanilla* focused research, existing microbiome metabarcoding studies are focused on the crop plant *V. planifolia* and *Vanilla* crop pathogens. For example, the recent study by Carbajal-Valenzuela *et al.* 2021, discussed microbial diversity of cultivated and feral *V. planifolia* affected by stem and rot disease. This study also concentrated primarily on *Fusarium* fungi, a notoriously problematic pathogen on monocultural *Vanilla* crops.

**Species-Species Contrast** - Our results demonstrate *Vanilla* species do not strongly influence mycobiome composition assembly, and consequently no distinction as a result of the leafy or leafless habit. In an analysis of variance (ANOVA) *V. barbellata* and *V. phaeantha* species indicate approximately 95% of variation is found within groups, suggesting there is a greater difference of OTUs between samples of the same species than between each species cumulatively (Table 3B). However, *V. barbellata* displays the highest variance (1.426), revealing steep differences in OTUs among samples of this species, as opposed to the much lower among-sample variance of *V. phaeantha* (0.04) or the Bulk Soil (0.049). Relative abundances of order-level fungal OTUs between mycobiomes of the two species hold many similarities, for instance Chaetothyriales, Capnodiales, Tremellales, Agaricales, Pleosporales, and Xylariales (Figure 5A). This appears appropriate as these orders encompass numerous ubiquitous fungi and prevalent

endophytes. These fungal community orders are also present in the top 20 most abundant genera identified by OTUs in Figure 6. These are well-known endophytes that have a wide range of ecological functions. In orchid fungal research studies, fungi of these orders are commonly observed and identified, this includes *Fusarium* (Hypocreales), *Colletotrichum* (Glomerellales), *Cladosporium* (Capnodiales), *Coprinellus* (Agaricales), *Diaporthe* (Diaporthales), and *Pestalotiopsis* (Xylariales) and in this study they are found in a higher abundance for few samples (Figure 6).

Although, there are a few noticeable differences of the mycobiomes between *Vanilla* sp., such as higher abundances of Hypocreales, Helotiales, and Polyporales in *V. phaeantha* (Figure 5A). Distribution of measurable fungal OTUs is exhibited by our Venn diagram in which 68 OTUs are shared by both species, 930 OTUs were unique to *V. barbellata* and 273 OTUs were unique to *V. phaeantha* (Figure 3). Additionally, while OTUs and diversity in general is observed to be slightly higher in most *V. barbellata* sample types, it is necessary to note that richness and diversity comparisons are sensitive to smaller sample sizes. The sampling size of *V. phaeantha* in this study is smaller than ideal for a sample size, especially compared to the depth of *V. barbellata* sampling.

**Plant Compartment Contrast Within Species-** Interestingly, we did find significant differences in richness and diversity of the Shannon index between plant compartments (compartment-compartment) and between similar plant compartments of the two species (species-species compartments). This may suggest host-driven specificity of mycobionts for each compartment and reveal this biotic factor as the main driver of mycobiont selection. Host-driven specificity has been witnessed in previous orchid research where three Mediterranean species show an ability to influence endophytic bacterial communities (Alibrandi *et al.*, 2020). This

ability was also exhibited in bacterial and fungal communities of *V. planifolia* in a study of microbial diversity of orchids affected by stem and rot disease (Carbajal-Valenzuela *et al.*, 2021).

As one of our primary curiosities of this study, compartment-specific differences in mycobiome composition patterns is not only intriguing but informative. We observed differences in OTU composition in *V. barbellata*'s terrestrial rhizosphere and terrestrial root endosphere. These terrestrial compartments exhibit a larger number of shared OTUs between each other (187 OTUs in common) than with the aerial root endosphere (40 OTUs and 79 OTUs, respectively) (Figure 3B). Distinctions characterizing *V. barbellata*'s compartments are further reiterated by the NMDS of fungal communities, where a small amount of crossover occurs in aerial root endospheres and terrestrial compartments. Even fewer commonalities exist between the aerial root endosphere and the bulk soil. Whereas the terrestrial compartments and bulk soil show significant overlap (Figure 7).

Interestingly, taxonomic distribution of *V. barbellata*'s terrestrial root endospheres and aerial root endospheres reported similar order-level OTU abundances of Capnoidales, which contains many dark-septate endophytes, and Hypocreales, which contains genera such as *Fusarium*. Terrestrial rhizospheres also share OTU orders but not of comparable abundances to aerial root endospheres. Rather, terrestrial rhizospheres are more congruent with the bulk soil. The abundance of Thelophorales was similar, yet other shared orders differed in abundance. As expected, rarefied orders revealed greater representation in the terrestrial rhizosphere (Figure 5B). For instance, Boletales known for the common boletes, Sebaciales that includes known orchid mycorrhizae, and Gaestrales renowned for unique morphologies such as earth stars (Spatafora *et al.*, 2017). The aerial root endosphere's of *Vanilla barbellata* also exhibited unique

order-level OTUs in higher abundance than other compartments, such as Xylariales, Tremallales, Diaporthales, and Pleosporales (Figure 5B). Consistent with the measured variance in the NMDS, these differences are observed in variance of the aerial root endosphere (0.098) which is lower than the variance of terrestrial root endosphere (0.134) and much lower than the terrestrial rhizosphere (0.213) (Table 3A).

By comparison, the variance among *V. phaeantha* compartments is quite low, likely due to the lower sampling of this group. In all likelihood, this also resulted in the lower significance of the p-value of the plant compartment ANOVA,  $P=0.065$  (Table 3A). Another likely effect of low sampling is the disproportionately high representation of Hypocreales OTUs in *V. phaeantha*'s terrestrial root endosphere comprising just over 75% of the total relative abundance. Likewise, Helotiales in the aerial root endosphere represents 34% of the total relative abundance (Figure 5B). Within both species, the Shannon and Simpson diversity indices are found to be highest in their terrestrial rhizospheres and most comparable to the overall bulk soil diversity. The Shannon diversity index of *V. barbellata* terrestrial rhizosphere was 0.7 higher than the terrestrial root endosphere. Likewise, the *V. phaeantha* terrestrial rhizosphere is 1.49 higher than the terrestrial root endosphere (Table 2).

Ultimately, there are distinct and evident compartment-compartment variations in both *Vanilla* species. Notably a greater variability of fungi resides outside of the orchid root than inside, which is true for both species. This outcome was anticipated considering the rhizosphere is known to be rich in nutrients compared to the bulk soil. Typically, a plant releases a variety of organic compounds that are used by microorganisms, which increases microbial growth and intensifies activity much higher than the bulk soil (Ligaba *et al.*, 2004; Lugtenberg and Kamilova

2009). The direct influence of these complex interactions between the microbes and root system, is known as the “rhizosphere effect” (Sylvia *et al.*, 1998).

**Plant Compartment Contrast Between Species** - Broadly speaking, both *V. barbellata* and *V. phaeantha* compartments of the same type demonstrated several similarities. The estimated Shannon diversity index of OTUs were similar between each compartment of the two species (Table 2 & Figure 4). Relative OTU abundances of aerial root endospheres are roughly similar, exhibiting close abundances of Xylariales, Pleosporales, and Hypocreales, for example (Figure 5B). Major differences between aerial root endospheres of the two species are observed in the orders Helotiales and Hypocreales which are over expressed as a result of singular samples. We reason that these two examples are products of the low sampling of *V. phaeantha* in conjunction with the possibility that these endophytes may have recently experienced a shift latency. Environmental changes have the ability to alter latency of pathogens (Ghelardini *et al.*, 2016). It is conceivable that at the time of collection, the *V. phaeantha* was not yet expressing visible symptoms giving no indication of pathogen shift. With this in mind, this pattern of similarity is consistent with data presented in the NMDS, where aerial root endospheres of both species show cross over, but aerial root endospheres compared to terrestrial root endospheres demonstrate much less crossover (Figure 7). These patterns are expected, as we expect ecological roles and diversity of terrestrial or soil fungi to be broader than those of aerial fungi that are more exposed to UV light, dehydration, and other harmful environmental influences (Carvalho & Castillo, 2018).

**Conclusions** – Overall differences of fungal community composition are demonstrated by distribution and abundance of OTUs. Plant organs between species exhibit differences in mycobiome taxa abundance, while plant organs of same species show similarities. Similarities of

mycobiome taxa abundances are also exhibited between *V. barbellata* terrestrial root samples (terrestrial rhizosphere and terrestrial root endosphere) and bulk soil samples (Figure 5 A&B). Conversely, there are differences noticeable between terrestrial root samples and aerial root samples (Figure 7). Although many OTUs are associated with known endophytic fungal orders, very few were identified to orders known to contain orchid mycorrhizal fungi. All of the orchid mycorrhizae orders were found exclusively from terrestrial rhizospheres and terrestrial root endospheres.

These are not entirely the outcomes we expected, but this study affords future research important insights into the types of endophytic and mycorrhizal fungi leafless and leafy *Vanilla* orchids. It appears that *V. barbellata* and *V. phaeantha* form similar fungal relationships as whole and even at the plant compartment level. The latter being more diverse but similar to bulk soil from the same habitat. This work suggests that similar fungal ecosystems may benefit from a wide range of species of the *Vanilla* genus and perhaps their mycobiont needs are not as specific as initially hypothesized. In essence, this would permit the ability to undertake conservation efforts for multiple species in aggregate and at the same location. Data acquired in this study provides a foundational baseline for the conservation efforts for the *Vanilla* genus that integrates their fungal ecology. To that end, future work should also include greater sampling of *V. phaeantha* and others.

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**Tables:**

**Table 1:** Mycobiome metabarcoding sampling method of both *V. barbellata* and *V. phaeantha*, location of the collection and the number of collections made per site. Completed collections are marked with ‘\*’. Sample types collected: P - Phyllosphere, PE – Phyllo-endosphere, AR – Aerial Root, ARE – Aerial Root Endosphere, TR – Terrestrial Root, TRE – Terrestrial Root Endosphere, BS – Bulk Soil, only one sample per site. A total of 133 samples for Illumina MiSeq Sequencing.

*Microbiome Sampling*

<i>Species</i>	<b>Location</b>	<b>Number of Individuals</b>	<b>P</b>	<b>PE</b>	<b>AR</b>	<b>ARE</b>	<b>TR</b>	<b>TRA</b>	<b>BS</b>
<i>Vanilla barbellata</i>	FL Keys	9	*	*	*	*	*	*	3
	Puerto Rico (Guánica SF & Susúa SF)	6	*	*	*	*	*	*	2
	U.S. Virgin Islands NP	3	*	*	*	*	*	*	1
<i>Vanilla phaeantha</i>	Puerto Rico (Maricoa SF)	3	*	*	*	*	*	*	1

**Table 2:** Estimated diversity based on overall fungal communities of both *Vanilla* species by community type (plant compartment) and Bulk Soil.

<i>Plant compartment</i>	Community Type	<i>Vanilla barbellata</i>			<i>Vanilla phaeantha</i>		
		Number of OTUs	Shannon Index (H')	Simpson Index	Number of OTUs	Shannon Index (H')	Simpson Index
<i>Terrestrial</i>	Terrestrial Root Endosphere	605	2.53	0.81	32	2.3	0.67
	Terrestrial Rhizosphere	1105	3.23	0.91	236	3.79	0.94
<i>Aerial</i>	Aerial Root Endosphere	79	2.66	0.78	164	2.77	0.76
	Phyllosphere	-	-	-	122	2.42	0.79
<i>All</i>	Overall	3232	2.9	0.84			
<i>Soil</i>	Bulk Soil	380	3	0.86			

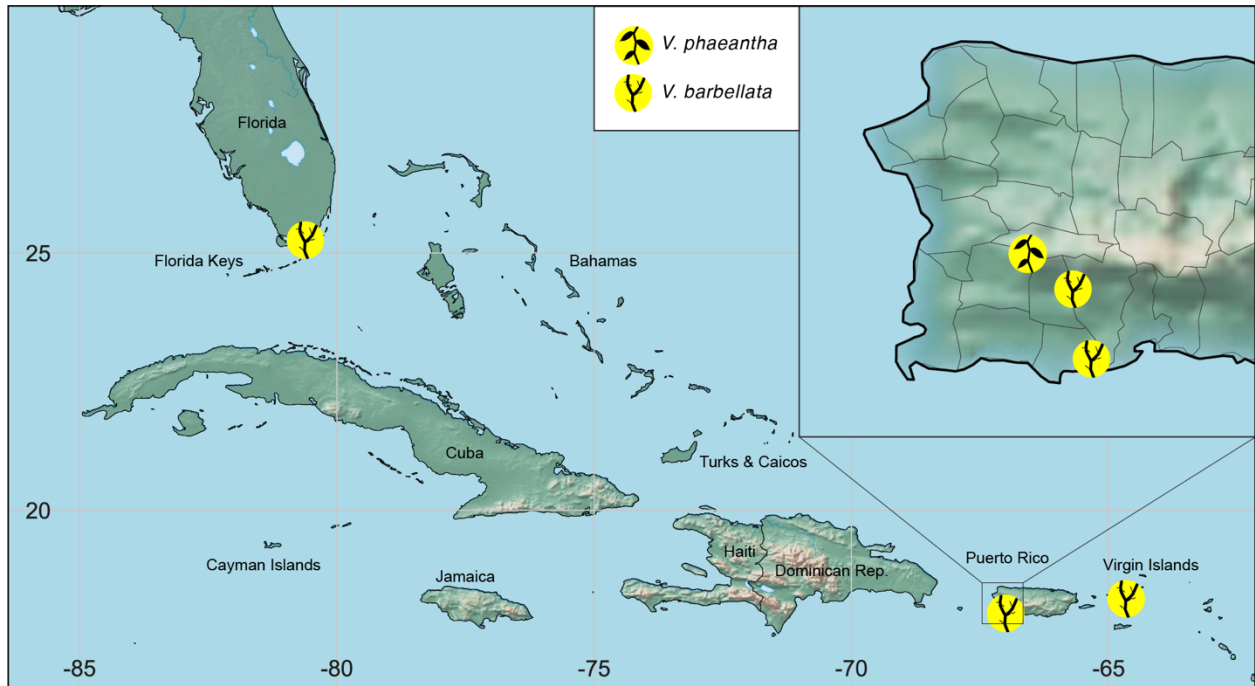
**Table 3:** A) Single Factor Analysis of Variance between species and plant compartment. B) ANOVA between *Vanilla* species and bulk soil.

<b>A</b>						
<b>ANOVA: Single Factor - Summary</b>						
<i>Species</i>	<i>Plant compartment</i>	<i>Count</i>	<i>Sum</i>	<i>Average</i>	<i>Variance</i>	
<i>Vanilla barbellata</i>	Terrestrial Root Endosphere	64	5.994	0.094	0.134	
	Terrestrial Rhizosphere	64	9.57	0.15	0.213	
	Aerial Root Endosphere	64	6.552	0.102	0.098	
<i>Vanilla phaeantha</i>	Terrestrial Root Endosphere	64	1.015	0.016	0.009	
	Terrestrial Rhizosphere	64	2.029	0.032	0.006	
	Aerial Root Endosphere	64	2.008	0.031	0.011	
<i>Soil</i>	Bulk Soil	64	3.964	0.062	0.049	
<b>ANOVA: Single Factor</b>						
<i>Source of Variation</i>	<i>SS</i>	<i>df</i>	<i>MS</i>	<i>F</i>	<i>P-value</i>	<i>F crit</i>
<i>Between Groups</i>	0.889	6	0.148	1.99	0.065	2.119
<i>Within Groups</i>	32.759	441	0.074			
<i>Total</i>	33.648	447				

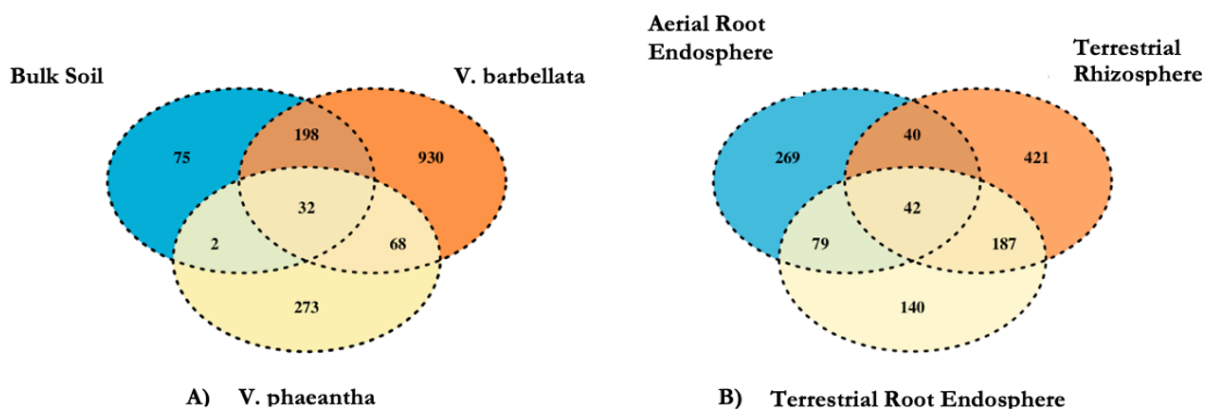
<b>B</b>						
<b>ANOVA: Single Factor - Summary</b>						
<i>Groups</i>	<i>Count</i>	<i>Sum</i>	<i>Average</i>	<i>Variance</i>		
<i>Vanilla barbellata</i>	64	25.287	0.3956	1.426		
<i>Vanilla phaeantha</i>	64	5.052	0.079	0.04		
<i>Bulk Soil</i>	64	3.964	0.062	0.049		
<b>ANOVA: Single Factor</b>						
<i>Source of Variation</i>	<i>SS</i>	<i>df</i>	<i>MS</i>	<i>F</i>	<i>P-value</i>	<i>F crit</i>
<i>Between Groups</i>	4.507	2	2.253	4.461	0.013	3.044
<i>Within Groups</i>	95.467	189	0.505			
<i>Total</i>	99.973	191				

**Figures:**

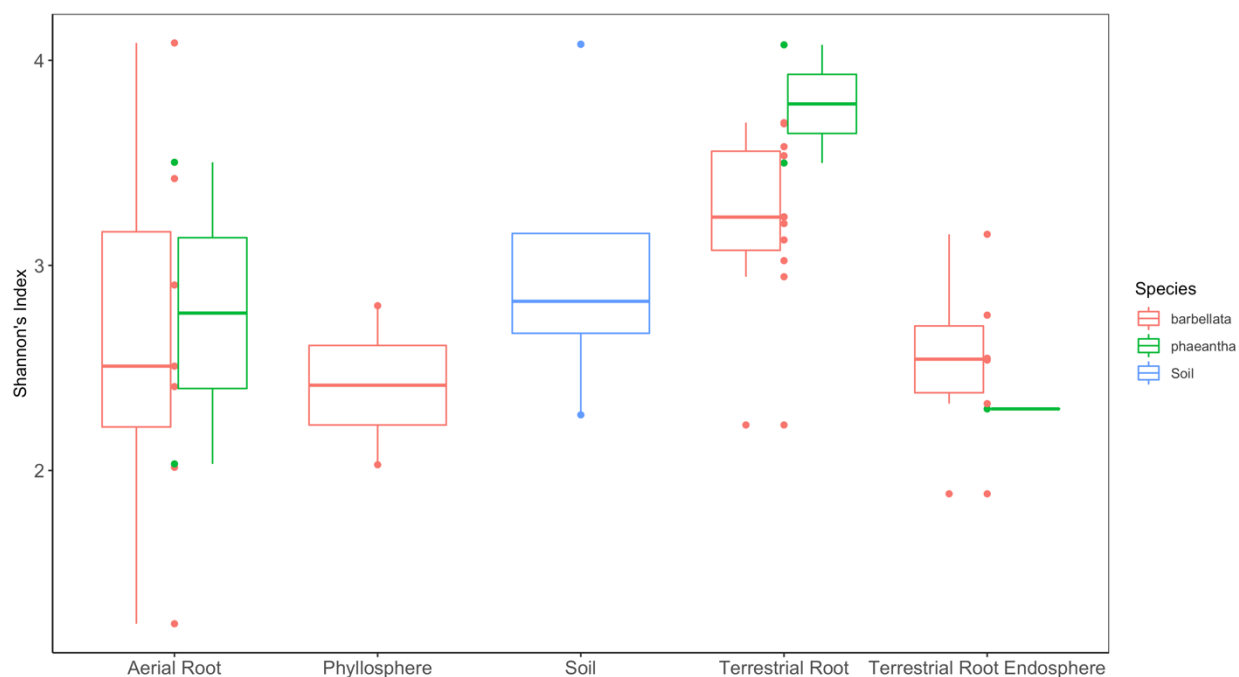
**Figure 1:** A) *Vanilla barbellata* in U.S. Virgin Islands National Park – coastal thick hammock habitat. B) *Vanilla barbellata* of the Florida Keys- coastal thick hammock with slightly sandier soil. C) Puerto Rico Susúa State Forest – higher elevation (not sea-level) montane forest with rocky soil. D) *Vanilla phaeantha* growing Puerto Rico’s Maricao State Forest a verdant montane forest with rocky soils.



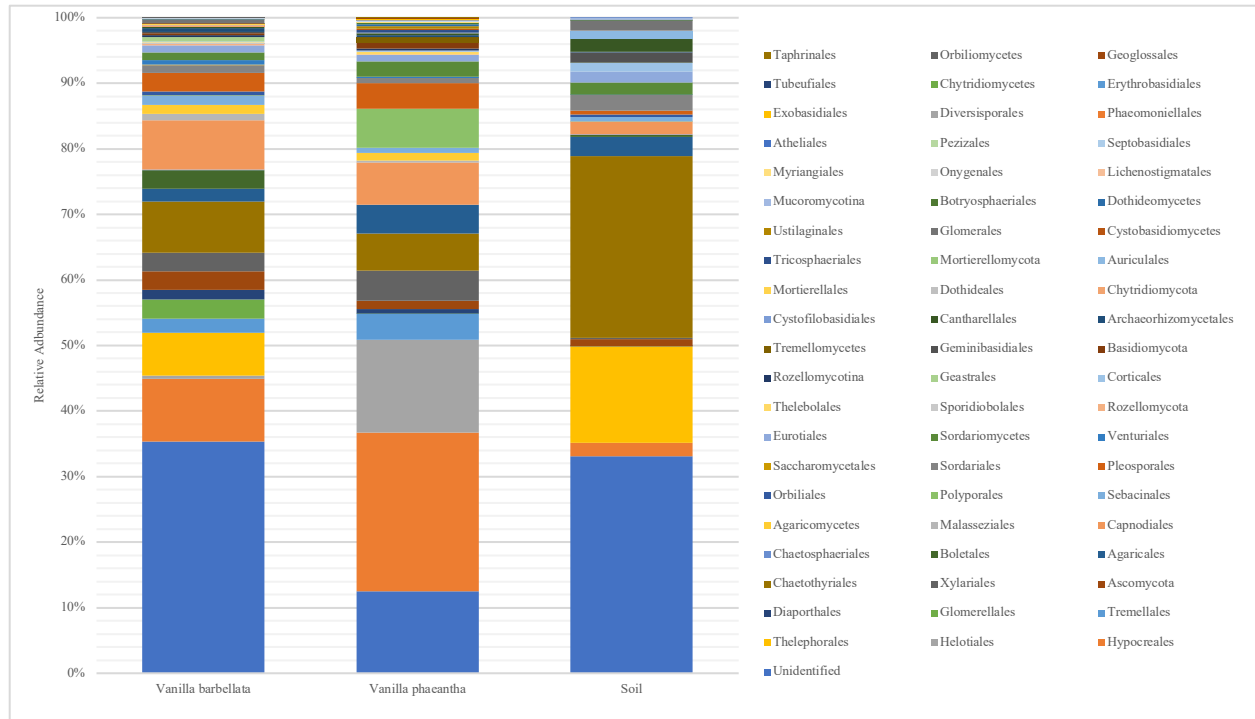
**Figure 2:** Map of sampling distribution throughout the Caribbean including four populations of the leafless *Vanilla barbellata* (Florida Keys, Puerto Rico [Susúa SF & Guánica SF], and U.S. Virgin Islands NP) and one population of the leafy *Vanilla phaeantha* in Puerto Rico's Maricao SF.



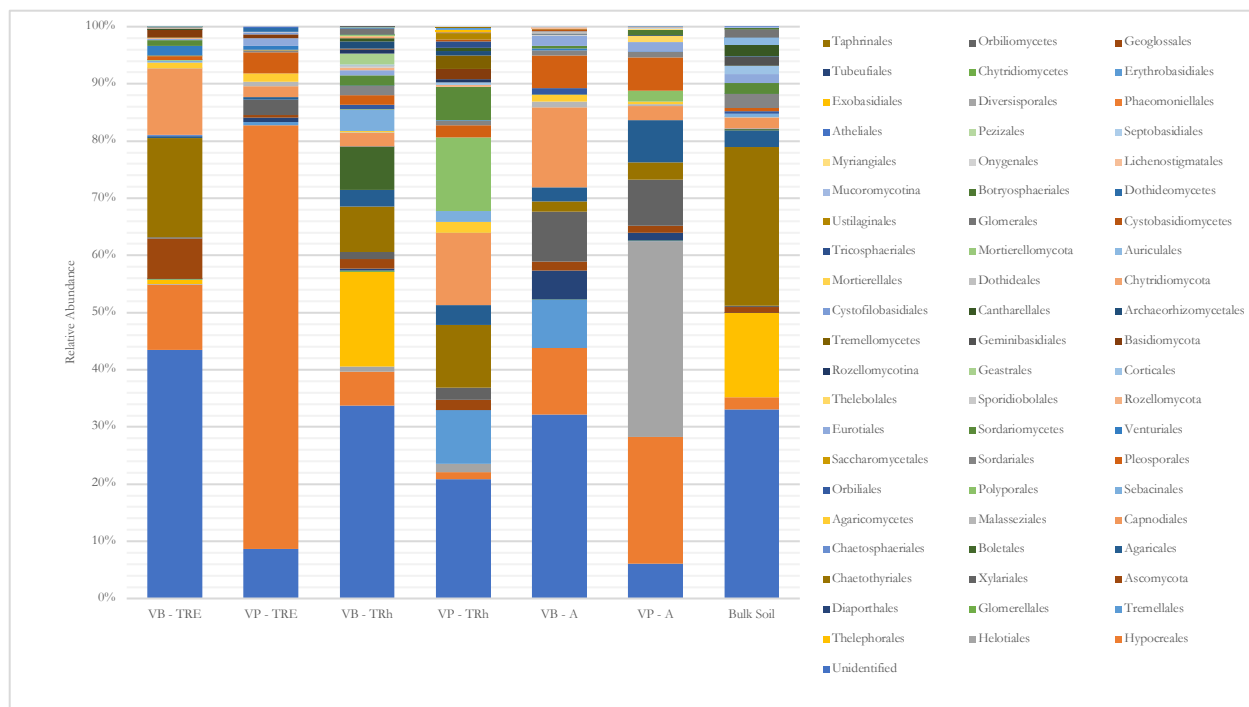
**Figure 3:** Venn diagrams showing the distribution of fungal measurable OTUs associated with A) *V. barbellata*, *V. phaeantha*, and adjacent bulk soil and B) fungal OTUs associated with different root sample types. Aerial root, terrestrial rhizosphere, and terrestrial root endosphere of *V. barbellata* as these sample types yielded the highest quality OTUs.



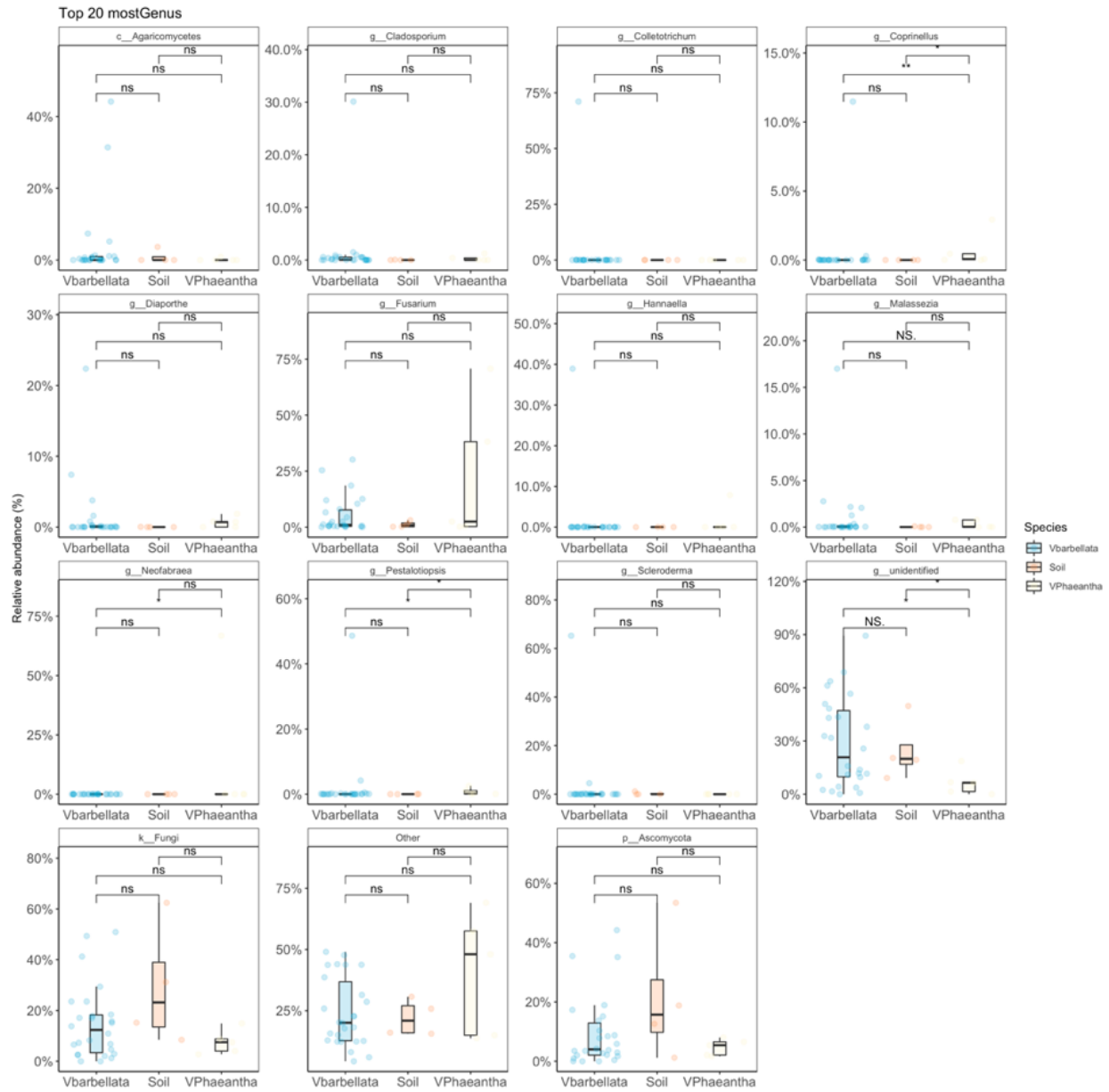
**Figure 4:** Estimated Shannon ( $H'$ ) index of fungal communities associated with each compartment type for the two *Vanilla* species, shown with SE.



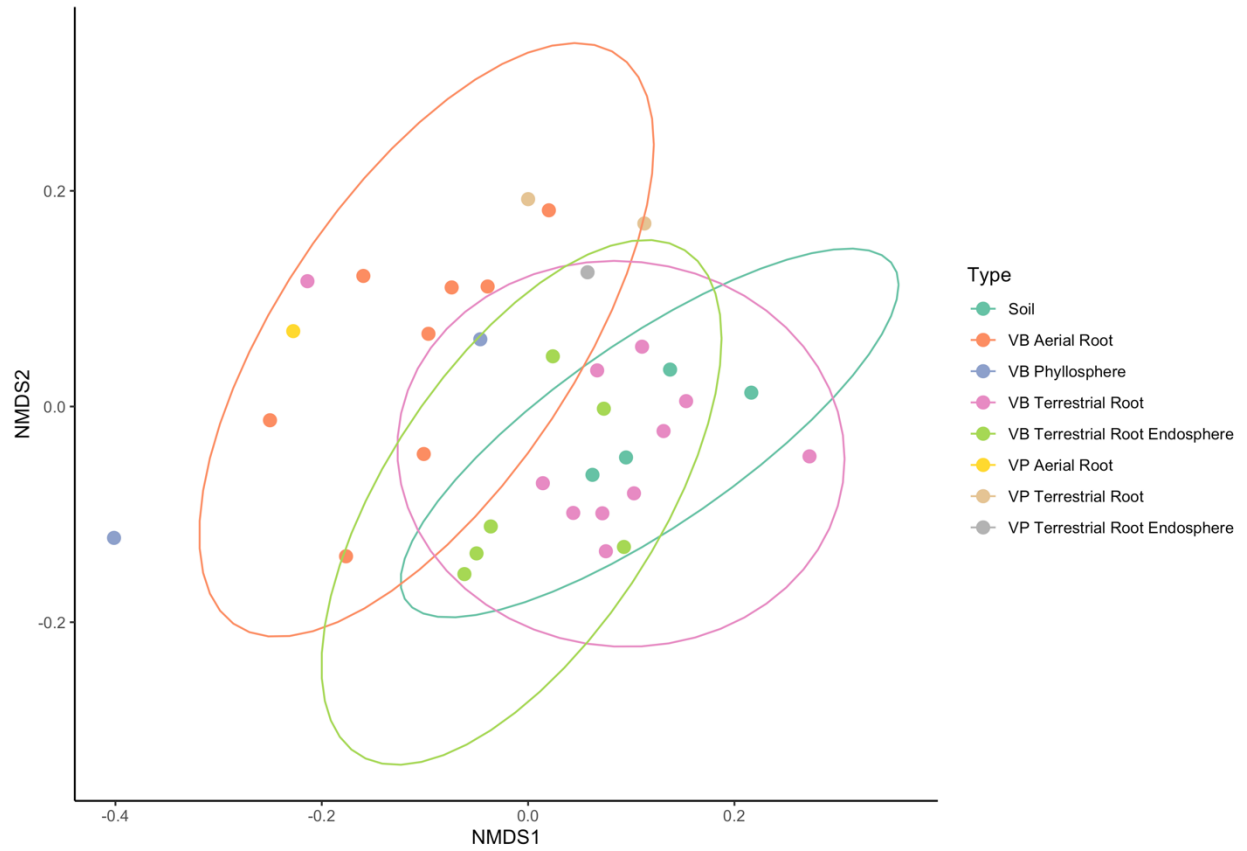
**Figure 5:** A) Order-level relative abundance plots of fungal communities (64 OTUs) by species and bulk soil organized by taxonomic bins containing operational taxonomic units indicate order-level.



**Figure 5: B)** Taxonomic distribution (64 OTUs) at order-level of fungal groups' relative abundance associate with the two *Vanilla* species based on OTU profiles. The rows are paired by the plant compartment of the two species for comparison. *Vanilla barbellata* abbreviated as VB and *Vanilla phaeantha* abbreviated as VP. Compartment type abbreviations: Terrestrial Root Endosphere (TRE), Terrestrial Rhizosphere (TRh), Aerial Root Endosphere (A).



**Figure 6:** The most abundant genera identified based on major operation taxonomic unit (OTUs) associated from all plant organs and both species.



**Figure 7:** Nonmetric multidimensional scaling (NMDS) plot for Bray-Curtis distance of the fungal communities from *Vanilla barbellata* and *Vanilla phaeantha* between plant organs.

## **Appendix I: Forensic Conservation: E-commerce & the Illegal Trade of Orchids**

### **Abstract:**

As technology continues to advance and human society becomes more global, conservation management and enforcement for endangered and rare species must also. Currently, endangered, and highly protected orchids are sold online to a global market on retail platforms such as Ebay. Currently, there is little to no regulation or conservation efforts aimed at preventing the poaching and illegal sale of orchids on e-commerce websites. This study intends to determine; 1) what orchids are being sold internationally, 2) who is selling them, and 3) what orchids with legal protections are being sold? With the application Octoparse, a web-based data extraction tool designed for the retail and a variety of other industries, data was scraped monthly for one year from Ebay using search terms such as ‘orchid’, ‘live orchid’, ‘orchid plant’, etc. A subset of the initial data (1k/8K) demonstrated high sales from Florida which has many orchid nurseries and is home to the Everglades National Park (Figure 1). Thailand & India are currently facing issues with large scale deforestation which is promoting orchid poaching and illegal trade. Our data shows the highest numbers of international orchid sales are from these two nations (Figure 2). Certain orchids found for sale that are of interest to current conservation efforts in the state of Florida include *Dendrophylax lindenii*, *Vanilla dilloniana*, and *Encyclia tampensis*. These orchids and my others currently for sale on the e-commerce platform Ebay are under the protection of the State of Florida, the Endangered Species Act, CITES, and the IUCN Redlist.

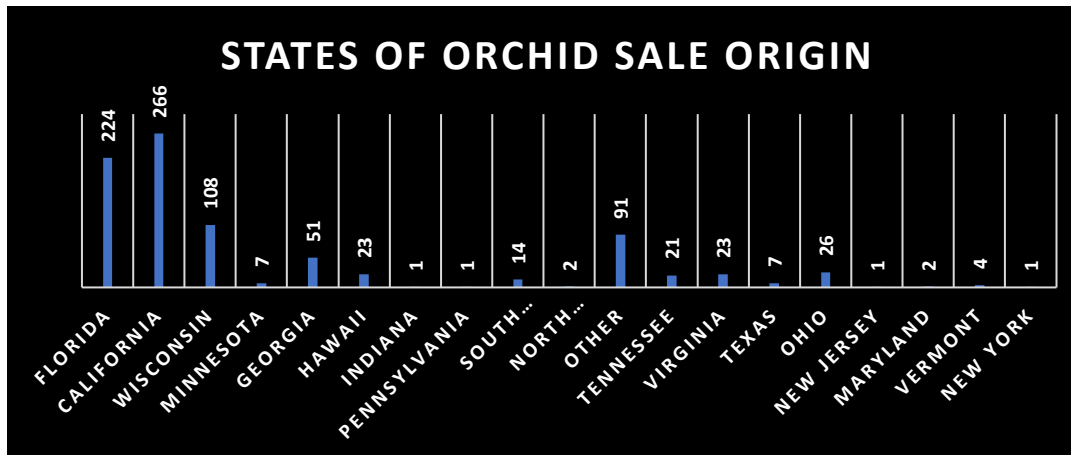


Figure 1: Origin of orchid sales by state from a subset of data, showing high sales from Florida, California, & Wisconsin.

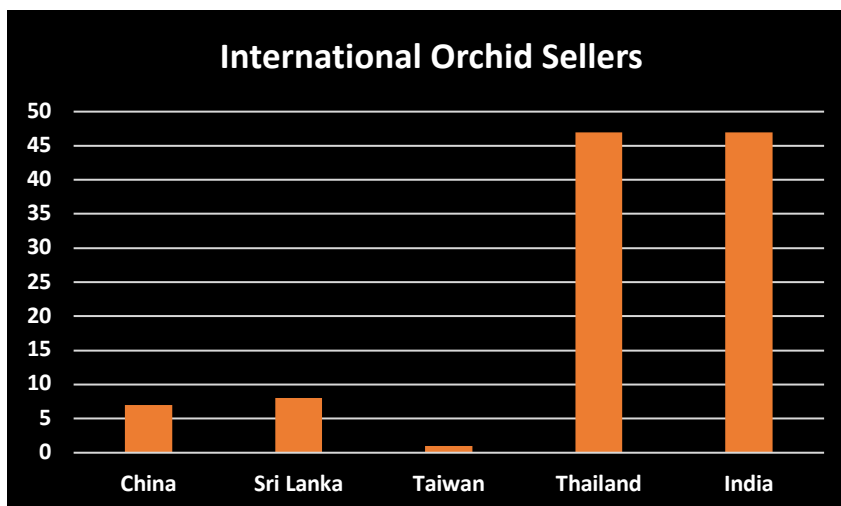


Figure 2: International orchid sellers, demonstrating a high concentration from Thailand and India.