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RESEARCH

Distinct orchid mycorrhizal fungal communities among co-occurring *Vanilla* species in Costa Rica: root substrate and population-based segregation

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Abstract

Despite being the second largest family of flowering plants, orchids represent community structure variation in plant-microbial associations, contributes to niche partitioning in metacommunity assemblages. Yet, mycorrhizal communities and interactions remain unknown for orchids that are highly specialized or even obligated in their associations with their mycorrhizal partners. In this study, we sought to compare orchid mycorrhizal fungal (OMF) communities of three co-occurring hemiepiphytic *Vanilla* species (*V. hartii*, *V. pompona*, and *V. trigonocarpa*) in tropical forests of Costa Rica by addressing the identity of their OMF communities across species, root types, and populations, using high-throughput sequencing. Sequencing the nuclear ribosomal internal transcribed spacer (*nr*ITS) yielded 299 fungal Operational Taxonomic Units (OTUs) from 193 root samples. We showed distinct segregation in the putative OMF (pOMF) communities of the three coexisting *Vanilla* hosts. We also found that mycorrhizal communities associated with the rare *V. hartii* varied among populations. Furthermore, we identified Tulasnellaceae and Ceratobasidiaceae as dominant pOMF families in terrestrial roots of the three *Vanilla* species. In contrast, the epiphytic roots were mainly dominated by OTUs belonging to the Atractiellales and Serendipitaceae. Furthermore, the pOMF communities differed significantly across populations of the widespread *V. trigonocarpa* and showed patterns of distance decay in similarity. This is the first report of different pOMF communities detected in roots of wild co-occurring *Vanilla* species using high-throughput sequencing, which provides evidence that three coexisting *Vanilla* species and their root types exhibited pOMF niche partitioning, and that the rare and widespread *Vanilla* hosts displayed diverse mycorrhizal preferences.

Keywords Niche partitioning · Root types · Hemiepiphyte · Rare · Widespread · Atractiellales · Orchids

Introduction

Mycorrhizal fungi and the roots of terrestrial plants interact to derive benefits bilaterally. Fungi typically receive photosynthetically derived carbohydrates and lipids from their host plants (Behie and Bidochka 2014). In return, plants can gain nutrients, water, and pest and disease resistance (Behie and Bidochka 2014; Siefert et al. 2018; Fernández et al. 2019). Therefore, productivity, diversity, and distribution of plant species and their communities can be linked to the diversity, specificity, and distribution of their mycorrhizal partners (Ishida et al. 2007; Zangaro et al. 2012; Mori et al. 2023). Within each type of mycorrhizal interaction, associations can range from specific to generic, meaning that either a host forms very narrow associations with a few fungal taxa, or it may be less selective in its choice of fungal partners. Regardless of the type and specificity of the interaction, mycorrhizal associations are known to assist with regulating host health and distribution (Awaydul et al. 2023; Horsch et al. 2023). Among the major types of mycorrhizal interactions, arbuscular mycorrhizae (AM) and ectomycorrhizae (EcM) are not specific to their host plants (van der

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Heijden et al. 2015). In contrast, orchid mycorrhizae (OM), are often confined to the family Orchidaceae as the name suggests (Rasmussen and Rasmussen 2014) and represent the strongest host-specific interaction compared to other mycorrhiza types (Dearnaley et al. 2012; van der Heijden et al. 2015; Põlme et al. 2018).

Being a group of plants with rudimentary embryos, the family Orchidaceae has an obligatory association with orchid mycorrhizal fungi (OMF) during germination and early development, when the fungi supply carbon to the seed (Cameron et al. 2006; Rasmussen et al. 2015). The fungi involved belong primarily to the basidiomycete families Ceratobasidiaceae, Sebacinaceae, Serendipitaceae, and Tulasnellaceae (Dearnaley et al. 2012; Weiß et al. 2016). Members of the Atractiellales have also been identified in the roots of tropical orchids and may have an active role as a symbiont of orchids (Kottke et al. 2010; Herrera et al. 2019; Qin et al. 2020; Fernández et al. 2023). While orchids show high dependency on OMF, previous studies have suggested varying degrees of mycorrhizal preferences among host orchids, ranging from highly specialized to generalistic (Shefferson et al. 2019). This varied specificity could influence the host plant distribution among habitats. For instance, *Dactylorhiza lapponica* and *D. alpestris* occupied alpine-boreal habitats which showed specialized association with the fungi which belonged to Tulasnellaceae and Sebacinaceae, while *D. viridis* occurred in wetlands by associating with Ceratobasidiaceae (Jacquemyn et al. 2016b). In some cases, the variation of the OMF community composition and the mycorrhizal specificity might affect the growth habit of co-occurring epiphytic and terrestrial orchids in tropical forests (Martos et al. 2012; Xing et al. 2019). For example, Xing et al. (2019) reported that epiphytic orchids had a higher specificity toward Tulasnellaceae in comparison to terrestrial orchids. Specialized interactions with a narrow group of fungi may provide competitive advantages by allowing more efficient nutrient exchange between orchids and their OMF partners (Nurfadilah et al. 2013; Oktalira et al. 2019). This can subsequently increase seed germination rates and might facilitate the orchid's growth and recruitment (Bonnardeaux et al. 2007; Nurfadilah et al. 2013; Oktalira et al. 2019; Kendon et al. 2020). On the other hand, being a generalist might enhance the host's adaptability, potentially allowing it to expand to colonize new spatial niches (Swarts and Dixon, 2009; Downing et al. 2020; Xing et al. 2020). Evidence also suggests that specificity towards OMF facilitated the orchid coexistence through niche partitioning (Jacquemyn et al. 2014; Xing et al. 2020). Co-occurring tropical orchids and their OMF communities are not well understood, even though the composition and structure of OMF in coexisting orchid communities have been intensively studied in temperate regions (Jacquemyn et al. 2012, 2014, 2015, 2016a; Pellegrino et al. 2014). While a few studies have shown low specificity towards OMF among two coexisting tropical orchids, *Epidendrum marsupiale* and *Cyrtochilum pardinum* with overlapping OMF (Cevallos et al. 2018; Herrera et al. 2019), more focused investigations in coexisting tropical orchids can provide strong evidence of the associated OMF communities and mycorrhizal specificity.

Rare orchids show high degrees of specificity to their OMF partners (Swarts et al. 2010; Kaur et al. 2019; Kendon et al. 2020). For example, *Platanthera praeclara*, a rare terrestrial orchid that is native to the midwestern tallgrass prairies of North America, is exclusively associated with a single Ceratobasidiaceae OTU throughout its natural distribution range (Kaur et al. 2019). However, a study suggested that the host orchid distribution is not necessarily limited by its associated OMF (Waud et al. 2017) as the case in *Liparis loeselii*, a rare terrestrial orchid distributed in the dune slacks in France and Belgium, associates with a wide range of OMF from families Ceratobasidiaceae, Sebacinaceae, and Tulasnellaceae. The variation in mycorrhizal communities of *L. loeselii* was found to be affected by the edaphic condition which limited its distribution (Waud et al. 2017). However, these studies examined the degree of specificity based on an individual rare orchid species. In another example, the mycorrhizal communities among the rare *Orchis canariensis* and the widespread *O. provincialis* were dominated by a widely distributed fungus, *Tulasnella helicospora* (Calevo et al. 2020). This result suggested that the rarity of host orchids is not necessarily limited by the distribution of associated OMF (Calevo et al. 2020). Additionally, in the context of orchid coexistence, in which OMF taxa are generally partitioned among co-existing orchids (Jacquemyn et al. 2014; Xing et al. 2020), the degrees of mycorrhizal communities between coexisting rare and common orchid hosts is not well understood.

The diversity of orchid mycorrhizal communities in both terrestrial and epiphytic orchids can also vary across soil characteristics, including nutrient content (Han et al. 2016; Mujica et al. 2016), texture (Tran et al. 2021), environmental heterogeneity (Duffy et al. 2019), and altitudinal gradients (Ren et al. 2021; Liang et al. 2022). For example, Duffy et al. (2019) documented high abundance of Sebacinaceae, Serendipitaceae, and Tulasnellaceae OTUs in roots of a widely distributed terrestrial orchid, *Spiranthes spiralis*, at the lower latitude of its habitat with lower precipitation and lower soil nitrogen in comparison to its habitats at higher latitudes. Likewise, the epiphytic orchid *Bulbophyllum tianguii* was reported to associate with multiple OTUs across habitats located at different altitudes, showing the abundance of OMF was highest at lower elevations (950 m) in comparison to middle (1198 m) and higher (1281 m)

elevations (Liang et al. 2022). Since the effect of spatial heterogeneity is expected to promote differences in OMF community composition, quantification of fungal symbionts across the distribution range of orchids is expected to provide a more complete picture of the mycorrhizal community, which can then be correlated with the degree of mycorrhizal specificity.

While most studies have focused on the mycorrhizal associations of either terrestrial or epiphytic orchids (McCormick et al. 2018; Li et al. 2021; Ogura–Tsujita et al. 2021), significantly less attention has been paid to hemiepiphytic orchids, which are characterized by a unique growing habit and specialized root types (Karremans et al. 2020; de Lima and Moreira 2022). This bias may merely be a result of the rarity of the hemiepiphytic habit in orchids. Genus *Vanilla* Miller comprises the largest radiation of hemiepiphytic orchids, comprising ca. 120 species (Karremans et al. 2020). Species belonging to genus *Vanilla* have stems modified into vines, with two types of roots. Whereas the terrestrial roots extend into the soil, epiphytic roots cling to the phorophyte. Comparison of mycorrhizal communities among these two types of roots and their associated microhabitats in hemiepiphytic *Vanilla*, or in other hemiepiphytic orchids for that matter (e.g., *Erythrorchis altissima*) is limited (Ogura–Tsujita et al. 2018). Just as occurs with other important ecological interactions, including pollination (Watteyn et al. 2021, 2023; Ackerman et al. 2023) and seed dispersal (Karremans et al. 2022, 2023), such studies in *Vanilla*, and other hemiepiphytic orchids, remain rare. OMF community assessments in *Vanilla* are virtually nonexistent despite its pantropical distribution. A few studies that have reported OMF from roots of naturally growing *Vanilla* were based on the isolation of fungi from a limited number of root samples (Porras–Alfaro and Bayman, 2003, 2007; Mosquera-Espinosa et al. 2010; Ordóñez et al. 2012). A culture-based method has been shown to be good for obtaining physical isolates and identifying fast-growing abundant OMF (O'Brien et al. 2005; Paul et al. 2018; Cale et al. 2021). However, this method does not allow a detailed assessment of the richness and abundance of fungal communities that are possible with tools such as high-throughput sequencing (Creer et al. 2016). It is worth noting that determining the functionality of fungal taxa is even more challenging for high-throughput sequencing data than for isolates cultured from pelotons.

To understand the variability in the OMF community structure across members of *Vanilla*, we studied the orchid-mycorrhizae association of three species, out of 13 native *Vanilla* species to Costa Rica (Karremans et al. 2020), using a high-throughput sequencing approach. These include the rare *V. hartii* Rolfe and the more common *V. trigonocarpa* Hoehne, along with *V. pompona* Schiede which represents the middle of the spectrum. We identified six sites where these species occurred singly and one site where all three of them co-occurred. One of these orchid populations was separated by distinct geographic barriers. In this study, we sought to determine the OMF communities of these three co-occurring orchids in Costa Rica by addressing the following research questions: **(a)** Do the three co-occurring congeners host non-overlapping OMF communities? **(b)** Does the rare *V. hartii* have a specialized OMF community in comparison to the two common congeners? **(c)** Do the terrestrial and epiphytic roots of the three *Vanilla* hosts have distinct OMF communities? and **(d)** Do the widely separated populations of the common *V. trigonocarpa* host distinct OMF communities? We hypothesized that the three co-occurring *Vanilla* species host distinct OMF communities. We also hypothesized that the OMF community in the rare *Vanilla* will show little overlap with those of the two more common congeners regardless of whether they co-occur or are geographically separated. The terrestrial roots are enveloped by organic matter and mineral-rich soil, while the epiphytic roots are attached to the phorophyte only on one side, hosting less diverse microbial communities than the substrate rich in organic matter (Cook et al. 2022). Hence, we further hypothesized that both root types will host distinct OMF communities with little overlap between the two. Finally, we expect that the four widely separated populations of *V. trigonocarpa* would show spatial segregation of their OMF communities.

Materials and methods

Study species and study site

The study focused on three *Vanilla* species, *V. hartii*, *V. pompona*, and *V. trigonocarpa*, sharing the same habitat, but with different ecological interactions and preferences. *Vanilla hartii* is characterized by the leaf length which is shorter than the length of the internode, acuminate leaf apex; dark green and subterete stem and the almost glabrous lip of the flower (Soto-Arenas and Dressler 2010; Karremans et al. 2020). Compared to the two congeneric species, *V. hartii* is relatively rare in the study sites in Costa Rica, although it has been found to occur from Mexico to Brazil (Karremans et al. 2020). The abundance of this species is low in its distribution range, where it occurs only in deeply shaded forest understories (Soto-Arenas and Dressler 2010; Karremans et al. 2020), and differs from its congeners in offering nectar as a reward to its pollinators (Watteyn et al. 2023) and seeds dispersed by bees (Karremans et al. 2023). *Vanilla pompona* is comparatively common within the study sites. This species is broadly distributed from Mexico to Paraguay where

it occurs in old-growth forests, disturbed evergreen forests or even on the roadside (Karremans et al. 2020). It features robust plants, with the leaf length longer than the internodes, thick stem spotted with white dots, and yellow flowers with a glabrous lip (Soto-Arenas and Dressler 2010). Differing from *V. hartii* in the aromatic, nectar-less flowers that attract fragrance-collecting bees and the seeds being dispersed by mammals (Watteyn et al. 2021; Karremans et al. 2022). *Vanilla trigonocarpa* is the most common among the three species within the study sites. This species can be identified based upon a long acuminate apex and a leathery leaf blade, which is elliptic in shape and bears the largest flowers among the three study species (Soto-Arenas and Dressler 2010). *V. trigonocarpa* is distributed from Belize to Brazil and frequently found in habitats including wet rainforests and disturbed forests (Soto-Arenas and Dressler 2010; Karremans unp.). It is suspected to be pollinated through food deception and having seeds dispersal by arboreal and flying mammals (Watteyn et al. 2023; Karremans et al. 2023).

This study was conducted in the Pacific and Atlantic coastal regions of Costa Rica belonging to the Mesoamerica Biodiversity Hotspot (CEPF 2023). These two watersheds are separated by the rugged highland topography of three mountain ranges (Cordillera Central, Cordillera de Talamanca, and Fila Costeña) with varying precipitation patterns and annual average temperatures (Kirby 2011; Calvo-Alvarado et al. 2014; Herrera 2016; Fick and Hijmans 2017). Seven study sites were chosen, namely, one site on the Atlantic side of the country, coded as Atlantic – with a single population *V. trigonocarpa*, and six sites in the Osa Peninsula within the Área de Conservación Osa (ACOSA) on the southern Pacific coastal region of Costa Rica. These six sites are coded as Mogos and Piro 1 – with a single population of *V. trigonocarpa* at either site; Piro 2 – with a single population of three co-occurring *V. hartii*, *V. pompona*, and *V. trigonocarpa*; Vaha_S1 and Vaha_S2 – with a single population of *V. hartii* at each site, and Vapo_S – with a single population of *V. pompona* (Fig. 1A). The seven sites represent seven *Vanilla* populations. The minimum distance between two populations (Vaha_S1 and Piro 1) was 32 m. We considered these two populations as independent because we wanted to maximize our sampling effort. Also, these populations had no physical connection being well-separated by an artificial barrier, i.e., a road. The ACOSA region comprises a biodiversity rich landscape exhibiting mangrove swamps, marine ecosystems, and the largest continuous area of tropical lowland rainforests that occur in Central America (Gilbert et al. 2016). Of the total 13 species of *Vanilla* that occur in Costa Rica, ACOSA is also home to half of them (Watteyn et al. 2020). The average annual temperature is 24 °C (Fick and Hijmans 2017), and the average annual precipitation ranges from 3,000 to 7,000 mm, with the heaviest rainfall occurring from May to November (Taylor et al. 2015; Gilbert et al. 2016) (Fig. 1B). The Atlantic region where *V. trigonocarpa* occurred singly was located 217 km apart from Piro 2. Atlantic is characterized by a tropical wet forest (McClearn et al. 2016). The mean annual temperature is 26 °C (Fick and Hijmans 2017), and the average annual rainfall is 6,000 mm with the rainy season typically from December to May (Herrera 2016) (Fig. 1B).

Root sampling, processing, DNA extraction, and PCR

Terrestrial roots (penetrating the soil) and epiphytic roots (attached to the bark of phorophytes) were sampled from 10 to 12 well-separated plants at distance of more than 5 m in the seven study sites. Root sampling of all three *Vanilla* was completed at Atlantic, Piro 1, Piro 2, Vaha_S1, Vaha_S2, and Vapo_S in July 2018. We later sampled terrestrial and epiphytic roots of *V. trigonocarpa* in Mogos in January 2019, located about 45 kms from Piro 2. Sampling in Mogos not only confirmed the wider distribution of *V. trigonocarpa*, but also allowed us to compare the OMF communities in the roots of this species along a wider distance gradient. For each individual, 3 to 10 terrestrial and epiphytic root fragments, approximately 5 to 10 cm in length, were collected, which were later combined into single samples based on root types. However, the epiphytic roots in Mogos had to be discarded due to contamination during sample transportation. Altogether, 193 healthy roots were sampled to represent two root types from all study sites (Table 1). All root samples were placed in plastic bags and processed within 24 h.

Prior to DNA extraction, both terrestrial and epiphytic roots of *V. hartii* and *V. trigonocarpa* were surface sterilized by exposing the roots to a 2-minute rinse in 70% ethanol, followed by a 4-minute wash in a 1.65% solution of sodium hypochlorite, then a 2-minute rinse in 70% ethanol, and finally three washes in sterile deionized water to remove all chemical residues. The terrestrial and the epiphytic roots of *V. pompona* were surface sterilized similarly except that we increased the exposure time in 1.65% sodium hypochlorite solution to 5 min due to the presence of thicker roots in this species. Subsequently, the epidermis of all root samples was removed. The surface sterilized root fragments from all three *Vanilla* species were then minced and lyophilized.

Total genomic DNA from each lyophilized root sample was extracted in 96-well plates using DNeasy 96 Plant kits as per the manufacturer's instructions (Qiagen, Hilden, Germany). A two-step PCR approach was used to prepare the library as described by Berry et al. (2011). Briefly, in the first PCR, the fungal nuclear ribosomal internal transcribed spacer (ITS2) region of DNA was targeted using primer

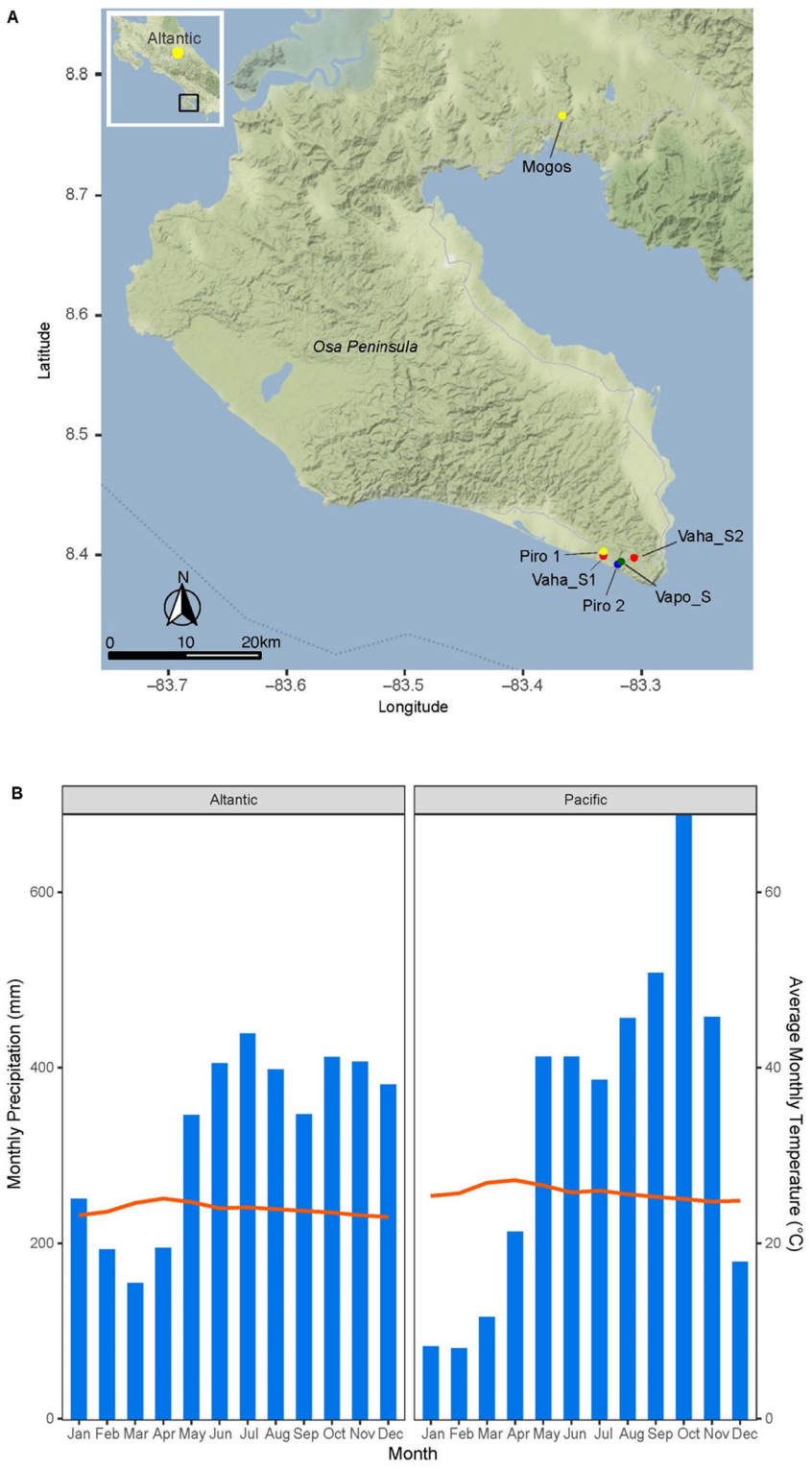

Fig. 1 Study sites of three *Vanilla* species and climatic variation at two regions. (A) The location of study sites where three *Vanilla* species were sampled in Costa Rica. Sampling sites where the host *Vanilla* occur singly (*Vanilla hartii*: red; *V. pompona*: green; *V. trigonocarpa*: yellow) and co-occur in a population (blue); (B) Walter and Lieth climate diagram showing the monthly precipitation (blue bars), and the average monthly temperature (red lines) in the Atlantic and Pacific region in Costa Rica (Fick and Hijmans 2017)

pair ITS3 and ITS4-OF (Waud et al. 2014). Products from the first PCR were used as DNA templates in the second PCR, which added Illumina index and flow cell sequences to the amplicons. The second PCR products were cleaned using AMPure XP magnetic beads (Beckman Coulter Inc., Denmark). Purified products were quantified using a Bio-Tek FL600 fluorescent plate reader (Biotek Instruments, Inc., Vermont, USA) and pooled in equimolar quantities to obtain a library. The library also included PCR positive controls representing a mock fungal community, and PCR

Table 1 Number of root samples from the three *Vanilla* species across seven populations in Costa Rica

Study species	Root type	Population	Number of roots sampled
Vanilla hartii	Epiphytic	Vaha_S1*	3
	Terrestrial		3
	Epiphytic	Vaha_S2*	3
	Terrestrial		3
	Epiphytic	Piro 2	8
	Terrestrial		8
Vanilla pompona	Epiphytic	Vapo_S*	13
	Terrestrial		13
	Epiphytic	Piro 2	16
	Terrestrial		16
Vanilla trigonocarpa	Epiphytic	Piro 1*	14
	Terrestrial		14
	Epiphytic	Piro 2	16
	Terrestrial		16
	Epiphytic	Mogos*	0
	Terrestrial		9
	Epiphytic	Atlantic*	19
	Terrestrial		19

Asterisk indicates the study species occurred individually

negative controls for detection of possible contamination during the PCR (Taylor et al. 2016). Finally, the amplicon library was purified using the above-mentioned cleaning method and paired-end sequenced (2 × 300 bp) with the Illumina Miseq platform (Illumina Inc., CA, USA) at the Center for Biotechnology and Genomics at Texas Tech University.

Sequence processing

Raw sequences were first processed using CUTADAPT to remove the adapter sequences and primers (Martin 2011), followed by merging the pair-end reads into a single sequence with the 'fastq_join' command with a minimum overlap (*-m*) set to 30 bp and selection of the 8% of the maximum difference (*-p*) in the overlapping region (Aronesty 2013). Merged sequences with a maximum expected error of 1 (*-fastq-maxee*), Ns (*-fastq-maxns*), and less than the length of 150 bp (*-fastq_minlen*) were filtered using the 'fastq_filter' command (Edgar 2010). Filtered reads were dereplicated and clustered into Operational Taxonomic Units (OTUs) at a 97% similarity threshold using UPARSE version 11 (Edgar 2013). The taxonomy of OTUs was then retained based on NCBI and UNITE reference database with 80% bootstrap confidence using three classifiers, including RDP Classifier *(--classify --conf 0.8*), "sintax" (*--sintax_cutoff*), and "utax" (*--utax_cutoff*) algorithms in USEARCH, implemented in CONSTAX v.1 (Edgar 2016; Gdanetz et al. 2017; Nilsson et al. 2018; NCBI 2020). To improve the taxonomic resolution of the OTUs that were not assigned to taxon-level (genus, family, and order level), we used the BLAST algorithm (*--blastn*): > 95% query cover, < 85% identity, and < 1E-50 E-value, and downloaded the closest related sequences in GenBank, which served as reference sequences (Camacho et al. 2009; Raja et al. 2017). The unassigned OTUs and their reference sequences were aligned using MUSCLE in MEGA X version (Edgar 2004; Stecher et al. 2020). Subsequently, maximum likelihood (ML) trees were generated for each fungal family and order with RAxML, and visualized using FigTree v.1.4.4 (Stamatakis 2014; Rambaut 2018). Finally, a taxonomic identity was assigned when OTUs formed monophyletic clades with the reference sequences supported by ≥70% bootstrap values.

Phylogenetic analyses

For the phylogenetic reconstruction of putative OMF (pOMF) OTUs obtained from the roots of three *Vanilla* species, we generated maximum likelihood (ML) phylograms for each pOMF family and order separately. We aligned the study OTUs (4 Atractiellales, 22 Ceratobasidiaceae, 16 Serendipitaceae, and 32 Tulasnellaceae) along with the reference sequences from each pOMF family and order that were previously documented from terrestrial or epiphytic host orchids across tropical or temperate regions (20 Atractiellales, 39 Ceratobasidiaceae, 21 Serendipitaceae, and 44 Tulasnellaceae) using T-coffee (Di Tommaso et al. 2011). Midpoint-rooted ML phylogenetic trees of each family and order were subsequently generated using IQ-Tree and bootstrapping of 1,000 replicates (Nguyen et al. 2015). Each phylogenetic tree was visualized using FigTree v.1.4.4 (Rambaut 2018).

Community analyses

Community analyses were carried out using the abundance-based OTU table combined with taxonomic placement in 'phyloseq' and 'vegan' packages in R version 4.0.0 (Oksanen et al. 2012; McMurdie and Holmes 2013; R core Team, 2022). Initially, we identified and removed potential contaminant OTUs by performing decontam analyses with the frequency-based method using sample DNA concentrations and the prevalence-based method using sequenced negative controls in 'decontam' R package (Davis et al. 2017). Non-fungal OTUs were excluded. OTUs occurring in very low abundances (i.e., containing fewer than ten sequences) were treated as possibly low-frequency artifacts and removed (Brown et al. 2015). We then conducted the following statistical analyses at two levels of fungal communities: (1) endophytic fungi, which includes all fungal families and orders retained from the roots of the study species, and (2) pOMF, comprising OMF families

(Ceratobasidiaceae, Serendipitaceae, and Tulasnellaceae) and an order (Atractiellales) that were previously reported to form pelotons in terrestrial and epiphytic orchids in the tropics. To evaluate the sufficiency of the sampling efforts in the detection of fungal communities that associate with three *Vanilla* species, two root types, and four populations of *V. trigonocarpa*, rarefaction curves were generated using 'iNext' R package (Hsieh et al. 2016).

The following analyses were used to determine the fungal community assembly in three co-occurring host taxa, among two root types, and four populations of *V. trigonocarpa*. We first estimated the alpha diversity of OTUs based on the Shannon-Wiener (H) diversity index (Whittaker 1972), followed by a comparison of the significant differences in diversity using non-parametric Kruskal-Wallis tests (Chao et al. 2014). To explore differences in the fungal community composition, we performed the permutational analysis of variance (PERMANOVA) with 999 runs (Anderson 2017). For this, we used the weighted Bray-Curtis dissimilarity index matrix computed based on the Hellinger transformed abundance data (Bray and Curtis 1957). Additionally, we carried out post hoc tests with adjusted *p*-values using the Bonferroni corrections in 'stats' and 'pairwiseAdonis' R packages (Martinez Arbizu 2020). Variations in the fungal community composition were then visualized with Principal Coordinate Analysis (PCoA) (Gower 1998). To test whether the OMF communities were predominantly clustered by three co-occurring host taxa, we performed hierarchical clustering and heat maps by using the above-mentioned Bray-Curtis dissimilarity matrix. We also performed Indicator Species Analysis to identify significant OTUs that showed significant mycorrhizal association in each host taxa using 'indicspecies' R package (De Cáceres et al. 2011).

To further identify the shared and unique OTUs in each host *Vanilla* and population of *V. trigonocarpa*, Venn diagrams were created to visualize the number of OTUs that are unique and common in each host taxa and populations using 'VennDiagram' R package (Chen and Boutros 2018). We subsequently used a ternary plot to identify the 12 most abundant OTUs that are shared and unique in each study host in 'ggtern' R package (Hamilton and Ferry 2018). To identify the highly abundant OTUs that were common and unique to the populations of each host *Vanilla*, we generated alluvial diagrams to depict the changes in the relative abundance of the dominant OTUs (accounted for a combined > 60% in each population) recovered in roots of each host taxon using 'ggalluvial' R package (Brunson 2020). To show the relationship between the OTU abundance and richness across each *Vanilla* hosts and populations of each of the *Vanilla* hosts, Spearman's correlations were performed based on the OTU abundance and richness using 'stats' R package (R Core Team 2024). Additionally, we performed the Mantel test to assess the relationship between the geographic distances among populations of *V. trigonocarpa* and the Bray-Curtis dissimilarity matrix of pOMF communities among populations.

Results

Overall fungal community composition

Illumina MiSeq sequencing generated 22,492,612 sequences from 193 healthy epiphytic and terrestrial root samples belonging to three *Vanilla* species. Paired-end reads were joined which produced 9,856,172 reads. Of these, 7,261,268 sequences passed quality filtering and chimeric filtering steps. Filtered reads were dereplicated and clustered into 1,580 OTUs based on a 97% similarity threshold. After the removal of contaminant, artifacts, and non-fungal OTUs, a total of 533,181 fungal sequences (299 OTUs) were retained for further analyses of the fungal community compositions in the roots of the three *Vanilla* species. Rarefaction curves based on sampling effort was close to saturation for three *Vanilla* species, two root types, and four populations of *V. trigonocarpa* (Figure S1), suggesting that the sampling effort was adequate to investigate the fungal community compositions in the roots of the three *Vanilla* taxa.

Of the 299 OTUs recovered from roots of the three *Vanilla* species, 231 OTUs belonged to Basidiomycota (96% of all fungal relative abundances – RAs), 30 OTUs belonged to Ascomycota (1% RA), while 38 OTUs belonged to other divisions or could not be assigned to any taxonomic level (3% RA). From these Basidiomycota and Ascomycota OTUs, 261 were identified as the endophytic fungal families or orders (525,148 sequences). Of these endophytic fungi, 74 pOMF OTUs make up 53% of endophytic fungal sequences. These OTUs belong to the dominant pOMF families, Tulasnellaceae (32 OTUs; 163,228 sequences), Ceratobasidiaceae (22 OTUs; 60,229 sequences), Serendipitaceae (16 OTUs; 28,357 sequences), and order Atractiellales (4 OTUs; 30,430 sequences). The dominant pOMF families and order allow us to present the biological results of the diversity and abundance of fungal communities in the three *Vanilla* hosts.

Phylogenetic analyses of each putative OMF family or order

The Atractiellales phylogram showed a broad phylogenetic breadth, with four OTUs associated with reference sequences from tropical terrestrial and epiphytic orchids in African, Asian, and Neotropical regions in three separate clades (Figure S2). The most abundant OTU 11 showed

close phylogenetic proximity to the fungus detected in Ecuadorian epiphytic *Epidendrum* and *Stelis* species. Additionally, ML tree for Ceratobasidiaceae showed that the 22 OTUs from this study formed well-supported clades with reference Ceratobasidiaceae sequences obtained from orchids sampled in different substrate types and distribution regions (Fig. 2). This phylogram also displayed a broad phylogenetic breath. OTU 9 with high RA in this study showed a phylogenetic affinity towards the fungus found in terrestrial *Paphiopedilum villosum* from Thailand, as well as in terrestrial *Limodorum abortivum* from Italy. The Serendipitaceae tree contained 16 OTUs recovered from this study, supported by reference sequences from terrestrial and epiphytic orchids across the temperate and tropical regions (Figure S3). The most abundant OTU 19 was closely related to the reference sequences obtained from the Costa Rican epiphytic *Epidendrum firmum*. Furthermore, the Tulasnellaceae phylogram showed wide phylogenetic breadth supporting three distinct clades, reported here as A (containing eight OTUs), B (eight OTUs), and C (16 OTUs) (Fig. 3). The majority of these OTUs of this study were well-supported by sequences previously sampled from terrestrial and epiphytic orchids in the tropical and temperate regions. For example, one of the most abundant OTU 8, showed proximity to the reference sequences collected from three terrestrial orchids in the tropical and temperate regions, *Paphiopedilum callosum*, *Cypripedium parviflorum*, and *C. japonicum*. However, four OTUs (OTU 35, 73, 441, and 633) from clade B were segregated into a separate sub-clade that did not nest within Tulasnellaceae OTUs previously reported from orchid roots.

Putative OMF community composition, structure, and diversity in the three co-occurring *Vanilla* species

To minimize information loss in the relative abundances of fungal OTUs in the co-occurring host *Vanilla*, and, to maximize the sampling efforts, we also ran analyses on the dataset combining populations of singly occurring *Vanilla* and the three co-occuring *Vanilla*. We found that the results from both datasets (the combined datasets with all populations versus only the co-occurring population) displayed a similar pattern. Thus, we chose to present the results from

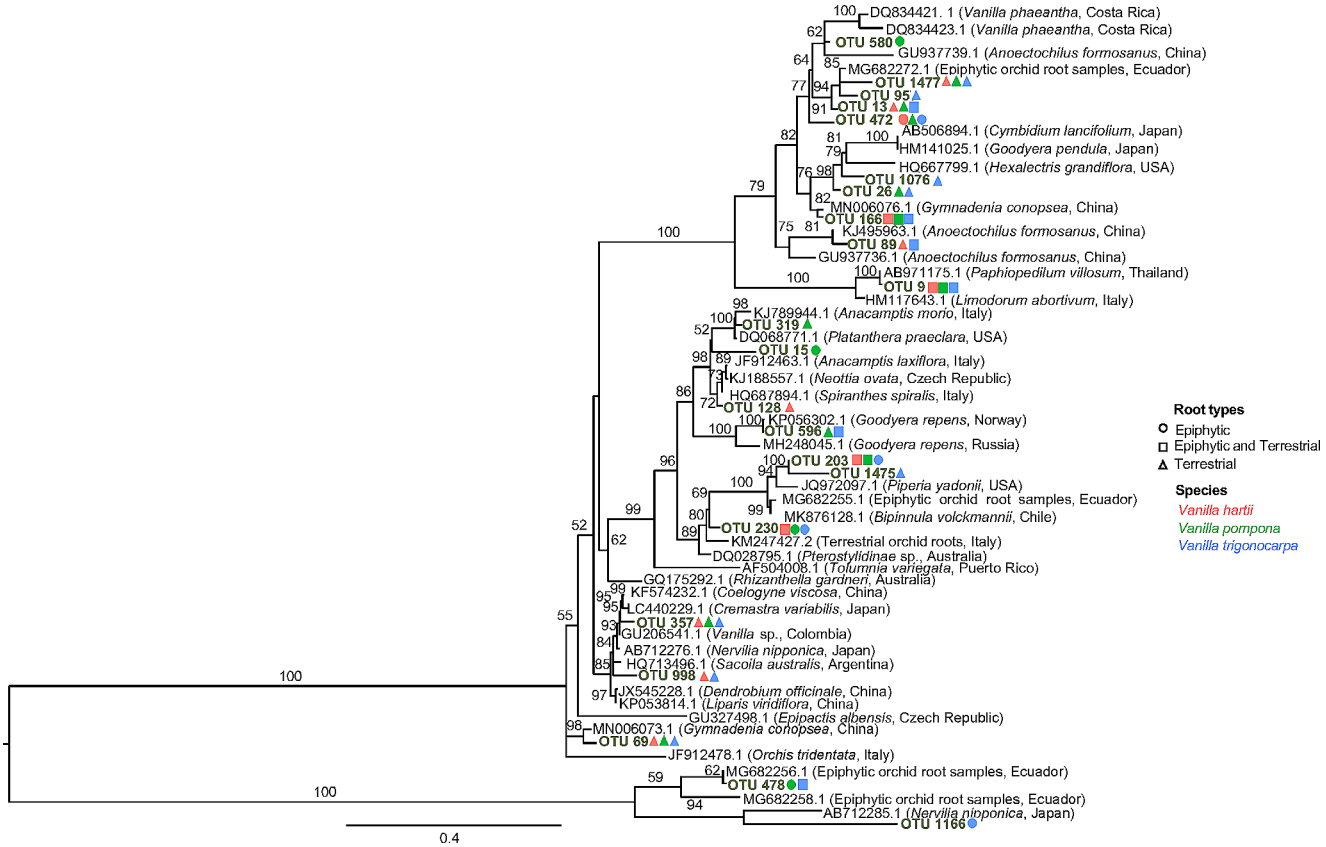

Fig. 2 Phylogeny of Ceratobasidiaceae operational taxonomic units (OTUs) within roots of three *Vanilla* species identified from nuclear ribosomal internal transcribed spacer (*nrI*TS) using high-throughput sequencing. Phylogram was generated with a maximum likelihood (ML) method with mid-point rooting. Bootstrapping values ≥ 50% are shown above branches from 1,000 replicates. Reference fungal sequences recovered from terrestrial or epiphytic host orchids from tropical or temperate regions are indicated by the corresponding GenBank accession numbers, their host orchid(s), and sampling location

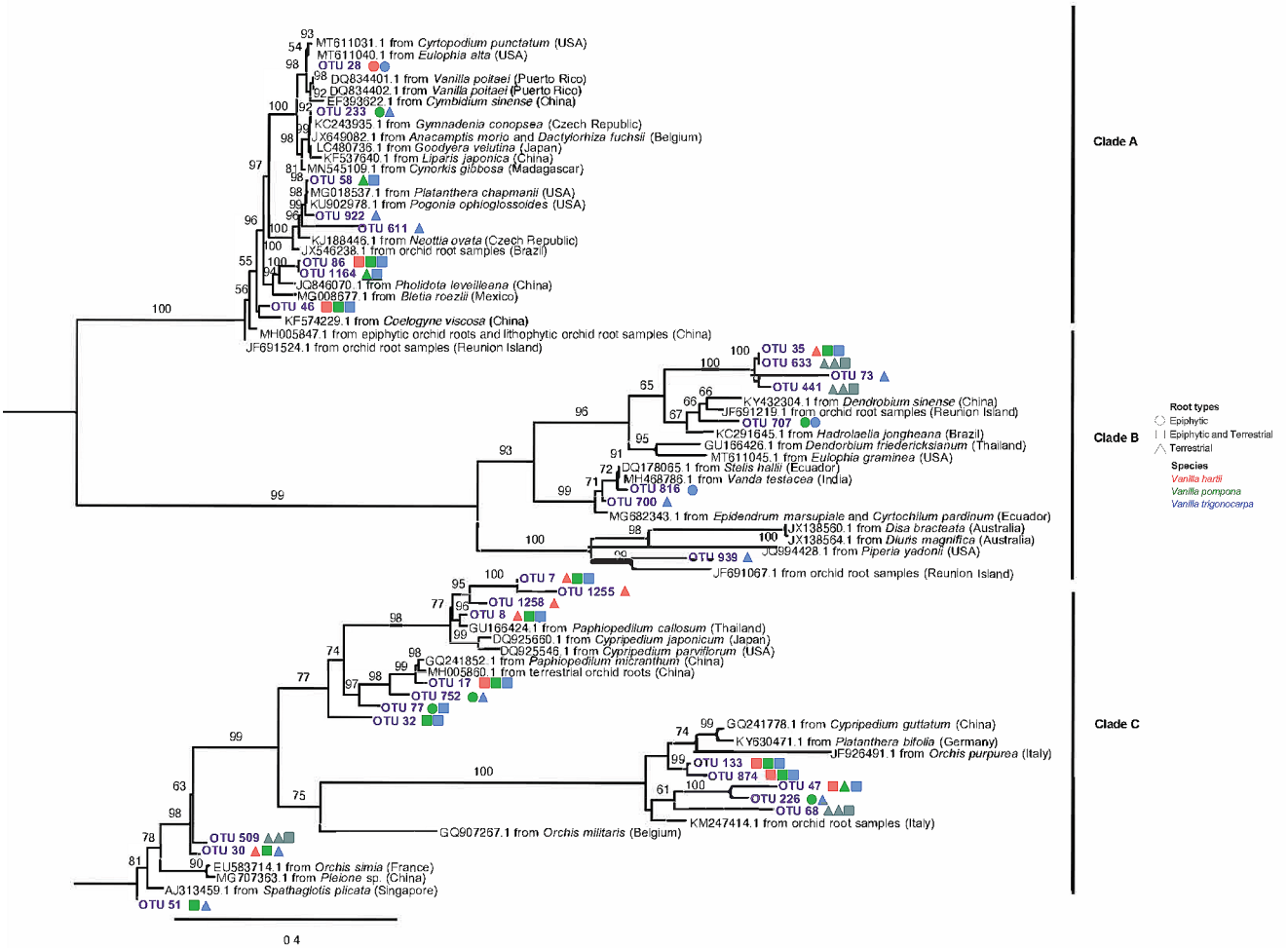

Fig. 3 Phylogeny of Tulasnellaceae operational taxonomic units (OTUs) within roots of three *Vanilla* species identified in nuclear ribosomal internal transcribed spacer (*nr*ITS) using high-throughput sequencing. Phylogram was mid-point rooted and generated with a maximum likelihood (ML) method with mid-point rooting. Bootstrapping values ≥ 50% are shown above branches from 1,000 replicates. Reference fungal sequences recovered from terrestrial or epiphytic host orchids from tropical or temperate regions are indicated by the corresponding GenBank accession numbers, their host orchid(s), and sampling location

the analyses from all populations combined (Piro 1, Piro 2, Vaha_S1, Vaha_S2, and Vapo_S) representing a larger root sample size with greater robustness to present the pOMF community in roots of the three co-occurring host *Vanilla*.

The root samples of the three co-occurring host *Vanilla* comprised 70 pOMF OTUs in populations (Piro 1, Piro 2, Vaha_S1, Vaha_S2, and Vapo_S). These pOMF were dominated by Tulasnellaceae (31 OTUs) and Ceratobasidiaceae (19 OTUs) that collectively accounted for 81% of all pOMF OTUs sequences, and to a lesser extent with OTUs of the Atractiellales and Serendipitaceae (Table S1). Of these pOMF OTUs, *V. hartii* was mostly associated with Tulasnellaceae (17 OTUs) and Ceratobasidiaceae (12 OTUs) with a combined pOMF RA of 88%, whereas *V. pompona* were largely dominated by Tulasnellaceae (23 OTUs) and Serendipitaceae (13 OTUs), which jointly accounted for 73% pOMF RA combined, and *V. trigonocarpa* was mostly associated with Tulasnellaceae (25 OTUs) and Atractiellales (2 OTUs) which comprised 80% pOMF RA altogether (Fig. 4A).

The alpha diversity of pOMF did not show a significant difference among the three host orchids (Kruskal–Wallis test, $p > 0.05$; Table 2); however, the pOMF community structure of the three host taxa was distinct based on PCoA (PERMANOVA, Pseudo-$F = 1.43$, df = 2, $R^2 = 0.02$, $p < 0.05$) (Fig. 4B). Hierarchical clustering separated the rare and common host taxa (Fig. 4C). Such separation could be contributed by a highly abundant Tulasnellaceae OTU 7 in roots of rare *V. hartii*. While *V. pompona* appeared to be influenced by two frequently detected Tulasnellaceae OTUs (OTU 32 and 51), *V. trigonocarpa* was linked by two Tulasnellaceae OTUs (OTU 8 and 35). Species indicator

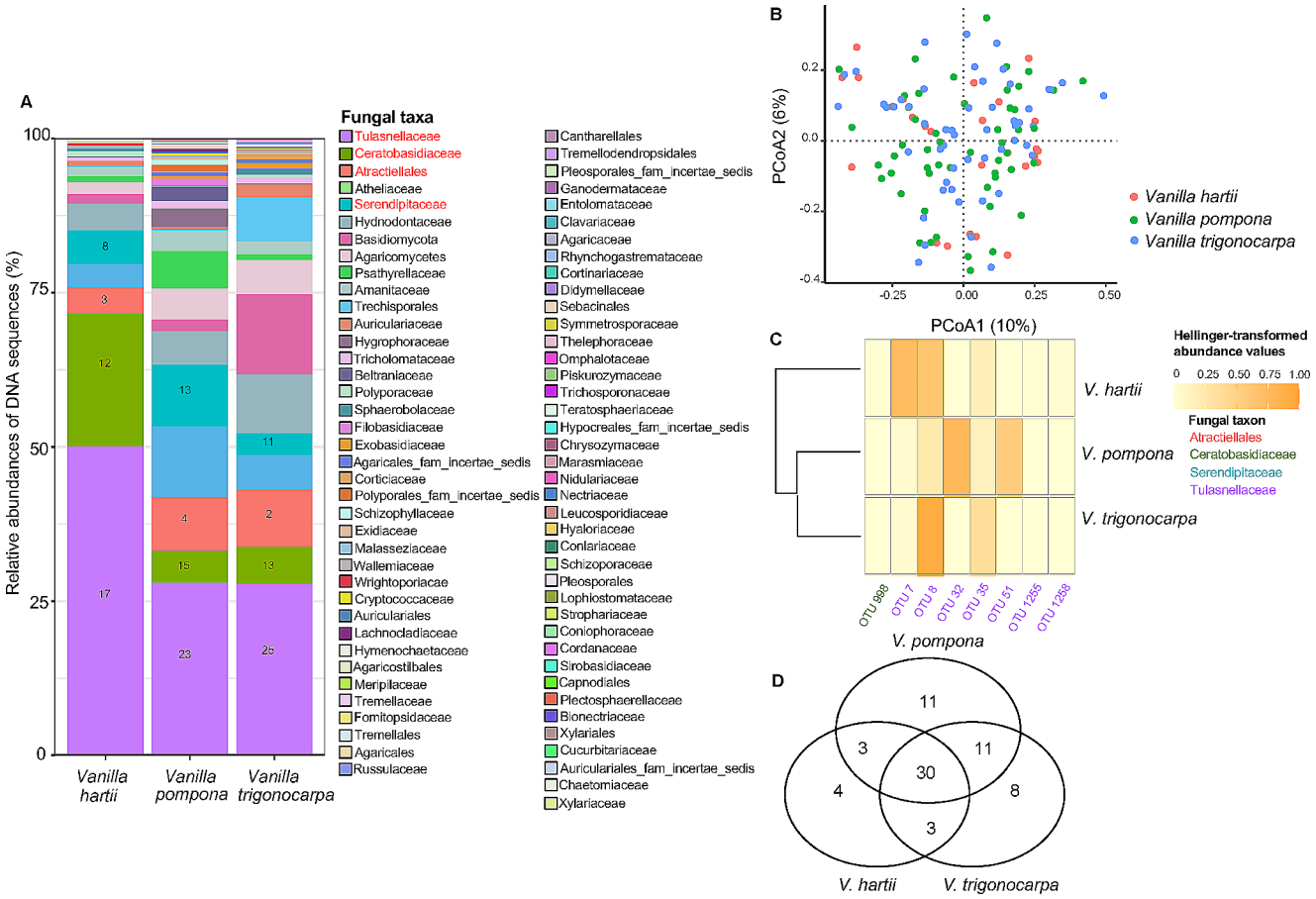

Fig. 4 Characterization of fungal communities recovered from roots of three *Vanilla* species (*V. hartii*, *V. pompona*, and *V. trigonocarpa*) in the populations (Piro 1, Piro 2, Vaha_S1, Vaha_S2, and Vapo_S) in the Osa peninsula when subjected to high-throughput sequencing of the nuclear ribosomal internal transcribed spacer (*nr*ITS). (A) Relative abundances of fungal taxa (families or orders) detected in the roots of three *Vanilla* species. Numbers inside bars represent the number of OTUs defined at a 97% sequence similarity threshold) within the respective pOMF families or orders, which are highlighted in red; (B) Principal coordinate analysis showing difference in OMF community across the three *Vanilla* species based on OTU relative abundance (PERMANOVA, Pseudo-$F = 1.43$, df = 2, $R^2 = 0.02$, $p < 0.05$); (C) Hierarchical clustering based on Bray-Curtis dissimilarity index of pOMF communities recovered from roots of three *Vanilla* species. The heat-map shows the Hellinger-transformed abundances of OTUs that were significantly associated with each *Vanilla* species using Species Indicator Analysis. The clustering pattern is likely to correspond to the rarity and commonness of *Vanilla* species; (D) Venn diagram showing the overlap in number of pOMF OTUs among three *Vanilla* species

Table 2 Shannon diversity index per three *Vanilla* species, two root types, and four populations of *V. trigonocarpa* in Costa Rica

	Shannon	Kruskal-Wallis test	df	*p*
Species		5.5	2	0.07
Vanilla hartii	2.1			
Vanilla pompona	2.8			
Vanilla trigonocarpa	2.7			
Root types		0.005	1	0.9438
Epiphytic	2.6			
Terrestrial	2.6			
Populations		6	3	0.1118
Atlantic	3.3			
Mogos	3.5			
Piro 1	2.9			
Piro 2	2.6			

analysis revealed that *V. hartii* was significantly associated with one Ceratobasidiaceae OTU and four Tulasnellaceae OTUs ($p < 0.05$), while two Tulasnellaceae OTUs were recognized as indicator taxa in *V. pompona* ($p < 0.05$), and one Tulasnellaceae OTU was characteristic for *V. trigonocarpa* ($p < 0.05$).

All three orchid hosts shared 30 OTUs belonging to pOMF families (Ceratobasidiaceae, Serendipitaceae, and Tulasnellaceae) and order (Atractiellales) (Fig. 4D). Of these, 24 OTUs were less abundant among orchid hosts representing < 10% RA (Table S1). These 30 shared OTUs accounted for unequal pOMF RAs in each host taxa: 60% RA in *V. hartii*, 39% RA in *V. pompona*, and 54% RA in *V. trigonocarpa*. On the other hand, each host orchid retained unique OTUs, belonging to the family Ceratobasidiaceae,

Serendipitaceae, and Tulasnellaceae. Four unique OTUs were detected in *V. hartii* representing < 1% RA. In *V. pompona*, 11 unique OTUs were detected. Among these, one Ceratobasidiaceae, and two Tulasnellaceae represented a combined pOMF RA of 18%. On the other hand, *V. trigonocarpa* exclusively harbored eight OTUs.

Species abundance distribution showed a significant positive relationship between the OTU abundance and OTU richness across three *Vanilla* species ($p < 0.05$) (Figure S4). Such positive relationships were also observed in each of the populations (Atlantic, Vaha_S1, Vapo_S, Mogos, Piro 1, and Piro 2) ($p < 0.05$) (Figure S4). Of these populations (Piro 1, Piro 2, Vaha_S1, Vaha_S2, and Vapo_S), one to four abundant pOMF OTUs were detected and accounted for a combined > 60% RA of the total pOMF community in the roots of the host taxon (Figure S5). Across three populations of *V. hartii* (Vaha_S1, Vaha_S2, and Piro 2), the pOMF community structure was distinct (PERMANOVA, Pseudo-$F = 1.46$, df = 2, $R^2 = 0.11$, $p < 0.05$). Two OTUs belonging to Ceratobasidiaceae (OTU 9) and Tulasnellaceae (OTU 7) were dominant in Piro 2, of which OTU 9 occurred exclusively there. In Vaha_S1 and Vaha_S2, where *V. hartii* occurred singly, there was a difference in dominant OTUs. Two Tulasnellaceae OTUs (OTU 7 and 8) were abundant in Vaha_S1 accounting for 75% RA, whereas OTU 8 solely represented 80% of RA in Vaha_S2. The pOMF community structure in the two populations of *V. pompona* (Vapo_S and Piro 2) was significantly different (PERMANOVA, Pseudo-$F = 2.4$, df = 1, $R^2 = 0.05$, $p < 0.05$). Atractiellales OTU 11 was abundant in both populations, representing 15–18% RA. Serendipitaceae OTU 19 showed higher abundance in Vapo_S than in Piro 2. In contrast, Tulasnellaceae OTU 30 was highly abundant in Piro 2, but lower in Vapo_S. Tulasnellaceae OTU 17 (32% RA) and two Tulasnellaceae OTUs (OTU 32 and 51) (collectively accounting for 36% RA) were uniquely found in Piro 2 and Vapo_S respectively. Furthermore, the pOMF community structure was distinct between the two populations (Piro 1 and Piro 2) of *V. trigonocarpa* (PERMANOVA, Pseudo-$F = 1.59$, df = 1, $R^2 = 0.03$, $p < 0.05$). Atractiellales OTU 11 and two Tulasnellaceae OTUs (OTU 8 and 17) were highly abundant which showed 12–27% RA; however, OTU 17 only had 4% RA in Piro 1. Tulasnellaceae OTU 47 accounted for 11% RA in Piro 2 but was absent in Piro 1, whereas Ceratobasidiaceae OTU 69 was exclusively found in Piro 1 and accounted for 19% RA.

Putative OMF community composition, structure, and diversity across two root types

The pOMF community of two root types revealed the presence of 48 OTUs in the epiphytic roots and 58 OTUs in the terrestrial roots of the three *Vanilla* host in populations (Piro 1, Piro 2, Vaha_S1, Vaha_S2, and Vapo_S). In epiphytic roots, Atractiellales (2 OTUs) and Serendipitaceae (12 OTUs) collectively accounted for 78% RA. They were the most abundant fungi representing more than 15% RA in each host taxon. In contrast, the majority of the OTUs in terrestrial roots belonged to Tulasnellaceae (26 OTUs) and Ceratobasidiaceae (16 OTUs) representing 90% of pOMF RA collectively (Fig. 5A).

The alpha diversity did not show any significant difference between root types of the three *Vanilla* host in populations (Piro 1, Piro 2, Vaha_S1, Vaha_S2, and Vapo_S) (Kruskal–Wallis test, $p > 0.05$; Table 2). However, a significant variation in the pOMF community composition was found between root types (PERMANOVA, Pseudo-$F = 7.49$, df = 1, $R^2 = 0.06$, $p < 0.05$), which was also supported by the PCoA plot (Fig. 5B). A similar pattern was observed within the same population for each host orchid. So, we used the combined data from all populations. Hierarchical clustering consistently grouped the two root types separately (Fig. 5C). Regarding the clustering pattern, the presence of Atractiellales OTU 11 and Serendipitaceae OTU 19 possibly defined the pOMF community in epiphytic roots, whereas the pOMF community in terrestrial roots was likely to be influenced by the presence of Tulasnellaceae and Ceratobasidiaceae OTUs. Indicator Species Analysis identified 16 OTUs that were significantly associated with root types ($p < 0.05$). Atractiellales OTU 11, Serendipitaceae OTU 19, and additional three Tulasnellaceae and Ceratobasidiaceae OTUs were characteristic for epiphytic roots of three host *Vanilla* ($p < 0.05$). In contrast, 11 Ceratobasidiaceae and Tulasnellaceae OTUs were significantly associated with terrestrial roots of all three host *Vanilla* ($p < 0.05$).

A comparison of the pOMF OTUs between the two root types showed that 36 OTUs (out of the total of 70) were common to both. Of which, 12 OTUs accounted for ≥ 5% of the pOMF community of each *Vanilla* species (Fig. 5D). Of these 12 OTUs, Atractiellales OTU 11 and Serendipitaceae OTU 19 were the most abundant in epiphytic roots detected in the three host orchids. Tulasnellaceae OTU 7 has the highest RA in *V. hartii* when compared to *V. pompona* and *V. trigonocarpa*. This OTU was readily detected in the soil roots of *V. hartii* (Fig. 5D). In the roots of *V. pompona*, the two Tulasnellaceae OTUs (OTU 32 and 51) were readily detected in both root types, while Ceratobasidiaceae OTU 580 and Serendipitaceae OTU 146 were nearly exclusively found in its epiphytic or terrestrial roots, respectively. The Tulasnellaceae OTU 47 and Serendipitaceae OTU 190 almost exclusively occurred in both root types of *V. trigonocarpa*, and three Tulasnellaceae and Ceratobasidiaceae OTUs were readily detected in the terrestrial roots of *V. trigonocarpa*.

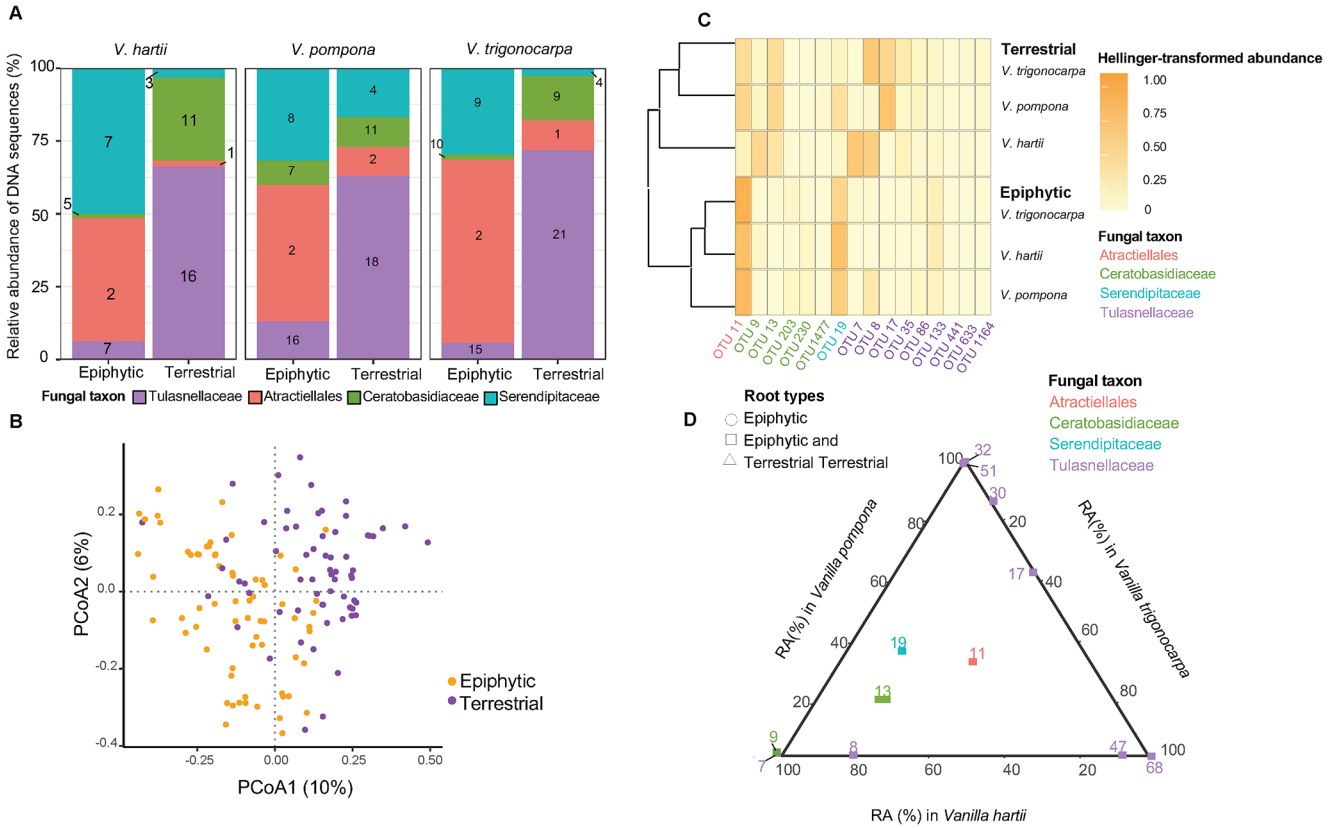

Fig. 5 Characterization of pOMF communities detected in two root types (epiphytic and terrestrial roots) from the three *Vanilla* species in the populations (Piro 1, Piro 2, Vaha_S1, Vaha_S2, and Vapo_S) in the Osa peninsula using high-throughput sequencing of the nuclear ribosomal internal transcribed spacer (*nr*ITS). (A) Relative abundances of pOMF families or orders in two root types from each *Vanilla* species. Numbers inside bars represent the number of OTUs, defined at a 97% sequence similarity threshold; (B) Principal coordinate analysis based on relative abundance of pOMF OTUs showed distinct clustering between epiphytic and terrestrial roots (PERMANOVA, Pseudo-$F = 7.49$, df = 1, $R^2 = 0.06$, $p < 0.05$); (C) Hierarchical clustering of pOMF communities recovered from two root types of each *Vanilla* species with Bray-Curtis dissimilarity index. The heat-map shows the Hellinger-transformed abundances of OTUs that were significantly associated with each root type of each *Vanilla* species using Species Indicator Analysis. The clustering separated epiphytic roots apart from terrestrial roots; (D) The ternary plot comparing the relative abundances of pOMF OTUs (accounting for ≥ 5% RAs) in three host *Vanilla* species

Putative OMF community composition, structure, and diversity across populations of *Vanilla trigonocarpa*

Of 65 pOMF OTUs recovered from the roots of *Vanilla trigonocarpa* across four populations (Atlantic, Mogos, Piro 1, and Piro 2), the most abundant family was Tulasnellaceae (30 OTUs) accounting for 52% RA, followed by Ceratobasidiaceae (18 OTUs) and Atractiellales (3 OTUs) representing 19% RA each, while Serendipitaceae (14 OTUs) made up the remaining 10%. In Piro 1, the pOMF community was almost equally shared by the pOMF family, which represented an average of 25% RA each (Fig. 6A). Piro 2 was dominated by Tulasnellaceae (19 OTUs) and Atractiellales (3 OTUs), which made up 83% of pOMF RA. The majority of pOMF OTUs, in Mogos, belonged to Tulasnellaceae (16 OTUs) and Serendipitaceae (7 OTUs) which represented 70% of pOMF RA, and Atlantic hosted an abundance of Tulasnellaceae (15 OTUs) and Ceratobasidiaceae (10 OTUs) which accounted for a combined RA of 79% (Table S1).

Across the four populations of *V. trigonocarpa* (Atlantic, Mogos, Piro 1, and Piro 2), Shannon alpha diversity did not show any significant difference (Kruskal–Wallis test, $p > 0.05$; Table 2), but the pOMF communities were generally differentiated (PERMANOVA; Pseudo-$F = 2.46$, df = 3, $R^2 = 0.09$, $p < 0.05$). The PCoA plot also showed a similar pattern in the variation in pOMF community compositions across populations (Fig. 6B). Furthermore, in the hierarchical clustering, while the two populations (Piro 1 and Piro 2) grouped together, Mogos and Atlantic remained distinct (Fig. 6C). Atlantic might have separated apart from the other three populations due to the presence of Ceratobasidiaceae OTU 957 and two Tulasnellaceae OTUs (OTU 46 and 441), while Mogos appeared to be influenced by the Atractiellales OTU 196, Ceratobasidiaceae OTU 998, and Tulasnellaceae

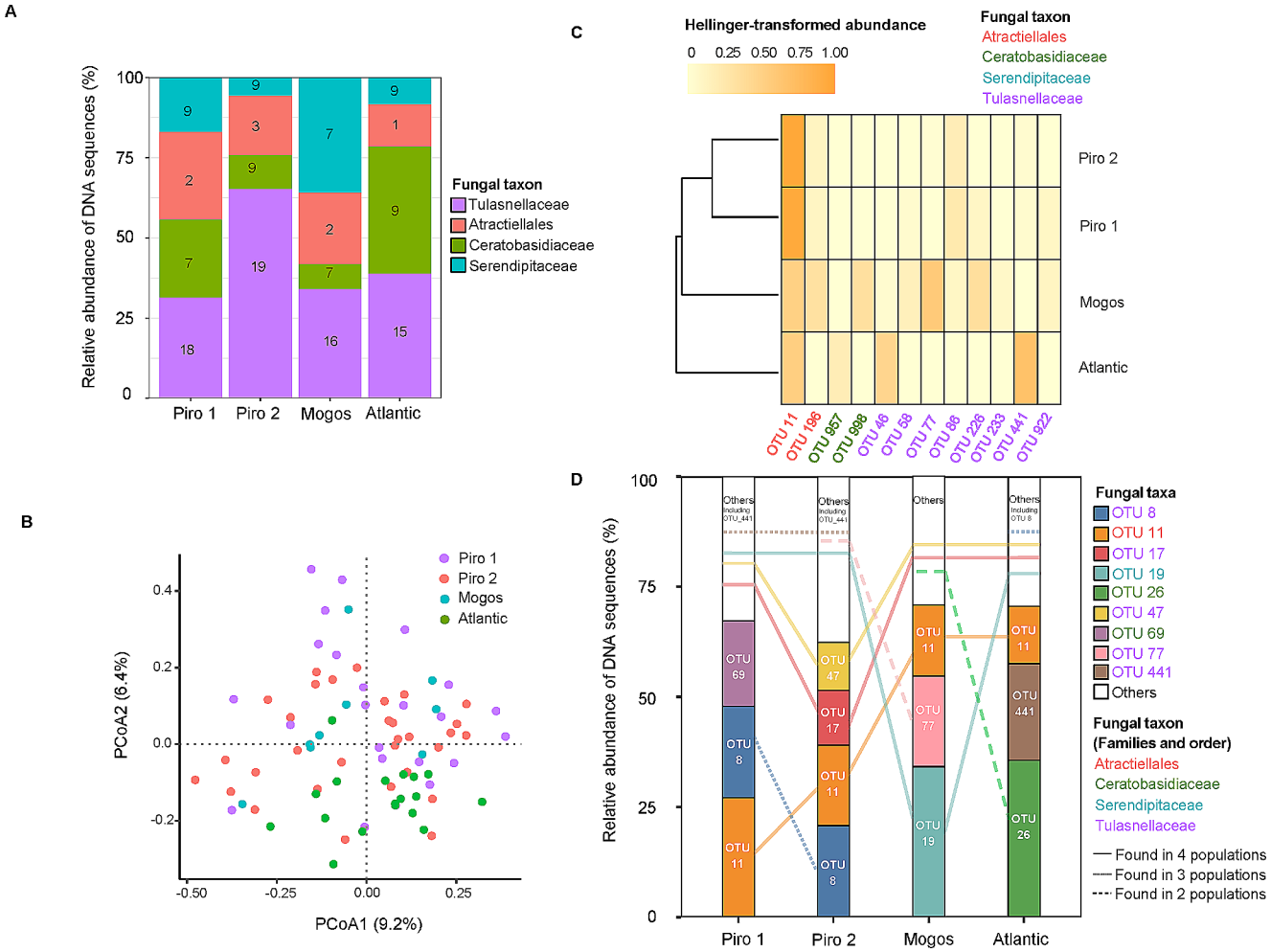

Fig. 6 Characterization of putative orchid mycorrhizal fungal (pOMF) communities within roots of *Vanilla trigonocarpa* across the four populations (Atlantic, Mogos, Piro 1, and Piro 2) in Costa Rica using operational taxonomic units (OTUs; defined at a 97% sequence similarity threshold) identified in the ITS2 region sequencing in a high-throughput sequencing platform. (A) Relative abundances (RAs) of OMF families or orders recovered from roots of *V. trigonocarpa* in each population. Numbers inside bars represent the number of OTUs within the respective family or order; (B) Principal coordinate analysis displayed distinct pOMF community composition across the four populations of *V. trigonocarpa* based on OTU relative abundance (PERMANOVA; Pseudo-$F = 2.46$, df = 3, $R^2 = 0.09$, $p < 0.05$); (C) Hierarchical clustering of pOMF communities recovered from roots of *V. trigonocarpa* across the four populations with Bray-Curtis dissimilarity index. The heat-map shows the Hellinger-transformed abundances of OTUs that were significantly associated with each population using Species Indicator Analysis. The pOMF communities showed dissimilarity in the distance among populations; (D) The alluvial diagram using OTU relative abundance in each population shows the most abundant OTUs accounted for a combined RA of ≥ 60% in each population

OTU 77. Indicator Species Analysis identified 12 OTUs belonging to Atractiellales, Ceratobasidiaceae, and Tulasnellaceae that were significantly associated with all four populations ($p < 0.05$). Two OTUs were uniquely associated with Piro 1. In Piro 2, one OTU was recognized as the indicator ($p < 0.05$), seven OTUs were characteristic of Mogos ($p < 0.05$), and three OTUs were significantly associated with Atlantic ($p < 0.05$).

Nine OTUs were most abundant and collectively accounted for 72% RAs of the total pOMF community of the four populations (Atlantic, Mogos, Piro 1, and Piro 2) (Fig. 5D). In addition, eleven OTUs were geographically widespread across populations (Figure S6). While multiple OTUs were shared among populations, unique OTUs were also found (Figure S6). Seven unique OTUs were exclusively found in Atlantic, but represented only < 5% RA. In addition, seven OTUs were unique in Piro 1, especially Ceratobasidiaceae OTU 69 representing 19% RA. In Piro 2, seven unique OTUs represented a combined 9% RA, whereas four unique OTUs were detected in Mogos, representing 8% RA. The Mantel test showed a significant distance-decay pattern that the similarity in pOMF communities among populations decreased with the increased geographical distance among populations ($R^2 = 0.03$, $p < 0.05$).

Discussion

The diversity and distribution of host orchids could be strongly structured by their mycorrhizal fungi; however, this conclusion is based on most studies undertaken on the temperate terrestrial orchids (Jacquemyn et al. 2012, 2014, 2015, 2016a; Pellegrino et al. 2014). Thus, orchid mycorrhizal associations are still facing a paucity of research to identify the fungi or the fungal community among the coexisting tropical orchids, in the terrestrial and epiphytic roots of the same species, as well as across different populations of the same species, using a high-throughput NGS-based approach. Herein is the first report describing the variation in the pOMF communities in three co-occurring host *Vanilla* species spanning common and rare host plant species, root types, and the populations that are spatially partitioned by geographic barriers, by using highly comprehensive individual root samples collected in the tropical rainforests in Costa Rica.

A diverse putative OMF community occurs across the three host *Vanilla* species

Our result shows that the fungal community obtained from the roots of three *Vanilla* were largely dominated by the pOMF families (Ceratobasidiaceae, Serendipitaceae, and Tulasnellaceae) and order (Atractiellales). Among the detected pOMF families, Tulasnellaceae and Ceratobasidiaceae are the dominant mycobionts, which is following earlier findings within the genus *Vanilla* (Porras–Alfaro and Bayman, 2007; Mosquera-Espinosa et al. 2010; Ordóñez et al. 2012). However, Serendipitaceae and Atractiellales were reported for the first time as abundant mycobionts in wild *Vanilla* species. The finding was consistent with previous studies on tropical terrestrial and epiphytic orchids (Kottke et al. 2010; Martos et al. 2012; Suárez et al. 2008, 2016; Cevallos et al. 2017, 2018; Xing et al. 2017, 2020; Herrera et al. 2019; Qin et al. 2019, 2020; Pecoraro et al. 2021; Wang et al. 2022). However, we did not detect Sebacinaceae, which was reported from many other orchids distributed in various continents (Pandey et al. 2013; Jacquemyn et al. 2017; Liang et al. 2022). In general, the failure to recover some pOMF taxa might happen as a result of the PCR primer bias. However, this bias can be ruled out in the current study as the primers we utilized were optimized to characterize pOMF communities including Sebacinaceae OTUs in many temperate and tropical orchids (Waud et al. 2014; Han et al. 2016; Jacquemyn et al. 2017; Wang et al. 2022), and have outperformed other primer pairs (Waud et al. 2014). Therefore, our results truly reflect the overall pOMF diversity in *Vanilla* species.

The three co-occurring *Vanilla* species host non-overlapping putative OMF communities

Among the four pOMF families and order detected across three coexisting *Vanilla* species, their pOMF community compositions were different as also supported by the PCoA, hierarchical clustering, and PERMANOVA (Fig. 4B, C). This differentiation in the pOMF communities might be influenced by the unique OTUs associated with each of the host plants. In our study, although a subset of OTUs were shared by the coexisting *Vanilla* species, they constituted unequal RAs in each host *Vanilla*. Our results also demonstrated that a single or a few OTUs were constantly more abundant in one host *Vanilla* than others. These results were congruent with the increasing evidence of niche partitioning among co-occurring orchids (Waterman et al. 2011; Jacquemyn et al. 2012, 2014, 2015, 2017; Martos et al. 2012; Těšitelová et al. 2013; Cevallos et al. 2018; Chen et al. 2019; Mennicken et al. 2023; Zhang et al. 2023). Such variation in pOMF communities among coexisting orchid hosts showed a possible resource-acquisition strategy, which could be the result of the different metabolic resource uptake patterns between fungi and even among multiple strains of the same OMF (Huynh et al. 2009; Pellegrino et al. 2014; Mennicken et al. 2023). For example, while some Ceratobasidiaceae OTUs were found to be effective in using nitrate (Nurfadilah et al. 2013; Novotná et al. 2023), increasing nitrate concentration suppressed the metabolic ability of other groups, namely the Serendipitaceae and Tulasnellaceae OTUs (Figura et al. 2021). The different metabolic behavior depicted by the OMF may confer competitive fitness to host orchids by enhancing nutrient access (Mennicken et al. 2023; Zhang et al. 2023). Therefore, the OMF with varied metabolic behavior is likely to favor the orchid coexistence (Jacquemyn et al. 2014; Cevallos et al. 2017; Põlme et al. 2018).

A significant positive relationship between the abundance and richness of OTUs across three *Vanilla* species ($p < 0.05$), as well as in each population is observed (Atlantic, Mogos, Piro 1, Piro 2, Vaha_S1, and Vapo_S) ($p < 0.05$) (Figure S4). Among co-occurring (Piro 2) and singly occurring populations (Piro 1, Vaha_S1, Vaha_S2, and Vapo_S) of each *Vanilla* host, their pOMF community compositions were different which comprised several locally unique OTUs (Figure S5). Unlike the shared dominant *Tulasnella helicospora* in roots of *Orchis canariensis* between their populations (Calevo et al. 2020), the mycorrhizal divergence was observed among populations of each of the *Vanilla* hosts, suggesting the important role of locally abundant OTUs in roots of orchid hosts that could support the host plant persistence in the habitat. The results of this study also suggests that the orchid coexistence led to the

mycorrhizal segregation to mediate the host plant's competition at the co-occurring site (van der Heijden et al. 2015; Tedersoo et al. 2020). Furthermore, even at shorter physical distances between populations of each *Vanilla* hosts, there were differences in the mycorrhizal communities between populations. Such differences at shorter distances were also observed in a previous study, although they focused on two congeneric species (Jacquemyn et al. 2016a). *Epipactis neerlandica* occurred in the drier locations, whereas *Epipactis palustris* typically occurred in the wetter locations within dune slacks. These host orchids had different mycorrhizal communities (Jacquemyn et al. 2016a). This may suggest that the spatial pattern of mycorrhizal segregation at a small scale is associated with environmental conditions at the sites. Further research is also needed to assess the fine-scale environmental variables between the co-occurring population and the singly occurring populations of each *Vanilla* host, to understand what drives spatial patterns at such small distances among populations.

The rare *Vanilla hartii* has some overlap with putative OMF communities of the other two common congeners

There was some overlap in pOMF communities among the common and rare *Vanilla* species as they shared 30 OTUs. This result supports the observations from earlier findings of OTU sharing between common and rare orchids (Oktalira et al. 2019; Calevo et al. 2021). For example, the mycorrhizal community of the widespread *Orchis provincialis* shared 16 common OTUs belonging to Ceratobasidiaceae, Serendipitaceae, and Tulasnellaceae with the rare congeneric *O. patens* (Calevo et al. 2021). Besides that, the widely distributed fungus, *Tulasnella helicospora*, was found to be dominant in the roots of *O. patens* (Calevo et al. 2020). This displays the apparent mycorrhizal generalism, showing the association with a dominant OTU that contributed unique resources and several sporadically occurring OMF that are responsible for redundant resources (Shefferson et al. 2019). In the current study, the mycorrhizal community in the roots of rare *V. hartii* showed a similar pattern. The phylogenetic breadth of the OTUs associated with this species showed a phylogenetic affinity with fungi isolated from widely distributed terrestrial *Gymnadenia conopsea*, *Neottia cordata*, or epiphytic *Coelogyne viscosa* (Figs. 2 and 3; Figure S3), which indicated that the rare *V. hartii* may not harbor a rare pOMF community. Furthermore, *V. hartii* associated with the highly abundant Tulasnellaceae OTU 7 and several low abundant unique OTUs (Fig. 4D). This suggests that *V. hartii* associates with a wide range of pOMF OTUs of which some may occasionally occur in their roots. Moreover, the Tulasnellaceae OTU 8 took on the role of the dominant pOMF when Tulasnellaceae OTU 7 was barely present in Vaha_S2 (Figure S5; Table S1). This suggests that the rarity of *V. hartii* may not necessarily be influenced by the mycorrhizal specificity, but may be dependent on the local-scale availability of the OTUs. Studies have found that the rarity of orchid hosts could be related to a lot of other aspects, including variation in edaphic factors (Phillips et al. 2011, 2014). However, whether the soil/substrate pOMF compositions are variable in the study populations, and to what extent these soil/substrate pOMF OTUs are related to the recruitment of the rare host orchid, require more investigation.

Epiphytic and terrestrial root types of the *Vanilla* host distinct putative OMF communities

The pOMF communities associated with epiphytic and terrestrial roots were differentiated in all *Vanilla* hosts, which were mainly influenced by the dominant OTUs present in both root types. The terrestrial roots of these *Vanilla* hosts showed a preference for Tulasnellaceae and Ceratobasidiaceae in our study, in which Ceratobasidiaceae was also known to occur in terrestrial roots of *V. planifolia* (Porras–Alfaro and Bayman 2007). Moreover, this result agrees with many other studies on terrestrial orchids that associated predominantly with Tulasnellaceae and Ceratobasidiaceae (Jacquemyn et al. 2016b; Waud et al. 2016; Kaur et al. 2021; Xing et al. 2020; Zhang et al. 2023). On the other hand, within the epiphytic roots, Atractiellales OTU 11 and Serendipitaceae OTU 19, showed high abundance (Fig. 5A). Atractiellales and Serendipitaceae are shown for the first time to form mycorrhizal associations in epiphytic roots of three *Vanilla* species, although Ceratobasidiaceae has also been detected on the epiphytic roots of *Vanilla* species (Porras–Alfaro and Bayman 2007). Indeed, the dominance of Ceratobasidiaceae in epiphytic roots is only supported by one study focusing on a single *Vanilla* taxon, *V. planifolia*. The dominance of Atractiellales and Serendipitaceae has been reported in the epiphytic roots of orchids. For example, high abundance of Atractiellales has been shown in the roots of *Dichaea eburnea*, *Masdevallia nidifica*, and *Oncidium klotzschianum* in Costa Rica as well as *Stelis* and *Epidendrum* species in Ecuador (Kottke et al. 2010; Fernández et al. 2023). On the other hand, the Costa Rican *Epidendrum firmum* and *E. odontochilum*, and the Eucadoran *Cytrochilum*, *Maxillaria*, and *Stelis* frequently detected the presence of Serendipitaceae OTUs (Suárez et al. 2008; Kartzinel et al. 2013; Cevallos et al. 2017; Fernández et al. 2023). In the current study, the phylogenetic analysis of the Atractiellales showed that OTU 11 clustered into a group with a bootstrap value of 99% with a fungus associated with epiphytic orchids, such as *Epidendrum* species collected in the

Neotropics and *Bulbophyllum odoratissimum* in the tropics (Kottke et al. 2010; Xing et al. 2019) (Figure S2). Further, the phylogram of Serendipitaceae showed that Serendipitaceae OTU 19 formed clades with epiphytic orchid fungus associated with Costa Rican *Epidendrum firmum*, with a 92% bootstrap value (Kartzinel et al. 2013). This suggests the ability of these Atractiellales and Serendipitaceae OTUs to associate with multiple epiphytic orchids, and thus they were likely to be found in the epiphytic roots of orchids. Nonetheless, we are just beginning to understand the pOMF community composition in root types of *Vanilla* species and their pOMF communities which were thus far examined by only a few studies. However, we cannot exclude the possibility of mycorrhizal role of Atractiellales and Serendipitaceae OTUs in roots of *Vanilla*. Thus, additional molecular, physiological, and morphological evidence on the pOMF mycorrhizal community in *Vanilla* species is needed.

The differentiation of the pOMF communities between root types may reflect a growing strategy of the host *Vanilla*. The associated pOMF in different root types may bring adaptive benefits which facilitate the host orchids to fulfill the specific requirement and strengthen the tolerance of host orchids, to adapt to different environmental conditions (McCormick et al. 2006; Xing et al. 2015, 2019; Jasinge et al. 2018). Microhabitat conditions have been proposed as one of the factors explaining the variation in the OMF community (Rasmussen and Rasmussen 2018). Several studies have previously reported that epiphytes are likely to experience extreme environmental conditions, such as physical weathering, water shortage, nutrient limitation, or high exposure to sunlight (Martos et al. 2012; Xing et al. 2013, 2015; Nakamura et al. 2017; Oliveira and Scheffers 2019). On the other hand, the conditions on or below ground (i.e., terrestrial) are likely to be comparatively stable as a refuge in contrast to the stressed epiphytic microhabitat (Oliveira and Scheffers 2019). In our study, the epiphytic roots of *Vanilla* were attached to the trunk of phorophytes, while their terrestrial roots were exposed to the soil, emphasizing the different ecological conditions that may create the distinct OMF communities stratified vertically between root types. Recent investigations into the root morphology and anatomy in *Vanilla* have shown that the root structure between epiphytic and terrestrial roots varies in nutrient acquirement (de Lima and Moreira 2022). As such, the distinct root structure between root types in plants might alter the resource acquisitive strategies for plant hosts to survive in both terrestrial and epiphytic microhabitats (McCormack and Iversen 2019) and potentially shape the fungal colonizers in the plant root tissue (Deveautour et al. 2018; McCormack and Iversen 2019; de Lima and Moreira 2022). The variation of pOMF communities between root types, therefore, may facilitate the host *Vanilla* in fulfilling the nutritional requirements in the adaptation to terrestrial and epiphytic microhabitats. A few other studies in tropical forests have reported that the fungal communities from the substrate along the phorophyte branches could vary across the submeter distances (Petrolli et al. 2021; Cook et al. 2022). More molecular studies on the fungal communities in roots are likely to elucidate the spatial pattern of the pOMF OTUs associated with the growth of their host *Vanilla* along the phorophyte trunk.

Spatial segregation among populations of *Vanilla trigonocarpa* facilitates hosting of distinct putative OMF communities

Vanilla trigonocarpa has mycorrhizal preference for multiple pOMF OTUs across four populations. While unique pOMF OTUs were found in each population, other OTUs were shared (Fig. 6D). This pattern could be interpreted as an apparent generalist approach (Shefferson et al. 2019), in which the orchid host associated with one or few unique OTUs, but also exhibited spatial pOMF replacement when associating with multiple OTUs across different populations. A similar pattern has been found in the widespread *Gymnadenia conopsea* across populations in European and Asian continents (Xing et al. 2020) and in the populations of Cypripedioideae subfamily from the Northern Hemisphere (Shefferson et al. 2019) as well as the *Pleione* species at multiple populations in southwestern China (Qin et al. 2019). Hence, the fungal replacement in host orchid roots under various environmental conditions might support the host orchid's growth and survival (McCormick et al. 2006; Mujica et al. 2016; Mennicken et al. 2023). A recent study reported that the number of reads generated from high-throughput sequencing could be reliable to reflect the biological abundance of fungi in the samples (Wang et al. 2023). In each population of *V. trigonocarpa*, the highly abundant OTUs were likely to contribute unique resources, whereas the less abundant unique OTUs may play a secondary role in contributing functionally redundant resources. As such, the acquisition of these OTUs in their respective population may be advantageous to improve the plant's establishment. In addition, Mennicken et al. (2023) showed that different mycorrhizal specificity across study sites with distinct climatic patterns. Similarly, the elevation, precipitation, and rainy season were different among populations of *V. trigonocarpa* (Gilbert et al. 2016; McClearn et al. 2016). Atlantic was located at a higher elevation (349 m) and was drier during the warmest month in contrast to the three populations (Fick and Hijmans 2017), which suggests different hydric levels among populations. Thus, the differences in climatic patterns among the four populations of *V. trigonocarpa* may lead to the result of the multiple OTUs

associating with *V. trigonocarpa*. Additionally, Atlantic and the other three populations are separated by three mountain ranges (i.e., ca. 217 km apart between Atlantic and Piro 2). Our result was similar to a previous study investigating the pOMF community of *Spiranthes spiralis* across > 3,000 km which exhibited the distance-decay pattern (Duffy et al. 2019). This result further supported the spatial heterogeneity as a significant factor affecting the pOMF community in host orchids (Kartzinel et al. 2013; Davis et al. 2015; Duffy et al. 2019; Shefferson et al. 2019; Mennicken et al. 2023).

Conclusions

Based on this study, we conclude the pOMF OTUs were dominant among the endophytic fungal communities of three co-occurring *Vanilla* taxa. Three co-occurring *Vanilla* hosts distinct pOMF communities. This result was consistent with the recent study on four co-occurring tropical orchids from different genera in Costa Rica (Fernández et al. 2023), indicating that niche partitioning of mycorrhizal fungal communities may reduce the competition between coexisting orchids. We also conclude that widespread *V. trigonocarpa* has more diverse pOMF communities that may support plant growth and survival in different habitats. Unexpectedly, the rare *V. hartii* with restricted range also showed a similar pattern of the generalistic approach in its mycorrhizal association. Thus, the rarity of *V. hartii* could be the result of aspects, like the edaphic factors (Phillips et al. 2011, 2014; Kaur et al. 2021), or the substrate-based variation in the pOMF community composition (Cook et al. 2022). The variation of pOMF communities between two root types possible to provide nutrient requirements for the host *Vanilla* to adapt to both terrestrial and epiphytic microhabitats. More germination experiments are needed to understand the functional role these fungi play in the germination and growth of host *Vanilla*.

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Author contributions JS, SW, AK, and PK designed the study. SW, JS, and AK conducted the fieldwork. SW carried out the lab work. SW and JK performed the bioinformatics analyses. SW carried out biostatistical analyses. SW and PK wrote the first draft of the manuscript. SW, JK, AK, and PK participated in reviewing and editing the manuscript. SW, PK, AK, and JS participated in revising the manuscript. All authors agreed to the final version of the manuscript.

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Data availability Raw sequences generated by Illumina MiSeq are available on GenBank through BioProject PRJNA772268.

Declarations

Accession numbers Raw sequences generated by Illumina MiSeq are available on GenBank through BioProject PRJNA772268.

Conflict of interest The authors declare that they have no conflict of interest.

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