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A rare temperate terrestrial orchid selects similar Tulasnella taxa in ex situ and in situ environments

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Abstract Mycorrhizal symbiosis in orchids is unique in that fungal presence is considered a requirement for germination as well as for further development. Additionally, orchid fungal associations can exhibit high specificity in nature. Yet, an important ecological question remains unanswered: 'With which orchid mycorrhizal fungi (OMF) do un-inoculated orchid seedlings form symbiosis when cultured ex situ?' Simultaneously, it is asserted that orchid conservation efforts involving ex situ plant culture should exclusively utilize natural symbionts of the respective orchid taxa. We present a first comparison of OMF communities within the roots of asymbiotically cultured plants of the rare orchid *Platanthera chapmanii* grown ex situ (ES), and those occurring naturally in situ (IS). Nuclear ribosomal internal transcribed spacer (nrITS) barcoding region was used to identify peloton forming OMF from roots collected between 2012 and 2014 from both growing environments. Our 114 sequences clustered into 11 operational taxonomic units (OTUs) belonging to four closely related clades of the fungal family Tulasnellaceae. Shannon–Wiener (H) and Simpson diversity (D) indices were similar ($p = 0.81$ for both) for ES and IS OMF communities. Beta diversity comparisons also showed similarity between ES and IS treatments based on weighted ($p = 0.10$) and unweighted ($p = 0.20$) Bray–Curtis dissimilarity matrices. Bayesian and Maximum Likelihood (ML) phylograms clustered ES and IS derived fungal OTUs into the same clades. Our data suggest that *P. chapmanii*: (1) forms symbiosis with taxonomically similar fungi in ex situ culture and in its native soil, and (2) exhibits a narrow phylogenetic breadth of mycorrhizal fungal OTUs within the Tulasnellaceae.

Introduction

Orchid mycorrhizal associations are different from other types of mycorrhizae in that mycorrhizal symbiosis is considered necessary for seed germination, and early plant development, in a majority of the 35,000 orchid species (Dressler 1981), whereas other mycorrhizal associations form after early plant development (Rasmussen 1995; Smith and Read 2008). Further, a majority of fungal species that form orchid mycorrhizal (OM) symbiosis belong exclusively to the

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phylum Basidiomycota with few belonging to Ascomycota (Dearnaley 2007; Dearnaley et al. 2012, 2016). Globally, a large majority of the reported orchid mycorrhizal fungi (OMF) belong to the basidiomycete families Tulasnellaceae, Sebacinaceae, and Ceratobasidiaceae (Shefferson et al. 2005; Dearnaley 2007). Their role in inducing germination (Rasmussen 1995) combined with the phylogenetic specificity (Taylor et al. 2002; McCormick et al. 2004; Shefferson et al. 2005, 2007; Pandey et al. 2013) essentially mandates considering OMF distribution, diversity, and specificity in understanding orchid ecology and in conservation applications.

It is generally known that carbon acquisition strategies of the host orchid (i.e., partial or full mycoheterotrophy), geographic range, and abundance influence their specificity towards OMF (McCormick et al. 2004, 2016) even though only limited generalizations can be made (Pandey et al. 2013; McCormick et al. 2016). Thus, knowledge of species-specific OMF partners is necessary to explain mycorrhizal ecology of the Orchidaceae, and for guiding ex situ and in situ conservation activities including orchid propagation, reintroduction, and restoration. For example, considerations for optimizing fungal compatibility in the orchid propagules can include: (1) introducing asymbiotically propagated seedlings directly into their natural habitats so they can acquire the preferred fungi on their own, (2) subjecting asymbiotically propagated seedlings to ex situ conditions for further growth before they are introduced into the wild habitats, and (3) first corroborating and isolating fungi that the orchid taxon prefers in its natural habitat and then utilizing the isolates in symbiotic propagation to generate symbiotic propagules. While conservation scientists transferring asymbiotically propagated plants directly in natural habitats assume that the suitable OMF partners are available at the recipient site, it is possible that this may not always be the case at sites where the plants have been extirpated. However, recent literature presents evidence for widespread distributions of OMF for both common (McCormick et al. 2016) and rare orchid taxa (Waud et al. 2017). Historically, symbiotic germination has often been considered superior to asymbiotic germination especially considering the sometimes faster germination and development of protocorms (Johnson et al. 2007), and it is also presumed to introduce the suitable fungal strains along with the propagules

(Zettler 1997; Stewart and Zettler 2002; Johnson et al. 2007). Further, it is feared that during ex situ culture of the asymbiotically grown seedlings, the propagules may form mycorrhizal associations with fungal strains that are not available in the natural habitat and that these 'foreign' strains could be introduced into natural habitats. Subsequently, while the orchid plants may succeed, the non-native mycorrhizal fungal strains might otherwise disturb the microbial community dynamics of the habitat. While these concerns are theoretically valid, empirical evidence does not exist to support them. On the contrary, symbiotically propagated plants are often first exposed to greenhouse environment prior to their relocation in situ and thus likely become mycorrhizal with additional fungi even if sterilized soil-less medium is utilized initially. Consequently, symbiotically germinated seedlings also have the potential for introducing non-native fungal strains in the native environment.

Clearly, there remains a large gap in our understanding of OMF ecology especially when orchids are cultured ex situ for conservation applications. It seems that to minimize the potential for introducing undesirable mycorrhizal fungi along with the orchid plants, the most important step that researchers can include is to compare the fungi that form mycorrhizae with the orchid taxon of interest ex situ and in situ to inform conservation decisions. To our knowledge, empirical comparisons of mycorrhizal fungal communities within orchid roots from un-manipulated ex situ and in situ environments are not available for any orchid taxa. A preliminary study by Richards and Sharma (2014) reported peloton forming fungi belonging to the Tulasnellaceae within the roots of asymbiotically germinated plants of *Platanthera chapmanii* that were cultivated in a peat-based substrate for > 1 year in a greenhouse. Considering that OMF can be saprophytic outside the orchid roots and can have wide geographic distributions (McCormick et al. 2004; Davis et al. 2015), it is possible that the organic components (peat, milled sphagnum, and fine fern fiber) of the substrates used for cultivating *Platanthera* species ex situ contain these saprophytic fungi. The same study by Richards and Sharma (2014) also reported Tulasnellaceae in the roots of naturally occurring plants. However, the phylogenetic relationships among OMF from plants cultivated ex situ after sterile in vitro germination and those that occur naturally in their wild habitats were not reported.

We used the temperate terrestrial orchid taxon *Platanthera chapmanii* to test the hypothesis that plants generated via asymbiotic germination and subsequent ex situ culture in peat-based growing medium will have a different suite of mycorrhizal fungi within their roots in comparison to plants of the same species occurring in situ in their native habitat. *Platanthera chapmanii* is a suitable model for testing the study questions because it responds relatively quickly to both in vitro and greenhouse culture conditions. To test our hypothesis, we quantified the influence of growing environment (i.e., ex situ cultivated plants that were never exposed to the natural habitat and those occurring in situ in their natural habitat) on root OMF community composition.

Materials and methods

Study species

Platanthera chapmanii is a temperate terrestrial orchid native to North America, where its few known, disjunct populations are restricted to southern Georgia, northern Florida, and southeastern Texas within the United States (Liggio and Liggio 1999; Poole et al. 2007; Richards and Sharma 2014). The rare perennial occurs in mesic and wet pine flatwoods, barrens, and savannas in sandy loam soils. The few known populations are often small with ≤ 10 individuals, and so far, only one population is known to host ≥ 100 plants (Richards and Sharma 2014). Individuals of *P. chapmanii* typically emerge aboveground between late February and early March, and flower from late July to early August when they produce a single raceme with ≥ 60 orange flowers. Flowering plants can range in height between 30 and 60 cm. Capsules dehisce from mid to late October when aboveground organs begin to senesce and the belowground organs enter dormancy. The species is recognized and protected as a 'rare' taxon in each of the three states. All plant material used in this study was obtained from the largest known population in Texas to prevent any potential negative impact on the remaining few and much smaller populations. The study population occurs in a longleaf pine savanna bog in southeast TX (georeferenced locations or site names of protected plant populations cannot be provided here),

where ≥ 100 flowering plants are observed each year within a 50 m^2 area.

Root collection and processing

Roots were collected between 2012 and 2014 from both the naturally occurring in situ (IS) plants and from ex situ (ES) cultured plants. For ex situ (ES treatment) plant culture, we used seeds from the same natural population (IS treatment) that was utilized for detecting variation in natural fungal diversity to avoid the artifact of ecotypic variation in dictating the mycorrhizal diversity associated with *P. chapmanii* in two disparate growing environments. Seeds were first surface sterilized and cultured in sterile culture in vitro as described in Richards and Sharma (2014). Once the plantlets had produced both shoots and roots, they were transplanted in a peat-based growing medium composed of peat moss (Premier, Quakertown, PA), builder's sand, and milled sphagnum moss (Richards and Sharma, 2014). We did not sterilize, nor add fungal inocula in, the growing medium. Plants were grown by following the culture methods that have been developed and used routinely for growing 'bog' orchids (Richards and Sharma 2014). Cultured plants are maintained in nursery greenhouse environment where they undergo normal growth and flowering, and are known to form fungal pelotons in their roots (Richards and Sharma 2014). Once ex situ cultured plants enter dormancy, they are exposed to vernalization conditions at 4 °C for 5 months prior to replanting in the peat-based medium in the following spring for another growth cycle. Each growing environment (i.e., IS and ES) was represented by three root sampling events (IS1, IS2, IS3; ES1, ES2, and ES3) during their reproductive stage over 3 years to obtain replicate data for both IS and ES treatments.

To compare root-associated OMF communities of the rare *P. chapmanii* in the two disparate growing environments, we sampled roots from 36 plants, of which 15 represented the IS treatment while 21 represented the ES environment. Up to 30 cm root tissue from each of the haphazardly selected plants was collected by using non-destructive sampling methods. The root system of each plant to be sampled was first gently exposed by removing the soil around the base of each plant growing either in the native bog habitat (IS) or in containers (ES). Roots were severed and stored at 4 °C immediately after collection.

Within 48 h of collection, roots were washed free of soil and other debris and examined for the presence of pelotons. Peloton containing roots were surface sterilized by exposing the tissues to: (1) a 30 s rinse in 70% ethanol, (2) a 30 s rinse in 0.6% sodium hypochlorite, and (3) a 30 s rinse in 70% ethanol. The root pieces were then washed with sterile ultrapure water until they were free of the residues of ethanol and sodium hypochlorite. After surface sterilization, the epidermis was removed before the roots were divided into smaller ($\sim$ 3 cm) pieces for further processing. Finally, 178 individual peloton containing root segments (93 from IS, and 85 from ES) were finely minced and stored at -80 °C until DNA was extracted. We also isolated and cultured peloton forming fungi from a few roots collected from the native habitat (IS). The root fragments that were used to culture fungi on nutrient medium were surface sterilized and processed similarly as described above, except instead of ultralow freezing the finely macerated tissue was suspended in molten potato dextrose agar (PDA) contained within 14 cm petri plates. The plates were examined every 1 to 3 days for growth of fungi with characteristics (e.g., moniliod cells) of orchid mycorrhizal fungi. Hyphal tips were harvested from actively growing fungi and cultured in individual plates until 10 pure cultures were retained for further processing.

DNA extraction, amplification, and sequencing

Nuclear DNA was extracted from each minced root sample by using the DNeasy Plant Mini Kit (Qiagen, Germany) and protocol with a few modifications. Immediately prior to lysing the tissues, samples were freeze-dried with liquid nitrogen. Lysing was carried out for 3 min with 30 disruptions/s. Subsequently, 400 μl of lysis buffer with 3.3% polyvinylpyrrolidone (PVP) was added to each sample. Finally, the incubation time during the cell wall disruption step was increased to 2 h at 65 °C during which each sample was shaken every 30 min. Simultaneously, fungal mycelium from pure cultures was examined and presence of moniliod cells was confirmed prior to subjecting each fungus to direct polymerase chain reaction (PCR).

Fungal nuclear ribosomal internal transcribed spacer region (nrITS) region was amplified in each sample by using ITS1-OF/ITS4-OF or ITS1/ITS4-Tul

primer pairs (Taylor and McCormick 2008; Sigma-Aldrich, Missouri, USA) by using previously published methods (Pandey et al. 2013). To verify the size of the amplicons, electrophoresis was carried out in a 2% agarose gel. Samples that showed a clear, single band between 600 and 800 bp were further cleaned using DNA Clean and Concentrator 5 kit (Zymo Research, Irvine, USA). Samples that showed multiple bands or bands that were wide and/or unclear underwent a gel extraction protocol by using the Genelute gel extraction kit (Sigma-Aldrich, St. Louis, Missouri). Sequencing reactions were prepared by using the cleaned amplicons, and were sent for sequencing at the DNA Analysis Facility on Science Hill at Yale University (New Haven, CT).

Data analyses

We generated 117 (56 from ES, and 61 from IS) raw useable sequences from 178 root fragments. Editing and assembly of 117 raw sequences was performed with CodonCode Aligner version 6.0.2 (CodonCode Corporation, Centerville, Massachusetts). Sequences were trimmed at both ends by removing 25 bp sections that had $\geq$ 3 bases with phred scores below 20. Any trimmed sequence that was shorter than 400 bp was not included any further. After quality filtering, 114 sequences were retained for analyses. Taxonomy was assigned to retained sequences at family level by using BLAST (NCBI Genbank, http://www.ncbi.nlm.nih.gov/genbank/). We then extracted the homologous regions from all sequences by aligning in T-Coffee version 11.00 (Notredame et al. 2000) with M-Coffee mode. Subsequently, Operational Taxonomic Unit (OTU) clustering was performed with Cd-hit (Li and Godzik 2006) at 97% similarity threshold which is the most used and recommended value for orchid mycorrhizal communities (Nguyen et al. 2015). The longest sequence in each OTU was used as its representative sequence. To estimate whether OTU diversity had been saturated during sampling, species accumulation curves were generated individually for ES and IS growing environments, and for *P. chapmanii* as a whole in R version 3.2.3 (RStudio Team 2015) by using all OTUs.

To compare the alpha diversity of OMF OTUs between the two growing environments (ES and IS), Shannon–Wiener (H) and Simpson (D) diversity indices were first calculated for each of the six

sampling events (ES1, ES2, ES3, and IS1, IS2, IS3) in R using the phyloseq package (McMurdie and Holmes 2013). These two indices account for both species richness and abundance. The diversity values were then subjected to the Kruskal–Wallis test to compare the alpha diversity between the two growing environments (ES and IS) using the stats package in R. Next, beta diversity was estimated for the six sampling events by generating the 6×6 unweighted richness-based and weighted abundance-based Bray–Curtis dissimilarity matrices. Prior to generating the beta diversity estimates, the OTU counts were normalized by using Hellinger transformation in the vegan package in R (Oksanen et al. 2007). To compare the community structures of ES and IS, a Permutational Multivariate Analysis of Variance (PERMANOVA) was performed separately on the richness-based and abundance-based dissimilarity matrices using the vegan package in R. Finally, the same two dissimilarity matrices were also used for average hierarchical clustering with the stats package in R to visualize the clustering of ES and IS replicates.

To examine the placement of Tulasnellaceae OTUs from *P. chapmanii* among previously known orchid mycorrhizal fungal sequences obtained from NCBI GenBank (http://www.ncbi.nlm.nih.gov/genbank/), we generated both Maximum likelihood (ML) and Bayesian phylograms. Thirty-five (35) sequences including the 11 Tulasnellaceae OTUs and 24 reference GenBank derived sequences were first aligned using the program T-coffee. Maximum likelihood trees were generated with RAxML-HPC2 version 7.4.2 (Stamatakis 2006), and the best tree from 1000 random parsimonious trees were assigned clade support values based on 1000 bootstrap replicates. Bayesian trees were constructed by using Kimura 2-parameter substitution model with gamma distribution, which was deemed as the best DNA substitution model in MEGA7 (Tamura et al. 2013), using MrBayes version 3.2.6 (Ronquist et al. 2012) with > 1 million generations until the standard deviation of split frequencies between two runs was < 0.01. Trees from the initial 25% generations were discarded before generating the consensus tree. Both ML and Bayesian trees were midpoint rooted due to a lack of a defensible related outgroup for Tulasnellaceae, and both consensus trees were visualized using FigTree version 1.4.2 (Rambaut 2007).

We also conducted sequence divergence analyses first with the individual 114 Tulasnellaceae sequences recovered from *P. chapmanii* in this study to compare the two growing environments, and then to compare sequence divergence within *P. chapmanii* sequences recovered from IS with the means generated for other terrestrial orchid taxa. Sequences were first aligned and then subjected to distance analyses by selecting 'pairwise deletion' of gaps using Kimura 2-parameter (K2P) DNA substitution model and gamma distribution of rate of substitution at each site in MEGA7. Mean pairwise K2P sequence distances between two growing environments were compared with PERMANOVA. Finally, to compare the phylogenetic breadth of Tulasnellaceae sequences detected in *P. chapmanii* with those of other terrestrial species, we generated mean pairwise sequence distances within reference orchid taxa by using publicly available sequences reported in previously published literature. Phylogenetic breath of *P. chapmanii* and reference orchid taxa was compared with Kruskal–Wallis test.

Results

We identified 11 fungal OTUs, which belonged to the fungal family Tulasnellaceae, from the roots of *P. chapmanii* across the ES and IS treatments (Table 1). Two (T4 and T9) of the 11 OTUs were singletons and six (T2, T3, T7, T8, T10, and T11) were observed in the roots of multiple individuals (Table 1). Two Tulasnellaceae OTUs (T2 and T7) were shared across in situ and ex situ environments, while five (T5, T6, T8, T9, and T11) were exclusive to the ex situ environment and four (T1, T3, T4 and T10) were exclusive to the in situ environment. Species accumulation curves revealed sufficient sampling effort for the orchid host species and for both growing environments (Online Resource 1). The estimated OTU richness was similar to the observed OTU richness for each sampling event except IS3, which showed a potential 0.5 increase over the observed value of 6 with increased sampling.

We did not detect differences in either Shannon–Wiener or Simpson diversity indices ($p = 0.81$ for both indices) between ex situ ($H = 0.53$, $D = 0.29$) and in situ ($H = 0.44$, $D = 0.22$) environments. Beta diversity comparisons with PERMANOVA also did not show differences in either OTU richness

Table 1 Number of sequences representing 11 nuclear ribosomal internal transcribed spacer (nrITS) based fungal operational taxonomic units (OTUs) identified from the roots of ex situ cultured (ES1, ES2, ES3), and in situ occurring (IS1, IS2, IS3) plants of *Platanthera chapmanii*. Each plant root source was sampled three times between 2012 and 2014. Values in parentheses are the number of plants in which a specific OTU was documented. Mean Shannon–Wiener (*H*) and Simpson (*D*) diversity indices based on OTU frequencies were estimated using replicated sampling events representing each growing environment. Estimates of OTU alpha diversity in ES and IS environments were similar with both *H* ($p = 0.81$) and *D* ($p = 0.81$)

| OMF source	Ex situ (ES)			In situ (IS)		
	ES1	ES2	ES3	IS1	IS2	IS3
OTU						
T1						2 (1)
T2		1 (1)	13 (8)			7 (3)
T3					15 (5)	18 (5)
T4						1 (1)
T5	8 (1)					
T6		3 (1)				
T7			3 (2)			1 (1)
T8		21 (4)				
T9			1 (1)			
T10				6 (1)		8 (2)
T11		6 (2)				
Mean *H* (*n* = 3)	0.53			0.44		
Mean *D* (*n* = 3)	0.29			0.22		

($p = 0.20$) or OTU abundance ($p = 0.10$) between ES and IS growing environments. Similarly, average hierarchical clustering did not reveal clades specific to any growing environment (Figs. 1a, b).

Both maximum likelihood (ML) and Bayesian trees showed similar relationships among the Tulasnellaceae OTUs representing the two growing environments (Fig. 2). The 11 Tulasnellaceae OTUs representing both ES and IS environments grouped in four clades along with reference sequences obtained from GenBank. Clade 1 included three ES OTUs (T6, T8, and T11), two IS OTUs (T3 and T4), and one OTU (T2) which was shared by ES and IS environments. This clade included reference fungal species *Tulasnella bifrons* along with an unknown *Tulasnella* species isolated from roots of the North American terrestrial orchid *Tipularia discolor*. Clade 2 consisted of an ES specific OTU (T9), and an OTU (T7) shared by ES and IS; this clade grouped with *Tulasnella calospora*, an uncultured Tulasnellaceae, and two unknown *Tulasnella* species from fungal cultures of unknown geographic origin and from roots of

Anoectochilus formosanus from China (Online Resource 2). A third clade (Clade 3) was formed by two OTUs (T1 and T5) from IS and ES, respectively, and associated with *T. calospora* isolated from the orchid host *Eulophia epidendraea* in India. Clade 4 diverged from the other three and consisted of a single OTU (T10) representing IS. All four clades containing the OMF OTUs from *P. chapmanii* clearly segregated from several *Tulasnella* originating from orchid hosts from multiple continents including North America, South America, Europe, Africa, Asia, and Australia (Fig. 2, Online Resource 2).

Mean pairwise sequence distance among the 114 *P. chapmanii* Tulasnellaceae sequences was 0.195. The absolute values for the same metric in ES plants was lower (0.082) than IS individuals (0.268). Regardless, pairwise comparisons of genetic distances with PERMANOVA did not reveal genetic divergence between OTUs associated with ES and IS ($p = 0.40$).

When the mean pairwise OMF sequence distance for *P. chapmanii* was compared to means in other orchid taxa, the Tulasnellaceae sequences from *P.*
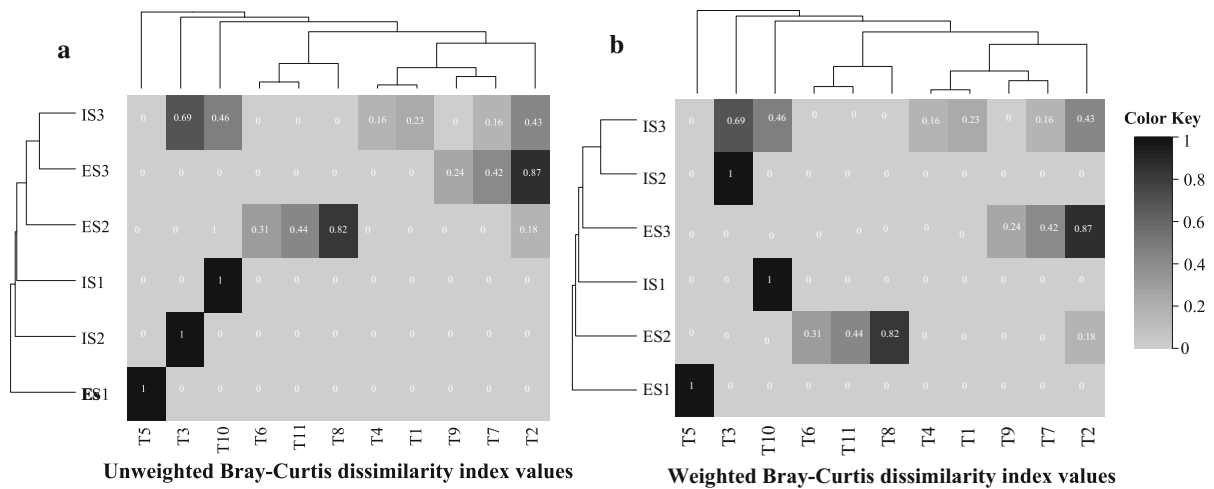
Fig. 1 Two-way hierarchical clustering of replicate sampling events based on 11 Operational Taxonomic Units (OTUs T1 to T11) identified from the nuclear ribosomal internal transcribed spacer (nrITS) sequences amplified from the roots of ex situ cultured (ES1, ES2, and ES3), and in situ occurring plants (IS1, IS2, and IS3) of *Platanthera chapmanii*. Bray–Curtis dissimilarity index was used to generate pairwise distances: **a** a dendrogram based on OTU richness dissimilarities among the six sampling events, and **b** a dendrogram based on OTU abundance dissimilarities among the same six sampling events. The grayscale shade bar provides the Bray–Curtis dissimilarity values

chapmanii were more diverse (0.268) than some widely distributed temperate terrestrial orchids (e.g., *Cypripedium japonicum* and *Cypripedium candidum*), and less diverse than others (e.g., *Anacampis laxiflora,* and *Ophrys fuciflora*) (Table 2). Conversely, *Platanthera (Piperia) yadonii*, an orchid species with a narrowly restricted geographic range showed wider phylogenetic breadth of Tulasnellaceae (0.383) in comparison to *P. chapmanii*. However, Kruskal–Wallis did not detect differences in mean sequence divergences representing the various orchid taxa ($p = 0.44$).

Discussion

Mycorrhizal specificity is known to be variable within the Orchidaceae, and while several orchid mycorrhizal fungi are free-living saprophytes with cosmopolitan distributions, orchid-fungus partnerships can be highly specific (Selosse et al. 2002; McCormick et al. 2004; Shefferson et al. 2005, 2007; Nomura et al. 2013) or general (Pandey et al. 2013; Bonnardeaux et al. 2014) depending on the life history, geographic distribution, or the adaptive traits of an orchid taxon (McKendrick et al. 2002; Otero et al. 2004). Considering that there are approximately 35,000 taxa distributed across the

planet, broad generalizations are difficult to make and are likely inappropriate until a majority of the taxa are studied individually and extensively. Further, a majority of the orchid species is considered rare in nature and is often the target of conservation efforts including augmentation and restoration of natural populations. Because orchid mycorrhizal fungi are necessary for germination and development of orchid individuals, consideration of fungal specificity, and maintaining the integrity of local genotypes is considered important.

Our results show that *Platanthera chapmanii* plants cultured ex situ select similar fungi as in situ, and both groups are narrowly specific toward fungi from Tulasnellaceae lineages. Of the four clades formed by the 11 *P. chapmanii* OMF OTUs, the one containing six (Clade 1) associated most closely with *Tulasnella bifrons* (AY373290) and a *Tulasnella* species (AY373299) isolated from the roots of a North American orchid *Tipularia discolor* (Fig. 2, Online Resource 2). Both ex situ (ES) and in situ (IS) growing environments were represented in this clade (Fig. 2). The remaining five *P. chapmanii* OMF OTUs were distributed across three clades, two of which (Clades 2 and 3) included both ES and IS derived OTUs and grouped with *Tulasnella calospora* from two sources including *Paphiopedilum charlesworthii*
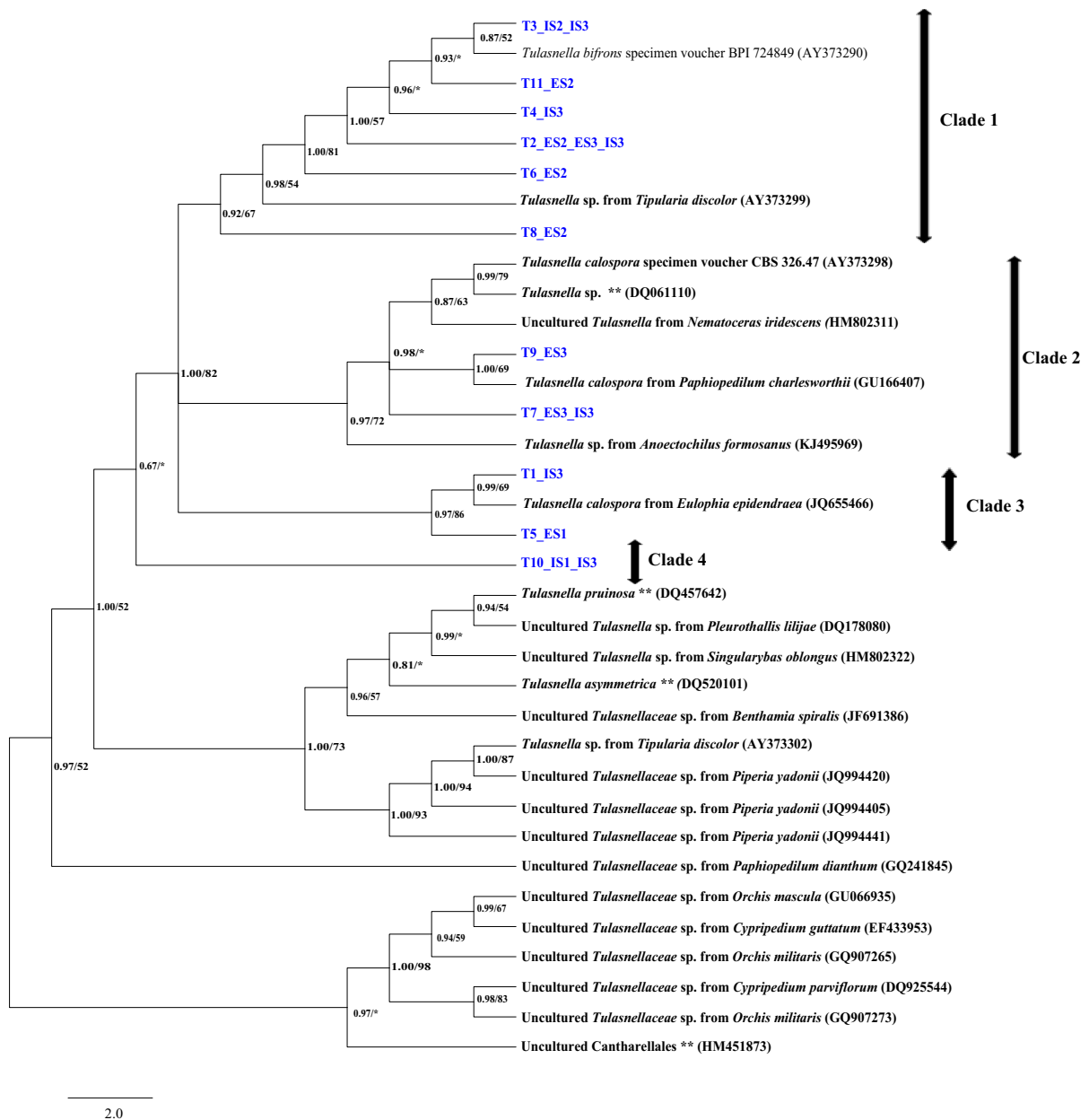
Fig. 2 A combined midpoint rooted Bayesian and maximum likelihood (ML) tree of the fungal family Tulasnellaceae built with orchid mycorrhizal fungal (OMF) Operational Taxonomic Units (OTUs; T1-T11) identified from nuclear ribosomal internal transcribed spacer (nrITS) sequences generated from roots of *Platanthera chapmanii* representing ex situ (ES1, ES2, and ES3) and in situ (IS1, IS2, and IS3) growing environments (blue font). The first value among branch support values represents the Bayesian clade support, and the second value represents the ML bootstrap value. Weak branch support values (≤ 0.5 for Bayesian and ≤ 50 for ML) were replaced with *. Scale bar represents estimated number of nucleotide substitutions. The reference sequences from unknown sources are represented by **. Please see Online Resource 3 for additional details on the reference GenBank sequences

from Thailand, an uncultured Tulasnellaceae from New Zealand, and unknown *Tulasnella* species from fungal cultures of unknown geographic origin and

from roots of *Anoectochilus formosanus* from China. The remaining clade that included a single *P. chapmanii* OMF OTU (T10; Clade 4) diverged from all

Table 2 Mean pairwise genetic distance within nuclear ribosomal internal transcribed spacer (nrITS) region of Tulasnellaceae sequences of in situ sampled *Platanthera chapmanii* individuals in comparison to those obtained from other orchid species. Sequence distances were calculated after the pairwise deletion of gaps based on Kimura 2-parameter model with gamma distribution of rates of variation among sites; n gives the number of sequences. Standard Error (SE) was obtained after 1000 bootstraps. Natural distribution ranges of each orchid taxon were classified as either wide, moderate, or restricted based on the geographic area across which its populations are distributed. The original source of sequences is listed for each taxon

Orchid Species	Tulasnellaceae		Geographic range	References
	n	Distance $\pm$ SE		
Platanthera chapmanii (In situ)	58	0.268 $\pm$ 0.031	Wide	This study
Anacampis laxiflora	12	0.517 $\pm$ 0.068	Wide	Girlanda et al. (2011)
Chiloglottis trapeziformis	12	0.010 $\pm$ 0.002	Wide	Roche et al. (2010)
Cypripedium candidum	7	0.000 $\pm$ 0.002	Wide	Shefferson et al. (2007)
Cypripedium japonicum	18	0.045 $\pm$ 0.007	Wide	Shefferson et al. (2007)
Ophrys fuciflora	12	0.641 $\pm$ 0.087	Wide	Girlanda et al. (2011)
Orchis purpurea	9	0.114 $\pm$ 0.012	Wide	Girlanda et al. (2011)
Serapias vomeracea	27	0.220 $\pm$ 0.025	Wide	Girlanda et al. (2011)
Cypripedium calceolus	7	0.298 $\pm$ 0.027	Moderate	Shefferson et al. (2007)
Cypripedium fasciculatum	20	0.212 $\pm$ 0.054	Moderate	Shefferson et al. (2007)
Chiloglottis aff. jeanesii	14	0.013 $\pm$ 0.002	Restricted	Roche et al. (2010)
Platanthera (Piperia) *yadonii*	63	0.383 $\pm$ 0.031	Restricted	Pandey et al. (2013)

other fungi, and exclusively represented IS growing environment. Altogether, the four clades containing the OMF OTUs from *P. chapmanii* formed a narrow grouping that clearly separated from several orchid derived *Tulasnella* from multiple continents (Fig. 2, Online Resource 2). While encountering narrow phylogenetic breadth among the OMF is not unusual (Selosse et al. 2002; McCormick et al. 2004; Shefferson et al. 2005, 2007; Nomura et al. 2013), it is notable that *P. chapmanii* is able to auto-select the same fungi in a peat-based substrate in ex situ culture conditions as it does in its native habitat. Both alpha and beta diversities (Fig. 1) of OMF communities were similar in the roots of *P. chapmanii* collected from ES and IS environments. Phylogenetic analysis further supported the results that the OMF communities from the two growing environments (Fig. 2) did not segregate and grouped narrowly when placed among the previously known representatives of Tulasnellaceae. The lack of genetic separation of OTUs from ES and IS plants coupled with the overlap of OTUs across the two treatments underpin the specificity of *P. chapmanii* toward its preferred OMF regardless of the growing substrate and environment. It is, however, possible that while the OMF may be

cosmopolitan, the Texas ecotypes of the orchid specifically seek the recovered OTUs of Tulasnellaceae. Studies including additional populations to represent additional provenances of *P. chapmanii* could expand further our knowledge of whether the phylogenetic breadth of OMF associated with the orchid increases with samples that represent the entire geographic distribution of *P. chapmanii*.

Our study provides the first evidence, to our knowledge, that orchid mycorrhizal fungal communities are similar in roots of plants cultured asymbiotically in vitro and then subsequently grown in ex situ conditions in peat-based substrate, and in roots of plants occurring naturally in their native habitat. Surely, similar studies are warranted for additional orchid taxa by also considering their geographic ranges to account for ecotypic variation in OMF alliances. It is conceivable that the strong overlap of ex situ and in situ OMF communities observed in this study varies across orchid taxa and further across their geographic ranges. In the meantime, ex situ culture of *P. chapmanii* for population augmentation and other conservation activities is unlikely to threaten the OMF communities at its native site. Moreover, utilization of seeds from the site that eventually becomes the

recipient site for the ex situ cultured plants should be the preferred method until a range-wide study of the OMF in roots of *P. chapmanii* can be conducted. Asymbiotically propagated seedlings exposed to ex situ culture conditions appear to represent a more natural mycorrhizal colonization considering that the orchid roots likely select the preferred OMF from a larger community of fungi in the growing substrate. We suggest that this biological acclimation might also have implications for the survival of plants first ex situ and then in situ. And while symbiotic propagation by using single fungal isolates in vitro holds merit in cases when orchid seeds respond more favorably to symbiotic culture in comparison to asymbiotic culture (Johnson et al. 2007), their OMF communities before and after translocation in situ and their performance in the native habitats is not yet known.

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