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High specificity of a rare terrestrial orchid toward a rare fungus within the North American tallgrass prairie

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ABSTRACT

The Orchidaceae are globally distributed and represent a diverse lineage of obligate mycotrophic plants. Given their dependence on symbiotic fungi for germination and/or plant development, fungal community structure in substrates is expected to influence the distribution and persistence of orchid species. Yet, simultaneous characterization of orchid mycorrhizal fungal (OMF) communities in roots and in soil is rarely reported. To explain the co-distributions of OMF in roots, orchid-occupied, and bulk soil, we characterized mycorrhizal fungi associated with *Platanthera praeclara* over multiple years across its entire natural distribution within the North American tallgrass prairie. Root derived OMF communities included 24 Ceratobasidiaceae and 7 Tulasnellaceae operational taxonomic units (OTUs) though the orchid exhibited high spatio-temporal specificity toward a single Ceratobasidiaceae OTU, which was strongly stable across population sizes and phenological stages of the sampled individuals. The preferred OMF OTUs were primarily restricted to orchid-occupied locations while infrequent or absent in bulk soil. Variation in soil OMF assemblies was explained most by soil moisture, magnesium, manganese, and clay. In this first study of coupled root and soil OMF communities across a threatened grassland ecosystem, we report a strong relationship, further nuanced by soil chemistry, between a rare fungus and a rare orchid.

1. Introduction

Orchids exemplify diversity in specialized biological interactions including mycorrhizal associations, and this diversity of interactions is also implicated in the taxonomic diversity within the Orchidaceae. Simultaneously, high taxonomic specificity is documented toward orchid mycorrhizal fungi (OMF), where most of the fungal associates belong to Ceratobasidiaceae and Tulasnellaceae along with Sebacinales within the Basidiomycota (Dearnaley et al., 2012; Weiß et al., 2016; Jacquemyn et al., 2017). Other basidiomycete fungi including Atheliaceae, Auriculariaceae, Cantharellaceae, Cortinariaceae, and Thelephoraceae have also been documented as OMF, though not as frequently. Some OMF are also known from Atractiellomycetes and even outside of Basidiomycota (e.g. Ascomycota, Dearnaley et al., 2012). Irregular distributions of OMF in the substrate, combined with mycorrhizal strategies ranging from generalist to highly specific could explain the high species diversity within the Orchidaceae (Pandey et al., 2013; Mccormick and Jacquemyn, 2014; Merckx, 2013), and perhaps also the distribution patterns of plants (Jacquemyn et al., 2017). However, the nature of biotic and abiotic predictors that can explain the distribution of orchid individuals and populations is generally not understood, especially with respect to OMF (Jacquemyn et al., 2017). Simultaneously, orchid decline is global, and successful conservation strategies may benefit from a better understanding of drivers of orchid distribution (Swarts and Dixon, 2009; Djordjević et al., 2016; Jacquemyn et al., 2017).

Patchy distribution of individuals and populations is common within the Orchidaceae (Dressler, 1993). Species that have wide geographic ranges and seemingly occupy a similar habitat aboveground also exhibit localized and disjunct distribution of individuals across the landscape. Whether this pattern is linked to microhabitat conditions including the local distribution of preferred OMF in suitable soil substrates has not been well-studied. While it has been suggested that orchid distribution may be limited by the distribution and abundance of appropriate soil OMF (Mccormick et al., 2009; Jacquemyn et al., 2017), the independence between soil OMF and orchid distribution has also been reported (Bonnardeaux et al., 2007; McCormick et al., 2016; Voyron et al.,

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2017). It is possible that the OMF have an extremely patchy and heterogeneous occurrence in soil, or that a high abundance of a preferred fungal partner is not a prerequisite for seed germination or persistence of an adult orchid individual. Also, despite the global distribution of OMF families, local scale availability of preferred strains within the large OMF families might still constrain endemic orchid recruitment and distribution, especially when coupled with extreme specificity in the orchid-fungus partnerships (Mccormick and Jacquemyn, 2014; Waud et al., 2016). Besides, in-situ orchid seed germination studies have observed the lack of germination despite the high abundance of OMF in soil, suggesting that germination might be limited by ambient microhabitat (Diez, 2007; Swarts et al., 2010; McCormick et al., 2016). However, few studies have reported the influence of macro or micro-scale physical environment on root and/or soil OMF or orchid distributions (McCormick et al., 2006, 2012; Bunch et al., 2013; Pandey et al., 2013; Mujica et al., 2016; Geml, 2017).

Along with spatial variability, temporal variability (inter-annual variation) in root and soil OMF communities is likely to occur but has not been documented for any habitat (Jacquemyn et al., 2017). It is conceivable that the OMF communities within the roots of orchids and in rhizosphere soil show coupled changes across time in response to the rhizosphere and/or aboveground conditions. Moreover, temporal shifts in OMF communities could potentially be exaggerated in ecosystems that experience periodic disturbances such as fire. In fact, soil fungal communities including, but not limited to, mycorrhizae are known to fluctuate in response to vegetation management such as prescribed fires and grazing (Lauber et al., 2008; Vályi et al., 2015; Egidi et al., 2016; Dove and Hart, 2017; Kyaschenko et al., 2017). And while the edaphic characteristics can also change in response to ecosystem management practices, it is not clear if, or how, they may affect biogeographical patterns in soil microbial or mycorrhizal communities (Lauber et al., 2008; Oberwinkler et al., 2017). Regardless, diversity patterns of OMF in most habitats, including the grasslands of North America, are not known despite the uniqueness, rarity, and high biodiversity of many such ecosystems. The iconic North American tallgrass prairie is one of the most threatened natural ecosystems on Earth while hosting at least 20 orchid taxa along with >500 other plant species. Historically, it is a fire and grazing-driven grassland ecosystem where both the frequency of fire events and persistent grazing by large herbivores impact nitrogen dynamics and overall ecosystem structure and function (Collins et al., 1998) including communities of arbuscular mycorrhizal fungi (Hiiesalu et al., 2014; Jumpponen and Jones, 2014; House and Bever, 2018). Among the several rare orchids in the tallgrass prairie, *Platanthera praeclara* Sheviak and Bowles is a large-flowered and notable species occurring in mesic calcareous prairie swales with sandy soil and a high water table. The taxon is considered an indicator of undisturbed tallgrass prairie habitat and is one of the focal taxa for designing management programs at the prairie preserves where it occurs (USFWS, 1996; USFWS, 2009) in at least five midwestern states in the United States and the province of Manitoba in Canada (Fig. 1A). With respect to their fungal partners, photosynthetic orchids native to grasslands can be generalists or specialists (Jacquemyn et al., 2010; Oja et al., 2015) but are primarily associated with the same Agaricomycete families as most other photosynthetic orchids from both temperate and tropical ecosystems (Dearnaley et al., 2012). However, whether orchid distributions are linked to the structure of OMF communities in soil via their root OMF communities in North American grasslands remains unknown. Additionally, correspondence between the ambient rhizosphere and aboveground conditions and OMF distribution in soil is not established even though both environments are expected to influence soil microbial communities.

Accordingly, we hypothesized that the discontinuous and limited distribution of a rare orchid across the tallgrass prairie ecosystem is a result of its high specificity toward OMF taxa that show narrow phylogenetic breadth and are distributed patchily across a seemingly homogeneous habitat. To test this hypothesis, we documented the variation in the root and soil (both, orchid-occupied and bulk) OMF community structure in response to geolocation, population size, edaphic characters, and phenology of individuals across three years. And, since each sampled population experiences some form of vegetation management, we availed the opportunity to gain a first, albeit introductory, insight into the effects of vegetation management on OMF community structure by utilizing an ongoing long-term vegetation management experiment at one large population in Minnesota. We chose the emblematic tallgrass prairie orchid, *P. praeclara*, to test our hypothesis given the coupling of its rarity and a wide geographic distribution of both the orchid and its habitat.

2. Materials and methods

2.1. Study species, sites, and root collection

P. praeclara is an herbaceous, obligate outcrossing, perennial terrestrial orchid endemic to the North American tallgrass prairie. The species is rare and listed Threatened by the U.S. Fish and Wildlife Service under the Endangered Species Act of 1973 in the United States and as Endangered in Canada (USFWS, 2009; Environment Canada, 2006). Disjunct populations of *P. praeclara* occur in six mid-western states in the United States (Kansas, Missouri, Iowa, Nebraska, Minnesota, and North Dakota) and in the province of Manitoba, Canada (USFWS, 1996, Fig. 1A). Like all other terrestrial orchids, seed germination and seedling development in *P. praeclara* cannot progress without mycorrhizal fungi (Sharma et al., 2003b). Although mycorrhizal associations in *P. praeclara* have been studied by using culture-based methods to identify both Ceratobasidiaceae and Tulasnellaceae in its roots, the geographic representation was generally low, ranging from one to five populations (Zelmer and Currah, 1995; Zelmer et al., 1996; Sharma et al., 2003a; Genbank accession# DQ068771, DQ068772 and DQ068773).

For the current range-wide, multi-year study, we sampled roots from individuals from eight populations (Fig. 1A; Table 1) representing the entire natural distribution of *P. praeclara* in 2013 and again in 2014. The largest geographic distance between any two populations (IA and MB) was 838 km whereas MNB1 and MNB2 were the closest with 3 km between them. The tallgrass prairie habitat that host the species are typically dominated by *Andropogon gerardii* (bigbluestem), *A. scoparius* (littlebluestem), and *Sorghastrum nutans* (Indiangrass) while *Deschampsia caespitosa* (tufftedhairgrass) and *Panicum virgatum* (switchgrass) are the common associates in wetter sites. Historically, the tallgrass prairie is a fire-dependent ecosystem, which keeps the woody shrub cover low. Specifically, *P. praeclara* generally occurs within the wetter facies of such prairies or in the associated sedge meadows (USFWS, 1996). Between mid-June and mid-July in both 2013 and 2014, roots were sampled from at least two seedlings, two vegetative, and two reproductive individuals at each population except MNP where each of the vegetation management treatments was sampled individually. Collectively, 148 individual plants were sampled. Up to six individual roots were severed from each sampled reproductive and vegetative individual without compromising the whole plant. Seedlings were collected whole because of the extremely reduced size of their root systems, which often included a single root of $\leq$1 cm. Roots were stored at 4 °C until they were shipped overnight to the laboratory at Texas Tech University.

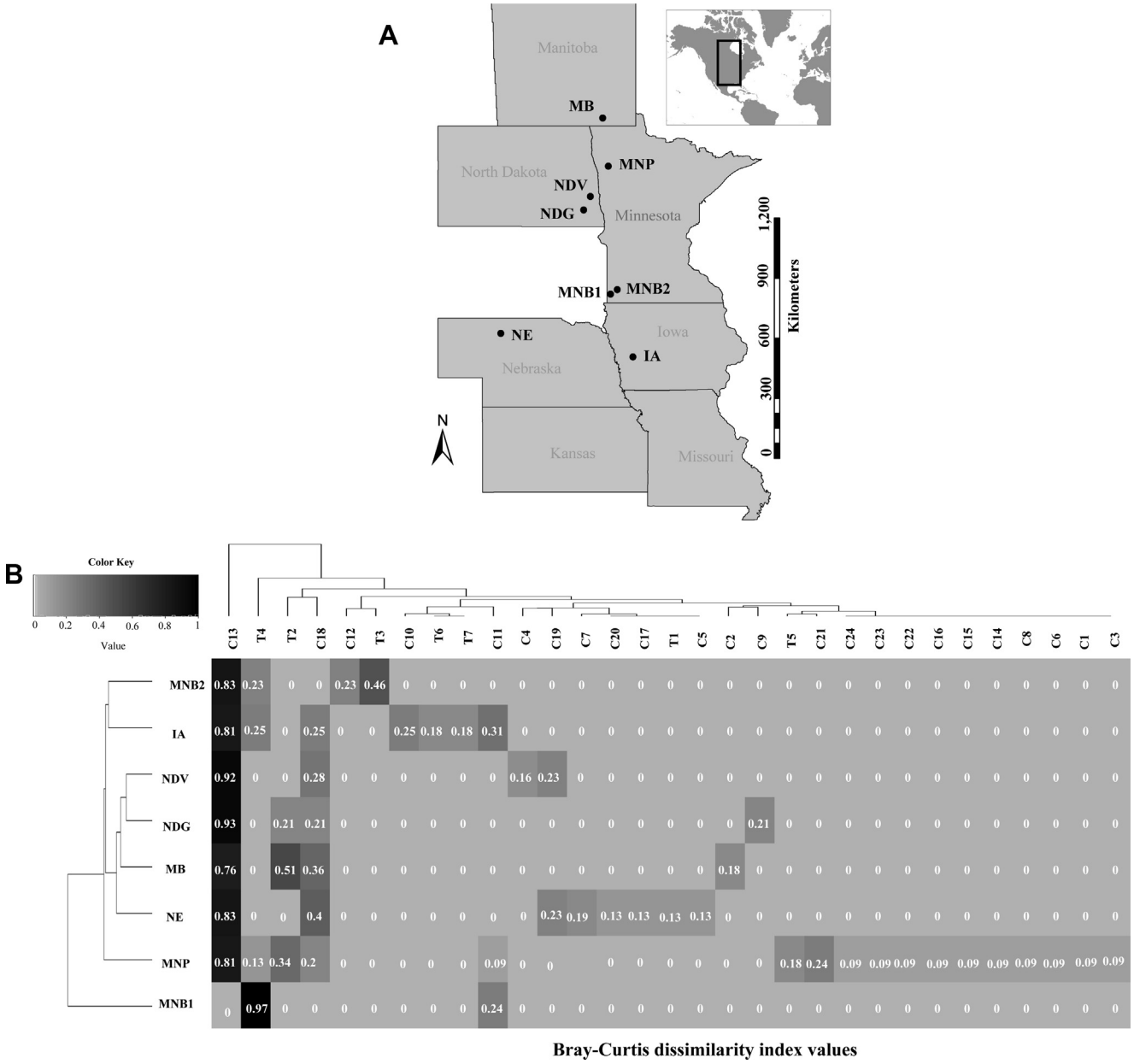

Fig. 1. Characterization of orchid mycorrhizal fungal (OMF) communities within roots of *Platanthera praeclara* by using operational taxonomic units (OTUs) identified from 286 Ceratobasidiaceae and 55 Tulasnellaceae internal transcribed spacer (ITS) locus Sanger sequences. (**A)** A partial map of the US and Canada showing the locations of study populations in Iowa (IA), Nebraska (NE), Minnesota (MNB1, MNB2 and MNP), North Dakota (NDG and NDV), and Manitoba (MB) where roots were sampled in 2013 and 2014. (**B)** Two-way hierarchical clustering of *P. praeclara* populations with weighted Bray–Curtis dissimilarity index values; OTUs identified from individual plants were pooled for each population before clustering.

2.2. Soil fungi and edaphic characters

To compare the OMF diversity in soil at locations where orchid plants occurred and those where the plants were absent, we collected approximately 30 g soil in 2013, 2014, and 2015 across eight populations. For each population, soil was sampled from, 1) the center of the area where the plant density was highest (orchid-occupied), and 2) a location approximately 100 m from the orchid patch where plants of the target species neither occur nor have historically been documented (bulk soil). Three individual cores to a depth of 15 cm (root depth) were collected, cleaned of large roots, homogenized, and frozen until DNA was extracted. Our design yielded a pair of aggregate samples to represent orchid-occupied soil and bulk soil (orchid absence) in each year at each population except MNP (Table S1). At MNP, orchid-occupied soil was represented by one aggregate sample from each of the four vegetation management experimental treatments (MNP_C, MNP_F, MNP_S, and MNP_H) while bulk soil was represented by a single aggregate sample. In year 2013, eight additional soil cores were collected from seedling rhizosphere of four populations (IA, NE, MNB2, MB) and four vegetation management treatments at MNP (MNP_F, MNP_H, MNP_S and MNP_C) that represented orchid-occupied locations afterwards. Altogether, 65 soil samples were collected, of which 41 represented orchid-occupied soil and 24 represented bulk soil (Table S1). At each of these locations, we also collected up to 500 g of soil by aggregating three individual samples for physicochemical characterization. Organic matter (OM), pH, cation exchange capacity (CEC), concentration of macronutrients (NO_3–N, P, K, Mg, Ca, S), micronutrients

Table 1
Description of *Platanthera praeclara* populations sampled for plant roots in 2013 and 2014. The grouping of populations into northern (N) and southern (S) latitude classes was based on the geographic distance between them. Populations hosting <50 flowering individuals were categorized as small, whereas populations with >150 flowering individuals were categorized as large. Sub-populations within MNP represent prescribed fire in spring every 4 y (MNP_S), prescribed fire in fall every 4 y (MNP_F), annual haying in fall (MNP_H), or no management (MNP_C). Total 341 Sanger sequences were used to analyze root orchid mycorrhizal fungal (OMF) communities, which represented 92 individual plants. The last column provides the number of OMF sequences representing seedling (S), vegetative (V), or reproductive (R) plants. Numbers within parentheses presents the number of plants from which each set of sequences was generated.

Population name county and state	Population Code	Population size	Latitudinal Group	Sampling Year	No. of sequences (no. of plants yielding the sequences)		
					S	V	R
Manitoba Tallgrass Preserve	MB	>150	N	2013	3 (1)	9 (3)	3 (1)
Manitoba				2014	11 (3)	2 (1)	3 (2)
Sheyenne National Grasslands	NDV	<50	N	2013		7 (1)	6 (1)
Ransom County, North Dakota				2014	10 (2)	16 (4)	
	NDG	>150	N	2013		9 (2)	9 (2)
				2014		1 (1)	4 (1)
Pembina Trail Preserve	MNP	>150	N				
Polk County, Minnesota	MNP_F			2013	3 (2)	11 (2)	5 (2)
				2014		7 (3)	12 (2)
	MNP_S			2013	1 (1)	3 (1)	6 (2)
				2014	2 (2)	2 (2)	2 (2)
	MNP_H			2013	4 (1)		3 (2)
				2014		14 (2)	17 (2)
	MNP_C			2013	3 (1)	6 (1)	11 (2)
				2014		4(2)	7(2)
Bluemound State Park	MNB1	<50	S	2013		2(2)	11(2)
Rock County, Minnesota				2014		1 (1)	3 (1)
	MNB2	<50	S	2013	1 (1)	4 (1)	10 (2)
				2014	1 (1)	2 (2)	1(1)
Dinesen Prairie	IA	<50	S	2013	7(1)	3(2)	7(2)
Shelby County, Iowa				2014			15 (2)
Valentine National Wildlife Refuge	NE		S	2013	2 (1)	17 (2)	19 (2)
Cherry County, Nebraska				2014		9 (2)	10 (2)

(Na, Zn, Mn, Fe, Cu, B), and textural components (% sand, silt and clay) were measured in each sample. Finally, at the time of soil sample collection, three readings of soil moisture (Soil Moisture Tester 6405, Spectrum Technologies, Inc, Aurora, USA) and three readings of soil compaction (Soil Compaction Tester 6120, Spectrum Technologies, Inc, Aurora, USA) were recorded.

2.3. DNA extraction, PCR, and sequencing

Roots were prepared for DNA extraction as described in Pandey et al. (2013). Total DNA was extracted using Qiagen DNeasy Plant Mini Kit (Qiagen, Valencia, CA, USA) and was used for 25 μL PCR amplification reactions using Promega GoTaq Flexi Polymerase kit (Promega, Madison, Wisconsin, USA) and two primer pairs: ITS1-OF/ITS4-OF and ITS1/ITS4-TUL (Taylor and McCormick, 2008). PCR reaction mixture and thermocycler profiles were as described by Pandey et al. (2013). Samples that showed a single amplicon between 600 and 800 bp were cleaned using DNA Clean and Concentrator 5 kit (Zymo Research, Irvine, USA), while samples with multiple bands were processed for 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). All sequences used in this study were obtained by performing PCR directly on root DNAs. Soil genomic DNA was extracted by using 'PowerSoil™ DNA Isolation Kit' (MoBio Laboratories, Carslbad, USA), following the manufacturer's protocol. Two technical replicates with 0.25 g soil were used for DNA extraction from each soil sample and these replicates were subsequently pooled during DNA binding step. The details on soil fungal library preparation and its sequencing on MiSeq Desktop Sequencer (Illumina Inc, San Diego, USA) are provided in appendix 1. The raw sequence reads from soil samples were deposited in GenBank under project PRJNA421973 (Accession# SAMN08174307-SAMN08174371).

2.4. Data analyses

2.4.1. Quality filtering and OTU delineation of Sanger-based root OMF sequences

Quality filtering parameters for root OMF sequences are provided in appendix 1. The sequences retained after quality filtering were deposited in GenBank (Accession# MG662718-MG663058). Afterwards, taxonomic identity of each sequence was determined at family level by using BLAST (NCBI GenBank, http://www.ncbi.nlm.nih.gov/genbank/). To extract the homologous regions, sequences belonging to each family were aligned separately in T-Coffee version 11.00 (Notredame et al., 2000) with M-Coffee mode. Operational Taxonomic Unit (OTU) clustering was performed with UCLUST (Edgar, 2010) at 97 % similarity threshold. The longest sequence in each cluster served as the representative sequence for each OTU. Additionally, the OMF sequences recovered from *P. praeclara* roots were trimmed to remove ITS1 and 5.8S regions to enable identification of *P. praeclara*-specific OMF OTUs in high-throughput soil sequencing data (described below).

2.4.2. Quality filtering and OTU delineation of NGS-based OMF sequences from soil

The quality filtering parameters for soil fungal sequences are provided in appendix 1. For OTU clustering, OMF sequences identified within *P. praeclara* roots were merged with soil fungal sequences after retaining only ITS2 region. Clustering of OTUs was performed *de novo* with UCLUST at 97 % similarity threshold (Edgar, 2010). Taxonomy was assigned using the BLAST implementation in QIIME (Altschul et al., 1990) (e-value $\leq$0.001, percent similarity $\geq$90 %). An OTU table was built in QIIME and filtered to include generally known OMF families (Sebacinaceae, Thelephoraceae, Tulasnellaceae, Ceratobasidiaceae, Pezizaceae, Pyronemataceae, Tuberaceae, Agaricaceae, Clavulinaceae, Corticiaceae, Inocybaceae, Marasmiaceae, Russulaceae, Tricholomataceae, Typhulaceae) (Waud et al., 2017). The OTU table was further filtered to retain OTUs that clustered with

OMF sequences obtained from *P. praeclara* roots, and subsequently these *P. praeclara*-specific soil-associated OTUs were used for soil OMF community analyses.

2.4.3. Community and phylogenetic analyses

We primarily used *Phyloseq* (McMurdie and Holmes, 2013) and *vegan* packages (Oksanen et al., 2007) in R version 3.4.0 (R Core Team, 2017) to compare the targeted fungal communities among latitudinal gradient, population size, populations, years, phenological stages (for root OMF), and sampling locations (for soil OMF). Additionally, fungal communities in roots and soil were compared separately for the four sampling locations (MNP_F, MNP_S, MNP_H, and MNP_C) within MNP representing manipulative vegetation treatments. To determine the OTU diversity saturation for all comparison groups, sample-based and sequence abundance based rarefaction and exploration curves were generated with *iNEXT* package ($q = 0$) (Hsieh et al., 2016). Alpha diversity was estimated using Simpson diversity index, which was subsequently compared with non-parametric Kruskal–Wallis test. OTU tables were transformed based on Hellinger transformation. Bray–Curtis dissimilarity index was used to generate abundance based pairwise dissimilarity matrix among individual samples, which was then subjected to PERMANOVA (Anderson, 2001) with the *vegan* package, and hierarchical clustering with the *stats* package. The p-values from a model with multiple comparisons were adjusted with Bonferroni correction. We further used ANCOM (analysis of composition of microbiomes) to determine the differentially abundant OTUs with adjustment of multiple comparisons with false discovery rate (Mandal et al., 2015). Influence of soil physicochemical properties on soil OMF community structure was tested by first selecting the variables with forward selection (forward.sel) with *adespatial* package (Dray et al., 2018), and subsequently by performing Redundancy Analysis (RDA) with the *vegan* package. Multiple Regression on distance Matrices (MRM) was used for variance partitioning in soil OMF OTU abundances (Bray–Curtis dissimilarity matrix) as a function of forwardly selected edaphic variables (Euclidean distance matrix) and the geographic distance matrix calculated from geo-coordinates of sampling locations. MRM was also conducted for variance partitioning in root OMF OTU abundances as a function of soil OMF OTU abundances and geographic distances. Finally, to compare the phylogenetic breadth of OTUs detected in *P. praeclara roots* with OMF OTUs of other terrestrial orchids, we generated Maximum-likelihood (ML) and Bayesian phylograms. Further information on selection of DNA substitution models, and phylogram generation parameters is provided in appendix 1.

3. Results

3.1. Root associated OMF communities

Across two years, eight populations, and three phenological stages of *P. praeclara*, we generated 791 raw Sanger-based ITS sequences from 148 *P. praeclara* individuals and 498 root pieces. We discarded 57 % of the raw sequences during the quality filtering step to retain only the highest quality sequences. The root associated OMF communities were thus represented by 341 sequences encompassing 92 individual plants. The number of sequences per individual varied from 1 to 15 (Table S2). We detected two fungal familieswhereby Ceratobasidiaceae was represented by 286 (84 %) of all sequences while 55 belonged to the Tulasnellaceae. Subsequent OTU clustering yielded 24 Ceratobasidiaceae OTUs, a majority (18) of which were singletons, and 7 OTUs of Tulasnellaceae (Fig. 1B). The number of OTUs identified within a single plant varied from 1 to 4 (Table S2). Of the 31 OTUs from both fungal families, C13 was the most abundant incorporating 78 % of all Ceratobasidiaceae sequences from 60 individuals (Table S2); it was recovered from all populations except MNB1 and occurred under all four vegetation management practices and all three phenological stages in both sampling years. Among the seven Tulasnellaceae OTUs, two (T1 and T7) were singletons while T2 and T4 were encountered most frequently representing 43 % and 39 %, respectively, of all Tulasnellaceae sequences. The extrapolated sampling curves based on sequence abundance showed higher number of OTUs across all comparison groups that might be due to the high number of singletons and OTUs with low abundances in our dataset (Fig. S1). Sample-based rarefaction and extrapolation curves showed similar trends (Fig. S2).

Simpson diversity index for root associated OMF communities did not vary across space and time, nor by population size or plant phenological stage ($P > 0.05$ for all). Alpha diversity was also similar for the four vegetation management treatments at MNP ($P = 0.400$) (Fig. S3). Conversely, beta diversity was variable across latitude (Pseudo-$F_{1,42} = 3.92$, $P = 0.025$) and populations (Pseudo-$F_{5,42} = 2.89$, $P = 0.010$), although no apparent effect of latitude or populations was observed on the two most abundant OTUs of Ceratobasidiaceae (C13 and C18) (Fig. 1B). By contrast, the two most abundant OTUs of Tulasnellaceae (T2 and T4) exhibited variation across latitude (Pseudo-$F_{1,42} = 6.46$, $P = 0.045$), and populations (Pseudo-$F_{5,42} = 3.07$, $P = 0.025$) (Fig. 1B). Additionally, ANCOM also detected only T2 and T4 as the differentially abundant OTUs across latitudinal extent ($P < 0.05$ for both), and only T4 as variably abundant across populations ($P < 0.05$). Besides these differences, beta diversity did not vary across time, population size, host phenology or vegetation management treatments. Hierarchical clustering grouped the eight study populations into four clusters based on the abundances of 31 OTUs: 1) MNB2 and IA; 2) NDV, NDG, MB and NE; 3) MNP; 4) MNB1 (Fig. 1B). However, the clustering pattern of populations did not correspond to latitude. Variance partitioning with MRM also showed that geographic distances among sites did not influence root-associated OMF OTU abundances ($P = 0.37$).

Both the ML and Bayesian trees showed similar topologies and grouped 24 Ceratobasidiaceae OTUs into four clades. The clade support values were moderate to low (26–85) in the ML tree, and high for the Bayesian tree (0.72–0.99) (Fig. 2A). Despite their segregation, the four clades exhibited narrow phylogenetic breath where Clades 2, 3, and 4 did not associate with any of the reference OMF sequences excepting those previously recovered from *P. praeclara* (Fig. 2A). However, Clade 1 containing only C10, associated closely with OMF sequences recovered from *Platanthera chlorantha* and a sequence generated from UAMH 6440 fungal culture isolated from *Platanthera obtusata*. In contrast, Tulasnellaceae OTUs showed a much wider phylogenetic breadth, and segregated in six clades in both the ML and Bayesian trees though the clade support values were lower (31–47) in the ML phylogram compared to the Bayesian (0.72–0.89) tree. Unlike the Ceratobasidiaceae OTUs, the seven Tulasnellaceae OTUs showed association with previously known OMF from other terrestrial orchids (Fig. 2B).

3.2. Potential OMF communities in soil

After read pairing and demultiplexing, 7,756,032 sequences were retained from which 15 %, 61 % and 2 % sequences were discarded during quality filtering, dereplication, and chimera filtering, respectively. After clustering 1,731,788 sequences at 97 % similarity threshold, a total of 69,423 fungal OTUs were recovered across all samples. Of the 69,423 fungal OTUs, 44 % were singletons which were removed before the downstream analyses and after this, total

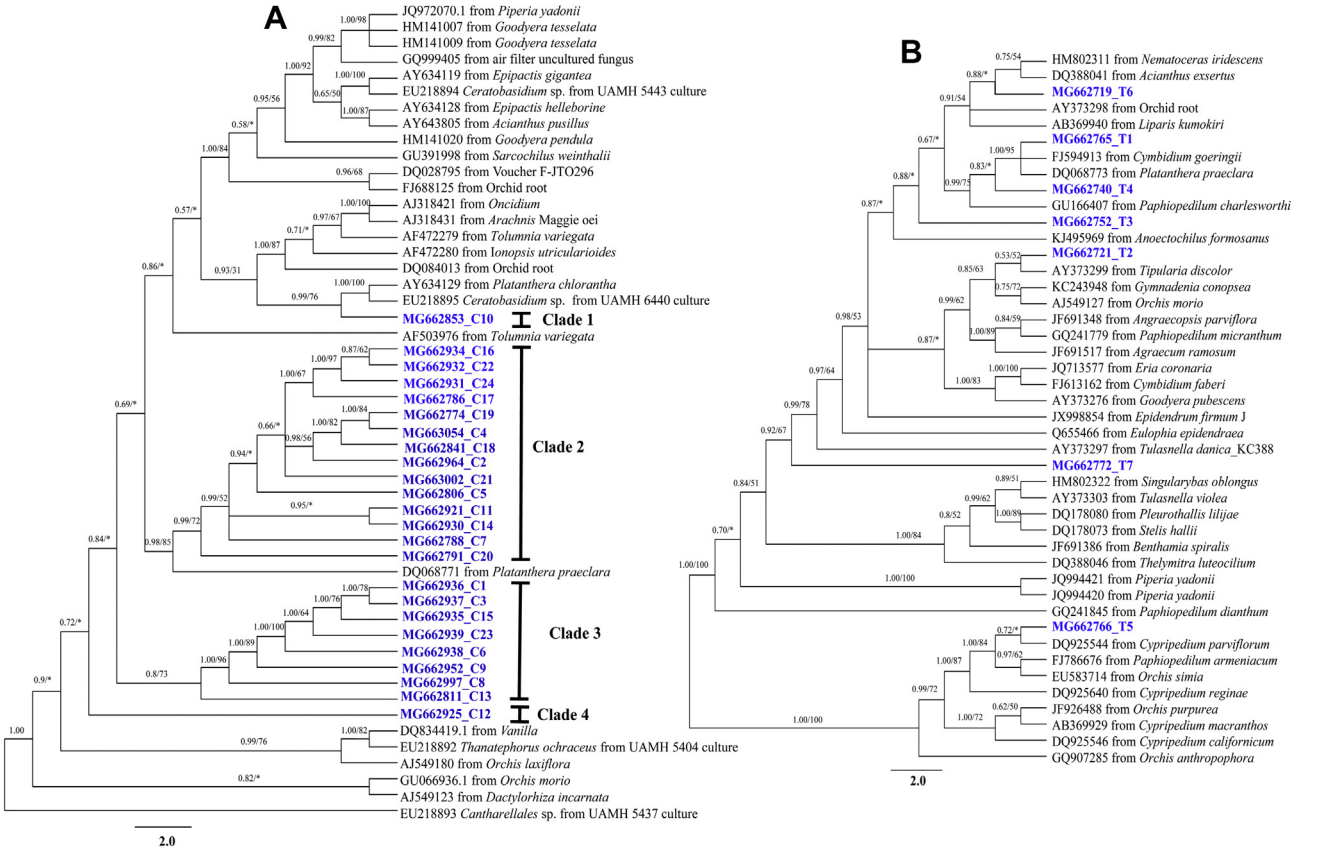

Fig. 2. Phylogeny of fungal operational taxonomic units (OTUs) recovered from roots of *Platanthera praeclara* by using nuclear ribosomal internal transcribed spacer (nrITS). Phylograms were generated with Bayesian Analysis and Maximum Likelihood methods, and clade support values from both methods are shown for each clade (an * indicates <50 % bootstrap support). (**A)** Phylogenetic positions of Ceratobasidiaceae OTUs (C1 to C24) rooted with a sequence generated from a culture (UAMH 5437) of Cantharellales sp. (**B)** Phylogeny of Tulasnellaceae OTUs (T1 to T7) with mid-point rooting. The GenBank accession numbers of the OTU representative sequences are provided along with OTU names in blue font. All reference sequences are coded by using GenBank accession# followed by the name of their host orchid species. (For interpretation of the references to color in this figure legend, the reader is referred to theWeb version of this article.)

number of sequences and OTUs per soil sample varied from 12,212 to 37,060, and 1037 to 2,917, respectively (Table S1). Rarefaction and extrapolation curves revealed sufficient sampling effort across predictor categories based on the number of sequences (Fig. S4), whereas these curves did not show diversity saturation based on number of soil cores collected (Fig. S5). From the 39,157 fungal OTUs retained after singleton removal, 8 % (3088 OTUs with 192,687 sequences) belonged to 15 fungal families that are generally known to include root-associated orchid mycorrhizal fungi. Simpson alpha diversity index was similar across latitudinal class, populations, population size, orchid-occupied/bulk soil, years or vegetation management treatments ($P > 0.05$ for all; Fig. S6).

From OMF families, Tulasnellaceae was the most OTU-rich, and abundant (1754 OTUs represented by 86,902 sequences) comprising between 20 % and 75 % of the soil OMF communities at majority of the sampling locations (Fig. 3A). Despite the family's high abundance in soil, only one Tulasnellaceae OTU (sT5) was encountered at single location (MNP_P) that clustered with root-associated Tulasnellaceae OTU T5. The OMF families Tricholomataceae, Sebacinaceae, Agaricaceae, Inocybaceae and Ceratobasidiaceae followed Tulasnellaceae in abundance at a majority of the sampling locations (Fig. 3A). Ceratobasidiaceae, which was the most abundant OMF family in roots of *P. praeclara* in this study, was represented only by 223 OTUs (9790 sequences) among the potential soil OMF communities. Further, only five of these soil-associated Ceratobasidiaceae OTUs showed overlap with root-associated OTU C13 (s13.1-s13.5), and occasionally with other OTUs. The abundances of six *P. praeclara*-specific OTUs identified in soil accounted for <0.5 % (2438 sequences) of the total OMF community across sampling locations. Beta diversity comparisons indicated that abundances of *P. praeclara*-specific soil-associated OMF OTUs varied only between two sampling locations (Pseudo-$F_{8,47} = 5.10$, $P = 0.005$), whereas no differences were observed at the population level. In contrast, ANCOM did not detect significant differences in abundances of *P. praeclara*-specific soil-associated OMF OTUs across sampling locations. In addition, temporal, population size, latitudinal variation and four experimental vegetation management treatments at MNP did not affect *P. praeclara*-specific OMF OTU diversity in soil. Overall, hierarchical clustering separated the orchid-occupied sites from bulk soil samples (Fig. 3B). Hierarchical clustering made three clusters where first cluster included all of the orchid-occupied locations together with bulk soil samples of IA and NE, and the second cluster comprised only of bulk soil samples of MB, MNB2, MNP and NDG, and the third cluster consisted only of bulk soil sample of MNB1 and NDV (Fig. 3B).

Soil moisture (SM), magnesium (Mg), clay and manganese (Mn) emerged as the significant edaphic variables explaining the variation in soil OMF communities between orchid-occupied sites and bulk soil (Fig. 3C; Table S3). The scores of RDA1 axes for SM, Mg, clay and Mn were 0.21, −0.49, 0.30 and −0.37 respectively, whereas scores of the same variables for RDA2 were −0.34, −0.69, −0.72 and 0.03 respectively. These analyses revealed the restricted occurrence of soil-associated *P. praeclara*-specific OTUs at locations with relatively high SM and clay along with low levels of Mn and Mg

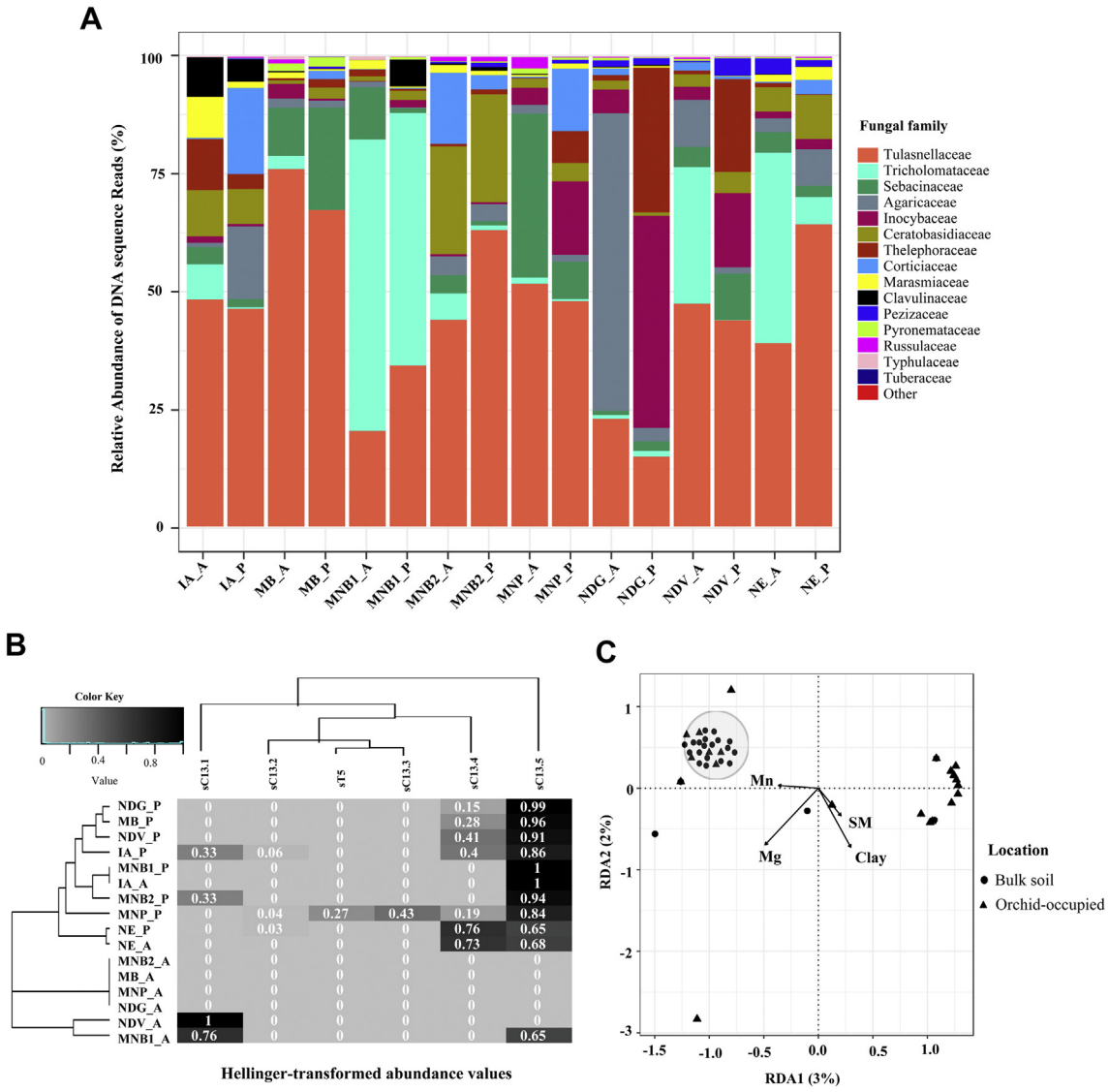

Fig. 3. Characterization of potential orchid mycorrhizal fungal (OMF) communities in habitat soil of *Platanthera praeclara* using operational taxonomic units (OTUs) of OMF families identified with internal transcribed spacer 2 (ITS2) sequences generated through an Illumina platform. Soil was sampled in Iowa (IA), Nebraska (NE), Minnesota (MNB1, MNB2 and MNP), North Dakota (NDG and NDV), and in Manitoba (MB) in 2013, 2014, and 2015 to represent orchid-occupied locations (P) and bulk soil where orchids were absent (A). **(A)** Relative abundances of DNA sequence reads belonging to potential OMF families in soil. **(B)** Two-way hierarchical clustering with weighted Bray–Curtis dissimilarity index values based on the abundances of six *P. praeclara*-specific OMF OTUs recovered from soil. **(C)** Redundant Analysis (RDA) biplot showing clustering of *P. praeclara* sampling locations based on the abundances of *P. praeclara*-specific OMF OTUs identified in soil and soil physicochemical characteristics, whereby soil moisture (SM), magnesium (Mg), clay (Clay), and manganese (Mn) were forwardly selected. The circle shows the overlapped points by the point adjacent to it.

(Fig. 3C). Further, the species score of RDA1 was highest for sC13.5 (−0.34), whereas RDA2 score was also highest for sT5 (−0.26) (Fig. 3C). These data clearly support the separation of orchid-occupied sites from the bulk soil along RDA1 axes with respect to the abundance of OTU sC13.5. However, the MRM procedure suggested that soil-associated OMF OTU abundances were independent of edaphic variables (SM, Mg, clay and Mn) ($P = 0.61$), and geographic distances ($P = 0.59$). Further, the MRM procedure also showed that the distributions and diversity of *P. praeclara*-specific OTUs recovered from roots are influenced by the abundances of *P. praeclara*-specific OTUs identified from soil ($r^2 = 0.33$, $P = 0.00$).

4. Discussion

Coupled distributions and diversity of orchid mycorrhizal fungi (OMF) in roots and soil have only recently been published (Oja et al., 2017; Waud et al., 2017; Voyron et al., 2017) while North American ecosystems remain absent from this literature. Spanning three years of sampling initiated in the year 2013, ours is the first study in one of the most imperiled ecosystems, the North American tallgrass prairie, to attempt to explain the temporal (inter-annual), spatial, and phenology-associated diversity of OMF in roots and soil by also including a series of abiotic predictors. We show that a rare temperate terrestrial orchid, *P. praeclara*, selects a narrow group of mycorrhizal fungi across time, space, and phenological development. Simultaneously, the mycorrhizal fungi associated with *P. praeclara* exhibit patchy and localized distribution in soil corresponding to the presence of the orchid host.

4.1. Platanthera praeclara *exhibits near-exclusivity in its mycorrhizal associations*

The OMF communities within orchid roots were dominated by members of Ceratobasidiaceae and Tulasnellaceae. Root-associated

Ceratobasidiaceae OTUs recovered in this study grouped in the same clade with a fungus previously sequenced from *P. praeclara* roots (Sharma et al., 2003b; GenBank accession # DQ068771). Our results align with prior observations that species within the genus *Platanthera* prefer fungi from the Ceratobasidiaceae and Tulasnellaceae OMF families (Sharma et al., 2003a; Yagame et al., 2012; Esposito et al., 2016; Oja et al., 2017). Interestingly, the symbionts of *P. praeclara* also did not share clade-space with majority of the Ceratobasidiaceae sequences obtained from other orchid taxa (Fig. 2A), indicating the orchid host's exclusive selection of fungi from a narrow clade within Ceratobasidiaceae. Remarkably, despite the narrow phylogenetic breadth of the Ceratobasidiaceae OTUs recovered in this study, *P. praeclara* was uniquely exclusive in associating with a single Ceratobasidiaceae OTU (C13) that dominated the OMF communities in the roots of seedlings, vegetative, and reproductive individuals at seven of the eight populations across two years (Fig. 1B). Although root OMF communities varied across populations and latitudinal gradient, abundances of the two most dominant root OMF OTUs (C13 and C18) did not vary across populations or latitude. It appears that the population- and latitude associated variation in OMF communities may be attributed to OTUs that were present in low abundances. The prairie remnants that *P. praeclara* often restricts itself to across its natural range are typically managed by, or experience, fire, haying, or grazing. While it is possible that the detected spatial variability in root OMF communities is a manifestation of selection pressures induced by vegetation management (Jasinge et al., 2018), the insensitivity of the root OMF communities to prescribed fire and haying at MNP suggests otherwise for *P. praeclara* although it could be debated with a larger manipulated experiment conducted over multiple decades.

The affinity of *P. praeclara* for C13, or vice-versa, suggests a tight co-evolutionary relationship as well as physiological interdependence between the host orchid and its mycobiont. Moreover, our data supports the observations of Sharma et al. (2003b) that reported the higher seed germination in *P. praeclara* with one isolate of Ceratobasidiaceae (UAMH 9847, GenBank accession # DQ068771) from three Ceratobasidiaceae and three Tulasnellaceae isolates tested in this study. While prior data from *P. praeclara* also suggested strong specificity toward Ceratobasidiaceae (Sharma et al., 2003a), the geographical, temporal, and phenological scope of this study was limited, and broader and deeper investigations were proposed. The evidence of specificity between *P. praeclara* and Ceratobasidiaceae was further strengthened by the presence of 15 OMF families in soil precluding the possibility of pseudo-specificity due to the unavailability of other potential OMF in soil. There are few other orchid taxa, including *Cypripedium candidum* (another rare and protected taxon) and *Liparis loeselii* that exhibit a similar phenology and may co-occur with *P. praeclara* at one or two sites. Of these, *C. candidum* was found to prefer Tulasnellaceae (unpublished data) in parallel to the genus *Cypripedium* that also typically associate with Tulasnellaceae (Shefferson et al., 2005, 2007); while information on the OMF of *Liparis* species native to the tallgrass prairie is non-existent, although *Liparis lillifolia* sampled from the eastern and mid-western US showed an affinity for Tulasnellaceae (McCormick et al., 2004).

An analysis of multiple co-occurring and phylogenetically allied orchids within the tallgrass prairie is warranted to resolve these questions. In this study, Tulasnellaceae OTUs showed lower abundances within *P. praeclara* roots, but exhibited wider phylogenetic breadth when compared to Ceratobasidiaceae OTUs. Despite their relative dominance in soil, low affinity of *P. praeclara* toward Tulasnellaceae indicated an orchid-fungal coupling that is independent of the relative abundances of potential OMF sequences in soil, even though the orchid exhibits a capacity to otherwise utilize Tulasnellaceae as OMF in seedling, vegetative, and reproductive stages, indicating that there is some stability in the relationship. In contrast, other rare and endangered terrestrial orchids, such as *Platanthera (Piperia) yadonii* and *Liparis loeselli* are known to adopt a generalist or opportunistic approach in selecting a wider array of OMF from two or three fungal families (Pandey et al., 2013; Waud et al., 2017).

4.2. *Preferred fungal associates of* P. praeclara *are rare and patchily distributed in soil*

The six *P. praeclara*-specific OTUs (s13.1 to s13.5, and sT5) were nearly absent in bulk soil at locations unoccupied by the orchids but exhibited higher relative abundances in soil at orchid-occupied locations suggesting their patchy distribution and the correspondence of orchid occurrence with that of its preferred fungal partners (Fig. 3B). While the relative abundances of these OTUs were higher at orchid-occupied locations in comparison to the locations where orchids were absent, the *P. praeclara*-specific OTUs generally accounted for <0.5 % of the potential OMF sequences at both types of locations indicating their rarity. Similar patterns of low abundances of orchid preferred OMF have also been reported in habitat soil of endangered orchids in southern China and southeastern Australia whereby the root associated OMF of both *Paphiopedilum spicerianum* (Han et al., 2016) and *Diuris fragrantissima* (Egidi et al., 2018) showed ≤1 % relative abundance in their habitat soils. Conversely, root associated OMF OTUs of a commonly occurring orchid *Ophrys sphegodes* were more abundant in soil in comparison to the associates of the rare *Anacamptis morio* in northern Italy (Voyron et al., 2017). Similarly, the preferred fungi of another three common orchid taxa (*Goodyera pubescens*, *Liparis liliifolia* and *Tipularia discolor*) in eastern US were widely, albeit patchily, distributed in soil (McCormick et al., 2016). Altogether, our results combined with those of others (McCormick et al., 2016; Oja et al., 2015, 2017; Egidi et al., 2018) indicate that the species-specific OMF decrease in abundance with increasing distance from host plant and that the limited distribution of the rare orchids is likely linked to this patchy and sparse occurrence of their specific OMF although stronger evidence for this linkage is still pending. For example, patterns of density and distribution of spore banks of OMF partners of an orchid host are not yet known in its occupied or potential habitats.

Simultaneously, while the distributions of *P. praeclara*-specific OMF are not uniform across orchid occupied locations vs. non-occupied bulk soil, we observed comparable abundances of these OTUs at both locations in IA, MNB1 and NE (Fig. 3B). The presence of potentially suitable OMF fungi in bulk soil represents available empty niches for *P. praeclara* where its distribution might be limited by seed dispersal or other factors. These areas present viable opportunities for experiments designed to isolate the effects of dispersal.

4.3. *Overlap between root and soil OMF communities*

A question could be raised that the low overlap between the root and soil OMF OTUs might be attributed to their respective sequencing platforms (Sanger vs. Illumina, i.e.). Notably, studies using the same platforms for both communities have failed to show high overlap (Voyron et al., 2017) suggesting that the observed patterns are biological in nature, especially that the distributions of terrestrial orchids and their preferred fungi are patchy, localized, and sparse. For instance, Oja et al. (2015), Han et al. (2016), and Egidi et al. (2018) utilized high-throughput sequencing for both root and soil OMF community delineation, and reported incongruence whereby rhizosphere soil exhibited reduced proportions of root associated OMF OTUs. In contrast, many other potentially OMF OTUs from both fungal families, Ceratobasidiaceae and

Tulasnellaceae, are detectable in high abundances in soil. More importantly, comparisons of Sanger sequencing and high-throughput sequencing for ectomycorrhizal and OM fungi established the potential of both methods to equally detect the dominant taxa associated with roots, while high-throughput sequencing enabled detection of rare fungal taxa with low abundances (Tedersoo et al., 2010; Huang et al., 2014).

Another potential concern is the influence of primer selection on fungal community compositions. In our study, the forward primers used for amplifying Ceratobasidiaceae from roots (ITS1-OF) and soil (ITS3) were different, whereas the reverse primer (ITS4-OF) was the same. When we checked the alignment of ITS3 primer sequence with Ceratobasidiaceae sequences and OTUs obtained from roots, a complete match was found with 80 % of the 286 sequences and 14 out of 24 OTUs (Fig. S7). Therefore, primer bias is very unlikely to have major influence on the overlap of Ceratobasidiaceae communities in roots and soil. In contrast, this was not the case with Tulasnellaceae sequences and OTUs (Fig. S7), which were amplified with two primer pairs (ITS1-OF/ITS4-OF and ITS1/ITS4-TUL) from roots and only one primer pair from soil (ITS3/ITS4-OF). Thus, the low detection of *P. praeclara*-specific Tulasnellaceae OTUs in soil may be attributed to primer bias and it is commonly expected because Tulasnellaceae host high variation in ITS sequences, which makes their detection with any single primer pair (even when degenerate) difficult (Linde et al., 2014). Regardless, primer bias against Tulasnellaceae in soil is unlikely to change the findings of this study given the dominance of Ceratobasidiaceae OMF family within the roots of *P. praeclara*.

4.4. Impact of soil physicochemical profiles on soil OMF communities

Our study adds to the evidence for the influence of edaphic environment on the distributions of putative OMF in soil whereby the influence of soil physicochemical profiles (SM, Mg, clay and Mn) was evident in governing the abundances of *P. praeclara*-specific OTUs in soil (Fig. 3C). No other study has reported the effect of Mg, clay and Mn on OMF dynamics in roots or soil. However, higher soil moisture at orchid-occupied locations has been observed by others (Diez, 2007; Jacquemyn et al., 2015; Waud et al., 2017) suggesting that soil moisture is key in orchid recruitment directly or indirectly. Outside of these few reports, evidence for correlations or cause and effect between soil physicochemical properties and OMF communities in soil is absent.

5. Conclusions

Our study provides evidence that the rarity and distribution of the host orchid is a consequence of its rigidity in selecting a few mycobionts through space, time, and phenology that are rare and patchily distributed in soil. This combination is certain to introduce bottlenecks, especially during recruitment of *P. praeclara* individuals, by narrowing its fundamental functional niche (Sharma et al., 2003b; Swarts et al., 2010; Davis et al., 2015). An empirical test of in-situ recruitment and subsequent plant growth in strategically selected or manipulated soil conditions can, however, allow a direct test of the influence of fungal abundance. Finally, it remains unknown why some OMF are rarely encountered in soil, and exhibit patchy distributions despite their saprotrophic nutritional modes. In grass-dominated ecosystems such as the tallgrass prairie, Basidiomycete fungi supporting orchids likely rely on saprotrophy for their existence outside of orchid roots. It is possible that a lack of an alternate carbon source, such as forming mycorrhizal relationships with other plants, might lead to their rarity. A soil fungal community analysis that incorporates fine local scale spatial correlations with root OMF could clarify the current spatial structure of OMF communities within the tallgrass prairie to serve as a benchmark in the future.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.funbio.2019.09.010.

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