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A narrowly endemic photosynthetic orchid is non-specific in its mycorrhizal associations

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Abstract

Mycorrhizal association is a common characteristic in a majority of land plants, and the survival and distribution of a species can depend on the distribution of suitable fungi in its habitat. Orchidaceae is one of the most species-rich angiosperm families, and all orchids are fully dependent on fungi for their seed germination and some also for subsequent growth and survival. Given this obligate dependence, at least in the early growth stages, elucidating the patterns of orchid–mycorrhizal relationships is critical to orchid biology, ecology and conservation. To assess whether rarity of an orchid is determined by its specificity towards its fungal hosts, we studied the spatial and temporal variability in the host fungi associated with one of the rarest North American terrestrial orchids, *Piperia yadonii*. The fungal internal transcribed spacer region was amplified and sequenced by sampling roots from eight populations of *P. yadonii* distributed across two habitats, *Pinus radiata* forest and maritime chaparral, in California. Across populations and sampling years, 26 operational taxonomic units representing three fungal families, the Ceratobasidiaceae, Sebacinaceae and Tulasnellaceae, were identified. Fungi belonging to the Sebacinaceae were documented in orchid roots only at *P. radiata* forest sites, while those from the Ceratobasidiaceae and Tulasnellaceae occurred in both habitats. Our results indicate that orchid rarity can be unrelated to the breadth of mycorrhizal associations. Our data also show that the dominance of various fungal families in mycorrhizal plants can be influenced by habitat preferences of mycorrhizal partners.

Keywords: Ceratobasidiaceae, endemic, fungal specificity, internal transcribed spacer, mycorrhizae, orchid, Orchidaceae, *Piperia yadonii*, Sebacinaceae, Tulasnellaceae

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Introduction

The symbiotic relationship between roots of plants and fungi, known as mycorrhizal association, is a common yet complex phenomenon in plants. Approximately, 90% of land plant families have some type of mycorrhizal association, and in many cases, this association is critical for survival, growth and the evolution of both fungi and plants because plants depend on fungi for nutrition and fungi on plants for energy (Fitter & Moyersoen 1996; Smith & Read 1997). Various types of mycorrhizal interactions have evolved including mutualistic (Alexander *et al.* 1989; Smith & Read 1997), partially exploitative (Alexander & Hadley 1985; Rasmussen 1995) and fully exploitative associations (Leake 1994; Taylor & Bruns 1999). Specificity of mycorrhizal partnerships, defined as the phylogenetic breadth of fungi associated with a particular species (Thompson 1994), particularly in rare plants, is considered to be an important ecological and evolutionary trait (Taylor & Bruns 1999). Identification of arrays of fungi that are important for survival and growth of plants, especially of threatened and endangered plants, allows targeting specific fungi for restoration and conservation programmes. Additionally, such data also provide insight

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into the evolutionary dynamics between plants and their fungal associates. However, the extent of mycorrhizal specificity and dependence on fungi for nutrition may also be influenced by environmental, ecological and evolutionary factors of both the plant and the fungus (Taylor & Bruns 1999; Brundrett 2002; Taylor *et al.* 2003a; Shefferson *et al.* 2005).

Assessments of plant ecological traits including growth, reproduction and biotic interactions that might contribute to the persistence of rare species have largely been lacking (Bevill & Louda 1999; Murray *et al.* 2002). Increasingly being recognized as one of the ecological traits directly and/or indirectly influencing plant distributions and abundance, mycorrhizal dependence has been linked to rarity in plants (Bevill & Louda 1999; van der Heijden 2002). Orchids, which comprise one of the largest angiosperm families with an estimated 20 000–35 000 species (Dressler 1981; Cribb *et al.* 2003), are known to have obligate mycorrhizal dependence for their initial development from the seed stage. Mycorrhizae continue to contribute to the subsequent growth and persistence of orchids (Waterman & Bidartondo 2008; Waterman *et al.* 2011) except in some epiphytic taxa that may not depend on fungi in the adult stage (Bayman *et al.* 1997, 2002). The high species diversity and relative rarity of most of the species within the Orchidaceae could partly result from the highly narrow interactions with pollinators and/or fungi (Taylor *et al.* 2003b; Cozzolino & Widmer 2005; Roche *et al.* 2010). All orchids are believed to be functional parasites (mycoheterotrophs), especially during the early protocorm development because of a combined lack of internal nutrient reserves and chlorophyll, which means that they depend entirely on fungi for carbon and nutrient supplies (Rasmussen & Rasmussen 2009). On the other hand, the status of the fungal relationships of adult plants varies from one orchid species to another (Leake 1994; Rasmussen 2002; Girlanda *et al.* 2011). Nonphotosynthetic orchids maintain this complete carbon dependence throughout their life (Leake 1994; McKendrick *et al.* 2000), while photosynthetic orchids not only become less dependent on fungi in the adult stage (Bruns & Read 2000; Bidartondo & Bruns 2001, 2005), some may also supply carbon to their fungal partners (Cameron *et al.* 2008).

Mycorrhizal alliances can range from highly specific to broad (i.e. non-specific). High specificity has been observed in uncommon nonphotosynthetic plants including orchids (Furman & Trappe 1971; Taylor & Bruns 1997, 1999; Taylor *et al.* 2002), while the association has been reported to be less specific in most photosynthetic species (Molina *et al.* 1992; Zelmer *et al.* 1996; Rasmussen 2002; Bidartondo *et al.* 2004; Selosse *et al.* 2004; Otero *et al.* 2007; Shefferson *et al.* 2008; Smith & Read 2008), with some exceptions (Perkins *et al.* 1995; Pritsch *et al.* 2000; McCormick *et al.* 2006; Shefferson *et al.* 2007; Roche *et al.* 2010). In some orchids, variation in the extent of mycorrhizal specificity across life history stages has been documented, whereby some plants utilize a wider range of fungi during germination than in the adult phase (Zelmer *et al.* 1996; Bidartondo & Read 2008), while in others, it is more specific in seedlings than in adult plants (McCormick *et al.* 2004). Moreover, habitat preference of mycorrhizal associations in terrestrial orchids has been documented (Masuhara & Katsuya 1994; Taylor & Bruns 1999; Shefferson *et al.* 2005). Several habitat characteristics such as altitude, soil characteristics and plant community also appear to play a significant role in the distribution of orchid mycorrhizal fungi (Ramsay *et al.* 1987; Masuhara & Katsuya 1994; Taylor & Bruns 1999). Although some studies have reported that rare orchid species associate with fewer mycorrhizal fungal species (Rasmussen & Whigham 1994; Shefferson *et al.* 2005), there are very few studies that have tested specificity in mycorrhizal association as the cause of rarity (Swarts *et al.* 2010; Phillips *et al.* 2011).

The photosynthetic endemic terrestrial orchid *Piperia yadonii* Rand. Morgan and Ackerman (family: Orchidaceae; subfamily: Orchidoideae) has an extremely narrow distribution in Monterey County, California, and was designated endangered by the U.S. Fish and Wildlife Service (USFWS) in 1998. It is believed that this species historically occurred throughout the Monterey Peninsula (USFWS 2004). However, most of its natural habitat has been lost and the remaining habitat is heavily fragmented due to extensive urbanization, agriculture, conversion of chaparral to oak or conifer forest and due to introduced exotic species (George *et al.* 2009). Approximately, 20 known *P. yadonii* populations remain distributed across two types of native habitats including *Pinus radiata* forests and maritime chaparral. In the maritime chaparral habitat, it grows along with several endemic species belonging to the genus *Arctostaphylos*, especially *Arctostaphylos hookeri* (George *et al.* 2009). The *P. radiata* forests support larger *P. yadonii* populations (mean individuals/population = 347), while four or five relatively isolated and smaller populations (mean individuals/population = 184) occur in maritime chaparral (George *et al.* 2009). *Piperia yadonii* is a perennial herb that can reach 50–60 cm tall when flowering, and it survives drought-induced dormancy between September and December via an underground tuber that supports a vegetative bud. New roots are produced in each growing season, which are infected by fungi anew during each growing season. Leaves emerge after autumn/winter rains, and flowering occurs between late May and early August (Vern L. Yadon and Jyotsna Sharma,

personal observation). Flowers are arranged in a dense, single vertical inflorescence spike. Only about 20% of plants emerging aboveground produce flowers in a given year. Pollination in this predominantly outcrossing species is primarily carried out by moths in the Pyralidae, Geometridae, Noctuidae and Pterophoridae (USFWS 2004).

Despite the imperilled status, its highly restricted distribution and the importance of fungi in orchid life cycles, fungal symbionts of *P. yadonii* remain largely unknown except for one study that reported a preliminary analysis of fungal diversity in a few populations of *P. yadonii* by sequencing the fungal internal transcribed spacer region (ITS) using the primer set ITS-1F/ITS-4 (Sharma *et al.* 2007). While generally allowing for broader fungal surveys, these primers can exclude the orchid-associated fungal lineage Tulasnellaceae (Taylor & McCormick 2008). By including more populations and a larger number of sampled plants and by using alternative primers, we sought to more fully characterize the mycorrhizal associations of the rare and endemic *P. yadonii*. This study was conducted to: (i) identify the mycorrhizal fungi associated with *P. yadonii* across its natural distribution and (ii) assess the differences in mycorrhizal specificity of *P. yadonii* across eight populations and two habitats. We hypothesized that: (i) because *P. yadonii* is distributed in a narrow geographical range, it is expected to show association with fewer taxa of fungi than observed in photosynthetic orchids with wider distributions (Selosse *et al.* 2004; Shefferson *et al.* 2008) and (ii) even though *P. yadonii* is expected to associate with few mycorrhizal fungi overall, we expect some of the fungi in *P. yadonii* roots to show habitat preference owing to differences between the two habitats, particularly with respect to plant communities.

Materials and methods

Sampling and processing of roots

Roots from 42 adult plants (average number of plants per population: 5.25 plants) of *Piperia yadonii* were collected from eight selected populations located within Monterey County, California, in 2006, 2007 and/or 2010 (Fig. 1, Table S1, Supporting information). Of these eight populations, four (AP, BC, PQR and SFB) represented the *Pinus radiata* forest habitat, and the other four (BLMR, JP, MP and PL) represented the maritime chaparral. Our sampling design included all of the isolated and farthest (north, south, west and east) populations of the species as well as several in the middle of the distribution range. The remaining unsampled populations are scattered in the middle of the distribution range where several of the sampled populations are also located (Fig. 1). The minimum and maximum distances separating the pairs of sampled populations are 2.14 km (between AP and JP) and 41.10 km (between BLMR and PL), respectively, while the mean distance among populations was 19.26 km.

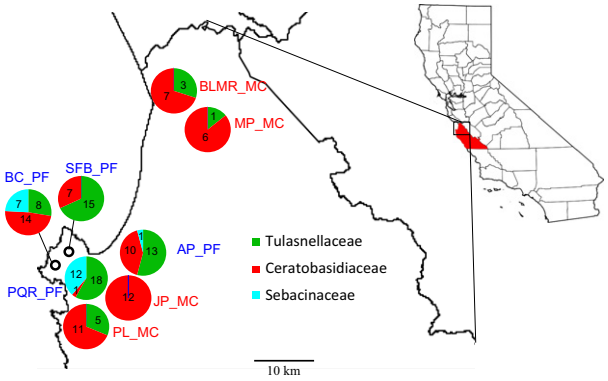

Fig. 1 Distribution of three orchid fungal families in eight populations of *Piperia yadonii* across two habitats: *Pinus radiata* forests (habitat code PF) and maritime chaparral (habitat code MC). The numbers in pie charts represent the number of individual internal transcribed spacer (ITS) sequences. Northern portion of Monterey County, California (highlighted on the map), containing all *P. yadonii* populations is enlarged.

We sampled AP in February 2006, February 2007 and June 2010; BC, MP, PL and PQR in February 2006 and June 2010; BLMR in February 2007 and June 2010; and JP and SFB only in February 2006. Four to five roots were collected from each of the 42 plants. Different individuals were sampled each time. Sampled roots were placed in individual plastic bags and transported at 4 °C to the laboratory. Roots were cleaned under running tap water to remove soil and other debris. Prior to inspection for fungal hyphal coils (pelotons) in cortical cells, roots were surface sterilized as follows: (i) a 2-min rinse under tap water followed by a 1-min rinse with 70% ethanol; (ii) a 30-s rinse in a 3% sodium hypochlorite solution; (iii) a 1-min rinse in 70% ethanol; and (iv) a 2-min rinse in sterile ultrapure water. Immediately before roots were inspected for peloton presence, the epidermis on larger root segments was shaved off using a sterile scalpel to remove additional microbes whose DNA may have survived the surface sterilization treatment. A thin root section (~0.5 mm) from each root piece was examined under a compound microscope at 100× magnification to verify the presence of pelotons. Once pelotons were located, adjacent pieces of 0.5–1.5 cm were individually placed in sterile 1.5-mL tubes and stored at −80 °C until DNA was extracted.

An aggregate soil sample from each population except BLMR was collected in 2006 and was analysed for 14 soil mineral nutrients [nitrate (NO_3), phosphorus

(P), potassium (K), calcium (Ca), magnesium (Mg), sulphate (SO_4), sodium (Na), iron (Fe), boron (B), copper (Cu), zinc (Zn), molybdenum (Mo), aluminium (Al) and manganese (Mn)], pH and electrical conductivity at Quality Analytical Laboratories (Panama City, FL, USA)] to examine correlations between orchid–fungal associations and soil variables.

DNA extraction, PCR and sequencing

Total DNA was extracted from peloton-containing root sections using the DNeasy Plant Mini Kit (Qiagen, Valencia, CA, USA). Quantity and quality of the DNA were measured using a NanoDrop ND1000 (Thermo Scientific, Wilmington, DE, USA). Polymerase chain reaction (PCR) amplification and sequencing of the fungal nuclear ribosomal ITS region were carried out using the primers ITS1-OF/ITS4-OF (Taylor & McCormick 2008). PCR amplifications were carried out in 25 μL reaction volumes using Qiagen HotStar HiFidelity *Taq* DNA polymerase kit (Qiagen) and run in an epGradients Master Cycler (Eppendorf, Hamburg, Germany). We used between 6 and 38 ng of total DNA. The thermocycling profile was as follows: initial denaturation step at 95 °C for 5 min, followed by 40 cycles of denaturation at 94 °C for 15 s each, annealing at 58 °C for 1 min, extension at 72 °C for 45 s and a final extension step at 72 °C for 10 min. Samples showing a single band of the expected size range (600–800 bp) were cleaned using DNA Clean and Concentrator 5 kit (Zymo Research, Irvine, CA, USA). In total, 275 root sections were assayed. Bidirectional Sanger sequencing of all cleaned PCR product was carried out by McLab (South San Francisco, CA, USA). For few samples, we obtain sequence data only from one side, and those were included only if they were of high quality.

Sequence editing and alignment

Editing and assembly of raw sequences were performed in CodonCode Aligner (http://www.codoncode.com/aligner/). Bases with phred scores below 20 were converted to Ns (i.e. 'masked') using an in-house script. Sequences that contained more than 2% Ns after masking and end trimming were excluded using our 'purge' tool (http://www.borealfungi.uaf.edu; Taylor & Houston 2011). After the quality control steps, we grouped the remaining sequences from 30 of the 42 plants sampled into operational taxonomic units (OTUs) at 97% sequence similarity criterion using the genome assembly program CAP3 (Huang & Madan 1999). The longest and highest quality representative sequence from each OTU was used for further analyses. FASTA files of sequences were submitted to the fungal metagenomics website, http://www.borealfungi.uaf.edu, for fungal identity searches. Sequences generated in this study were deposited in GenBank (Table S2, Supporting information).

Data analyses

To estimate sequence divergence within fungal families, first, a separate multiple alignment of all individual sequences belonging to each of the three fungal families, that is, Ceratobasidiaceae, Sebacinaceae and Tulasnellaceae, was constructed using CLUSTALW (Thompson *et al*. 1994) within MEGA4 (Tamura *et al*. 2007). Next, mean pairwise sequence distance (*pi*; Nei & Kumar 2000; also reported in other literature as π) among all individual sequences within a fungal family was estimated based on a combined transition and transversion substitution model using MEGA4. We also estimated distances for published data using the same program to compare them with the distances among fungal sequences found in *P. yadonii*. For this comparison, we selected only those studies that have reported at least one of the three fungal families observed in *P. yadonii* and have reported at least five sequences per fungal family and per orchid species.

To test the statistical significance of the variation in fungal diversity between the two habitats, Pearson chi-square contingency test was performed based on counts of OTUs (i.e. numbers of root sections in which a particular OTU was found) across the two habitats. The test was performed in R (R Development Core Team 2010) using only the five most abundant OTUs, so that no expected cell frequencies would be below 5. Two-way hierarchical ordination analysis (Bray & Curtis 1957) based on the occurrence of the 26 OTUs was performed at the population level using PC-ORD (MJM Software, Gleneden Beach, OR, USA). Statistical significance of habitat-based variation in fungal community composition in orchid roots was tested with a multiple response permutation procedure (MRPP) using the same program.

Cumulative OTU diversity curves within and across habitats using the sample-based Mao Tao and Chao 1 methods were computed using ESTIMATES (version 8.2.0, available at: http://viceroy.eeb.uconn.edu/biota) to examine whether the number of OTUs increases with increasing sample size. Spatial autocorrelation analysis (Smouse & Peakall 1999) within and across habitats was performed using GENALEX (Peakall & Smouse 2006) to assess whether the geographically proximal populations have more similar sets of fungi. For this analysis, population-level geographic and OTU distance matrices were used. The OTU distance matrix was estimated based on presence and absence of each OTU in each population.

To better identify the *Piperia*-associated fungi following BLAST searches, we first constructed separate alignments

in MUSCLE (Edgar 2004) for each of the three fungal families, that is, Ceratobasidiaceae, Sebacinaceae and Tulasnellaceae (as in Taylor & McCormick 2008). Known orchid mycorrhizal fungal sequences were added to the data used to generate the trees to place the fungi sequenced in this study in the context of previously known orchid mycorrhizal fungi. The resulting alignments were improved by eye in SeAl (Rambaut 1996). Phylogenetic relationships among the fungi within each family were estimated by determining the best-fit substitution models according to AIC criteria in ModelTest (Posada & Crandall 1998) and then using the most similar available model to construct maximum-likelihood trees using the program GARLI (Zwickl 2006). Support values were estimated via 1000 bootstrap replicates in GARLI. Due to accelerated diversification of *Tulasnella* nuclear ribosomal regions, we followed previously published strategies in first constructing a family-level alignment using only the conserved and alignable 5.8S regions extracted from the full-length ITS sequences (Shefferson *et al.* 2005; Taylor & McCormick 2008), then calculated a maximum-likelihood tree in GARLI (Zwickl 2006). We used this tree to identify subclades within Tulasnellaceae containing the *Piperia* OTUs and constructed full-length ITS alignments from these subclades. We attempted to retain several well-identified reference sequences in each of the three subalignments. However, due to extreme divergence from identified taxa, this was not possible for the third alignment, containing only OTU10. The 5.8S tree was midpoint rooted due to lack of a defensible a priori outgroup. Subclade trees were rooted based on relationships shown in the broader 5.8S tree.

Nonmetric multidimensional scaling was used to ordinate fungal communities in orchid roots using PC-ORD (MJM Software) and to test for relationships between soil chemical properties and orchid-associated fungi in the two habitats. In addition to ordination analysis, Pearson correlation coefficient between soil chemical properties and abundance of fungal families according to habitats was estimated using SAS 9.2 (SAS Inc., Cary, NC, USA). Before performing analysis of Pearson correlation coefficients, data were normalized using an arcsine transformation. Bonferroni correction was applied to *P*-values to avoid the possibility of occurrence of correlation between fungal diversity and soil variables by chance. Further, an analysis of variance (ANOVA) was performed to test for differences in soil properties between the two habitats using SAS 9.2 (SAS Inc.).

Results

Twenty-six OTUs, representing three families, that is, Ceratobasidiaceae, Sebacinaceae and Tulasnellaceae, were identified from roots of *Piperia yadonii* plants across eight natural populations (Fig. 1 and Table 1). Tulasnellaceae was the most OTU rich with 15 OTUs followed by Ceratobasidiaceae and Sebacinaceae with eight and three OTUs, respectively (Table 1). Of the three fungal families, Ceratobasidiaceae and Tulasnellaceae were most frequently represented in *P. yadonii* roots with frequencies of 45% (68 sequences) and 42% (63 sequences), respectively. Sebacinaceae was the least frequent fungal group observed in *P. yadonii* with a frequency of 13% (20 sequences; Fig. 1). Across all plants and populations, infection of a single root system by 2–6 fungal OTUs was observed in 40% of all sampled plants, while the remaining 60% of the plants contained only one OTU (Fig. S1, Supporting information). A majority of the plants (22 of 30 plants) possessed fungi belonging to a single family, while only one plant had fungi belonging to all three families (Fig. S1, Supporting information). Roots of one plant from population BC contained fungi belonging to five OTUs representing all three fungal families detected in *P. yadonii*. In *P. yadonii*, mean pairwise sequence distance ($\pi = 0.231$, SE $\pm$ 0.026) among fungi belonging to Tulasnellaceae was higher than the distance observed among Ceratobasidiaceae ($P = 0.077$, SE $\pm$ 0.006) or Sebacinaceae sequences ($\pi = 0.076$, SE $\pm$ 0.003; Table 2). Additionally, the mean pairwise distance among sequences belonging to Tulasnellaceae was higher in *P. yadonii* than in other orchids used for comparison, except *Cypripedium californicum* ($\pi = 0.325$, SE $\pm$ 0.015) and *Tipularia discolor* ($\pi = 0.273$, SE $\pm$ 0.042; see Table 2 for results and references). Similarly, except in *Goodyera hachijoensis* ($\pi = 0.102$, SE $\pm$ 0.009), the distance among Ceratobasidiaceae sequences was higher in *P. yadonii* than in any other orchid species. Sequences belonging to Sebacinaceae were more similar to each other in all other orchid taxa than in *P. yadonii* (Table 2).

Except for the absence of Tulasnellaceae in the population JP, Ceratobasidiaceae and Tulasnellaceae were represented in roots of *P. yadonii* from all populations. Sebacinaceae was absent from maritime chaparral, and it was represented only in three populations (AP, BC and PQR) from the *Pinus radiata* forest habitat. The contingency test with the five most abundant OTUs was highly significant ($\chi^2 = 50.1194$, d.f. = 4, $P < 0.0001$) revealing that *P. yadonii* roots host different fungal communities in the two habitats. Results were equally significant when all 26 OTUs were analysed (data not shown), although low abundance of many OTUs violates the assumptions of the test. Similarly, population-wise two-way hierarchical ordination analysis (Bray & Curtis 1957) based on the 26 OTUs showed clustering of three maritime chaparral (BLMR, MP and PL) and three pine forest populations (BC, PQR and

Table 1 Number of root sections (i.e. sequences) representing each of the 26 operational taxonomic units (OTUs) of *Piperia yadonii* mycorrhizal fungi in eight populations from two habitats

	Pinus radiata forest habitat				Maritime chaparral habitat			
Fungal family/OTU type	AP	BC	PQR	SFB	BLMR	JP	MP	PL
Ceratobasidiaceae								
C1	6 (2)							
C2						8 (1)		
C3	4 (2)	12 (3)		7 (2)	2 (1)			
C4							8 (2)	
C5		2 (2)	1 (1)		5 (2)		3 (1)	6 (2)
C6						2 (1)		
C7						1 (1)		
C8						1 (1)		
Sebacinaceae								
S1	1 (1)	6 (2)	8 (2)					
S2			4 (2)					
S3		1 (1)						
Tulasnellaceae								
T1		7 (1)	4 (1)					
T2		1 (1)			3 (1)		4 (1)	
T3	9 (2)							
T4	3 (1)							
T5				8 (1)				
T6	1 (1)		12 (3)					1 (1)
T7				2 (1)				
T8							1 (1)	
T9			1 (1)					
T10			1 (1)					
T11				1 (1)				
T12				1 (1)				
T13				1 (1)				
T14				1 (1)				
T15				1 (1)				
Total	24 (4)	29 (6)	31 (6)	22 (3)	10 (4)	12 (1)	16 (3)	7 (3)

The first letter of an OTU name represents a fungal group: C, Ceratobasidiaceae; S, Sebacinaceae; T, Tulasnellaceae; and the number represents individual OTUs within a fungal group.
Values in parentheses indicate the total number of individual plants in which an OTU was represented.

SFB) according to the two habitat types (Fig. S2, Supporting information). The multiple response permutation procedure also showed segregation ($P = 0.010$) of OTUs between the two habitats (data not shown). Thus, fungal associations in *P. yadonii* are strongly influenced by habitat.

Sample-based cumulative OTU diversity curves showed that estimates of Chao 1 (44 OTUs) are higher than the estimates of Mao Tau (26 OTUs), indicating that OTU diversity has not been saturated with current sampling and that more OTUs would be found with additional sampling (Figs 2 and S3, Supporting information). Spatial autocorrelation analysis did not show any significant clustering (data not shown) of populations within and across habitats, indicating no grouping of fungi from geographically proximal populations.

A majority of the nodes in the maximum-likelihood trees were supported with bootstrap values $\geq 80\%$ (Figs 3 and S4, Supporting information). Among the Ceratobasidiaceae, 75% of the OTUs were closely related to uncultured Ceratobasidiaceae isolates known to occur in *Goodyera pendula* (Shefferson *et al.* 2010), *Goodyera tesselata* (Shefferson *et al.* 2010) and *Epipactis gigantea* (Bidartondo *et al.* 2004). *Goodyera tesselata* and *E. gigantea* are terrestrial orchids native to the United States and Canada, while *G. pendula* has a wide distribution covering Scandinavia to East Asia, the Pacific Islands, New Guinea and Australia to coniferous forests in North America (Bidartondo *et al.* 2004; Shefferson *et al.* 2010). The remaining OTUs of Ceratobasidiaceae were related either to uncultured Ceratobasidiaceae found in ectomycorrhizal root tips of tropical forests of Malaysia (Peay *et al.* 2010) or to an uncultured fungus

Table 2 Mean pairwise fungal internal transcribed spacer sequence distances (π; Nei & Kumar 2000), estimated based on combined transitions and transversions substitution model, within fungal families identified in roots of *Piperia yadonii*, and from previously published data

	Mean pairwise sequence distance, π (±SE)						
Orchid species	*N*	Ceratobasidiaceae	*N*	Sebacinaceae	*N*	Tulasnellaceae	References
Photosynthetic orchids							
*P. yadonii**	71	0.077 (0.006)	22	0.076 (0.003)	58	0.231 (0.026)	This study
Anacamptis laxiflora†	12	0.071 (0.006)			12	0.186 (0.009)	Girlanda *et al.* (2011)
Ophrys fuciflora†	7	0.063 (0.006)			12	0.225 (0.010)	
Orchis purpurea†					9	0.089 (0.008)	
Serapias vomeracea†	8	0.038 (0.004)			27	0.097 (0.007)	
Chiloglottis aff. *jeanesii**					14	0.014 (0.003)	Roche *et al.* (2010)
Chiloglottis trapeziformis†					12	0.006 (0.002)	
Goodyera foliosa†	7	0.062 (0.009)					Shefferson *et al.* (2010)
Goodyera hachijoensis‡	5	0.103 (0.009)					
Goodyera tesselata†	5	0.044 (0.006)					
Cypripedium arietinum‡					7	0.177 (0.010)	Shefferson *et al.* (2007)
Cypripedium calceolus‡					12	0.005 (0.002)	
Cypripedium californicum‡					20	0.325 (0.015)	
Cypripedium candidum†					7	0.003 (0.002)	
Cypripedium fasciculatum‡					17	0.094 (0.006)	
Cypripedium guttatum‡					6	0.109 (0.008)	
Cypripedium japonicum†					18	0.033 (0.006)	
Cypripedium macranthon†					35	0.133 (0.015)	
Cypripedium montanum‡					11	0.206 (0.010)	
Cypripedium parviflorum†					20	0.193 (0.012)	
Cypripedium reginae†					17	0.193 (0.012)	
C. fasciculatum‡					20	0.116 (0.009)	Shefferson *et al.* (2005)
C. montanum‡					10	0.087 (0.011)	
C. parviflorum†					8	0.097 (0.012)	
Goodyera pubescens†					18	0.008 (0.002)	McCormick *et al.* (2004)
Liparis liliifolia†					8	0.001 (0.001)	
Tipularia discolor†					17	0.273 (0.042)	
Nonphotosynthetic orchids							
Hexalectris arizonica‡			27	0.014 (0.002)			Kennedy *et al.* (2011)
*Hexalectris colemanii**			9	0.054 (0.007)			
Hexalectris grandiflora‡	6	0.023 (0.003)					
Hexalectris nitida‡			9	0.029 (0.004)			
Hexalectris spicata†			53	0.048 (0.005)			
Neottia nidus-avis†			23	0.050 (0.005)			Selosse *et al.* (2002)

N, number of sequences.
Values in parentheses are standard errors (±SE) obtained after 1000 bootstraps.
*Restricted distribution.
†Widely distributed.
‡Moderate distribution range.

collected from an air filter (Fröhlich 2009). All three Sebacinaceae OTU sequences were closely allied with a fungus known to occur in *Dactylorhiza incarnata* (Illyes *et al.* 2009), a temperate orchid native to Europe. A vast majority (11 of 15) of the Tulasnellaceae OTUs appeared to be novel phylotypes (Figs 3 and S4, Supporting information), as they were not closely related to any previously accessioned sequences. The remaining four Tulasnellaceae OTUs were related to either *Tulasnella* spp. (GenBank accession DQ061110) or uncultured Tulasnellaceae isolates known from roots of *Cypripedium guttatum* (Shefferson *et al.* 2007) or *Platanthera chlorantha* (Bidartondo *et al.* 2004). The OTU 26 was not included in the family-level 5.8S tree of Tulasnellaceae (Fig. S4, Supporting information) because it was missing the entire 5.8S region.

Nonmetric multidimensional scaling ordination analysis of fungal communities in orchid roots followed by analysis of correlations with soil chemical properties showed that distinction in orchid-associated fungi is

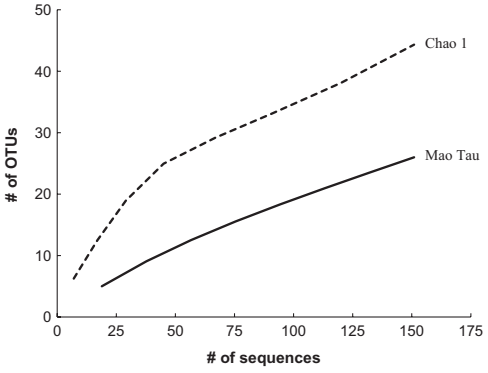

Fig. 2 Sample-based observed (Mao Tau) and rarefaction (Chao 1) cumulative operational taxonomic unit (OTU) diversity curves for *Piperia yadonii*.

most strongly related to aluminium (Al) and manganese (Mn) concentrations between the two habitats (Fig. S5, Supporting information). However, the Pearson correlation coefficient after Bonferroni correction (adjusted $P = 0.003$) did not show any significant association between any of the 16 soil variables and abundance of each of the three fungal families (Tables S3 and S4, Supporting information). Similarly, ANOVA also did not show any variation in the concentration of soil variables between the two habitats after Bonferroni correction (adjusted $P = 0.003$).

Discussion

Although we expected a narrow association with mycorrhizal fungi in the endemic and narrowly distributed *Piperia yadonii*, we detected a range of fungi including 26 OTUs across three fungal families, that is, Ceratobasidiaceae, Sebacinaceae and Tulasnellaceae (Table 2, Fig. 3). Hence, the mycorrhizal diversity of *P. yadonii* is remarkably high considering that these three Agaricomycetes families account for the vast majority of known photosynthetic orchid associates (Warcup & Talbot 1967; Dearnaley 2007; Taylor & McCormick 2008). Furthermore, the rarefaction curves (Mau Tao and Chao 1 in Figs 2 and S3, Supporting information) showed that higher number of OTUs might have been detected had we investigated additional roots and/or plants. This further confirms that *P. yadonii* has a wide range of mycorrhizal fungal associates. In agreement with the level of fungal diversity reported in our study, Sharma *et al.* (2007) also reported several taxa of fungi in *P. yadonii* in the same populations except BLMR.

The comparisons of mean pairwise distance among sequences belonging to *P. yadonii* and other orchid species demonstrate that *P. yadonii* is the least specific among the surveyed orchids. And the few orchids that have larger *P*-values are only associated with one or two fungal families despite the fact that they were sampled over a much larger range of habitats and/or geographic areas, which would tend to inflate their *P*-values relative to *P. yadonii*. This provides strong quantitative evidence that *P. yadonii* has low specificity compared to nearly all other similarly characterized orchids.

While not tested experimentally in this study, the high level of mycorrhizal diversity observed in *P. yadonii* indicates that the global rarity of this species is likely not linked to extreme mycorrhizal specificity. This suggests that *P. yadonii* is likely an opportunist in forming mycorrhizal associations, although some discernible habitat-specific patterns in fungal associations were documented (Table 1, Figs 1 and S2, Supporting information). Similar to our observations, Phillips *et al.* (2011) reported no link between mycorrhizal specificity and rarity of two photosynthetic terrestrial orchids *Drakaea elastica* and *Drakaea micrantha* although the formation of mycorrhizae being mostly restricted to the same fungal lineage, and a specific microhabitat was implicated. In contrast, specific mycorrhizal association was estimated to be the primary cause of rarity for the photosynthetic terrestrial orchid *Caladenia huegelii* (Swarts *et al.* 2010).

Presence of multiple fungal taxa in a single root system was detected in *P. yadonii*. This phenomenon has also been reported in other photosynthetic orchids, for example, *Platanthera* spp. (Zelmer *et al.* 1996); *Tipularia discolor* (McCormick *et al.* 2004); *Cephalanthera damasonium*, *Epipactis atrorubens* and *Platanthera chlorantha* (Bidartondo *et al.* 2004); *Aphyllorchis* spp. (Roy *et al.* 2009) and *Orchis* spp. (Jacquemyn *et al.* 2010; Lievens *et al.* 2010). Individual root systems hosting several fungal taxa are routinely documented in nonorchid species, which typically have much lower fungal specificity (Lodge & Wentworth 1990; Perotto *et al.* 1994, 1996; Monreal *et al.* 1999). In contrast, individual root systems of mycoheterotrophic orchids, for example, *Corallorhiza maculata* (Taylor & Bruns 1999), *Hexalectris revoluta* (Taylor *et al.* 2003b) and *Neottia nidus-avis* (Selosse *et al.* 2002) and photosynthetic orchid *Goodyera pubescens* (McCormick *et al.* 2006) were reported to be colonized by a single fungal taxon. Collectively, these observations indicate that multiple fungi in a single root system may occur more often in photosynthetic than in mycoheterotrophic orchids, but that this characteristic is not universal in orchid mycorrhizal systems.

Most of the known orchid mycorrhizal fungi are saprotrophic, meaning they do not depend on mycorrhizal

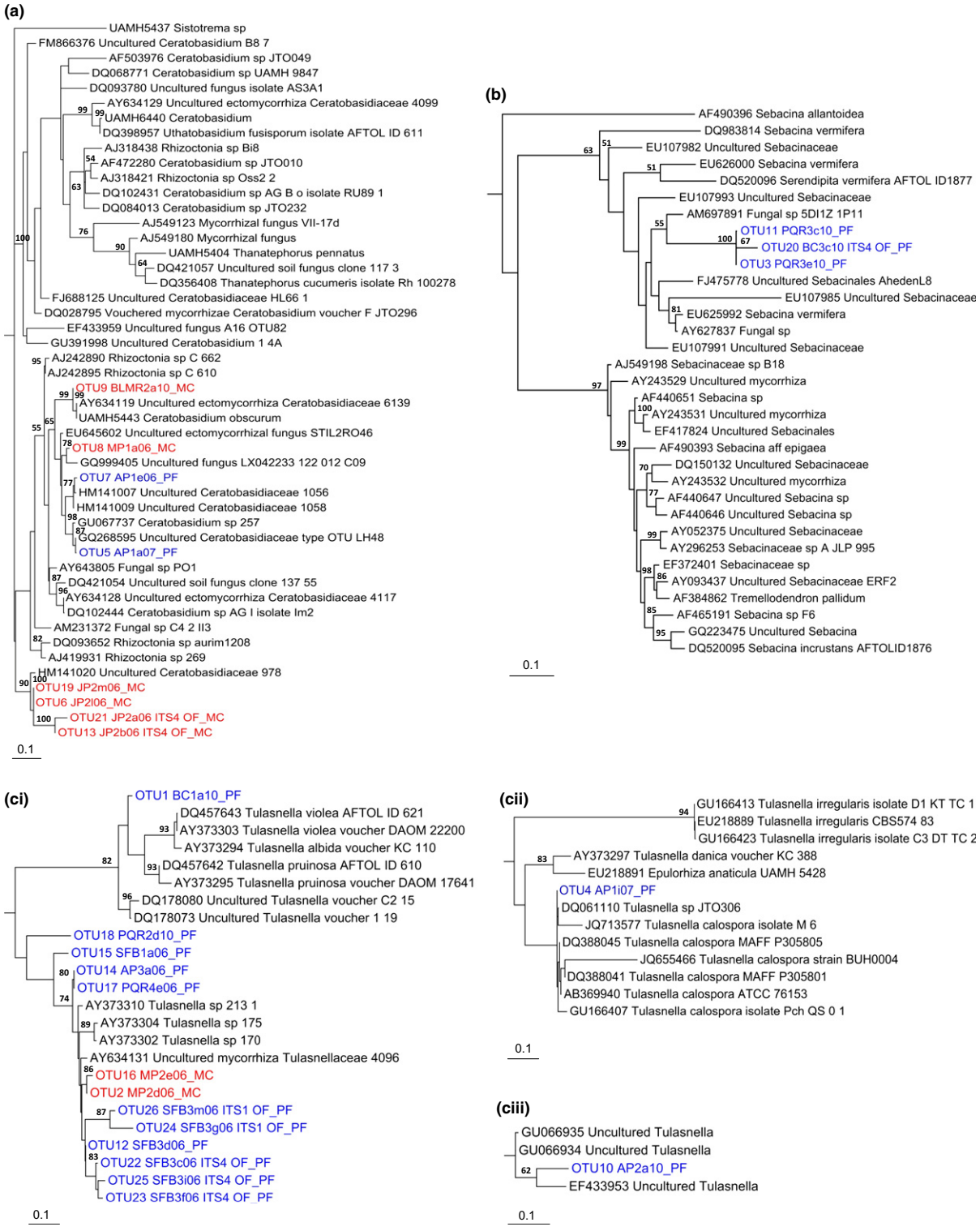

Fig. 3 Maximum-likelihood trees constructed with operational taxonomic unit (OTU) sequences from three fungal families, that is, Ceratobasidiaceae (a), Sebacinaceae (b) and Tulasnellaceae (c, subclade trees, i, ii and iii) observed in *Piperia yadonii* roots collected from *Pinus radiata* forest (habitat code PF) and maritime chaparral (habitat code MC) habitats. Ceratobasidiaceae (a) and Sebacinaceae (b) trees were rooted with *Sistotrema* sp. (GenBank accession UAMH5437) and *Sebacina allantoidea* (GenBank accession AF490396), respectively. Tulasnellaceae trees (c) were rooted with midpoint method. Only bootstrap values ≥ 50 are shown above nodes. Scale bar in the figure represents estimated number of DNA substitutions per site.

symbiosis for their survival and can live and reproduce in the soil freely (Roberts 1999; Rasmussen 2002). Fungi from the three fungal families detected in *P. yadonii* are also usually saprotrophic, although certain clades within the Tulasnellaceae and Sebacinaceae also form ectomycorrhizae (ECM; Selosse *et al.* 2002; Bidartondo *et al.* 2004; Weiss *et al.* 2004), while fungi belonging to Ceratobasidiaceae can occur as endophytes in some conifer trees (Eriksson & Ryvarden 1973; Sen *et al.* 1999). However, we observed the Ceratobasidiaceae OTUs primarily in the maritime chaparral habitat, although some of these were also found in the *Pinus radiata* forest habitat. While these fungi may not depend entirely on their partner plants, the abundance of mycorrhizal fungi is known to be influenced by that of their plant hosts (Johnson *et al.* 2006; Kulmatiski *et al.* 2008). Strong variation in the dominance of orchid mycorrhizal fungi across habitat types was observed in *P. yadonii* (results of contingency test, Table 2, Figs 1 and S2, Supporting information). The fungi from Sebacinaceae were absent in orchid roots collected from the maritime chaparral, but they were present in the roots from *P. radiata* forest habitat, indicating that the *P. radiata* forest offers more options to the orchids for forming mycorrhizal associations. Considering that these forests are ECM dominated, while the chaparral is ericoid/arbutoid fungi dominated, it may be that the observed Sebacinaceae fungi form ECM with *Pinus*. However, these three Sebacinaceae OTUs do not fall into the exclusively ectomycorrhizal clade-A identified by Weiss *et al.* (2004). Ramsay *et al.* (1987), using morphological observation, also reported variation across habitats in mycorrhizal associations in several photosynthetic terrestrial orchids in Australia; they observed that some fungal species were present only in dry habitats, others in wetter coastal regions and yet others in non-native pine plantations. Similarly, variation in fungal association across habitats was also observed in the mycoheterotrophic orchids *C. maculata* and *Corallorhiza mertensiana* when Taylor & Bruns (1999) compared the mycorrhizae of these orchids across vegetation and elevation gradients. We found that the influences of habitat on fungal associations in *P. yadonii* are stronger than have been shown previously in other orchids. This observation supports our contention that despite its extremely narrow distribution, *P. yadonii* has little mycorrhizal specificity, instead displaying promiscuous associations, at least in the adult phase.

A variable not tested in this study, but of relevance, is the successional variability in orchid–fungal associations in relation to the life stage of a plant. Specificity during germination and early development has been detected in orchid taxa, whereas the adult phase of plant growth in the same species can have less specific fungal alliances (Sharma *et al.* 2003; Bidartondo & Read 2008). Accordingly, fungi required for germination and recruitment of *P. yadonii* might be different from those associated with adult plants. This question deserves further study, although fungal amplifications from very young seedlings are not yet available and *P. yadonii* has so far resisted *in vitro* germination attempts (VLY, personal observation). Further, capsule and seed production is often low because of low fruit set and high herbivory of inflorescences, which limit seed availability for destructive *in situ* germination tests.

Considering the size of the orchid family, which is the largest among angiosperms, it is not surprising that results similar to those as ours, as well as those contrasting ours, have been reported before for various photosynthetic taxa. It is apparent that few generalizations can be made about orchid–fungal specificity, diversity, influence on rarity or commonness of the plant hosts and seasonal variation, etc., especially for photosynthetic orchids. For example, rarity in the Orchidaceae has been linked to pollen or nectar reward and/or deceit, pollinator specificity and pollination mechanisms (Neiland & Wilcock 1998). Nectariferous orchids in temperate regions tend to be more successfully pollinated and have higher fruit set, while nectarless temperate taxa in the British Isles are also generally rare; at the same time, these relationships are not obvious in tropical orchids (Neiland & Wilcock 1998). *Piperia yadonii* is a fragrant, nectariferous, putatively cross-pollinated temperate species that is pollinated by night-flying, short-tongued moths in the Pyralidae, Geometridae, Noctuidae and Pterophoridae. Other insects, including diurnal *Bombus* spp. and a night-flying mosquito, have also been observed carrying pollen in certain populations (USFWS 2004). Most of the pollinators for the orchid are known to be common or very common, which indicates that the orchid may not be pollinator limited although published data are not available to support these field observations.

Additionally, empirical studies have not generally correlated fruit set success with *in situ* seed germination and/or seedling recruitment success, which involve the fungal partners directly. This, indeed, highlights the importance of studies that combine the assessment of several biological traits, including biotic interactions such as mycorrhizal specificity, simultaneously to test cause-and-effect hypotheses for orchid rarity. It is also evident that taxon-specific studies within the Orchidaceae, especially for aiding conservation management of rare species, are necessary. Patterns of mycorrhizal associations in plants are likely more complex than previously thought. Our work, along with evidence from other studies, indicates that plant families considered

unique in their mycorrhizal associations can also exhibit a wide range of ecological strategies at the species level.

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M.P. performed laboratory analyses, parts of the data analysis and wrote the first draft of the manuscript; J.S. designed the research, collected samples, provided overall guidance and research direction, wrote sections of text and edited the complete manuscript; D.L.T. performed parts of the data analysis, wrote sections of text and edited the complete manuscript; V.L.Y. guided field collections, relayed taxon-specific information and assisted with field work.

Data accessibility

DNA sequences: GenBank accession numbers JQ972064–JQ972131 and JQ994393–JQ994475.

Supporting information for each sequence of fungal DNA obtained from roots of *Piperia yadonii* is presented in the Table S2 (Supporting information).

Data for soil variables are presented in the Table S3 (Supporting information).

Supporting information

Additional supporting information may be found in the online version of this article.

Fig. S1 Number of plants containing different number of fungal families (a) and OTUs (b) in single plants of *Piperia yadonii* across eight populations.

Fig. S2 Two-way hierarchical cluster tree and matrix coding based on 26 operational taxonomic units (OTUs) observed in eight populations of *Piperia yadonii* from the *Pinus radiata* forest (blue) and maritime chaparral (red) habitats.

Fig. S3 Sample-based observed (Mao Tau) and rarefaction (Chao 1) cumulative OTU diversity curves of *Piperia yadonii* in Pine forest and maritime chaparral habitats.

Fig. S4 Maximum likelihood tree of the fungal family Tulasnellaceae constructed with operational taxonomic unit (OTU) sequences observed in *Piperia yadonii* roots collected from *Pinus radiata* forest (habitat code PF) and maritime chaparral (habitat code MC) habitats.

Fig. S5 Population level non-metric multidimensional scaling (NMS) ordination of the fungal communities of *Piperia yadonii* in eight populations from *Pinus radiata* forest (blue) and maritime chaparral (red) habitats, with significant correlation of community axes to the 14 soil mineral nutrients, pH, and electric conductivity (EC) depicted by the labeled vectors.

Table S1 Population code, habitat, and year(s) of sampling for mycorrhizal fungi across eight populations of *Piperia yadonii* located in Monterey County, California.

Table S2 Supporting information for each sequence of fungal DNA obtained from roots of *Piperia yadonii*.

Table S3 Soil chemical characteristics of seven sites from where *Piperia yadonii* plants were sampled for mycorrhizal analyses.

Table S4 Pearson's correlation coefficients (*r*) for number of sequences belonging to each family and soil chemical characteristics.