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MYCORRHIZAL DIVERSITY OF AN ENDEMIC TERRESTRIAL ORCHID

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Mycorrhizal associations of the Orchidaceae - the largest and most diverse angiosperm family with an estimated number of taxa ranging between 17,000 and 35,000 - are highly complex. Plant-fungus interactions in this family can range from obligate- or facultative mycotrophy, mutualism, to minimal dependence on fungi in adulthood (Smith & Read 1997, Sivasithamparam *et al.* 2002, Taylor *et al.* 2002, Cameron *et al.* 2006). Terrestrial orchids also generally exhibit high habitat specificity. A unique ecological reliance upon mycorrhizal fungi is one of the reasons for habitat specificity in orchids. The fungal association is especially critical during the seed germination phase, but continues through the life-cycle of the plants, especially in the obligate mycotrophic species. It can therefore be expected that recruitment and persistence of orchids in their natural habitat depends on the availability of suitable fungi, which also form an underground network that connects orchids with the surrounding vegetation. Fungal ecology often is overlooked in orchid conservation projects, but if orchid-fungus relationships are very specific, the persistence of a population and/or success of a reintroduction program will be affected greatly by the presence or absence of suitable fungi in the soil, and by the habitat's ability to continuously support these fungi.

Piperia yadonii R. Morgan & J. Ackerman (a photosynthetic terrestrial) is endemic to Monterey County in California and is listed endangered by the US Fish and Wildlife Service. The Monterey area is part of the California Floristic Province Biodiversity Hotspot and represents the northern or southern limit of natural distribution of many plants, several of which co-occur with this orchid. *Piperia yadonii* occurs in two distinct habitats within its natural

range: 1) lower elevation *Pinus radiata* D. Don forests with an herbaceous, sparse understory; and 2) higher elevation ridges in maritime chaparral growing beneath dwarfed *Arctostaphylos hookeri* G. Don shrubs. Biodiversity Hotspots are characterized both by exceptional levels of plant endemism and by dangerous levels of habitat loss. Only a few extant populations of *P. yadonii* remain and/or are known, and these are seriously threatened because of the increasing pressure from urban and recreational development in the area. One of the goals of USFWS is to downlist the species as threatened instead of delisting, primarily because its biology is not yet well enough understood to set delisting as an objective of the Recovery Plan (U.S. Fish and Wildlife Service 2004). Prior to this work, mycobionts of *P. yadonii* have not been studied. The specific objectives of this research were to: (1) identify fungal diversity associated with *P. yadonii* at selected sites/populations along two, a longitudinal and a latitudinal, transects across its natural distribution, and (2) identify how the fungal diversity of the orchid is related to both habitat types, and to the size of the orchid populations.

Materials and Methods

Roots of *Piperia yadonii* were collected between February and March of 2005 and 2006 from several locations across its natural distribution. Soil samples also were collected from each site to determine the soil chemical properties at each location. All materials were transported at 4°C to the laboratory. Root sections were processed by using culture dependent and culture independent methods to assess the identity and diversity of the culturable and non-culturable mycobionts of *P. yadonii*. To obtain pure cultures of the peloton-forming fungi, we slightly modified the

method described in Sharma *et al.* (2003). When pure cultures of fungal isolates were obtained, DNA was extracted from these isolates. Fungal internal transcribed spacer (ITS-1) region then was amplified by using the primer pair ITS-1F/ITS-4 (Gardes & Bruns 1993). Digestion with restriction enzymes was used to obtain restriction fragment length polymorphism (RFLP) data. Based on the RFLP data, we subsequently selected isolates with unique profiles and subjected these to sequencing by using T7 and SP6 primers. For identifying the fungi by using culture independent method, thin sections of roots first were examined under the microscope to locate regions which contained fungal pelotons. We then extracted total DNA from these sections. In this case also, we amplified the fungal ITS-1 region by using the same primer pair as above, but followed with cloning the product. Clones were digested with *EcoRV* before selecting the unique profiles and subjecting these selected clones to sequencing with the same primers as above. In both cases, only those sequences whose T7 and SP6 (reverse complement) sequences matched completely were used further. Similar sequences were searched by using BLAST program (Altschul *et al.* 1997) by accessing the NCBI web-page (<http://www.ncbi.nlm.nih.gov/blast/Blast.cgi>). We used the default settings to conduct these searches.

Results and Discussion

Preliminary results show that fungi associated with *Piperia yadonii* primarily belong to the taxonomic groups Ascomycetes and Basidiomycetes, some of which have previously been reported as ectomycorrhizae of coniferous trees (Table 1). While some of the fungi identified thus far in our work have been reported from temperate terrestrial orchids, photosynthetic and non-photosynthetic, many have not previously been reported as orchid endophytes. Other fungi belonged to *Phomopsis* (Ascomycota), *Ceratobasidium* (Basidiomycota), *Neonectria* (Ascomycota), and *Phialocephala* (Ascomycota), which are reported to be coniferous ectomycorrhizae. *Ceratobasidium* spp., which are known to associate with many terrestrial orchids worldwide (Zettler *et al.* 2003), occurred at several sites including both *Pinus radiata* and *Arctostaphylos* spp. dominated habitats.

Insofar, few individual species or taxonomic groups of fungi were observed at locations excepting MC (Table 1). This is a site which is close to urban areas and harbors a very small population of the orchid. On the other hand, the other sites, including those which harbor large numbers of orchids and/or are more isolated, yielded a low diversity of fungi associated with these plants. Because we are now using additional primers to amplify (in order to identify fungi unidentified this far) the fungal ITS-1 region of the same DNA samples, our forthcoming results may reveal a different pattern of fungal diversity in these locations.

While conserving plant diversity in biodiverse habitats is recognized as an outstanding global priority, there has been limited recognition of the macro level conservation in relation to ecosystem functioning at the micro level (Sivasithamparam *et al.* 2002). This is a result of a lack of knowledge and understanding of the micro level communities. Our results presented herein indicate that Ascomycetes, especially those forming ectomycorrhizae with the surrounding vegetation, may be equally critical to the persistence of this orchid as might be the saprophytic fungi or the Basidiomycetes from the *Rhizoctonia* complex, which are generally believed to be the primary orchid endophytes.

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TABLE 1. Fungal ITS-1 region of the mycobionts associated with roots of *Piperia yadonii*, an endemic terrestrial species, was sequenced and matched with previously known, similar sequences by using the BLAST program (Altschul *et al.* 1997) by using the NCBI web-page (<http://www.ncbi.nlm.nih.gov/blast/Blast.cgi>).

Previously Listed Source/Host	Closest sequence(s) found in GenBank by BLAST	Taxonomic Classification	Reference
MC^z Pine O-horizon soil	Uncultured ascomycete	Eukaryota; Fungi; Ascomycota	O'Brian <i>et al.</i> 2005
<i>Picea abies</i> decayed root	<i>Phomopsis columnaris</i>	Eukaryota; Fungi; Ascomycota	Menkis <i>et al.</i> 2006
Isolated from roots of <i>Triticum</i> sp.	<i>Phomopsis</i> sp.	Eukaryota; Fungi; Ascomycota	Carter <i>et al.</i> 1999
Mycorrhizal root tips (host <i>Cephalanthera damasonium</i>)	Uncultured mycorrhizal ascomycete	Eukaryota; Fungi; Ascomycota	Julou <i>et al.</i> 2005
Cultured fungus from mycorrhizal hemlock root tip	<i>Phialocephala fortinii</i>	Eukaryota; Fungi; Ascomycota	Lim <i>et al.</i> unpubl. data
<i>Tricholoma matsutake</i> fairy rings in a natural <i>Pinus densiflora</i> forest	Uncultured ectomycorrhizal fungus	Eukaryota; Fungi	Lian <i>et al.</i> 2005 (published only in NCBI database)
Natural <i>Tuber magnatum</i> truffle-ground	Uncultured Nectriaceae	Eukaryota; Fungi; Ascomycota	Murat <i>et al.</i> 2005.
<i>Pinus sylvestris</i> decayed root	<i>Neonectria macrodidyma</i>	Eukaryota; Fungi; Ascomycota	Menkis <i>et al.</i> 2006
BC <i>Picea abies</i> wood disc	<i>Ceratobasidium</i> sp.	Eukaryota; Fungi; Basidiomycota.	Menkis <i>et al.</i> 2004
PQR Environmental sample	Uncultured ectomycorrhiza	Eukaryota; Fungi; Basidiomycota	Murat <i>et al.</i> 2005
SFB <i>Picea abies</i> wood disc	<i>Ceratobasidium</i> sp	Eukaryota; Fungi; Basidiomycota	Menkis <i>et al.</i> 2004
PL <i>Taxus chinensis</i> var. <i>mairei</i>	Fungal endophyte	Eukaryota; Fungi; Ascomycota	Wang & Wang Unpublished
Not listed	<i>Cercophora sparsa</i> voucher	Eukaryota; Fungi; Ascomycota	Miller & Huhndorf 2004
JP Soil	Uncultured soil fungus	Eukaryota; Fungi	Waldrop <i>et al.</i> unpubl. data
MP <i>Picea abies</i> wood disc	<i>Ceratobasidium</i> sp.	Eukaryota; Fungi; Basidiomycota	Menkis <i>et al.</i> 2004

^zMC, BC, PQR, SFB, PL, JP, and MP are codes for locations from which roots of *Piperia yadonii* were collected.

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Maria L. Ishida has technical expertise in microbiology, biochemistry, and molecular biology. She earned her Ph.D. in Biochemistry at the Federal University of Parana, Brazil, and currently is a Biological Scientist for the rhizosphere microbiology program in the Department of Environmental Horticulture at University of Florida NFREC.

Vernal L. Yadon is a botanist (*Piperia yadonii* is named after him) and Director Emeritus of the Pacific Grove Museum of Natural History where he served for 35 years. He received an M.S. from Oregon State University, Corvallis, Oregon, USA, in Fish and Game Management. He currently serves as a consultant to the review committee that passes on proposed changes for The Inventory of Rare and Endangered Plants of California. His research continues to be a database of vouchers of Monterey County plants. He has authored a Revision of the Vascular Plants of Monterey County, California, in press.