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Mycobiont overlap between two mycoheterotrophic genera of Monotropoideae (*Pterospora andromedea* and *Sarcodes sanguinea*) found in the Greater Yellowstone Ecosystem

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Abstract *Pterospora andromedea*, a mycoheterotroph, has been shown to form obligate symbioses with only three species of *Rhizopogon* in section *Amylopogon: R. salebrosus, R. arctostaphyli* and an undescribed molecular taxon. *Sarcodes sanguinea*, another my coheterotroph in Ericaceae, and sister taxon to *Pterospora andromedea,* has been found to form symbioses with two species of *Rhizopogon* in section *Amylopogon: R. ellenae* and *R. subpurpurascens*. To date no overlap has been recorded between these two achlorophyllous plants and their associated mycobionts. Tissue from *Pterospora andromedea* rootballs and *Rhizopogon spp.* basidiocarps were collected from the Greater Yellowstone Ecosystem. The mycobionts were identified using sequence analysis of the ITS locus and compared with sequences of *Rhizopogon spp.* section *Amylopogon* from GenBank. Sequences of two additional loci, ATP6 and RPB2 were also generated and analyzed. In addition to *Rhizopogon salebrosus*, *Pterospora andromedea* was found for the first time in association with a fourth mycobiont, *Rhizopogon ellenae*, a known associate of *Sarcodes sanguinea*. The discovery of a new symbiont may provide evidence for an undiscovered lineage of *Pterospora andromedea* inhabiting the Greater Yellowstone Ecosystem. In addition, overlap in obligate mycobionts between closely related mycoheterotrophs provides interesting new information on the phylogenetic history and coevolution of the mycoheterotrophs in the Monotropoideae (Ericaceae).

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1 Introduction

Monotropoideae (Ericaceae) is mainly comprised of mycoheterotrophic plants (Bidartondo and Bruns 2002; Hynson and Bruns 2009). These achlorophyllous plants form a tripartite relationship with an autotrophic host via an obligate mycorrhizal fungus to procure their carbon requirements. Although some generalist relationships do exist, (Martos et al. 2009; Roy et al. 2009; Hynson and Bruns 2009), many mycoheterotrophs are specific to certain genera, species groups or individual species of fungi (Leake 2004; Bidartondo and Bruns 2002). Three tribes of mycoheterotrophs are recognized in Monotropoideae: Pterosporeae, Monotropeae and Pyroleae. All species within Pterosporeae and Monotropeae form a specialized association with specific fungal taxa, genera or species, whereas some species of Pyroleae are now known to form more general associations with a broader range of mycobionts (Bidartondo and Bruns 2005; Bidartondo 2005; Bidartondo and Bruns 2002, 2001; Hynson and Bruns 2009, 2010; Tedersoo et al. 2007).

An interesting monospecific plant in Monotropoideae is *Pterospora andromedea* Nutt. in tribe Pterosporeae, (Hazard 2006; Massicotte et al. 2005; Bidartondo and Bruns 2005, 2002; Robertson and Robertson 1982; Bakshi 1959). *P. andromedea* forms highly specific associations with *Rhizopogon* species in section *Amylopogon* A.H.Sm. (Bidartondo and Bruns 2005, 2002; Bruns and Read 2000; Cullings et al. 1996) and can reproduce sexually or asexually (Bakshi 1959). This symbiotic relationship is

so tightly linked that seeds from *P. andromedea* plants only form mycorrhizal associations with *Rhizopogon* species associated with the parental plant, despite the fact that many closely related fungal species may induce the seeds to germinate (Bidartondo and Bruns 2005; Bruns and Read 2000). Furthermore, gene-for-gene selection has been hypothesized to occur between lineages of *P. andromedea* as well as within lineages of the different *Rhizopogon* species, potentially representing even higher levels of specificity than previously observed (Bidartondo and Bruns 2005).

To date only three species groups in *Rhizopogon* have been found in association with *P. andromedea* over its entire endemic range of North America, albeit through relatively limited sampling: *Rhizopogon salebrosus* A.H.Sm., *R. arctostaphyli* A.H.Sm., and an unidentified molecular taxon (Hazard 2006; Bidartondo and Bruns 2005; Kjøller and Bruns 2003; Cullings et al. 1996; Kennedy and Bruns 2005). These *Rhizopogon* species exhibit geographically limited distributions. Specifically, *R. arctostaphyli* is restricted to the west coast. The unidentified molecular taxon is the only mycobiont found in eastern North America. However, this has only been confirmed through limited sampling as it has only been found once in Quebec and several times within Michigan (Hazard 2006; Bidartondo and Bruns 2005). *R. salebrosus* has the widest geographic range, extending from the Pacific coast into the Rocky Mountains (Dowie et al. 2011; Bidartondo and Bruns 2005; Bidartondo and Bruns 2002).

Previous sampling of 97 *Pterospora andromedea*, rootballs distributed throughout the south-central Rocky Mountains (Dowie et al. 2011) conclusively identified the mycobiont as exclusively *Rhizopogon salebrosus*. The present study reports for the first time a fourth fungal symbiont of *P. andromedea*, *R. ellenae*, occurring in sympatry with *R. salebrosus* in the Greater Yellowstone Ecosystem (Fig. 1). This finding is important because this is the first documented instance of mycobiont overlap between *P. andromedea* and *Sarcodes sanguinea*, even though, *S. sanguinea* does not currently occur in the Yellowstone area. This location is considered a biodiversity hotspot situated near the southern extent of the Laurentide Ice Sheet, and directly in the zone of hypothesized active migration of both potential autotrophic hosts and their fungal associates following glaciation (Hanna et al. 2007; Carstens et al. 2005; Soltis et al. 1997). Overlap in obligate mycobionts between closely related mycoheterotrophs provides interesting data on the phylogenetic history and coevolution of the mycoheterotrophs in the Monotropoideae (Ericaceae). It also illustrates the need for more extensive surveys of mycoheterotrophs and associated mycobionts in order to accurately decipher the evolutionary ecology of these obligate associations.

2 Material and methods

2.1 Sample collection and preparation

Nine basidiocarps of *Rhizopogon spp*. and two tissue samples from distinct *Pterospora andromedea* mycorrhiza rootballs were collected in *Pinus ponderosa* var *scopulorum* Engelm, *P. contorta* var *latifolia* Engelm. and *P. albicaulis* Engelm. stands in the Greater Yellowstone Ecosystem (Table 1). A portion of each rootball was carefully exposed and mycorrhiza tissue was collected in situ and then the rootball was reburied. Each mycorrhiza sample was placed into two separate 2 ml tubes with 95% ethanol and kept at 4°C until processed. In the lab, mycorrhiza samples were thoroughly cleaned with DI H2O to minimize any potential DNA contaminants, and tissue was taken from basidiocarps.

2.2 Molecular methods

DNA was extracted following a CTAB protocol with a modified Gene Clean III DNA purification process (Bergemann and Miller 2002). PCR primers for three loci were successfully used to amplify and sequence the DNA for analysis, and included the nuclear Internal Transcribed Spacer region (ITS) using ITS-1F/ITS-4B (Gardes and Bruns 1993; White et al. 1990), the mitochondrial ATPase subunit 6 (ATP6) using ATP6-2/ATP6-3 (Kretzer and Bruns 1999), and the second largest subunit of RNA polymerase II (RPB2) using RPB2-5F/RPB2-7R (Liu et al. 1999; Matheny 2005). Both the noncoding ITS and coding RPB2 nuclear gene regions have fungal specific primers available; the coding mitochondrial ATP6 locus does not.

Rhizopogon ellenae, R. salebrosus and *R. subpurpurascens* basidiocarps in this study were used to confirm successful amplification of the target DNA. The protocol for amplification of ITS was an initial denaturing at 95°C for 2 min followed by 34 cycles: 95°C for 1 min, 55°C for 1 min, 72°C for 2 min and a final extension step of 72°C for 7 min. The protocol for amplification of ATP6 was an initial denaturing at 95°C for 5 min followed by 39 cycles: 95°C for 30 s, 50°C for 2 min, 72°C for 1 min. The protocol for amplification of RPB2 was an initial denaturing at 95°C for 4 min followed by 35 cycles: 95°C for 1 min, 50°C for 1 min, 72°C for 2.5 min and a final extension step of 72°C for 10 min. The RPB2 PCR product was electrophoresed on a 1% agarose gel and the bands corresponding to the target region were excised and purified using a modified GeneClean III protocol before sequencing (Bergemann and Miller 2002). A PCR cleanup protocol using ExoSAP-IT was used to remove unused primers and nucleotides for ITS and ATP6 PCR products prior to sequencing (USB Corporation Cleveland, OH USA).

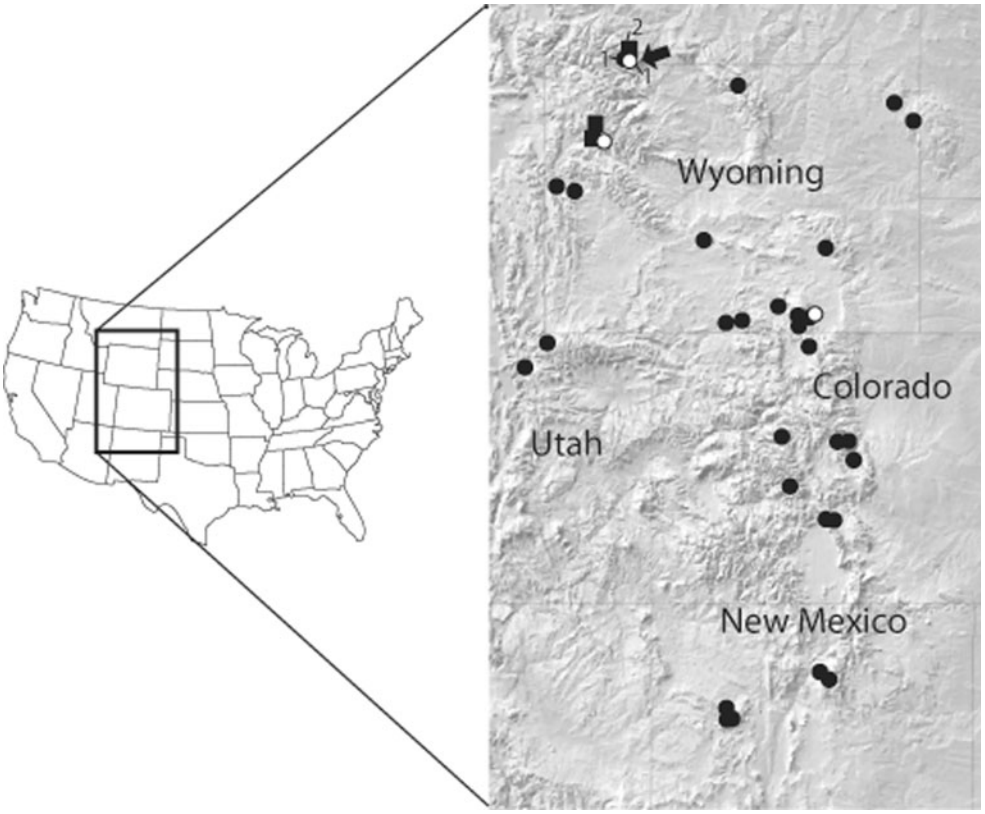


Fig. 1 Collecting locales for samples of monotropoid mycorrhizal tissue taken from *Pterospora andromedea* rootballs and *Rhizopogon spp*. section *Amylopogon* basidiocarps in the south-central Rocky Mountains. Closed circles indicate *R. salebrosus*, open circles indicate *R. ellenae* and closed squares indicate *R. subpurpurascens*. The arrow denotes the location of individual *Pterospora andromedea* plants found in association with either *R. ellenae*, *R. salebrosus*, or *R. subpurpurascens*, all growing in sympatry. Numbers indicate the number of original samples for this study at that locale

Sequencing was conducted on an ABI3130xl 16-capillary Genetic Analyzer (Applied Biosystems, CA USA) at the University of Wyoming Nucleic Acid Exploration Facility. Contigs were assembled from forward and reverse sequences and were manually checked and edited from chromatograms using Sequencher v4.7 (Gene Codes Corporation, MI USA).

2.3 Identification and phylogenetic analysis

The *Rhizopogon* basidiocarps and mycobionts of *Pterospora andromedea* taken from mycorrhiza samples were identified through analysis of ITS sequences of *Rhizopogon* species and comparison with sequences in *Rhizopogon* section *Amylopogon* deposited in GenBank. The data

Table 1 Eleven original samples used in this study. An * trailing the sample collection number indicates samples taken from mycorrhiza tissue

Rhizopogon species group	Sample	General location	GenBank Accession Numbers		
			ITS	ATP6	RPB2
R. subpurpurascens					
	CLC2171	Cooke City, MT	JF695021	JF695043	JF695032
	CLC2544	Beartooth Mountains, MT	JF695022	JF695044	JF695033
	R1AYSNP	Lewis Lake, Yellowstone, WY	JF695023	JF695045	JF695034
	R12YSNP	Craig Pass, Yellowstone, WY	JF695020	JF695042	JF695031
R. salebrosus					
	R11YSNP	Scaup Lake, Yellowstone, WY	JF695015	JF695037	JF695026
	RS8YSNP	Nez Perce Creek, Yellowstone, WY	JF695014	JF695036	JF695025
	175BTMT*	Beartooth Mountains, MT	JF695013	JF695035	JF695024
R. ellenae					
	RS9YSNP	Lewis Lake, Yellowstone, WY	JF695018	JF695040	JF695029
	RCPSRWY	Snowy Range, WY	JF695016	JF695038	JF695027
	176BTMT*	Beartooth Mountains, MT	JF695019	JF695041	JF695030
	RS2BTMT	Beartooth Mountains, MT	JF695017	JF695039	JF695028

matrix consisted of sequences from the *Rhizopogon* species groups identified by Bidartondo and Bruns (2002), including many holotype and paratype sequences. GenBank accession numbers for samples used in identification were: AF224276, AF351874, AF377122, AF377133, AF377134, AF377135, AF442136, AF377144, AF377154, AF377156, AF377119, AF377146, AF377148, AF377152, AF377157, AF377159, AF377166, AF377167, AF377169, AF377170, AF377171, AF377173, AF377175, AF377126 and DQ426676. The ITS sequences from the *Rhizopogon* basidiocarps found in this study were then used as a proxy for identification in ATP6 and RPB2 analyses, since neither is available for any *Rhizopogon* species on GenBank or AFTOL. All sequences in this study were aligned using ClustalW (Larkin et al. 2007) implemented in Geneious v3.5.7 (Drummond et al. 2006) and manually checked and corrected in BioEdit v7.0.9.0 (Hall 1999).

Independent Bayesian phylogenetic estimation was conducted for ITS, ATP6, and RPB2 sequence regions using Mr. Bayes 3.1.2 (Huelsenbeck and Ronquist 2001). Five independent runs of 10 000 000 generations were conducted under the GTR+G evolutionary model with a burn-in of 2 500 000 generations sampling every 100 generations. Consensus trees with posterior probabilities for each locus were generated from 375 000 trees. The *Rhizopogon arctostaphyli* and the eastern *Rhizopogon* species groups were used to root the ITS phylogeny. Because representative samples from these two species groups were unavailable for the ATP6 and RPB2 Bayesian reconstructions, the samples from the *R. ellenae* group were used in rooting the phylogenies.

3 Results

All loci successfully resolved each species group. ATP6 sequences were easy to align and effectively differentiated each species group, however, ATP6 exhibited little polymorphism, with seven polymorphic sites in 709 base pairs and provided no intra-cladal support. RPB2 was able to resolve the species groups well and provided strong intra-lineage resolution. The ITS locus was useful in determining the species groups for all *Rhizopogon* samples used in the present study.

Tree topologies resulting from Bayesian analysis were consistent across all loci and each clade, and therefore identification of the mycobionts found in association with *Pterospora andromedea*, was supported by posterior probabilities of 95% or greater (Fig. 2). For the ITS locus, 5 species groups were present in *Rhizopogon* section *Amylopogon*: *R. salebrosus*, *R. arctostaphyli*, the unidentified eastern *Rhizopogon* species group, *R. subpurpurascens* and *R. ellenae*. The trees for ATP6 and RPB2 contained 3 distinct clades, each clade representing a species group: *R. salebrosus*, *R. subpurpurascens* and *R. ellenae*.

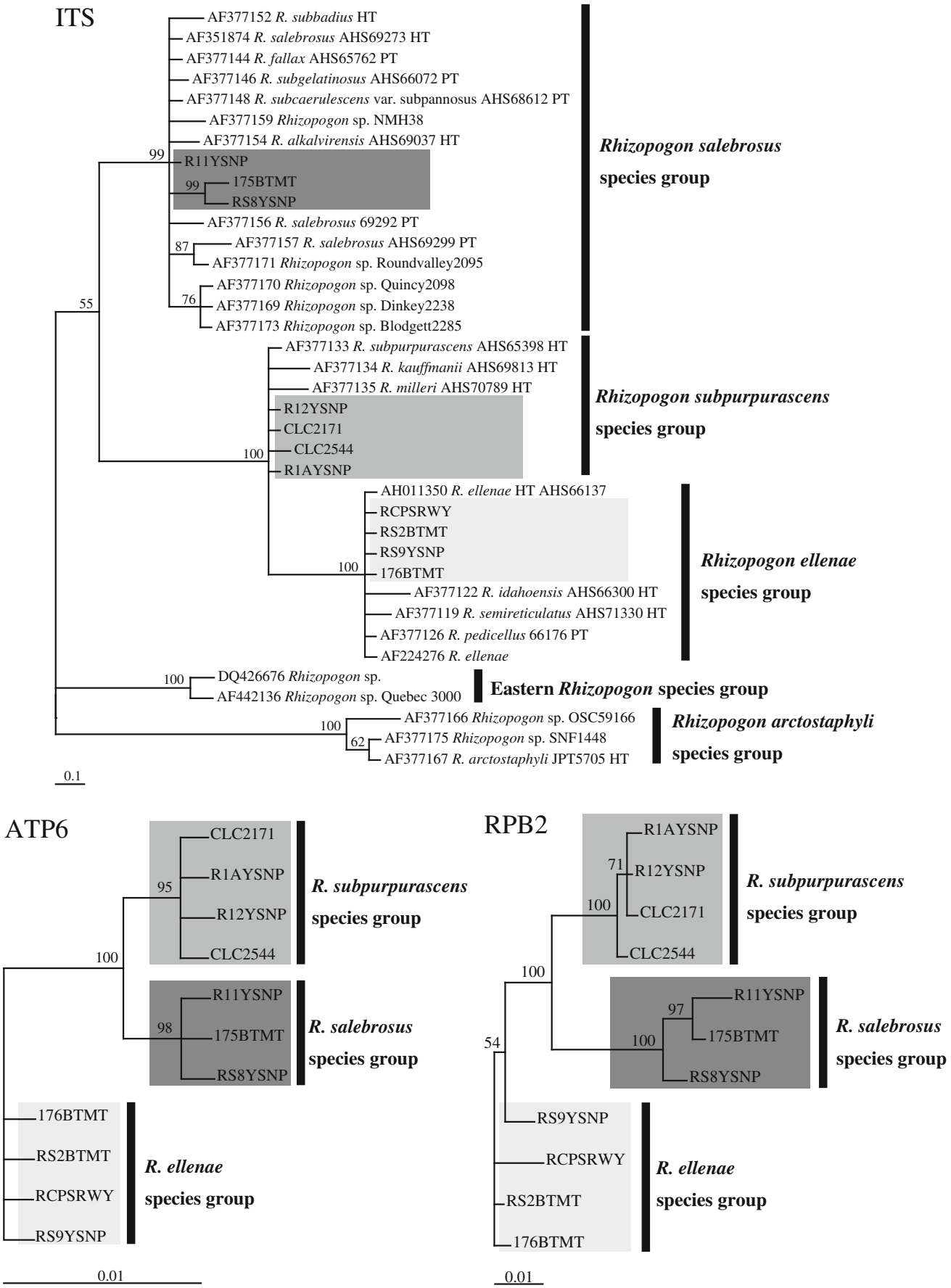
Fig. 2 Independent phylogenies constructed from three sequence loci, ITS, ATP6, and RPB2, using Mr. Bayes v3.1.2. Samples 175BTMT and 176BTMT were from *Rhizopogon* mycorrhiza tissue found in association with *Pterospora andromedea,* and all others were from *Rhizopogon* basidiocarps found opportunistically. Topologies represent majority consensus of five independent runs of 75 000 trees. Posterior probabilities greater than 50% are indicated by numerical values. The scale bar indicates % sequence divergence. All three phylogenies confirmed three species groups: *R. salebrosus*, *R. ellenae*, and *R. subpurpurascens*. *Rhizopogon arctostaphyli* and the eastern *Rhizopogon* species groups were used to root the ITS phylogeny. Because representative samples, and therefore sequences, from the two outgroup species groups were unavailable for the ATP6 and RPB2 Bayesian reconstructions, samples from the *R. ellenae* species group was used in rooting the phylogeny

Sample 176BTMT, taken from *Pterospora andromedea* mycorrhiza samples associated with *Pinus ponderosa*, fit solidly into the *Rhizopogon ellenae* clade in the results for each locus. In the ITS analysis, the *R. ellenae* clade also contained the holotype of *R. ellenae*, confirming the identification.

4 Discussion

Rhizopogon ellenae was found in association with *Pterospora andromedea* in the Greater Yellowstone Ecosystem and represents the only record of a fourth fungal symbiont identified thus far in the *P. andromedea/Pinus/Rhizopogon* tripartite system. Not only was a new mycobiont in this mycoheterotrophic relationship discovered, but this study also provides the first evidence for overlap in fungal symbionts between *P. andromedea* and sister taxon *Sarcodes sanguinea* Torr. (Bruns and Read 2000; Kretzer et al. 2000; Bidartondo and Bruns 2005). Interestingly, this novel observation was made outside the present endemic range of *S. sanguinea*, the west coast of North America, where *Pterospora andromedea* and *Sarcodes sanguinea* are often found in sympatry. Additionally, the unique incidence of *R. ellenae* with *P. andromedea* was found within 75 m of a *P. andromedea* plant symbiotic with *R. salebrosus*, the most common mycobiont in the Rocky Mountains (Dowie et al. 2011).

Both *P. andromedea* and *S. sanguinea* associate exclusively with *Rhizopogon* species in section *Amylopogon* (Bidartondo and Bruns 2005). *Pterospora andromedea* associates with three previously mentioned *Rhizopogon* species: *R. salebrosus* and *R. arctostaphyli* and one unidentified species, while *S. sanguinea* has been found to exclusively associate with two different *Rhizopogon* species: *R. ellenae* throughout the entire range of *S. sanguinea* and *R. subpurpurascens* in southern Oregon (Hazard 2006;


ITS
AF377152 R. subbadius HT
AF351874 R. salebrosus AHS69273 HT
AF377144 R. fallax AHS65762 PT
AF377146 R. subgelatinosus AHS66072 PT
AF377148 R. subcaerulescens var. subpannosus AHS68612 PT
AF377159 Rhizopogon sp. NMH38
AF377154 R. alkalvirensis AHS69037 HT
99
R11YSNP
99
175BTMT
RS8YSNP
AF377156 R. salebrosus 69292 PT
87
AF377157 R. salebrosus AHS69299 PT
AF377171 Rhizopogon sp. Roundvalley2095
AF377170 Rhizopogon sp. Quincy2098
55
76
AF377169 Rhizopogon sp. Dinkey2238
AF377173 Rhizopogon sp. Blodgett2285
Rhizopogon salebrosus
species group
AF377133 R. subpurpurascens AHS65398 HT
AF377134 R. kauffmanii AHS69813 HT
AF377135 R. milleri AHS70789 HT
R12YSNP
CLC2171
100
CLC2544
R1AYSNP
Rhizopogon subpurpurascens
species group
AH011350 R. ellenae HT AHS66137
RCPSRWY
RS2BTMT
RS9YSNP
100
176BTMT
AF377122 R. idahoensis AHS66300 HT
AF377119 R. semireticulatus AHS71330 HT
AF377126 R. pedicellus 66176 PT
AF224276 R. ellenae
Rhizopogon ellenae
species group
100
DQ426676 Rhizopogon sp.
AF442136 Rhizopogon sp. Quebec 3000
Eastern Rhizopogon species group
100
AF377166 Rhizopogon sp. OSC59166
62
AF377175 Rhizopogon sp. SNF1448
AF377167 R. arctostaphyli JPT5705 HT
Rhizopogon arctostaphyli
species group
0.1
ATP6
CLC2171
95
R1AYSNP
R12YSNP
CLC2544
R. subpurpurascens
species group
100
R11YSNP
98
175BTMT
RS8YSNP
R. salebrosus
species group
176BTMT
RS2BTMT
RCPSRWY
RS9YSNP
R. ellenae
species group
0.01
RPB2
R1AYSNP
71
R12YSNP
100
CLC2171
CLC2544
R. subpurpurascens
species group
100
R11YSNP
97
54
100
175BTMT
RS8YSNP
R. salebrosus
species group
RS9YSNP
RCPSRWY
RS2BTMT
176BTMT
R. ellenae
species group
0.01

Bidartondo and Bruns 2002, 2005, 2001). Although each of these *Rhizopogon* taxa are closely related, Bidartondo and Bruns (2005) found no fungal symbiont overlap between 93 *S. sanguinea* and 77 *P. andromedea* plants found in sympatry on the west coast of North America.

Out of nearly 300 collections of *P. andromedea* mycorrhizas that have been examined, this is the first time *R. ellenae* has been encountered (Dowie et al. 2011; Hazard 2006; Bidartondo and Bruns 2005, 2002; Kretzer et al. 2000). In addition, *R. ellenae* basidiocarps were found in areas where *P. andromedea/R. salebrosus* mycorrhiza samples have been collected, further demonstrating that *R. ellenae* can occur in sympatry with other *Rhizopogon spp*. The degree of reciprocal specificity in the *P. andromedea/R. ellenae* relationship is unknown. However, it is likely that the *P. andromedea/R. ellenae* symbiosis exhibits a high level of specificity as previously recorded for *P. andromedea/R. salebrosus* and *P. andromedea/R. arctostaphyli.*

One possible explanation is that widely disseminated seeds of *P. andromedea* may have allowed a fungal host switch by increasing the likelihood of encountering an *R. ellenae* population devoid of selective pressure such as naïve mycobionts not previously subjected to coevolutionary patterns (Bidartondo and Bruns 2005). Exceptions to parental specificity may occur in offspring under allopatric conditions (Bidartondo and Bruns 2005) and may be the case here, since *S. sanguinea* is not present in this area. Whatever the case, these findings suggest that it is likely that *P. andromedea* can associate with other species in section *Amylopogon*. One likely possibility would be the discovery of an association between *R. subpurpurascens* and *P. andromedea* since this fungus is more closely related to other identified mycobionts of *P. andromedea* than is *R. ellenae* (Grubisha et al. 2002, 2001; Kretzer et al. 2000; Bidartondo and Bruns 2002).

Hybridization between *P. andromedea* and *S. sanguinea* may also favor a symbiotic relationship with *R. ellenae*. Little is known about the reproductive lifecycle and speciation in Monotropoideae lineages (Klooster and Culley 2009; Bidartondo 2005), however hybridization between *Monotropa hypopithys* and *Pterospora andromedea* has previously been hypothesized to occur in Yellowstone National Park with no morphological distinction (Cullings 2000). Although *Sarcodes sanguinea* is not currently present in the Greater Yellowstone Ecosystem, it is found on the West Coast of North America and its present distribution extends eastward as far east as Nevada. It may have been possible for intergeneric *P. andromedea/S. sanguinea* hybrid seeds to develop a functional association with a suitable allopatric fungal symbiont such as *R. ellenae*. The West Coast contains the highest species diversity for Monotropoideae and other taxa, such as *Pityopus californicus,* may have arisen by similar hybridization events (Neyland 2005). Although this hypothesis is unlikely, little is known about the reproductive lifestyle and biogeographic history of any Monotropoideae. Alternatively, glaciation or region-wide drying periods may have impacted the potential distribution of *S. sanguinea* in the Rocky Mountains, whereas *Pterospora andromedea* was able to survive (Jackson et al. 2005).

Another explanation for the unique association is that *Rhizopogon ellenae* may have been the most recent common symbiont before the taxonomic split of *P. andromedea* and *S. sanguinea*. This would mean that a more ancestral genotype of *P. andromedea*, if one exists in the area, may still form specific associations with *R. ellenae*. However, little is known about the historic range limits or migration patterns of either mycoheterotroph and further study is required to test this assertion.

Identification of *Rhizopogon ellenae* was confirmed in analysis of three independent loci: ITS, ATP6 and RPB2. ITS has been extensively used in the past to determine *Rhizopogon* species groups (Kretzer et al. 2003; Grubisha et al. 2002, 2001; Bidartondo and Bruns 2002) and was useful in determining the species groups for all *Rhizopogon* samples used in the present study. This report is the first implementation of ATP6 and RPB2 for identification of *Rhizopogon* species from basidiocarps and environmental samples. These loci may be important tools for delineating *Rhizopogon* species groups and may help overcome the difficulties ITS has had in resolving *Rhizopogon* phylogenies due to alignment problems among the 100+ species (Bidartondo and Bruns 2002; Grubisha et al. 2002).

Studies elucidating the parasitic or mutualistic relationship between mycoheterotrophs and their fungal symbionts are fundamental in understanding the ecology and coevolution of specific mycoheterotrophic relationships. If *Rhizopogon* species associate with cryptic lineages of *P. andromedea* as previously hypothesized (Bidartondo and Bruns 2005, 2002), then the discovery of *R. ellenae* in symbiosis with *P. andromedea* will provide exciting avenues of research regarding the *P. andromedea/S. sanguinea* divergence.

There has never been a comprehensive sampling of a mycoheterotroph across its entire endemic range. So like much of the sampling that has been accomplished for *Pterospora* mycobionts, this result was based on one opportunistic sampling of a single *P. andromedea* and *R. ellenae* association. However, this does not diminish the importance of this finding and evidence of this novel association further advances knowledge and hypotheses regarding the evolution and ecology of this relationship. At the present time it is difficult to determine whether this relationship is causal or incidental, however this finding advances knowledge of the evolution and specificity

between two important mycoheterotrophic plants. A comprehensive study examining large scale and population level patterns throughout the entire endemic range of *P. andromedea* and its mycobionts, as well as *S. sanguinea* and its mycobionts is underway.

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