Variability of Ecological and Autotrophic Host Specificity in a Mycoheterotrophic System:
Pterospora andromedea and Associated Fungal and Conifer Hosts

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ABSTRACT

Few studies of tripartite mycoheterotrophic systems have examined ecological specificity across broad geographic ranges or addressed autotrophic host specificity. *Pterospora andromedea* was selected as an ideal candidate to examine ecological specificity of a mycoheterotrophic system as it is widely distributed, has been shown to have high levels of symbiont specificity with *Rhizopogon* subgenus *Amylopogon*, and is found with several autotrophic hosts. Pairs of *P.*

andromedea + *Rhizopogon* spp. samples were co-collected across North America and were sequenced using *trnL* and ITS, respectively. Bayesian phylogenetic reconstructions between the co-collected taxa were used to examine ecological specificity, and for subsequent tests for autotroph specificity. *Pterospora andromedea* lineages exhibited both high specificity and relaxed specificity for fungal symbionts and autotrophic hosts across the geographic landscape under allopatric and sympatric conditions. This strong evidence for geographic mosaics of specificity in mycoheterotrophic systems is an important future consideration in determining the evolutionary ecology of mycoheterotrophs.

Keywords: Mycoheterotroph, *Rhizopogon*, *Amylopogon*, *Pterospora andromedea*, specificity, *Pinus*, biogeography, North America, Monotropoideae

Introduction

Assessing levels of plant-fungus specificity has become a key characteristic for understanding mycoheterotrophic plants and their evolutionary ecology (Bidartondo and Bruns 2001, Bruns et al. 2002, Taylor et al. 2002, Merckx et al. 2009, Barrett et al. 2010, Hynson and Bruns 2010, Merckx and Freudenstein 2010, Merckx 2013). Mycoheterotrophs, which form an obligate relationship with their fungal mycobionts, for part or all of their lifecycle, can establish a symbiosis with free-living saprotrophic fungi or become a member of tripartite arbuscular or ectomycorrhizal mycorrhizal networks that include an autotrophic plant (Leake 1994, Bidartondo 2005). High levels of mycobiont specificity are commonplace in associations between mycoheterotrophs and their fungi (Leake 1994, Taylor 2004, Bidartondo 2005, Waterman et al. 2013). The mycobiont specificity in mycoheterotrophs has been characterized by

tracking genera, species or intraspecific lineages of associated fungi (Bidartondo and Bruns 2001, 2002, 2005, Merckx and Bidartondo 2008, Merckx et al. 2008, Merckx et al. 2009, Barrett et al. 2010).

Mycobiont specificity is common for many mycoheterotrophs in Monotropoideae, Ericaceae (Kretzer et al. 2000, Bidartondo and Bruns 2001, 2002, Hynson and Bruns 2009, Waterman et al. 2013). In this subfamily, mycoheterotrophy evolved independently two to three times, potentially once in each of the three tribes (Leake 1994, Bidartondo 2005, Hynson et al. 2009, Merckx and Freudenstein 2010). These tribes have diversified into five plant lineages and typically have distinct and high levels of mycobiont specificity. These mycobionts are from a diverse taxonomic range of ectomycorrhizal basidiomycetes, can occur at various geographic scales and are commonly found in sympatric conditions with co-occurring mycoheterotrophic sister taxa (Kretzer et al. 2000, Bidartondo 2005, Hynson and Bruns 2010, Merckx and Freudenstein 2010). The smallest tribe in Monotropoideae, *Pterosporeae*, is composed of only two monotypic genera, *Pterospora andromedea* and *Sarcodes sanguinea*. Both of these fully mycoheterotrophic plants exhibit distinct and extreme examples of symbiont specificity toward their obligate fungal associates in *Rhizopogon* subgenus *Amylopogon*, even in sympatric conditions (Kretzer et al. 2000, Bidartondo and Bruns 2001, 2002, Grubisha et al. 2002). Previous work addressing mycobiont specificity in *P. andromedea* and *S. sanguinea* provided evidence of highly specific associations with little to no mycobiont overlap (Bidartondo and Bruns 2002, Hazard et al. 2012). Mycobiont specificity occurs in many members of the species in tribe Pyroleae, some of which appear to be fully autotrophic, while others are partially or fully mycoheterotrophic (Tedersoo et al. 2007, Hynson et al. 2009, Jolles and Wolfe 2012, Matsuda et al. 2012). However, exceptions to high mycobiont specificity have been recorded in several

species of Pyroleae which are now considered to be generalists (Tedersoo et al. 2007, Zimmer et al. 2008, Hynson and Bruns 2009).

Although symbiotic specificity between species, lineages and haplotypes can vary across a biogeographic landscape and can result in a geographic mosaic of symbiont specificity, few studies have attempted to examine mycobiont specificity in mycoheterotrophic systems across large portions of their endemic ranges (Dieckmann and Doebeli 1999, Thompson 1999, Coyne and Orr 2004, Bidartondo 2005, Thompson 2005, Thrall et al. 2007, Barrett et al. 2010, Douglas 2010, Fedrowitz et al. 2012). This is unfortunate because comparative molecular studies over endemic ranges have recently been suggested as being critical to understanding the evolutionary ecology of mycoheterotrophs (Merckx et al. 2013, Taylor et al. 2013, Waterman et al. 2013). Barrett *et al.* (2010), who examined *Corallorhiza striata* complex, Orchidaceae, and its fungal associates in genus *Tomentella*, represents the most thorough research on mycoheterotroph associations throughout an endemic range. They found high levels of host-symbiont specificity in *C. striata* + *Tomentella* and had a pattern indicative of a geographic mosaic (Thompson 1999, Thompson 2005, Barrett et al. 2010).

Pterospora andromedea is an ideal candidate to assess broad-scale ecological specificity of an important and distinct mycoheterotrophic system. Specifically, *P. andromedea* lineages have been hypothesized to exhibit very high levels of mycobiont specificity for distinct *Rhizopogon* subgenus *Amylopogon* species, including *R. salebrosus*, *R. arctostaphyli*, and *R. kretzeriae*, and are found over a large area of North America (Bidartondo and Bruns 2002, Dowie et al. 2011, Hazard et al. 2012, Grubisha et al. 2014). The recent discovery of a *P. andromedea* + *R. ellenae* symbiosis provided evidence of a more complex pattern of interactions across a geographic mosaic of *P. andromedea* + mycobiont specificity. This finding, coupled with

sampling across a much broader geographic range, provides the ability for a more extensive and focused examination of host-symbiont specificity to determine if localized sampling is suitable to discern large-scale patterns.

This study examines host-symbiont specificity in *P. andromedea* and mycobionts using cophylogenetic methodologies for all currently known mycobionts throughout the endemic range of *P. andromedea*. This approach will provide evidence to previously accomplished informative research to determine if a consistent pattern emerges across the biogeographic landscape for all associations and if these patterns can be extended into the third member of the tripartite symbioses, namely the autotrophic conifer species (Bidartondo and Bruns 2002, Barrett et al. 2010, Hazard et al. 2012). Any evidence of the widespread distribution of these associations will help elucidate the evolutionary history and biogeography of these symbioses. Furthermore, this type of approach may be critical in determining potential extinction risks within complex symbioses.

Materials and Methods

Sample Collection, DNA Extraction and Sequencing

Pterospora andromedea and mycobionts were collected in pairs throughout the endemic range of *P. andromedea* in the United States: Michigan, New York, New Hampshire, New Mexico, Colorado, Wyoming, South Dakota, Montana, Idaho, Utah, Washington and Oregon. At each collection location, due to the possible extensiveness of tree roots and common mycorrhizal networks, all possible photoautotrophic hosts in the vicinity of the collection were recorded. This conservative determination was used to best prevent recording false positive associations, since

the autotrophic host cannot be easily discerned without a clear absence of the other possible autotrophic hosts. Extra efforts in the field were made to visit monoculture stands of *P. ponderosa*, *P. contorta*, *P. strobus*, *P. monticola*, *P. strobiformis*, *P. lambertiana*, *Abies magnifica*, and *Pseudotsuga menziesii*, for collections to examine the possible influence of autotroph species with the specificity found in the *P. andromedea* and mycobiont associations. Tissue samples were carefully taken from the monotropoid rootball as well as above ground plant tissue following the protocol of Dowie et al. 2012. *Pterospora andromedea* tissue samples typically included leaf bracts towards the base of the plant as well as plant shoot tips in several cases when present. Samples were cleaned, sorted and stored in 95% ethanol and kept at 4° C until processed. Remaining tissue was then stored at -20° C. DNA was extracted according to Bergemann & Miller (2002).

To have comparable results with previous studies (Bidartondo and Bruns 2002, Barrett et al. 2010, Hazard et al. 2012), the widely used multicopy internal transcribed spacer (ITS) region and the uniparentally inherited plastid *trnL* region were used in *Rhizopogon* spp. and *P. andromedea*, respectively. The ITS region of the second largest ribosomal subunit was amplified in all *Rhizopogon* spp. samples using ITS1f and ITS4b following previously established protocols (White et al. 1990, Gardes and Bruns 1993). The sequencing was performed using an ABI3131x1 16-capillary Genetic Analyzer at the Nucleic Acid Exploration Facility at the University of Wyoming. Chromatograms were visually inspected and manually edited using Sequencher 5.0 (Gene Codes Corporation).

In the paired *P. andromedea* samples, the plastid pseudogene tRNA-Leu intergenic spacer (*trnL*) region was amplified from monotropoid ectomycorrhizal tissue or plant tissue using *trnL-c* and *trnL-f* following the protocol of Taberlet *et al.* (1991). In some cases, obtaining

higher quality plastid DNA was easiest from mycorrhizal tissue, depending on the age and condition of the stalk.

Phylogenetic Reconstructions

Rhizopogon spp. datasets were aligned using ClustalW (Larkin et al. 2007) within the sequence alignment software platform Geneious v4.8.3 (Drummond et al. 2006). Additional holotype and paratype sequences used regularly for identifying species in subgenus *Amylopogon* were included (Bidartondo and Bruns 2002, Grubisha et al. 2002, Dowie et al. 2011, Dowie et al. 2012, Hazard et al. 2012). GenBank accession numbers 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). BioEdit v7.1.3.0 (Hall 1999) was used for all later manual adjustments. Identification of *Rhizopogon* spp. was accomplished from the ITS sequences using Neighbor-joining methods for 100 000 bootstrap replicates in PAUP 4b10 (Swofford 2002).

Representative paired samples of *Rhizopogon* spp. and *P. andromedea* were used to further analyze host-symbiont specificity. These samples were chosen based upon distinct geographic areas and discernible conifer host associations within the cladal assignments from the Neighbor-joining phylogeny of the *Rhizopogon* mycobiont. Intraspecific taxonomic information for *P. andromedea* is currently very limited, however intraspecific lineages have been hypothesized as being concordant with mycobiont lineages (Cullings et al. 1996, Bidartondo and Bruns 2001, 2002, Merckx et al. 2009, Hynson and Bruns 2010, Leake and Cameron 2010). Therefore, the inclusion of *P. andromedea* samples was dependent on *Rhizopogon* spp.

identification. Specifically, *P. andromedea* samples used in the final analyses were chosen based on species identification of the mycobionts, distinct areas across the entire geographic range of the collections and contained associated autotrophic hosts. In an attempt to hold biogeography neutral for tests of mycobiont specificity, samples were also selected based upon close geographic proximity in areas which *P. andromedea* had multiple *Rhizopogon* spp. associations. These included: R175 and R176; R206, R210 and R211; R283 and R284; R286 and R287; R288 and R290; R321 and R322; R383 and R384; R413 and R414; R447 and R448; R1092 and R1093; R1144 and R1146; R1222 and R1223 (Table 1). In many cases, collections were within 100 meters. Some bias is possible with this methodology and likely limited the total number of discovered haplotypes as well as additional associations. However, this approach was suitable for the selection of *P. andromedea* haplotypes from all collections while simultaneously addressing the premise of the study, namely ecological specificity.

Bayesian phylogenetic reconstructions were accomplished using MrBayesV3.2.5 (Huelsenbeck and Ronquist 2001, Ronquist and Huelsenbeck 2003, Ronquist et al. 2012) for both *Rhizopogon* and *P. andromedea* datasets. Nucleotide substitution models for ITS and *trnL* were estimated with jModelTest2.1.4 (Guindon and Gascuel 2003, Darriba et al. 2012) using the Akaike information criterion (AIC), the Akaike information criterion with correction (AICc), the Bayesian information criterion (BIC) and the decision theory method (DT) (Akaike 1974, Schwarz 1978, Hurvich and Tsai 1989, Minin et al. 2003). Multiple methods were used to determine if there was any disagreement in estimating nucleotide substitution models. For ITS, all methods determined the K80+G model to be the best, except for AIC which selected the SYM+G model, with K80+G being the second best. The difference in criterion values between SYM+ G and K80+G was negligible, therefore the K80+G model was selected. For *trnL*, all

methods determined the F81 model to be the most accurate, except for AIC which selected the F81+I as the best model. Again, there was almost no difference in criterion values between the two and the F81 model was selected. To obtain as many phylogenetically informative sites as possible, indels were recoded to binary format, as recommended in the MrBayes manual (Ronquist et al. 2005, Ronquist et al. 2011). IndelCoder (Müller 2006) was used in SeqState v1.4.1 (Müller 2005) and followed the simple indel coding method of Simmons and Ochoterena (2000). This resulted in two partitions to analyze per locus, the aforementioned model and a restriction site model for the indel coded partition. Each analysis consisted of ngen = 10 000 000, nruns = 5 and nchains = 4 for both datasets of 70 samples. Midpoint rooting was used as an acceptable outgroup but could not be selected for the monotypic *P. andromedeae*, as the *trnL* region in sister taxon, *Sarcodes sanguinea*, was approximately twice as long and not alignable to this dataset. Midpoint rooting was also used in *Rhizopogon* subgenus *Amylopogon*. Trees were viewed in FigTree v1.3.1 (Rambaut 2009) and manually edited in Adobe Illustrator v15.0.0 (Adobe Systems, Mountainview, CA).

Comparative Analyses

The compatibility of the alignments were analyzed using the Partition Homogeneity Test in PAUP 4b10 (Swofford 2002), which utilizes the Incongruence Length Difference test (ILD) developed by Farris *et al.* (1994, 1995). A heuristic search was performed with 1000 replicates and 5 random sequence additions using the tree-bisection reconnection branch swapping algorithm. The model for the ILD test can be of limited use and has often been stated as having a propensity for a type-1 error (wrongly rejecting the null) (Cunningham 1997, Graham et al. 1998, Dolphin et al. 2000, Yoder et al. 2001, Barker and Lutzoni 2002, Darlu and Lecointre

2002). In fact, Darlu and Lecomte, (2002), found that the ILD test is limited in usefulness in detecting incongruence due to tree topologies and in the variation of evolutionary conditions.

In addition to the ILD test, several additional comparative analyses were used to establish evidence for paired specificity by assessing phylogenetic congruence, e.g. co-collected samples using the *ITS* region in *Rhizopogon* spp. and the *trnL* region in *P. andromeda*. Bayesian phylogenetic reconstructions were converted into patristic distance matrices using Patristic (Fourment and Gibbs 2006). These distance matrices for *P. andromeda* and *Rhizopogon* spp. were compared using ParaFit (Legendre et al. 2002) within the CRAN APE package (Paradis et al. 2004) in the comprehensive R archive network (RDC Team, 2011). Although other software for examining cophylogenies are available, ParaFit is an important tool as trees do not have to be fully resolved and it is able to test the hypothesis of coevolution or independence between the phylogenies and within individual Host-Parasite associations (Legendre et al. 2002). For the purposes of the current study, this approach was used not to assess potential coevolution, but to examine topological concordance to more thoroughly assess mycobiont specificity. The significance of the global test and individual tests was assessed at $P = 0.05$ for 10 iterations of 9999 permutations. The Congruence Among Distance Matrices, CADM (Legendre and Lapointe 2004) was also performed using the patristic distance matrices to test the null hypothesis of completely incongruent phylogenies. This analyses has been shown to have an accurate type-I error rate and is useful in testing for congruence in phylogenomic studies (Campbell et al. 2011).

Mapping of the phylogenetic data was accomplished using GenGis 2.0 (Parks et al. 2009). The map was created by using preexisting geospatial data derived from Oak Ridge National Laboratory's Distributed Archive Center for Biogeochemical Dynamics (ORNL

DAAC). All maps were manually edited for illustrative purposes within GenGIS 2.0 and then subsequently finalized in Adobe Illustrator v15.0.0.

Data were also treated categorically to test for expected versus observed patterns for a variety of nonrandom associations. A log-likelihood G-test was used in R using a script developed by Peter L. Hurd found at <http://www.psych.ualberta.ca/~phurd/cruft/g.test.r>. An $r \times c$ contingency table was formed for each *Rhizopogon* spp., *P. andromedea* groups, as well as conifer stand type association. The single collections in *Pinus monticola* and *Abies magnifica* as well as mixed *P. contorta* and *P. ponderosa* stands were not included to prevent data bias.

Results

Phylogenetic Reconstructions

The Neighbor-joining tree (not shown) was useful for preliminary screening of specific *Rhizopogon* spp. and paired *P. andromedea* samples for Bayesian analysis. Of the 569 samples successfully sequenced, 523 were from *R. salebrosus*, 18 were from the *R. kretzerae*, 15 from *R. arctostaphyli* and the remaining 13 from *R. ellenae*. Ultimately, 70 paired samples were selected as representatives and were used in Bayesian phylogenetic reconstruction (Table 1). More *R. salebrosus* associations were included as it was the most prolific association, has a larger diversity and is found over a larger geographic area. Specifically, 49 *R. salebrosus* samples were used, as well as nine *R. arctostaphyli*, five *R. ellenae* and seven *R. kretzerae*. Reconstruction for the *ITS* region consisted of 130 unique sites for the first partition and 15 unique sites for the indel recoded partition. The *trnL* region for *P. andromedea* had 42 unique sites for the first partition and six unique sites for the indel recoded partition.

Bayesian reconstruction resulted in four distinct clades for *Rhizopogon* spp. and two distinct clades for *P. andromedea* (Figure 1). The four clades for *Rhizopogon* spp. represented the four distinct species groups within subgenus *Amylopogon* and will be hereafter referred to as: *R. salebrosus*, *R. ellenae*, *R. arctostaphyli* and the *R. kretzeriae* clades. Additionally samples R128 and R129 were contained within the *R. salebrosus* clade, however they had many shared single nucleotide polymorphisms (SNPs) with *R. ellenae* and *R. kretzeriae*. Among the single nucleotide polymorphisms that distinctly resolve *R. ellenae* and *R. kretzeriae*, R128 and R129 shared twelve states with *R. kretzeriae* and eleven with *R. ellenae*. Both clades found for the 13 recovered haplotypes of *P. andromedea* could be further broken down into two additional lineages. The resultant lineages will be hereafter referred to as: PTAN-Major, PTAN-Minor, PTAN-Arctostaphyli and PTAN-Eastern. Furthermore, the recoding of indels to binary format and treating these data as a new partition for *P. andromedea* did not result in any additional haplotypes. Out of the 5 previously known haplotypes of *P. andromedea* only one of these haplotypes (**AF377252**), which was discovered by Bidartondo and Bruns (2002), was not recovered in this study.

Comparative Analyses

Several comparative methods were used to test for congruence and incongruence to assess paired specificity between *P. andromedea* and *Rhizopogon* species. The null hypothesis of congruence between the *Rhizopogon* ITS locus and the *P. andromedea* trnL locus was rejected in the ILD test ($P = 0.01$). Despite this result, the phylogenies were visually symmetrical and patterns were present for many specific clade-to-clade associations. All ten ParaFit iterations rejected the global null hypothesis of both trees occurring independently ($P = 0.001$). Despite potential

support for the hypothesis of global coevolution under this model, there were many rejections between individual Host-Parasite links (Figure 1). Some type-I and type-II errors may be present, which may be due to a spurious conclusion of a strict pattern for coevolution, as the loci are not biologically linked and the branch lengths were much longer in the *Rhizopogon* spp. ITS than the *P. andromedea trnL*. Coevolutionary patterns were not the focus of this study but instead the congruency revealed from this analysis was useful in determining host-symbiont specificity between taxa. Congruence Among Distance Matrices also rejected the null hypothesis of complete incongruence between topologies at $P = 0.001$. Although the original intention of CADM was to determine when different loci may be combined in phylogenomic analyses (Legendre and Lapointe 2004), it was used as an additional test to address topological congruence between these unlinked neutral loci among different, obligately linked associates and has been used as such in multipartite symbioses (Roe et al. 2011).

Likelihood tests supported a nonrandom association between the four *P. andromedea* lineages, the four *Rhizopogon* species groups and the discernible autotrophic conifer hosts: *Pinus contorta*, *P. ponderosa*, and *P. strobus* with which they were associated. Specifically, *Rhizopogon* spp. had a nonrandom association with *P. andromedea* lineages ($P = 0.0001$); *Rhizopogon* spp. had a nonrandom association with *Pinus contorta*, *P. ponderosa* and *P. strobus* ($P = 0.0005$); and *P. andromedea* groups had a nonrandom association with *P. contorta*, *P. ponderosa* and *P. strobus* ($P = 0.0007$). Although the sampling scheme of targeting monotypic stands may have favored this outcome, the mycobiont *R. salebrosus* was never recovered in *P. ponderosa* stands and *R. arctostaphyli* was never recovered in *P. contorta* stands despite close geographic proximity.

Specific patterns of associations from the mycoheterotrophic perspective emerged. PTAN-Major and PTAN-Minor associated almost exclusively with *R. salebrosus*. PTAN-Major had four exceptions to a strict one-to-one relationship with its mycobiont: two in *R. ellенае*, P1223 and P1257, and two in *R. arctostaphyli*, P345 and P383. PTAN-Minor only had two mycobiont exceptions from *R. salebrosus*, both occurring within *R. ellенае*, P176 and P414. PTAN-Arctostaphyli had the least amount of diversity with only one haplotype and also exhibited the highest level of specificity as only one sample, P447, did not associate with *R. arctostaphyli* but instead associated with *R. ellенае*. All PTAN-Arctostaphyli samples were found with the autotrophic host *P. ponderosa*. PTAN-Eastern was found in the disjunct distributions in the Great Lakes and New England area and was found in association with *R. kretzeræ* and a novel association with *R. salebrosus*, P211 (Figure 2a). Also, PTAN-Eastern included a new *P. andromеdеа* haplotype from eastern New York (P217). This is only the second recorded haplotype ever recovered in the Eastern distribution of *P. andromеdеа*. However, this study included collections from New York and New Hampshire which were previously unstudied. PTAN-Eastern was also found throughout the eastern extremities of the western distribution, specifically the eastern-most portion of the south-central Rocky Mountains and associated exclusively with *R. salebrosus* within *P. ponderosa* stands.

Alternatively, patterns of mycoheterotroph specificity from an ectomycorrhizal perspective were present. Overall, *R. salebrosus* is the most widespread association, had the most intraspecific resolution associated primarily with PTAN-Major and PTAN-Minor. Six associations were recorded with PTAN-Eastern, including the novel discovery of an *R. salebrosus* association in the disjunct Eastern area, R211 (Figure 2B). *Rhizopogon salebrosus* did not associate with PTAN-Arctostaphyli. *Rhizopogon kretzeræ* only associated with PTAN-

Eastern of *P. andromedea*. Contrary to the two recovered haplotypes in PTAN-Eastern, there were many haplotypes present within *R. kretzeriae*. *Rhizopogon arctostaphyli* was highly specific with PTAN-Arctostaphyli, with only two other associations occurring with PTAN-Major, R346 and R383. As with PTAN-Arctostaphyli, *R. arctostaphyli* only occurred with *P. ponderosa* or in mixed stands with *P. ponderosa*. *Rhizopogon ellenae* had no specific pattern and appeared to be nonspecific within *P. andromedea*, with respect to the other *Rhizopogon* species. PTAN-Eastern was the only *P. andromedea* group with which *R. ellenae* did not associate, which could be due to the lack of geographic overlap or very low frequency if parapatric conditions exist.

Patterns of mycoheterotroph and mycobiont specificity for autotrophic hosts were also present. *Pinus contorta* was only found in association with PTAN-Major and PTAN-Minor as well as in *R. salebrosus* and *R. ellenae*. All *P. andromedea* and *Rhizopogon* species groups were found with *P. ponderosa*. *Pinus strobus* was observed as the only autotrophic host in the Eastern disjunct distribution of the symbioses which is consistent with previous findings (Hazard et al. 2012, Grubisha et al. 2014). Although PTAN-Eastern only associated with *R. salebrosus* within *P. ponderosa* stands in the eastern margins of the western distribution, not all of the samples collected in *P. ponderosa* stands in the eastern extremity of the western sampling were contained within PTAN-Eastern, P050 was in PTAN-Major and P045 was in PTAN-Minor. Several samples collected in *P. contorta* stands within the same margins were alternatively placed within both PTAN-Major and PTAN-Minor (P020, P094, P106, P101, P128 and P129, P131).

Discussion

Overall, the hypothesis of high levels of host-symbiont specificity in the symbioses between *P. andromedea* and *Rhizopogon* spp. mycobionts was supported across much of the endemic range

of this association. A high level of host-symbiont specificity and an example of relaxed host-symbiont specificity were observed in the association of *P. andromedea* with *R. arctostaphyli* and *R. ellenae*, respectively. Conifer specificity was also supported for the associated autotrophic species both in conjunction and independent of geography, as well as in both sympatric and allopatric distributions. High levels of specificity were also identified in the association with *R. kretzeriae*. However, this particular pattern could be attributed to an artifact of long term allopatric conditions given the disjunct distribution. Biogeography likely plays a prominent role in the variation of associations and specificity across the endemic range for this system. The most prolific and widespread association of *P. andromedea* + *R. salebrosus*, may be the most successful association due to the higher diversity for both the plant and the fungus in this relationship.

The PTAN-Arctostaphyli + *R. arctostaphyli* association was the most specific association and appears to be largely dependent on the presence of the autotrophic host, *P. ponderosa*. PTAN-arctostaphyli + *R. arctostaphyli* specificity was present in sympatry with all other mycobionts with overlapping geographic ranges, namely *R. salebrosus* and *R. ellenae*. This association was found in Oregon, southeastern Washington and western-central Idaho, and was centered in the Blue Mountains, which is a known refugium for many plant and animal species during the Pleistocene (Wallace 1975, Soltis et al. 1997, Demboski and Cook 2001, Carstens et al. 2005, Godbout et al. 2008). The geographic boundary of this specific association is interesting as it does not appear to extend eastward of the Rocky Mountains through a zone of secondary contact between the westerly distributed *P. ponderosa* var. *ponderosa* and the easterly distributed *P. ponderosa* var. *scopulorum* (Latta and Mitton 1999). Evidence for autotroph host specificity has been recorded for *Rhizopogon* spp. in the past and any host-symbiont specificity for *P.*

ponderosa var. *ponderosa* and *R. arctostaphyli* may be responsible for the increased mycoheterotroph specificity found in the association with *Pterospora andromedea* (Molina and Trappe 1982, Massicotte et al. 1994, Molina and Trappe 1994, Molina et al. 1997). Despite this pattern of specificity with *Pinus ponderosa*, *P. andromedea* + *R. arctostaphyli* has been found in association with *Abies magnifica* in the present study (Table 1) and has previously been observed in mixed *Pinus monticola* and *A. magnifica* stands, as well as monoculture stands of *A. magnifica* (Bidartondo, MI pers. comm., 2011). Two samples in *R. arctostaphyli* did deviate from a pattern of specificity with PTAN-Arctostaphyli. Bidartondo and Bruns (2002) also found mycobiont overlap, albeit small, for three of their five recovered *P. andromedea* haplotypes which associated with both *R. arctostaphyli* and *R. salebrosus*. One of these three haplotypes, haplotype #4 (Bidartondo and Bruns 2002, Bidartondo, MI pers. comm. 2015), was not recovered in the current study and its absence may have slightly biased the data. However, out of a 73 sample dataset this haplotype occurred ten times with *R. salebrosus* and only once with *R. arctostaphyli*, and these data still supported a nonrandom association between *P. andromedea* and its mycobiont (Bidartondo and Bruns 2002). Additionally, some exceptions to a one-to-one pattern of specificity within the system, and deviations, are expected with gene trees between two neutral/unlinked molecular markers, especially in the case of distinct evolutionary mechanisms between plastid and nuclear ribosomal DNA.

The recently discovered association between *P. andromedea* and *R. ellenae* has now been found to be much more widespread than initially thought and was the only group for which a specific pattern for host-symbiont specificity was not found. These samples seemed to occur randomly throughout Washington, Oregon, Idaho, Montana and Wyoming and were commonly found adjacent to samples associated with either/both *R. arctostaphyli* and *R. salebrosus*. Since

many samples have been collected from California, it is unlikely that *P. andromedea* associates with *R. ellenae* in this area (Bidartondo et al. 2000, Bruns and Read 2000, Bidartondo and Bruns 2001, 2002, 2005). The respective lack of specificity and widespread distribution in this relationship is perplexing as this association has not been found in sympatry with *Sarcodes sanguinea* + *R. ellenae* despite the entire distribution of *S. sanguinea*, which is found almost exclusively in California, overlapping with *P. andromedea* populations. This initial evidence may have several critical implications for the evolutionary ecology in *Pterosporeae*. Recent mycobiont switches in *P. andromedea* via vicariant events is theoretically possible and cannot be ruled out since it likely happened at some point in the evolutionary history of this symbiosis. *Pterospora andromedea* is considered to be a more prolific disperser of seeds than *S. sanguinea* (Bakshi 1959, Luoma 1987, Bruns and Read 2000), and the wind-dispersed dust-like seeds of *P. andromedea* would have more easily circumvented geographic barriers of the Great Basin Divide as well as between two distinct areas of refugia, the Sierra Nevada Mountains and the Klamath/Siskiyou Mountains (Soltis et al. 1997, Janzen et al. 2002, Swenson and Howard 2005, Eckert et al. 2008). It may also be possible that *S. sanguinea* is a better local competitor for mycobionts and dispersal distance is not a crucial trait for any significant selection pressure. Mycobiont host switching under sympatric conditions also may have happened and would likely have preceded specificity since ecological theory suggests that host jumps within highly specific parasitic interactions are rare events (Ellers et al. 2012). In fact, Merckx and Bidartondo (2008) concluded that in mycoheterotrophs there was a lack of mycobiont switching to distant fungal lineages due to the high specificity of interspecific physiological germination cues (Bruns and Read 2000, Leake et al. 2004, Bidartondo and Bruns 2005). Mycoheterotroph associations which are less specific would be the better candidates for mycobiont switching or perhaps represent

ancestral and more diverse lineages as previously hypothesized and may have experienced a regional extinction event (Dowie et al. 2011). Bidartondo and Bruns (2005) hypothesized that mycobiont shifts would also occur at higher frequency at the fringe of the geographic range and that allopatric settings would perpetuate the divergence of the different fungal taxa associated with *P. andromedea* and *S. sanguinea* thus resulting in divergence within *Pterosporeae*. Increased phylogenetic resolution within *Rhizopogon* subgenus *Amylopogon* and within *Pterosporeae* would likely resolve these discrepancies.

Rhizopogon salebrosus is the most abundant associate of *P. andromedea* and is likely the most important in terms of persistence and conservation. This association was found in almost all areas and, although severely limited in the eastern distribution, had the highest level of intraspecific phylogenetic resolution and associated with the greatest number of *P. andromedea* haplotypes. Most of the supported subclades within *R. salebrosus* and associations could be clumped into the Pacific Northwest, which has been hypothesized as the area of origin for both Monotropoideae and *Rhizopogon* due to the high species diversity (Smith and Zeller 1966, Wallace 1975, Molina and Trappe 1994, Massicotte et al. 1999, Merckx et al. 2013). This area has long been considered a hotspot of diversity for many organisms due to ancestral refugia during glacial cycles (Soltis et al. 1997, Runck and Cook 2005, Swenson and Howard 2005, Shafer et al. 2010). This specific association may be the one association that is the least vulnerable to environmental change, which may also be said of the population in the Blue Mountain refugia (Shafer et al. 2010, Dufresnes et al. 2013). Additionally, this study uncovered evidence for possible introgression or hybridization in the *Rhizopogon* mycobiont. R128 and R129 samples also were found in association with the most common *P. andromedea* haplotype

and occurred in an area previously found to contain putative hybrids of *R. salebrosus* from a northern and southern lineage (Dowie et al. 2012).

Biogeographic patterns were also found in PTAN-Eastern and *R. kretzeriae*. Overall, one dominant haplotype of *P. andromedea* was found to associate with *R. kretzeriae* and subsequently *P. strobilus*. This haplotype was also observed to associate with *R. salebrosus* in the eastern-most margins of the western distribution, extended from New Mexico to the Black Hills of South Dakota and was almost exclusively limited to *P. ponderosa*. This previously identified haplotype was thought to be common as it has been found on the western-most portion of the western distribution, namely the Sierra Nevada Mountains (Bidartondo and Bruns 2002, Hazard et al. 2012). However, there was a notable absence in the interior of the western distribution of *P. andromedea* and north of the Klamath Mountains. Additionally, the western-most distribution is the most established, likely ancestral, and provides new insight into a subsequent eastward migration of *P. andromedea*. This is also tangentially supported by the novel discovery of *R. salebrosus* in the western-most margins of the eastern distribution. It is also plausible that *R. kretzeriae* speciated from *R. salebrosus*, especially if *R. kretzeriae* and *R. salebrosus* are indeed sister species as hypothesized by (Grubisha et al. 2014). The newly discovered haplotype in the east also shared a unique character state with the haplotype recorded from Bidartondo and Bruns (2002) from California, not recovered in this study.

Conclusion

The various levels of specificity in this tripartite relationship revealed from this study of *P. andromedea* serve to emphasize the cryptic and complex evolutionary ecology in mycoheterotrophs. Specific to this relationship, *Pterospora andromedea* + *Rhizopogon* subgenus

Amylopogon spp. should now be considered as having a range of host-symbiont specificity within populations and communities across a geographic mosaic. This variation in specificity may also be represented in many mycoheterotrophic systems. Future extrapolations of system-wide specificity are cautioned as specific associations, geographic area, environmental factors, small-scale sampling or the comparative groups used in analyses may bias the results and provide misleading evidence. Further investigation into specific examples of symbiont overlap between lineages will prove to be invaluable to more fully understand the evolutionary events and potential coevolutionary history for mycoheterotroph specificity. Similarly, evidence for variability in specificity across a geographic distribution may be beneficial for conservation purposes such as assessing extinction risks or in determining the sensitivity or resilience to perturbation for specific associations at local levels. At this time it is unfortunate that more suitable molecular markers are unavailable to more thoroughly evaluate these symbioses. However, multiple anonymous nuclear loci for both *P. andromedea* and *Rhizopogon* subgenus *Amylopogon* are currently being developed, which will allow more comprehensive examination of the evolutionary ecology and phylogeography in this system while simultaneously addressing negative and positive consequences of specificity in this specific plant-fungus ectomycorrhizal symbiosis.

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Figure Captions

Figure 1: A tanglegram representing Bayesian phylogenetic reconstructions and comparison of *Pterospora andromedea* and associated *Rhizopogon* subgenus *Amylopogon* spp. throughout the United States. Lines are drawn connecting all pairs; green lines depict a positive host-parasite link assessed at $P = 0.10$, thickened lines were assessed at $P = 0.05$, thickest green lines were assessed at $P = 0.01$, a black line represents a deviation from an otherwise emergent association, thickened lines indicate significance at $P = 0.05$ from ten iterations of 9 999 permutations in ParaFit. Posterior probability is indicated by number. Support was derived from 375 000 trees after a burnin of 125 000 (25%).

Figure 2: Comparative phylogenies of *Pterospora andromedea* and associated *Rhizopogon* spp. overlaid on map of geographic areas using GenGis v2.0. Although both figures are similar, they are drawn from different perspectives i.e. the symbol shape and color of each group is oriented by perspective: (A) is drawn from a perspective of *P. andromedea*; (B) is drawn from the perspective of the associated *Rhizopogon* species.

Table 1 Sample identification and phylogenetic assignment for *Pterospora andromedea* and *Rhizopogon* spp. pairs as well as GenBank accession numbers and geographic area for collections.

<i>Pterospora</i>	Lineages	Genbank	<i>Rhizopogon</i>	<i>Rhizopogon</i>	Genbank	Geographic region and State	<i>Pinus</i> Species	Collector(s) of
Sample ID	Assignment	Accession	Sample ID	Species	Accession	(NF=National Forest)	Present	Samples
P016	PTAN-Major	KC170047	R016	<i>salebrosus</i>	HQ914255	Snowy Range, WY	<i>P. contorta</i>	N. Dowie
P020	PTAN-Minor	KC170048	R020	<i>salebrosus</i>	HQ914257	Snowy Range, WY	<i>P. contorta</i>	N. Dowie
P045	PTAN-Minor	KC170049	R045	<i>salebrosus</i>	HQ914274	Santa Fe NF, NM	<i>P. ponderosa</i>	N. Dowie
P050	PTAN-Major	KC170050	R050	<i>salebrosus</i>	HQ914279	Carson NF, NM	<i>P. ponderosa</i>	N. Dowie
P060	PTAN-Eastern	KC170051	R060	<i>salebrosus</i>	KJ555110	Carson NF, NM	<i>P. ponderosa</i>	N. Dowie
P078	PTAN-Minor	KC170052	R078	<i>salebrosus</i>	HQ914290	Wasatch NF, UT	<i>P. contorta</i>	N. Dowie
P079	PTAN-Major	KC170053	R079	<i>salebrosus</i>	HQ914291	Wasatch NF, UT	<i>P. contorta</i>	N. Dowie
P094	PTAN-Major	KC170054	R094	<i>salebrosus</i>	HQ914298	Laramie Mountains, WY	<i>P. contorta</i>	N. Dowie
P101	PTAN-Major	KC170055	R101	<i>salebrosus</i>	HQ914302	Roosevelt NF, CO	<i>P. contorta</i>	N. Dowie
P106	PTAN-Major	KC170056	R106	<i>salebrosus</i>	HQ914307	Roosevelt NF, CO	<i>P. contorta</i>	N. Dowie
P123	PTAN-Major	KC170057	R123	<i>salebrosus</i>	KC170117	Bridger Teton Mountains, WY	<i>P. contorta</i>	N. Dowie
P126	PTAN-Minor	KC170058	R126	<i>salebrosus</i>	HQ914321	Bridger Teton Mountains, WY	<i>P. contorta</i>	N. Dowie
P128	PTAN-Major	KC170059	R128	<i>salebrosus</i>	KC170118	Medicine Bow, WY	<i>P. contorta</i>	N. Dowie
P129	PTAN-Major	KC170060	R129	<i>salebrosus</i>	KC170119	Medicine Bow, WY	<i>P. contorta</i>	N. Dowie
P131	PTAN-Major	KC170061	R131	<i>salebrosus</i>	KC170120	Medicine Bow, WY	<i>P. contorta</i>	N. Dowie
P138	PTAN-Eastern	KC170062	R138	<i>salebrosus</i>	HQ914325	Pike NF, CO	<i>P. ponderosa</i>	N. Dowie
P140	PTAN-Eastern	KC170063	R140	<i>salebrosus</i>	HQ914326	Pike NF, CO	<i>P. ponderosa</i>	N. Dowie
p157	PTAN-Major	KC170064	R157	<i>salebrosus</i>	HQ914334	White River NF, CO	<i>P. ponderosa</i>	N. Dowie
P162	PTAN-Major	KC170065	R162	<i>salebrosus</i>	HQ914335	Green Mountains, WY	<i>P. contorta</i>	N. Dowie
P169	PTAN-Eastern	KC170066	R169	<i>salebrosus</i>	KC170121	Black Hills, WY	<i>P. ponderosa</i>	N. Dowie
P172	PTAN-Eastern	KC170067	R172	<i>salebrosus</i>	HQ914342	Black Hills, WY	<i>P. ponderosa</i>	N. Dowie
P173	PTAN-Major	KC170068	R173	<i>salebrosus</i>	KC170122	Yellowstone National Park, WY	<i>P. contorta</i>	N. Dowie
P174	PTAN-Major	KC170069	R174	<i>salebrosus</i>	KC170123	Yellowstone National Park, WY	<i>P. contorta</i>	N. Dowie
P175	PTAN-Minor	KC170070	R175	<i>salebrosus</i>	KC170124	Beartooth Mountains, MT	<i>P. contorta</i> , <i>P. ponderosa</i>	N. Dowie
P176	PTAN-Minor	KC170071	R176	<i>ellenae</i>	KC170125	Beartooth Mountains, MT	<i>P. contorta</i> , <i>P. ponderosa</i>	N. Dowie
P177	PTAN-Major	KC170072	R177	<i>salebrosus</i>	JF695013	Yellowstone National Park, WY	<i>P. contorta</i>	N. Dowie
P179	PTAN-Major	KC170073	R179	<i>salebrosus</i>	JF695019	Gallatin NF, MT	<i>P. contorta</i> , <i>P. ponderosa</i>	N. Dowie
P182	PTAN-Major	KC170074	R182	<i>salebrosus</i>	KC170126	Yellowstone National Park, WY	<i>P. contorta</i>	N. Dowie

P186	PTAN-Major	KC170075	R186	<i>salebrosus</i>	KC170127	Yellowstone National Park, WY	<i>P. contorta</i>	N. Dowie
P187	PTAN-Minor	KC170076	R187	<i>salebrosus</i>	KC170128	Outside of Missoula, MT	<i>P. ponderosa</i>	N. Dowie
P206	PTAN-Eastern	KC170077	R206	<i>kretzeriae</i>	KC170129	Ottawa NF, MI	<i>P. strobus</i>	N. Dowie/S. Miller
P210	PTAN-Eastern	KC170078	R210	<i>kretzeriae</i>	KC170130	Ottawa NF, MI	<i>P. strobus</i>	N. Dowie/S. Miller
P211	PTAN-Eastern	KC170079	R211	<i>salebrosus</i>	KC170131	Ottawa NF, MI	<i>P. strobus</i>	N. Dowie/S. Miller
P215	PTAN-Eastern	KC170080	R215	<i>kretzeriae</i>	KC170132	Sault Ste. Marie State Forest, MI	<i>P. strobus</i>	N. Dowie/S. Miller
P217	PTAN-Eastern	KC170081	R217	<i>kretzeriae</i>	KC170133	Point Au Roche, NY	<i>P. strobus</i>	N. Dowie/S. Miller
P220	PTAN-Eastern	KC170082	R220	<i>kretzeriae</i>	KC170134	South of Hanover, NH	<i>P. strobus</i>	N. Dowie/S. Miller/M. Klooster
P222	PTAN-Eastern	KC170083	R222	<i>kretzeriae</i>	KC170135	South of Hanover, NH	<i>P. strobus</i>	N. Dowie/S. Miller/M. Klooster
P223	PTAN-Eastern	KC170084	R223	<i>kretzeriae</i>	KC170136	South of Hanover, NH	<i>P. strobus</i>	N. Dowie/S. Miller/M. Klooster
P243	PTAN-Major	KC170085	R243	<i>salebrosus</i>	KC170137	Olympic NF, WA	<i>P. monticola</i>	N. Dowie/L. Grubisha
P248	PTAN-Major	KC170086	R248	<i>salebrosus</i>	KC170138	Okanogan NF, WA	<i>P. ponderosa</i>	N. Dowie/L. Grubisha
P257	PTAN-Major	KC170087	R257	<i>salebrosus</i>	KC170139	Okanogan NF, WA	<i>P. ponderosa</i>	N. Dowie/L. Grubisha
P283	PTAN-Major	KC170088	R283	<i>salebrosus</i>	KC170140	Wenatchee NF, WA	<i>P. ponderosa</i>	N. Dowie/L. Grubisha
P285	PTAN-Arctostaphyli	KC170089	R285	<i>arctostaphyli</i>	KC170141	Wenatchee NF, WA	<i>P. ponderosa</i>	N. Dowie/L. Grubisha
P286	PTAN-Arctostaphyli	KC170090	R286	<i>arctostaphyli</i>	KC170142	Wenatchee NF, WA	<i>P. ponderosa</i>	N. Dowie/L. Grubisha
P287	PTAN-Major	KC170091	R287	<i>salebrosus</i>	KC170143	Wenatchee NF, WA	<i>P. ponderosa</i>	N. Dowie/L. Grubisha
P288	PTAN-Major	KC170092	R288	<i>salebrosus</i>	KC170144	Wenatchee NF, WA	<i>P. ponderosa</i>	N. Dowie/L. Grubisha
P290	PTAN-Arctostaphyli	KC170093	R290	<i>arctostaphyli</i>	KC170145	Wenatchee NF, WA	<i>P. ponderosa</i>	N. Dowie/L. Grubisha
P321	PTAN-Arctostaphyli	KC170094	R321	<i>arctostaphyli</i>	KC170146	Umatilla NF, WA	<i>P. contorta, P. ponderosa</i>	N. Dowie
P322	PTAN-Major	KC170095	R322	<i>salebrosus</i>	KC170147	Umatilla NF, WA	<i>P. contorta, P. ponderosa</i>	N. Dowie
P346	PTAN-Major	KC170096	R346	<i>arctostaphyli</i>	KC170148	Umatilla NF, OR	<i>P. ponderosa</i>	N. Dowie
P355	PTAN-Minor	KC170097	R355	<i>salebrosus</i>	KC170149	Umatilla NF, OR	<i>P. ponderosa</i>	N. Dowie
P356	PTAN-Major	KC170098	R356	<i>Salebrosus</i>	KC170150	Umatilla NF, OR	<i>P. ponderosa</i>	N. Dowie
P383	PTAN-Major	KC170099	R383	<i>arctostaphyli</i>	KC170151	Ochoco NF, OR	<i>P. ponderosa</i>	N. Dowie
P384	PTAN-Major	KC170100	R384	<i>salebrosus</i>	KC170152	Ochoco NF, OR	<i>P. ponderosa</i>	N. Dowie
P413	PTAN-Minor	KC170101	R413	<i>salebrosus</i>	KC170153	Wallowa-Whitman NF, OR	<i>P. contorta</i>	N. Dowie
P414	PTAN-Minor	KC170102	R414	<i>ellenae</i>	KC170154	Wallowa-Whitman NF, OR	<i>P. contorta</i>	N. Dowie
P415	PTAN-Major	KC170103	R415	<i>salebrosus</i>	KC170155	Wallowa-Whitman NF, OR	<i>P. ponderosa</i>	N. Dowie
P439	PTAN-Arctostaphyli	KC170104	R439	<i>arctostaphyli</i>	KC170156	Boise NF, ID	<i>P. ponderosa</i>	N. Dowie
P447	PTAN-Arctostaphyli	KC170105	R447	<i>ellenae</i>	KC170157	Boise NF, ID	<i>P. ponderosa</i>	N. Dowie
P448	PTAN-Major	KC170106	R448	<i>salebrosus</i>	KC170158	Boise NF, ID	<i>P. contorta, P. ponderosa</i>	N. Dowie
P1036	PTAN-Major	KC170042	R1036	<i>salebrosus</i>	KC170112	Clearwater NF, ID	<i>P. contorta</i>	S. Miller

P1092	PTAN-Arctostaphyli	KC170043	R1092	<i>arctostaphyli</i>	KC170113	Klamath NF, OR	<i>P. ponderosa</i>	S. Miller
P1093	PTAN-Major	KC170044	R1093	<i>salebrozus</i>	KC170114	Klamath NF, OR	<i>Abies magnifica</i>	S. Miller
P1144	PTAN-Major	KC170045	R1144	<i>salebrozus</i>	KC170115	Deschutes NF, OR	<i>P. contorta</i>	S. Miller
P1146	PTAN-Arctostaphyli	KC170046	R1146	<i>arctostaphyli</i>	KC170116	Deschutes NF, OR	<i>P. ponderosa</i>	S. Miller
P1222	PTAN-Major	KC170037	R1222	<i>salebrozus</i>	KC170107	Bighorn Mountains, WY	<i>P. contorta</i>	S. Trowbridge
P1223	PTAN-Major	KC170038	R1223	<i>ellenae</i>	KC170108	Bighorn Mountains, WY	<i>P. contorta</i>	S. Trowbridge
P1242	PTAN-Major	KC170039	R1242	<i>salebrozus</i>	KC170109	Little Rockies, MT	<i>P. contorta</i>	S. Trowbridge
P1254	PTAN-Major	KC170040	R1254	<i>salebrozus</i>	KC170110	Beartooth Mountains, MT	<i>P. contorta, P. ponderosa</i>	S. Trowbridge
P1257	PTAN-Major	KC170041	R1257	<i>ellenae</i>	KC170111	Casper Mountain, WY	<i>P. contorta</i>	S. Trowbridge

Figure 1

Rhizopogon subgenus *Amylopogon* (ITS)

Pterospora andromedea (*trnL*) lineages

Figure 2a

Figure 2b

Figure 2a Black and white

Figure 2b black and white