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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. *P. 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.

1. 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 life-cycle, 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, 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

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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, 2005; Barrett et al., 2010).

P. 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 *Rhizopogon salebrosus*, *Rhizopogon arctostaphyli*, and *Rhizopogon kretzerae*, 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* + *Rhizopogon 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.

2. Materials and methods

2.1. Sample collection, DNA extraction and sequencing

P. 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 photoautrophic 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 *Pinus ponderosa*, *Pinus contorta*, *Pinus strobus*, *Pinus monticola*, *P. strobiformis*, *Pinus 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). *P. 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 and 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.

2.2. 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, 2012; Hazard et al., 2012). GenBank accession numbers were: AF224276, AF351874, AF377122, AF377133, AF377134, AF377135, AF442136, AF377144,