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Authors

Hynson, Nicole A

Weiß, Michael

Preiss, Katja

et al.

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Fungal host specificity is not a bottleneck for the germination of Pyroleae species (Ericaceae) in a Bavarian forest

NICOLE A. HYNSON,* MICHAEL WEIß,† KATJA PREISS,‡ GERHARD GEBAUER‡ and KATHLEEN K. TRESEDER§

*Department of Botany, University of Hawaii Manoa, 3190 Maile Way, Honolulu, HI 96822, USA, †Department of Biology, University of Tübingen, Auf der Morgenstelle 5, 72076 Tübingen, Germany, ‡Laboratory of Isotope Biogeochemistry, Bayreuth Center of Ecology and Environmental Research (BayCEER), University of Bayreuth, 95440, Bayreuth, Germany, §Department of Ecology and Evolutionary Biology, University of California-Irvine, Irvine, CA 92697, USA

Abstract

Plants that produce dust seeds can recruit fungi to meet their earliest requirements for carbon and other nutrients. This germination strategy, termed initial mycoheterotrophy, has been well investigated among the orchid family, but there are numerous other plant lineages that have independently evolved mycoheterotrophic germination strategies. One of these lineages is the tribe Pyroleae (Ericaceae). While the fungi associated with mature plants in Pyroleae have been fairly well documented, their mycobionts at the germination and seedling stages are largely unknown. Here, we use an *in situ* seed baiting experiment along with molecular fingerprinting techniques and phylogenetic tests to identify the fungi associated with seedlings of two Pyroleae species, *Pyrola chlorantha* and *Orthilia secunda*. Our results indicate that similar to adult plants, Pyroleae seedlings can associate with a suite of ectomycorrhizal fungi. Some seedlings harboured single mycobionts, while others may have been inhabited by multiple fungi. The dominant seedling mycobiont of both Pyroleae species was a fungus of unknown trophic status in the order Sebaciales. This taxon was also the only one shared among seedlings of both investigated Pyroleae species. We discuss these results juxtaposed to orchids and one additional *Pyrola* species in the context of ontogenetic shifts in fungal host specificity for mycoheterotrophic nutrition.

Keywords: dust seeds, Ericaceae, fungi, germination, mycoheterotrophy, mycorrhizae, Pyroleae, Sebaciales

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Introduction

Dust seeds have evolved independently in at least 12 plant families (Eriksson & Kainulainen 2011). Owing to their lack of carbohydrate reserves, all dust seeds are dependent on an external carbon source for germination (Eriksson & Kainulainen 2011). A subset of the plant families that produce dust seeds meet their carbon demands via symbiotic associations with fungi. This form of nutrition is referred to as initial mycoheterotrophy (Leake 1994). While some plant species are only ini-

tially mycoheterotrophic, others remain partially or completely dependent upon fungi to meet their carbon and other nutrient demands throughout the remainder of their life cycles. The most well-studied groups of mycoheterotrophic plants are the orchids, all of which are likely initially mycoheterotrophic (Dearnaley 2007), and the tribes Pterosporeae and Monotropeae (Ericaceae), which contain only fully mycoheterotrophic species (Bidartondo & Bruns 2005). A less-studied dust seed-producing tribe is Pyroleae (Ericaceae), which contains 37 species (Liu *et al.* 2010). All species in Pyroleae are initially mycoheterotrophic, with a few species remaining partially mycoheterotrophic (a form of mixotrophy) upon reaching adulthood (Tedersoo *et al.* 2007; Zimmer

Correspondence: Nicole A. Hynson, Fax: (808) 956 3923; E-mail: nhynson@hawaii.edu

et al. 2007; Matsuda et al. 2012). There is also a single species of Pyroleae, *Pyrola aphylla*, that is fully mycoheterotrophic (Hynson et al. 2009a). While recent studies have used molecular fingerprinting techniques to identify the fungi associated with adult plants in Pyroleae (Tedersoo et al. 2007; Zimmer et al. 2007; Vincenot et al. 2008; Hynson & Bruns 2009; Toftegaard et al. 2010), the fungi involved in the germination and seedling stages of these plants have remained largely unknown.

Mature plants within Pyroleae tend to associate with a diversity of fungi that also form ectomycorrhizae with trees (Tedersoo et al. 2007; Zimmer et al. 2007; Massicotte et al. 2008; Vincenot et al. 2008; Hynson & Bruns 2009; Toftegaard et al. 2010; Hashimoto et al. 2012). Pyroleae species (pyroloids) dependency on ectomycorrhizal fungi is similar to closely related fully mycoheterotrophic species in Monotropoideae and Pterosporeae (monotropes). The ectomycorrhizal symbionts of monotropes are shared with surrounding trees to create mycorrhizal networks that physically connect these unrelated plants via their fungal associates. These networks in turn support mycoheterotrophy, where the ultimate carbon source for fully mycoheterotrophic plants that associate with ectomycorrhizal fungi is autotrophic trees (Leake & Cameron 2010). Unlike pyroloids, monotropes tend to be highly specific to particular fungal hosts throughout their life cycles (Bidartondo & Bruns 2005). It has been suggested (Hynson & Bruns 2009) and recently demonstrated (Hashimoto et al. 2012) that pyroloids too require specific fungi, but only at their initial mycoheterotrophic stages. Thus, similar to some orchids (Bidartondo & Read 2008), the bottleneck for pyroloids' survival may involve encountering an appropriate fungal host at an early stage of development.

To date, there are only two published accounts of the fungi associated with Pyroleae seedlings. The first is from germinated *Pyrola chlorantha* and *Orthilia secunda* seeds from Europe (Smith & Read 2008). The second is a recent study of a single species of Pyroleae, *Pyrola asarifolia*, from northern Japan (Hashimoto et al. 2012). Both studies found that the primary mycobionts of seedlings were fungi of unknown trophic status in the order Sebaciales. However, the findings from Europe were based on a very small sample size (seven seedlings), and the investigations from Japan were on well-developed mycoheterotrophic seedlings. Thus, our goals were to analyse a more extensive sampling of *P. chlorantha* and *O. secunda* seedlings and to determine the fungi responsible for both the initial stages of germination and later stages of seedling development. We chose to focus on these two species of pyroloids because they are relatively common components of deciduous and evergreen forests in North America and Europe (Rydberg 1914; Düll & Kutzelnigg 1994), and previous

studies have identified the mycobionts of adult plants (Tedersoo et al. 2007; Zimmer et al. 2007). Also, *P. chlorantha* and *O. secunda* are photosynthetically active when mature, but are potentially capable of partial mycoheterotrophy (Tedersoo et al. 2007; Zimmer et al. 2007). To accomplish our goals, we used an *in situ* seed baiting technique, molecular and phylogenetic tools to identify the mycobionts associated with single seedlings of each Pyroleae species (Brundrett et al. 2003; Bidartondo & Bruns 2005).

Materials and methods

Seed packets

Seeds of *Pyrola chlorantha* and *Orthilia secunda* were collected in late August 2005 from a dense broadleaf forest in NE Bavaria, Germany (49°40'N and 11°23'E) dominated by *Fagus sylvatica* (for a detailed site description, see Zimmer et al. 2008). Harvested seeds were removed from mature capsules and sealed in nylon netting of 37-µm mesh enclosed in photographic slide mounts (50 × 50 mm) with coloured string attached. Each seed packet contained approximately 100 seeds of each Pyroleae species. In early September 2005 at the same site where seeds were collected, the packets were buried <0.5 m from adult plants of the same species. To minimize soil disturbance, we used a narrow planting spade to create a small fissure into which single packets were pushed into the soil to a depth of approximately 10–15 cm. Then, the spade was removed, soil moved back in place by hand, and the loose end of the string was staked above ground. For each species, we established five 10 × 10 blocks of packets, with each packet buried about 10 cm from another, for a total of 500 packets per species. In September 2007, the seed packets were harvested by pulling on their strings. Packets were then sealed in plastic bags, put on ice and brought directly back to the University of Bayreuth for further inspection. Using a dissecting scope, moistened seed packets were inspected for germination and fungal colonization. A seed was considered germinated when the testa was broken and the embryo was enlarged or emerging (Fig. 1B). Germinated seeds from individual packets were preserved in 100 µL of 2× CTAB buffer and stored at –20 °C for future molecular analyses. All seed packets were inspected within 3 days of harvest.

Molecular identification of Pyroleae seedling fungi

DNA was extracted from individual seedlings by boiling them in 10 µL of a basic Tris/EDTA 1 M solution (~pH of 8), after which 20 µL of 3% BSA buffer was added. Polymerase chain reaction was carried out in

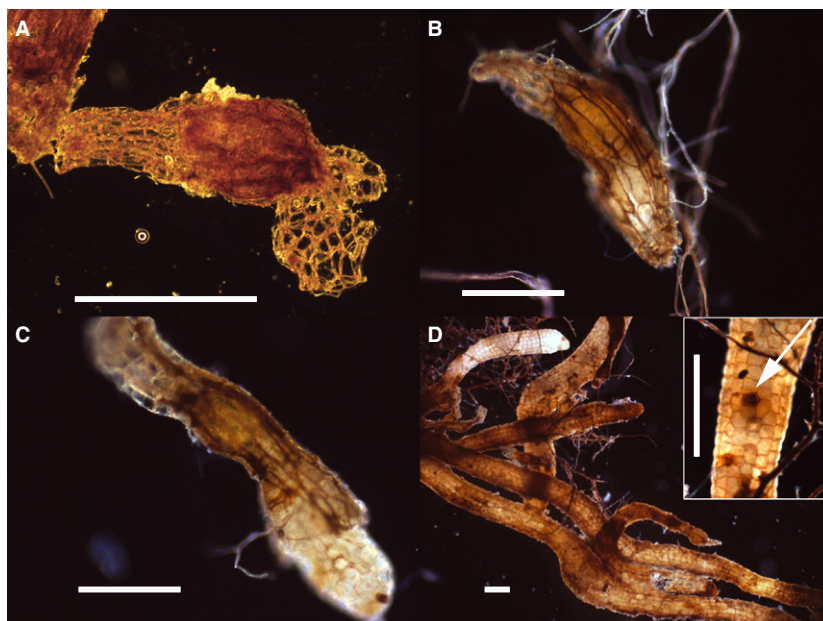


Fig. 1 Micrographs of *Pyrola chlorantha* seedlings, some surrounded by different morphotypes of fungal hyphae. White bars represent approximately 0.5 mm. (A) An ungerminated seed with no fungal colonization, (B) a seedling where the embryo has become swollen, but has not emerged from the testa, (C) a seedling that has broken the testa and (D) a well-developed seedling. We visually and molecularly detected fungal colonization at stages B–D. The seedling in photograph D was colonized by a member of the Sebaciniales clade B. The arrow in figure D (insert) highlights an example of colonization by Sebaciniales 1.

25- μ L total volume reactions that contained 2.5 μ L of DNA extract, 10 μ L of REDExtract-N-Amp PCR Ready-Mix (Sigma-Aldrich, St Louis, MO, USA), 0.6 μ L of one of two sets of fungal specific primers in 50 μ M concentrations and 11.9 μ L of purified deionized water. Following the thermal cycler protocol described in Gardes and Bruns (1993), the first primer pair, ITS1F and ITS4, was used to amplify the nuclear ribosomal internal transcribed spacer (nrITS) region. Successful amplicons were purified and unidirectionally sequenced by Beckman Coulter Genomics (Danvers, MA, USA) with the ITS1F primer. These sequences were used to determine the identities of the fungi associated with Pyroleae seedlings (see Data Analysis section below). In addition to the nrITS region, we used a second primer pair (ITS1F and TW14) to amplify part of the nuclear ribosomal large subunit (nrLSU) (Bidartondo & Duckett 2010) on a subset of seedlings found to associate with Sebaciniales fungi. These amplicons were also unidirectionally sequenced using the TW14 primer. The LSU amplicons along with all Sebaciniales ITS amplicons were used to examine the phylogenetic relationship of these sequences (see Results section).

Data analysis and phylogenetics

Trimmed ITS sequences with a quality score of >80% were imported into a Sequencher project (v 4.7) and binned at 95% similarity. The dominant haplotype from each bin, or singleton sequences, was compared to those in the GenBank database using the BLASTN algorithm. Unknown sequences from Pyroleae seedlings were considered matches to top hits in GenBank when there was

a Max Identity score of $\geq 95\%$. These hits were used to define our taxa at the generic or ordinal level (Table 1). Representative DNA sequences are deposited in GenBank (ITS Accession nos JX561229–JX561242).

Based on the analysis of ITS sequences described above, we identified a diversity of fungi from the order Sebaciniales associating with multiple seedlings of each Pyroleae species (see Results section and Table 1). To place these fungi in a phylogenetic context, a data set of reference sequences from GenBank was constructed as follows: first, we performed separate BLAST searches against the GenBank nucleotide database for each of the ITS sequence types found in this study and did so also for their respective LSU complements (GenBank LSU Accession nos JX623961–JX623965). Sequences of the closest matches for each of these BLAST searches were retrieved and stored. To increase taxonomic resolution in our data set, we additionally included a sampling of representative GenBank Sebaciniales sequences derived from named vouchers or cultures determined to species or genus. To facilitate phylogenetic analysis of ITS and LSU sequences in one integrated run, this data set was complemented by full-length ITS + LSU Sebaciniales sequences, mostly taken from Riess (2009) and Garnica *et al.* (2012). A preliminary alignment performed with MAFFT, v. 6.903b (Katoh *et al.* 2002; Katoh & Toh 2008), was used to separate this data set into three components: (i) ITS + LSU sequences, (ii) ITS sequences and (iii) LSU sequences. We then aligned the ITS + LSU sequences and used this alignment as a skeleton to subsequently add the ITS and finally the LSU sequences using the seed option of MAFFT 6.903b. Last, we added the sequences generated in this study to this profile alignment.

Table 1 Identity of fungi associated with *Pyrola chlorantha* and *Orthilia secunda* seedlings based on closest BLAST match of fungal ITS sequences. Query sequences were considered a match to reference ones in GenBank only if the Max identity score was >95%

Plant species	Fungus	Seed packet ID	Number of seedlings	Accession no. of best BLAST match	Name in GenBank
<i>O. secunda</i>	<i>Ilyonectria</i> *	OS08	1	JF735278.1	<i>Ilyonectria rufa</i>
	<i>Russula</i>	OS07	1	AM087279.1	<i>Russula mairei</i>
	Sebacinales 1	OS04	1	EU909214.1	<i>Sebacina</i> mycobiont of <i>Riccardia palmata</i>
		OS06	5	EU909214.1	
		OS07	3	EU909214.1	
	Sebacinales 2	OS03	2	EU668225.1	<i>Sebacina</i> isolate 7.8061.1.L
	Sebacinales 4†	OS05	1	EU668269.1	<i>Sebacina</i> isolate III.cm98.fs
	Sebacinales 5	OS01	1	FR852338.1	<i>Sebacina</i>
	<i>Tricholoma</i>	OS02	1	HQ184170.1	<i>Tricholoma</i>
	<i>Verticillium</i> *	OS03	1	FJ197918.1	<i>Verticillium</i> clone W23
<i>P. chlorantha</i>	<i>Cortinarius</i>	PC03	2	HQ604725.1	<i>Cortinarius junghuhnii</i>
	<i>Inocybe</i> 1	PC06	2	JF908088.1	<i>Inocybe nitidiuscula</i>
	<i>Inocybe</i> 2	PC09	8	AM882864.2	<i>Inocybe terrigena</i>
	Mycosphaerellaceae*	PC16	2	FJ553102.1	Mycosphaerellaceae
	Sebacinales 1	PC03	1	EU909214.1	<i>Sebacina</i> mycobiont of <i>Riccardia palmata</i>
		PC04	2	EU909214.1	
		PC05	1	EU909214.1	
		PC07	1	EU909214.1	
		PC09	4	EU909214.1	
		PC10	7	EU909214.1	
		PC11	2	EU909214.1	
	Sebacinales 3	PC12	1	EU909214.1	
		PC13	1	EU909214.1	
		PC14	2	EU909214.1	
		PC15	1	EU909214.1	
		PC04	2	AF440650.1	<i>Sebacina</i> endomycorrhiza of <i>Neottia nidus-avis</i>
		PC06	1	AF440650.1	
	Sebacinales 6†	PC02	1	EU668220.1	<i>Sebacina</i> isolate 2.7747.3.R
	Sebacinales 7	PC01	1	GQ219904.1	<i>Sebacina</i> clone SC_ITS_101
	<i>Sistotrema</i>	PC05	1	EU668295.1	<i>Sistotrema</i> isolate I.8268.2
	<i>Tomentella</i>	PC06	1	EU668274.1	<i>Tomentella</i> isolate I.8280.5
		PC08	1	EU668274.1	

Fungi highlighted with an asterisk (*) are plant parasites; the remainder are fungi known to form ectomycorrhizae with trees except for possibly Sebacinales 1. Sebacinales 1 belongs to Sebacinales clade B that contains a diversity of functional guilds including saprotrophic, ectomycorrhizal, orchid and arbutoid mycorrhizal fungi. Seed packets highlighted in bold contained more than one fungus. From all Sebacinales except those with a † symbol, we also amplified LSU fragments.

After trimming, we used the Gblocks server (Talavera & Castresana 2007; http://molevol.cmima.csic.es/castresana/Gblocks_server.html) to eliminate alignment regions too heterogeneous for phylogenetic analysis. Individual alignment positions had to be identical in more than 50% of the included sequences to be accepted as a conserved or a flanking position, the maximum accepted number of contiguous nonconserved positions was set to eight, and the minimum length of a block was set to five positions. Gaps were accepted if they occurred in less than half of the included sequences at a given position. Using these settings, the original MAFFT alignment of 1529 positions was reduced to 985 positions to be used in phylogenetic analysis.

Molecular phylogenetic analysis was performed using the maximum-likelihood (ML) approach (Felsenstein 1981) as implemented in RAXML (Stamatakis 2006; v. 7.3.1 using the GAMMACAT model of DNA substitution). These analyses were run with integrated fast bootstrap analysis (Felsenstein 1985; Stamatakis *et al.* 2008). Preliminary trees were calculated to reduce the initial sequence sampling by deleting sequences from groups of very similar or identical sequences that were not closely related to our query sequences. The final alignment contained 384 sequences and 965 sites. In the final analysis, we ran 2000 bootstrap replicates, after a preliminary analysis using the *autoFC* option in RAXML had yielded that at least 500 replicates would be needed for stable bootstrap results (Pattengale *et al.* 2010). The

resulting tree was displayed and edited using FigTree (Fig. 2, Rambaut 2009).

Results

After two years of burial *in situ*, we recovered germinated seeds of both Pyroleae species from two of the five plots for each species (four plots total). Of the 500 harvested packets of *Pyrola chlorantha*, only 22 (4.4%) contained germinated seeds. Germinated *Orthilia secunda* seeds were found in only seven of the 500 packets (1.4%). From these packets, we collected 97 germinated *P. chlorantha* and 53 *O. secunda* seeds (Table 1). From these seedlings, we had 63% positive PCR amplification of the fungal ITS. We were unable to amplify the fungal ITS from the remaining seedlings indicating that either PCR was inhibited or that these seedlings had very low, if any, fungal DNA templates present. Seventeen sequences of the total 53 positive ITS amplicons for *O. secunda* (32%) and 43 sequences of the 97 positive ITS amplicons for *P. chlorantha* (45%) met our stringent sequence quality (>80% quality score) and BLAST match affinity (>95% Max Identity) criteria (Table 1). This led to the identification of eight ectomycorrhizal fungi in the Cantharellales, Agaricales and Thelephorales (Table 1). In addition, we identified six Sebaciniales taxa in the Sebaciniales clade A. For two of our Sebaciniales clade A taxa (OTUs 4 and 6), we did not succeed in amplifying the LSU. Thus, for these taxa, only their ITS types were included in our phylogenetic analyses. We also found three fungal plant pathogens and a single Sebaciniales taxon (Sebaciniales 1) that falls within the Sebaciniales clade B (Table 1, Fig. 2). Sebaciniales clade B was previously thought to contain no ectomycorrhizal taxa (Weiß *et al.* 2004). However, based on our phylogenetic analyses, the subclade that includes Sebaciniales 1 contains ectomycorrhizal taxa, as well as saprotrophic and arbutoid mycorrhizal ones (Fig. 2e). Interestingly, two of the closest relatives to Sebaciniales 1 were identified from mature plants of *P. asarifolia* in Japan, but not seedlings (Hashimoto *et al.* 2012; Fig. 2e), and mature *Pyrola rotundifolia* plants from Estonia (Vincenot *et al.* 2008; Fig. 2e). Additional Sebaciniales clade B fungi from *P. asarifolia* adult plants as well as seedlings collected in Japan (Hashimoto *et al.* 2012) and Austria (Weiß *et al.* 2004) are included in our phylogeny, but do not fall within the same subclade as Sebaciniales 1 from this study (Fig. 2). Two of the Sebaciniales taxa from clade A, Sebaciniales 3 and 7, fell within separate subclades (Fig. 2a,b), and their most closely related taxa were primarily ectomycorrhizal Sebaciniales. Also in clade A, Sebaciniales 2 and 4 were grouped within a subclade and nested among ectomycorrhizal taxa (Fig. 2c). A similar result was found for Sebaciniales

5 and 6 (Fig. 2d). Of these subclades, only group 'b' had bootstrap support >50%. An additional Sebaciniales clade A sequence from adult *P. rotundifolia* roots collected in Estonia (Vincenot *et al.* 2008) is included in our phylogeny, but does not fall within any of the subclades that include sequences from this study (Fig. 2).

We found no correlation between early seedling developmental stages (i.e. Fig. 1B vs. 1C) and fungal specificity. For instance, 52% of the *P. chlorantha* seedlings colonized by Sebaciniales 1 were in stages similar to Fig. 1B or less developed, while the remaining 48% were in stages similar to Fig. 1C or D. However, the most common fungus found to associate with both *P. chlorantha* and *O. secunda* seedlings was Sebaciniales 1 (Fig. 2). This fungus was found both in the greatest total number of packets that contained germinated seeds (68%) and associating with the greatest percentage of seedlings (60%, Table 1). We also identified this fungus from a single well-developed *P. chlorantha* seedling where multiple cells harboured intracellular fungal coils (Fig. 1D). Although Sebaciniales 1 was the dominant seedling associate, it was not uncommon to find one or more ectomycorrhizal taxa colonizing other seeds within the same packets as this taxon (Table 1). Based on ITS sequences, there were no fungi shared among seedlings of the two Pyroleae species besides Sebaciniales 1. Of the ungerminated seeds, the majority of them had intact embryos, indicating that these species seeds have a potential viability of >2 years. However, we did not chemically test ungerminated seeds for viability.

Discussion

Until now, there was only one published record that used molecular tools to determine the fungi responsible for the mycoheterotrophic germination of *P. chlorantha* and *O. secunda* seeds (Smith & Read 2008). The results of the previous study were briefly reported in a textbook chapter and contained data from only seven seedlings that were found to be solely associated with a Sebaciniales taxon from clade B (M.I. Bidartondo pers. comm.). In the current study, we obtained a substantially larger sample size of seedlings from each species and revealed a greater diversity of fungal associates, including multiple Sebaciniales taxa. We identified six Sebaciniales taxa from clade A, which contains ectomycorrhizal taxa in addition to symbionts of terrestrial orchids, plant endophytes and arbutoid mycorrhizal species, as well as one Sebaciniales clade B taxon. Sebaciniales clade B taxa were the dominant fungi associated with *P. chlorantha* and *O. secunda* seedlings in both the current study and Smith and Read (2008). This clade contains a highly diverse group of species including numerous saprotrophic and endophytic strains, for

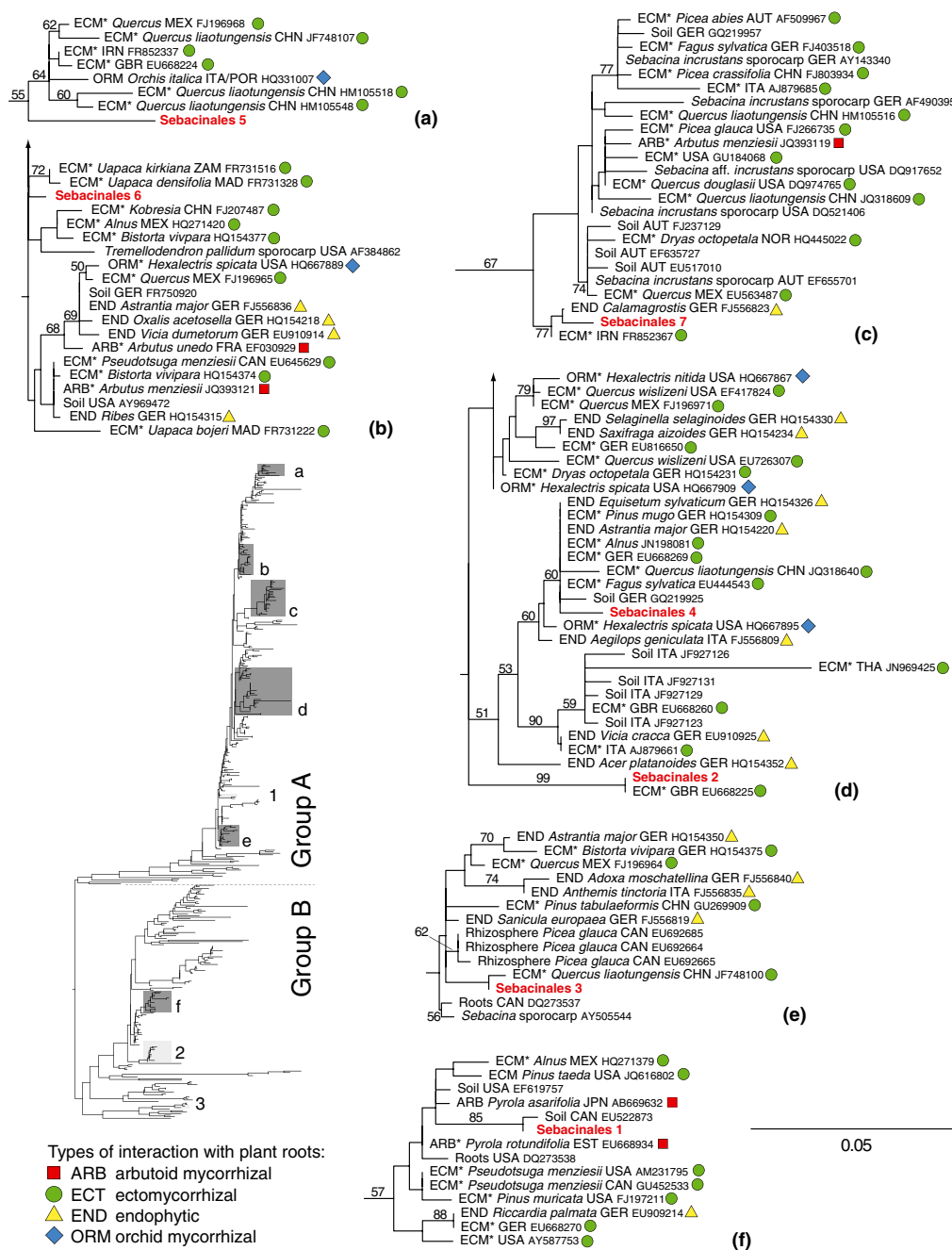


Fig. 2 Phylogenetic placement of Sebacinale mycobionts from *Pyrola chlorantha* and *Orthilia secunda* seedlings detected in this study. ITS sequences from each of seven sequence types found were complemented with LSU sequences except for Sebacinale 4 and 6, aligned to a representative sampling of GenBank reference sequences and analysed with maximum likelihood using RAxML. Branch support was derived from 2000 bootstrap replicates, and values below 50% were omitted. The tree was midpoint rooted. Branch lengths are given in terms of expected nucleotide substitutions per site. Note that branch lengths shown underestimate genetic distances, because hypervariable alignment regions were excluded from the alignment prior to phylogenetic analysis. Acronyms used for geographic provenance of sequences: AUS, Australia; AUT, Austria; CAN, Canada; CHN, P.R. China; EST, Estonia; ETH, Ethiopia; GBR, Great Britain; GER, Germany; IRN, Iran; ITA, Italy; JPN, Japan; KOR, South Korea; MAL, Malaysia; MEX, Mexico; NOR, Norway; POR, Portugal; SPA, Spain; SUI, Switzerland; TAS, Tasmania; ZAM, Zambia. *Type of plant–fungus interaction verified for this sequence (estimated otherwise). Branch 1 is a Sebacinale clade A sequence from *Pyrola rotundifolia* roots collected in Estonia (Vincenot *et al.* 2008), subclade 2 contains multiple Sebacinale clade B sequences from mycoheterotrophic seedlings and partially mycoheterotrophic adults of *Pyrola asarifolia* collected in Japan (Hashimoto *et al.* 2012), and branch 3 is a Sebacinale clade B sequence from the roots of *P. asarifolia* collected in Austria (Weiß *et al.* 2004).

example, the *Sebacina vermifera* species complex and species in the genus *Piriformospora* (Weiß *et al.* 2004; Basiewicz *et al.* 2012). In our study, Sebaciniales 1 was found to nest within a subclade that contains ectomycorrhizal species (Fig. 2e). Until now, Sebaciniales clade B was not known to include ectomycorrhizal taxa. This new finding might be explained by the fact that the majority of molecular ecology studies of ectomycorrhizal fungi have been based on ITS sequences, while phylogenetic analyses of Sebaciniales have been based on LSU sequences (Weiß *et al.* 2004, 2011; Selosse *et al.* 2009). To our knowledge, this study is the first to integrate ITS and LSU sequences in a single phylogenetic analysis. Alternatively, because Sebaciniales clade B fungi are common root endophytes (Selosse *et al.* 2009; Weiß *et al.* 2011), they may have been preferentially amplified from the ectomycorrhizal host trees named in Fig. 1E. To better resolve the trophic nature of taxa in Sebaciniales clade B, additional physiological studies and the inclusion of more ITS fragments in phylogenetic analyses of this order are necessary.

In addition to Sebaciniales fungi, the current study has revealed a hitherto undiscovered diversity of fungi that are capable of hosting pyrolids in their initially mycoheterotrophic stages. These include ectomycorrhizal species in the genera *Russula*, *Tricholoma*, *Cortinarius*, *Inocybe*, *Sistotrema* and *Tomentella*, all of which have also been found to associate with adult pyrolids (Tedersoo *et al.* 2007; Zimmer *et al.* 2007; Vincenot *et al.* 2008; Hynson & Bruns 2009; Toftegaard *et al.* 2010; Matsuda *et al.* 2012), whereas, Sebaciniales clade B taxa have not found to be among the dominant mycobionts associated with adult plants (Tedersoo *et al.* 2007; Zimmer *et al.* 2007; Vincenot *et al.* 2008; Hashimoto *et al.* 2012). Combined, these results indicate that fungi from Sebaciniales clade B are more commonly associated with Pyroleae seedlings than adults, but the overall richness of fungi associated with mature Pyroleae species is similar to that of *P. chlorantha* and *O. secunda* seedlings. Based on our results, another primary distinction of Pyroleae seedlings from adults is that single seedlings harboured single fungal colonists, while adult plants associate with multiple species of fungi. It should be taken into consideration that this result might be partially biased by our methodological approach. Because we employed direct sequencing of the fungal ITS region, sequences from seedlings colonized by multiple fungi may have been discarded owing to poor sequence quality. However, if this is the case, then we have actually underestimated the number of fungal taxa that are involved in the germination of *P. chlorantha* and *O. secunda*, and the diversity of single seedling's fungal associates may more closely mirror mature plants. Future studies on the fungi involved in Pyroleae germination may consider using

cloning or next-generation sequencing technologies to confirm whether seedlings are indeed specifically associated with single mycobionts.

One possible pathway for *P. chlorantha* and *O. secunda* seedling development includes germination triggered by a diversity of fungi that may or may not remain with the plants as they mature. Because pyrolids apparently continue to collect fungal associates as they mature, when the same fungus involved in germination is also detected in adult plants, it is difficult to say whether it remains an important symbiont. However, two of the genera we found to associate with pyrolid seedlings, *Russula* and *Tricholoma*, contain species that are the sole mycobionts at germination and beyond to the fully mycoheterotrophic species *Monotropa uniflora* (Bidartondo & Bruns 2005), *Allotropia virgata* (Bidartondo & Bruns 2002) and *Monotropa hypopitys* (Leake *et al.* 2004), which are in a sister tribe to Pyroleae (Kron & Johnson 1997). Species within *Russula* and *Tricholoma* have also been identified from the roots of numerous mature Pyroleae plants (Tedersoo *et al.* 2007; Zimmer *et al.* 2007; Vincenot *et al.* 2008; Matsuda *et al.* 2012), indicating that these fungi may play important roles in Pyroleae species development.

Our findings that a diversity of fungi can trigger Pyroleae germination are in contrast to the recent findings of Hashimoto *et al.* (2012) who determined that seedlings of *P. asarifolia* in Japan were solely associated with very closely related fungi within Sebaciniales clade B. The discrepancy between our results and those of Hashimoto *et al.* (2012) is likely owed to the following differences between the two studies: in the current study, we analysed Pyroleae seedlings at earlier stages of development than those from *P. asarifolia* in Japan (Hynson *et al.* 2012), also we did not investigate the same species of Pyroleae and the fungi associated with seedlings may vary among host plants, and our seed packet sowing strategies were slightly different. Hashimoto *et al.* (2012) sowed seeds either in the presence or in the absence of mature *P. asarifolia* plants and found that in general, seedlings did not germinate in close proximity to mature plants. In the current study, seeds of *P. chlorantha* and *O. secunda* were only sown in close proximity to adult conspecifics; thus, we cannot comment on how absence of adult plants may influence the interactions of seedlings with soil fungal communities. Though not within the same subclades, Sebaciniales clade B fungi were the only mycobionts shared among the mycoheterotrophic seedlings of our two investigated Pyroleae species and *P. asarifolia* seedlings from Japan (Hashimoto *et al.* 2012). Also, Sebaciniales 1 from clade B was the sole colonist of a well-developed seedling of *P. chlorantha* (Fig. 1D). This seedling was the only one from the current study that was at a similar developmental

stage as those analysed by Hashimoto *et al.* (2012) hinting at the importance of these fungi for seedling development past the initial germination stage.

Evidence of mycoheterotrophy in mature individuals of *O. secunda* and *P. chlorantha* is limited to two studies (Tedersoo *et al.* 2007; Zimmer *et al.* 2007). These studies used the naturally abundant carbon stable isotope values of Pyroleae species compared to nearby fully autotrophic plants as an assay for the degree of mycoheterotrophy. Both found that *O. secunda* plants were partially mycoheterotrophic (Tedersoo *et al.* 2007; Zimmer *et al.* 2007). Conversely, the dependency of adult *P. chlorantha* plants on mycoheterotrophy remains somewhat unclear as the same studies had conflicting results. Tedersoo *et al.* (2007) found evidence of partial mycoheterotrophy in mature *P. chlorantha* plants, while Zimmer *et al.* (2007) did not. The discrepancy between these two studies may be in part owed to the identities and functional relationships of the fungi associating with *P. chlorantha* plants. From our phylogenetic analyses, it appears that at least some Sebaciniales clade B fungi are potentially capable of forming ectomycorrhizae with host trees such as *Pinus* spp., *Pseudotsuga menziesii* and *Alnus* sp. (Fig. 1E). However, this needs to be corroborated with additional functional and morphological studies of Sebaciniales clade B fungi when in association with these tree species. If Sebaciniales clade B fungi associated with Pyroleae seedlings are not capable of forming functional ectomycorrhizae with surrounding trees, then gaining ectomycorrhizal fungal symbionts would be a critical step for these plants to adapt a partially mycoheterotrophic lifestyle.

Although a striking few seedlings were recovered in this study, this is not an uncommon trait among mycoheterotrophic dust seed-producing species. For example, after the seeds of nine orchid species were buried for 16–36 months *in situ*, Bidartondo and Read (2008) found that germination rates varied from 6% to 63%, and that of the species with the highest germination rates, only 1% produced mycorrhizal seedlings. Concurrently, Hashimoto *et al.* (2012) had very low germination rates for *P. asarifolia* seeds that were buried for 6 months in Japan. There, <0.02% of the total seeds were successfully sequenced for the fungal ITS. The fungi found to associate with Pyroleae species at our study site – especially the Sebaciniales clade B – are not particularly rare. Consequently, our finding that very few seeds germinated over a 2-year period buried *in situ* indicates that germination cues among Pyroleae species may include factors other than host recognition. Future investigations involving additional Pyroleae species are needed to assess the impact of such factors as the presence of conspecific adults on the germination and development of these species.

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Data accessibility

DNA sequences: GenBank ITS Accession nos JX561229–JX561242 and LSU Accession nos JX623961–JX623965.