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Extreme specificity in epiparasitic Monotropoideae (Ericaceae): widespread phylogenetic and geographical structure

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Abstract

The Monotropoideae (Ericaceae) are nonphotosynthetic plants that obtain fixed carbon from their fungal mycorrhizal associates. To infer the evolutionary history of this symbiosis we identified both the plant and fungal lineages involved using a molecular phylogenetic approach to screen 331 plants, representing 10 of the 12 described species. For five species no prior molecular data were available; for three species we confirmed prior studies which used limited samples; for five species all previous reports are in conflict with our results, which are supported by sequence analysis of multiple samples and are consistent with the phylogenetic patterns of host plants. The phylogenetic patterns observed indicate that: (i) each of the 13 plant phylogenetic lineages identified is specialized to a different genus or species group within five families of ectomycorrhizal Basidiomycetes; (ii) mycorrhizal specificity is correlated with phylogeny; (iii) in sympatry, there is no overlap in mature plant fungal symbionts even if the fungi and the plants are closely related; and (iv) there are geographical patterns to specificity.

Keywords: mycorrhiza, nonphotosynthetic, parasite, *rps*2, specialization, symbiosis

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Introduction

Epiparasitic plants are nonphotosynthetic and they obtain fixed carbon from other plants via a shared mycorrhizal fungus. This behaviour makes them cheaters of one of the most pervasive mutualisms in terrestrial ecosystems (Taylor & Bruns 1997; Perry 1998). There are several unique features of epiparasitic cheating that make it a system likely to yield novel insights into symbiotic interactions. First, epiparasitism involves a plant–fungal mutualism, whereas our understanding of cheating is based on animal (almost exclusively insect) interactions (Axelrod & Hamilton 1981; Soberon & Martinez 1985; Bull & Rice 1991; Thompson 1994; Connor 1995; Poulin & Vickery 1995; Maloof & Inouye 2000). Second, the photosynthetic host does not interact directly with its epiparasite. Thus, because there is no opportunity for the photosynthetic host to select against its epiparasite without selecting against its own mutualist, an 'unholy alliance' is forged between the epiparasitic plant and the mycorrhizal fungus. Third, it is a system that combines an intimate interaction (i.e. one with cell to cell contact) with a diffuse one (single fungi associated with multiple plants and vice versa).

A critical need for the study of any symbiosis is the ability to identify the interacting lineages in nature. In this study, we focus on the evolutionary history of epiparasitic association in the Monotropoideae (Ericaceae). Species in the subfamily Monotropoideae have only traces of chlorophyll *a* and no chlorophyll *b* (Cummings & Welschmeyer 1998), many are endangered and dependent on old-growth forests (United States Department of Agriculture 1993), and some are known to associate with fungi that are ectomycorrhizal with tree roots (Björkman 1960; Duddridge & Read 1982; Cullings *et al*. 1996; Kretzer *et al*. 2000). In a remarkably long history of study of the monotropoid symbiosis, putative identifications have included saprobic fungi (Oliver 1890; Peklo 1908; Rexhausen 1920; Riley & Eichenmuller 1970; Campbell 1971; Went 1971), pathogenic fungi (Campbell 1971), and various mycorrhizal fungi (Reess 1885; Francke 1934; Björkman 1960; Singer 1965; Khan 1972; Trappe 1976; Kernan & Finocchio 1983; Castellano & Trappe 1985; Martin 1985; Martin 1986; Cullings *et al*. 1996; Kretzer *et al*. 2000). The fungi associated with *Monotropsis*

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and *Pityopus* have not been previously examined. Even though detailed world-wide taxonomic treatments of the Monotropoideae are available (Wallace 1975; Wallace 1995), no comprehensive phylogenetic sampling of the group has been used in either plant- or fungal-focused studies (Cullings 1994; Cullings *et al*. 1996; Kron 1996; Cullings & Hileman 1997). Relationships within the subfamily remain controversial (Cullings 2000), and *Monotropsis* has been reported to belong outside the Monotropoideae (Cullings 1994).

We re-examined and expanded the sample size and geographical range of previous identifications to systematically evaluate mycorrhizal specificity in the Monotropoideae. To determine if different Monotropoideae lineages are specifically dependent on different lineages of fungi we identified the plant and fungal lineages involved in the monotropoid mycorrhizal symbiosis for 10 species over parts of their world-wide distribution. This sample represents all but two (*Cheilotheca* spp.) described species in the Monotropoideae. This allowed us to test whether within the Monotropoideae: (i) plant and fungal phylogenies are correlated; (ii) symbiotic fidelity is maintained in sympatry; and (iii) there are geographical mosaics of specificity (Thompson 1994). These patterns of association are widespread in parasitic associations (Price 1980; Thompson 1994). However, in the mycorrhizal symbiosis such patterns have only recently been reported for two congeneric non-photosynthetic orchids (Taylor & Bruns 1999), and they are in contrast with early predictions and patterns observed in photosynthetic plants (Harley & Smith 1983; Molina *et al*. 1992).

Materials and methods

Sampling of plant and mycorrhizal tissue

Plant tissue, at least one flower or scale and several roots, was obtained from a total of 104 populations and 331 plants. The rarity of some species precluded intensive sampling at most sites. Occasionally, when only senescent inflorescences were present, only roots were obtained from the perennating root mass. Roots were obtained by removing one or more 2.5 cm soil cores near emerging inflorescences or by excavating part of the root mass. Each root sample was sprayed with water over 2 mm and 500 μm stacked sieves to separate coarse and fine soil fractions. To find monotropoid roots, all the soil and roots collected in both sieves were spread thinly in Petri dishes and examined using stereomicroscopes. Inflorescence tissue and monotropoid roots were then lyophilized. Additionally, we obtained plant tissue samples from 17 photosynthetic relatives of the Monotropoideae within the family Ericaceae (University of California Botanical Garden collection). The localities at which Monotropoideae species were sampled were as follows: *Monotropa hypopithys* N. America: Albany County (WY), Benton Co. (OR), Chittenden Co. (VT), Lane Co. (OR), Klamath Co. (OR); *M. hypopithys* Sweden: Skåne Province (Sweden), Uppland Prov. (Sweden); *M. hypopithys* Eurasia: Derbyshire (UK), Isle of Anglesey (UK), Iwate Prefecture (Japan), Lappeenranta (Finland), North Yorkshire (UK), Skåne Prov. (Sweden); *Pityopus californicus*: Benton Co. (OR), Douglas Co. (OR), Lane Co. (OR), Tulare Co. (CA); *Allotropa virgata:* Curry Co. (OR), Douglas Co. (OR), Lane Co. (OR), Napa Co. (CA), Klamath Co. (OR); *Hemitomes congestum*: Lane Co. (OR), Klamath Co. (OR); *Monotropsis odorata*: Transylvania Co. (NC); *M. uniflora* N. Am. August Co. (VA), Benton Co. (OR), Chittenden Co. (VT), Lane Co. (OR), Lunenburg Co. (NS, Canada), Washington Co. (VT); *M. uniflora* Japan: Fukushima Pref.; *Monotropastrum humile*: Ibaraki Pref. (Japan); *Pleuricospora fimbriolata*: Douglas Co. (OR), El Dorado Co. (CA), Fresno Co. (OR), Lane Co. (OR), Plumas Co. (CA), Tulare Co. (CA); *Sarcodes sanguinea*: Curry Co. (OR), El Dorado Co. (CA), Fresno Co. (CA), Kern Co. (CA), Nevada Co. (CA), Plumas Co. (CA), San Bernardino Co. (CA), Ventura Co. (CA); *Pterospora andromedea*: Apache Co. (AZ), El Dorado Co. (CA), Fresno Co. (CA), Josephine Co. (OR), Klamath Co. (OR), Plumas Co. (CA), Tulare Co. (CA).

Identification of fungal lineages

We identified fungi using methods described by Gardes & Bruns (1996). In summary, we extracted genomic DNA from individual monotropoid roots and we amplified the internal transcribed spacer (ITS) of the nuclear ribosomal repeat using the polymerase chain reaction (PCR) with the fungus-specific primers ITS1F/ITS4 (White *et al*. 1990; Gardes & Bruns 1993). PCR products were screened by restriction fragment length polymorphisms (RFLP) using the restriction endonucleases *Alu*I, *Hin*fI and/or *Cfo*I (New England Biolabs Inc.). We estimated the molecular size of the restriction fragments obtained using the program GELREADER v.2.0.5 (National Center for Supercomputing Applications, Champaign, IL). As a primary family or genus-level screen we sequenced a fragment of the fungal mitochondrial large subunit (mtLSU) rDNA (Bruns *et al*. 1998). Two to five root samples per plant lineage were selected for this analysis following a criterion similar to that described below for plant samples. In most cases, the primer combination ML5/ML6 was used; in cases where PCR amplification was weak, or if sequencing proved difficult possibly due to the presence of introns, we used the primer combinations ML5/MLIN5R, CML5.5/ML6, or MLIN3/ML5.5 (Bruns *et al*. 1998). As a secondary species-level screen, we sequenced the ITS region for each ITS–RFLP type. These sequences were used to query the GenBank database via BLAST. When only distant relatives were retrieved (< 90% sequence identity), we used the mtLSU phylogenetic placement data to select members of the corresponding fungal families or genera from the basidiocarp collections at various Herbaria (specimen vouchers for matching taxa are listed in Table 1).

Table 1 Symbionts of the monotropoid mycorrhizal symbiosis. The 13 plant lineages and 31 fungal lineages involved were defined by nrITS sequence data. One-hundred and four populations, 331 plants, and 251 fungal basidiocarps were screened using molecular methods. Fungal nrITS pairwise sequence matches > 95% have been putatively assigned to the species of the matching sequence, and those ≤ 95% have been putatively assigned to the genus of the matching sequence with the nearest species between parentheses

Plant symbionts	Populations	Plants	Fungal symbionts* (% nrITS sequence identity)
Monotropa hypopithys N. Am. (Oregon, Vermont, Wyoming)	12	30	*Tricholoma portentosum* (99) *Tricholoma flavovirens* (99) *Tricholoma sejunctum* (99) *Tricholoma* sp. (*T. squarrulosum*, 95)
Monotropa hypopithys Sweden (Sweden)	4	4	*Tricholoma columbetta* (98) *Tricholoma portentosum* (98) *Tricholoma saponaceum* (97)
Monotropa hypopithys Eurasia (Finland, Japan, Sweden, UK)	6	9	*Tricholoma cingulatum* (99) *Tricholoma terreum* (99)
Pityopus californicus (California, Oregon)	4	12	*Tricholoma myomyces* (99) *Tricholoma* sp. (*T. mutabile*, 95) *Tricholoma* sp. (*T. atrosquamosum*, 95)
Allotropa virgata (California, Oregon)	7	18	*Tricholoma magnivelare* (99)
Hemitomes congestum (Oregon)	4	7	*Hydnellum diabolus* (99) *Hydnellum aurantiacum* (98) *Hydnellum* sp. (*H. diabolus*, 87)
Monotropsis odorata (North Carolina)	1	2	*Hydnellum* sp. (*H. geogenium*, 86)
Monotropa uniflora N. Am. (Nova Scotia, Oregon, Virginia, Vermont)	10	33	*Russula brevipes* (100) *Russula paludosa*† *Russula* sp. (*R. cremoricolor*, 95) *Russula* sp. (*R. postiana*, 93) *Russula* sp. (*R. integra*, 91) *Russula* sp. (*R. postiana*, 92) *Lactarius theiogalus* (100)
Monotropa uniflora Japan (Japan)	1	2	*Russula* sp. (*R. postiana*, 92)
Monotropastrum humile (Japan)	1	2	*Russula* sp. (*R. postiana*, 91)
Pleuricospora fimbriolata (California, Oregon)	13	42	*Gautieria monticola* (99)
Sarcodes sanguinea (California, Oregon)	19	93	*Rhizopogon ellenae* (100) *Rhizopogon subpurpurascens* (99)
Pterospora andromedea (Arizona, California, Oregon)	22	77	*Rhizopogon salebrosus* (99)‡ *Rhizopogon arctostaphyli* (100)§

*Basidiocarp vouchers: ACAD: KAH13873, KAH14014, KAH14017; L: Nordeloos95210; MICH: AHS69273; NY: Tracy5705; O: 51046; SFSU: HDT53493, HDT54300, HDT54614, KMS281, KMS285, KMS286, KMS304, KMS428, KMS435; T.D. Bruns herbarium: EAL2000501. ACAD, E.C. Smith Herbarium, Acadia University; L, National Herbarium of the Netherlands; MICH, University of Michigan Herbarium; NY, New York Botanical Garden Herbarium; O, Oslo University Herbarium; SFSU, Thiers Herbarium, San Francisco State University; T.D. Bruns Laboratory Herbarium, University of California at Berkeley. GenBank accessions for fungal sequences already in the database: AF241519, AF062614, AF071445, AF071446, AF062929, AJ277910, AF230898, AF230896.

†(Unpublished basidiocarp sequence, Dr S. Miller (University of Wyoming, Laramie).

‡Species group 1 in Kretzer *et al*. (2000).

§Species group 4 in Kretzer *et al*. (2000).

We extracted DNA from 251 basidiocarps and screened them in the manner described for monotropoid roots using ITS–RFLP. Matching basidiocarp and monotropoid fungi ITS–RFLP types were sequenced to compute BLAST pairwise distances (Tatusova & Madden 1999). We relied on sequence comparisons rather than ITS–RFLP comparisons (Kårén *et al*. 1997; Taylor & Bruns 1999) because ITS–RFLP alone can be insufficient among some closely related species (Kretzer *et al*. 2000).

Identification of plant lineages

We selected plants for sequence analysis by maximizing the geographical distance of specimens within each morphospecies and including disjunct geographical provenances based on available distribution maps (Wallace 1975). Plant shoot tissue for which we did not obtain matching root tissue was not included in this study. When only root tissue was obtained, we used it for plant and fungal identification. We extracted DNA from inflorescence tissue by the method mentioned above.

To investigate relationships within the Monotropoideae and the photosynthetic Ericaceae, we selected a plastid gene encoding ribosomal protein CS2, *rps*2, previously used for phylogenetic reconstruction of the parasitic Scrophulariaceae/Orobanchaceae (dePamphilis *et al*. 1997). We sequenced *rps*2 from 21 Monotropoideae and 17 photosynthetic Ericaceae. We used primers *rps*2-47F/*rps*2-661R (dePamphilis *et al*. 1997) or *rps*2-47F/*rps*2R. *rps*2R (tgc tga tca aga atr att aca a) is an internal primer designed from Monotropoideae sequences produced with the first primer combination.

To investigate relationships within the Monotropoideae in greater detail, two nuclear ribosomal repeat regions, the internal transcribed spacers and a portion of the 28s gene, were sequenced from an expanded set of 32 Monotropoideae with two outgroup photosynthetic Ericaceae. We used primers ITS1/ITS4 (White *et al*. 1990) for the ITS region and KJII/TW14 (Cullings 1994) for the 28s gene.

DNA sequencing

Sequencing of both strands was performed with an ABI model 377 Sequencer (Applied Biosystems Co.) using a Thermo Sequenase™ Dye Terminator Cycle Sequencing Pre-Mix Kit (Amersham Pharmacia Biotech), or a BigDye™ Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems Co.). We used DNA sequencing analysis v.2.1.2 and SEQUENCE NAVIGATOR v.1.0.1 (Applied Biosystems Co.) for processing raw data. The 181 DNA sequences generated have been deposited in GenBank under accession nos AF349686–AF349717 and AF351863–AF352013.

Phylogenetic analysis

Alignments were produced with CLUSTAL X (Thompson *et al*. 1997), corrected manually, analysed by parsimony and distance, and bootstrapped using PAUP 4.0b5. Options were 1000 random taxon addition sequences and 1000 bootstrap replicates. The distance method used was neighbour joining under a Jukes–Cantor one-parameter model. Decay analysis was performed with AUTODECAY 4.0 (Eriksson 1999). For the mtLSU, we used neighbour-joining on an updated version with 159 taxa (available at http://plantbio.berkeley.edu/~bruns/ftp/ML56DB.159.hqx) of the alignment by Bruns *et al*. (1998). The mtLSU was rooted with *Cantharellus*, *Clavulina* and *Tulasnella* (Bruns *et al*. 1998). The *rps*2 was rooted with *Enkianthus chinensis* (Ericaceae) (Anderberg 1992). The nuclear DNA (nrDNA) was rooted with *Pyrola picta* and *Arctostaphylos manzanita* (Ericaceae) based on *rps*2 results (see below) and results from prior analyses (Cullings 1996; Kron 1996). We analysed the *rps*2 data set to detect statistically significant variation in evolutionary rates. The likelihood of the most likely tree without assuming constant rates was compared with one of the same topology under a molecular clock using a likelihood ratio test (Felsenstein 1981). We tested congruence between *rps*2 and nrDNA with a partition homogeneity test (Farris *et al*. 1994) using 1000 random taxon addition sequences in PAUP excluding parsimony-uninformative characters.

Results

Fungal ITS

ITS–RFLP and sequence data are summarized in Table 1. All fungi were identified to species or species group, except for some members of the Russulales and Thelephorales which are taxonomically diverse and poorly sampled obligate ectomycorrhizal groups. Fungal nrITS pairwise sequence matches of > 95% have been putatively assigned to the species group of the matching sequence. Symbiont taxa with ≤ 95% sequence identity to an identified basidiocarp have been putatively assigned to the genus of the matching sequence. The nearest species and the sequence identity is indicated between parentheses in Table 1.

Fungal mtLSU

Most fungal symbiont sequences were placed with bootstrap support > 70% within Basidiomycete clades known to contain obligate ectomycorrhizal taxa such as the suilloid group, Russulales, Thelephorales, or Gomphales (clade designations from Bruns *et al*. 1998). The Tricholomatoid group is poorly resolved, but the symbionts of *Monotropa hypopithys*, *Pityopus californicus* and *Allotropa virgata* shared shortest distances with its members (Fig. 1).

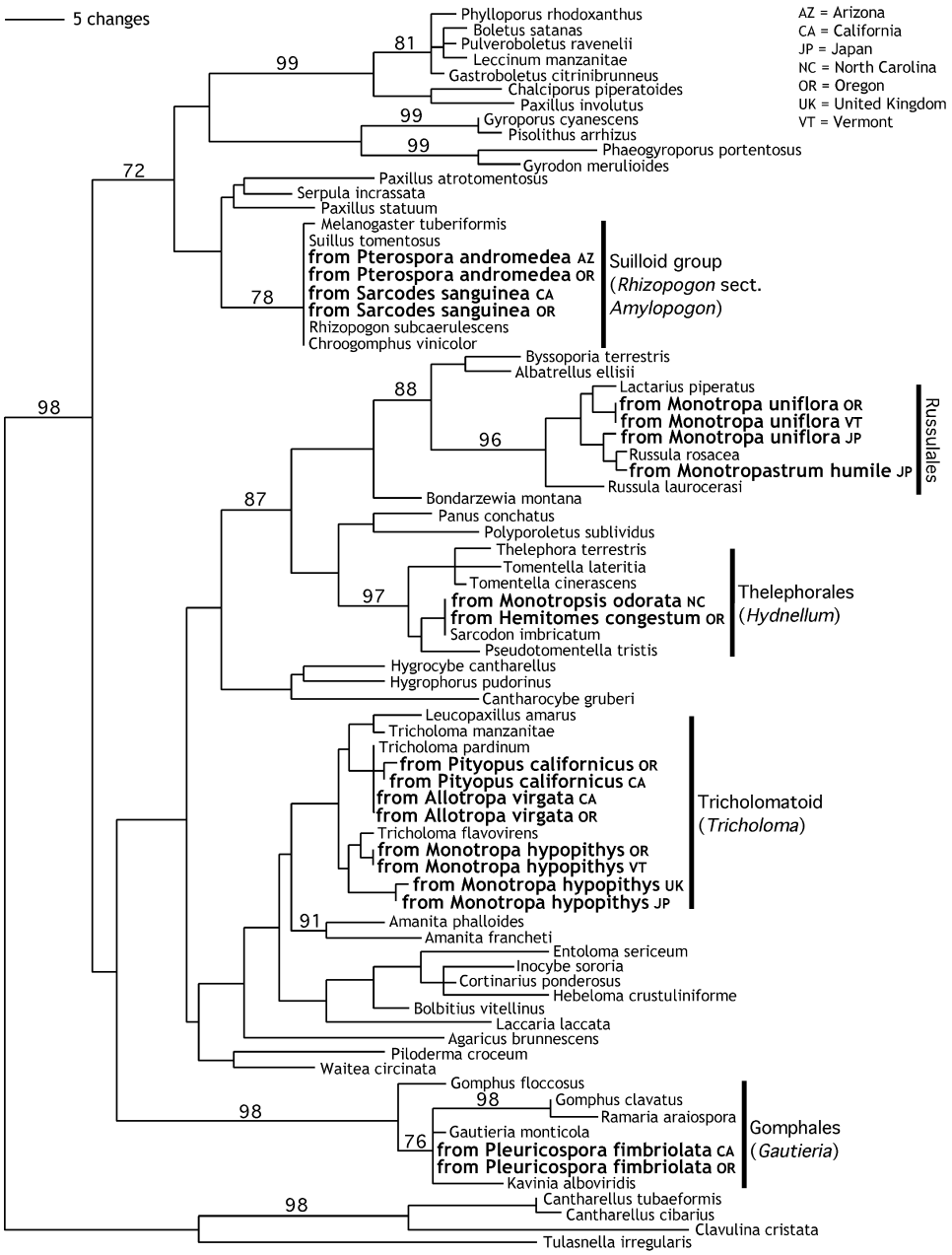

Fig. 1 Phylogenetic placement of Monotropoideae-associated (in bold) mycorrhizal fungi. Fungal clade names are to the right. Partial mtLSU sequences from mycorrhizal fungi of plants in the Monotropoideae (see text for taxon sampling criterion) were aligned with those from basidiocarps of 159 (mostly ectomycorrhizal) basidiomycete species, previously used for the identification of mycorrhizal fungi (Bruns *et al*. 1998). Analysis was performed by neighbour-joining with 1000 bootstrap replicates (values > 70% are shown near branches). *Cantharellus*, *Clavulina*, and *Tulasnella* were used as outgroups. After the analysis, we pruned 101 taxa from the tree, leaving representatives of every clade.

Plant rps2

Priming with *rps*2-47F/*rps*2-661R failed on *Sarcodes sanguinea*, *Pterospora andromedea* and *M. uniflora* N. Am. For these taxa, we used a reverse primer, *rps*2R, that produced a sequence ending at position 15211 with respect to *Arabidopsis thaliana* NC–000932.1. Overall, there were 213 parsimony informative characters in 543 aligned characters. The *rps*2 analysis supports a monophyletic vaccinioid radiation (*Gaultheria*, *Pernettya*, *Vaccinium*, *Leucothoe*, *Pieris*, *Oxydendron*), rhododendroid radiation (*Rhododendron*, *Ledum*) and arbutoid radiation (*Arbutus*, *Arctostaphylos*, *Comarostaphylis*) (Fig. 2). The relationships of monotropoid, arbutoid and pyroloid taxa are supported

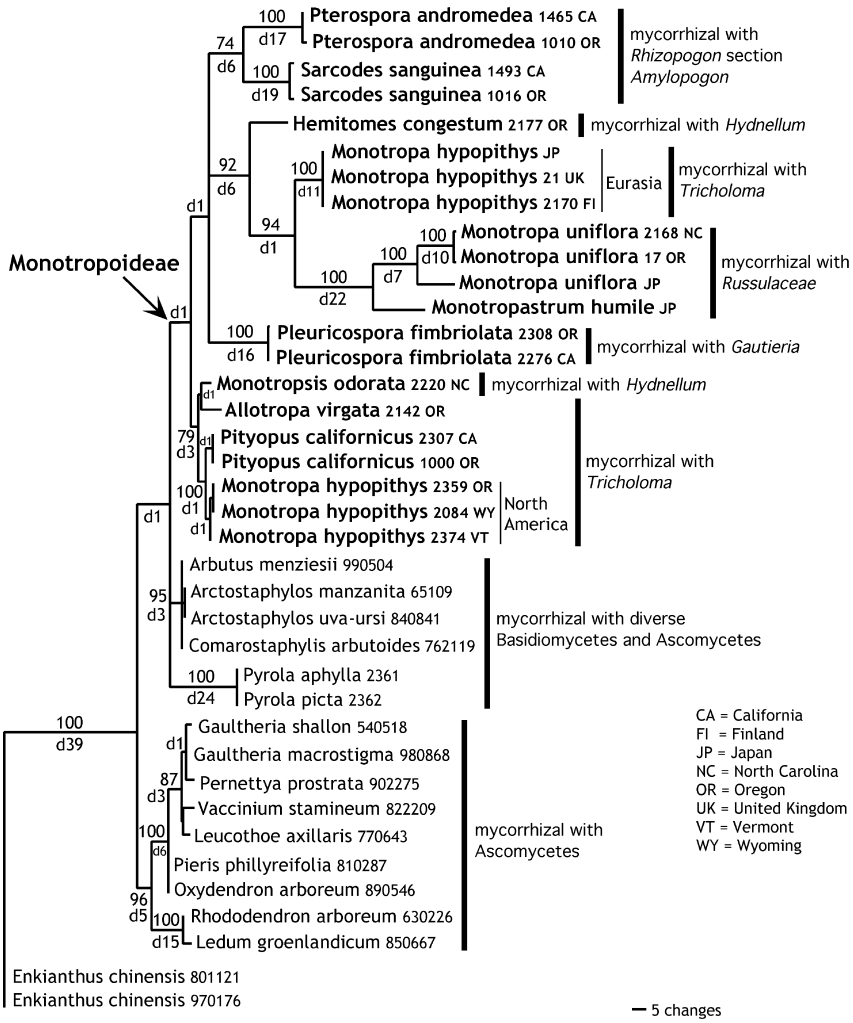

Fig. 2 Molecular phylogeny of nonphotosynthetic Monotropoideae (in bold) and selected photosynthetic relatives in the Ericaceae based on parsimony analysis of plastid *rps*2 sequence data. The mycorrhizal fungal associates of plants in each clade are indicated to the right (based on fungal mtLSU and nrITS sequence data). Only branches present in the strict consensus of the 12 most parsimonious trees are shown, all others were collapsed. Decay (dx) values and bootstrap values > 70% are near branches. *Enkianthus* was used as outgroup. Collection identifiers follow taxon names.

only by the parsimony consensus. *Monotropsis odorata* is nested within the Monotropoideae on a short branch near *A. virgata*. The genus *Monotropa* is polyphyletic, as shown by Cullings (1994). Unexpectedly, Eurasian *M. hypopithys* are distant from North American *M. hypopithys*.

Plant rps2 *rate analysis*

When parsimony trees are drawn with branch lengths proportional to mutations (Fig. 2), long branches lead to a large subset of the Monotropoideae (upper clades, Fig. 2). The hypothesis of equal rates was tested and rejected by a likelihood ratio test. The likelihood calculated under the assumption of a strict molecular clock was −3264.98, significantly lower than that obtained without enforcing a molecular clock −3192.01 (d.f. 42, $P = 0.002$). Accelerated *rps*2 evolutionary rates have been observed in other nonphotosynthetic plants, but accelerated rates and parasitism are not necessarily correlated (dePamphilis *et al*. 1997). Examination of the *rps*2 topology suggests the same may be true within the Monotropoideae.

Plant nrDNA

The Monotropoideae are depicted as monophyletic in the strict consensus of 160 most parsimonious (MP) trees (Fig. 3), but again this relationship is not supported by high bootstrap or decay values. There were 375 parsimony informative characters in 1225 aligned characters. All ingroup branches were supported by bootstrap and decay values and they were present in the parsimony consensus, except at the transition between *Hydnellum*- and *Tricholoma*-associated taxa (i.e. *Monotropsis odorata*, *Hemitomes congestum* and *A. virgata*). As shown earlier by Cullings *et al*. (1996) the genus *Monotropa* is polyphyletic. *Monotropsis odorata* is again nested within the Monotropoideae, in contrast to previous reports that placed it within the Vaccinioideae (Cullings 1994). Strongly supported subclades were detected in both

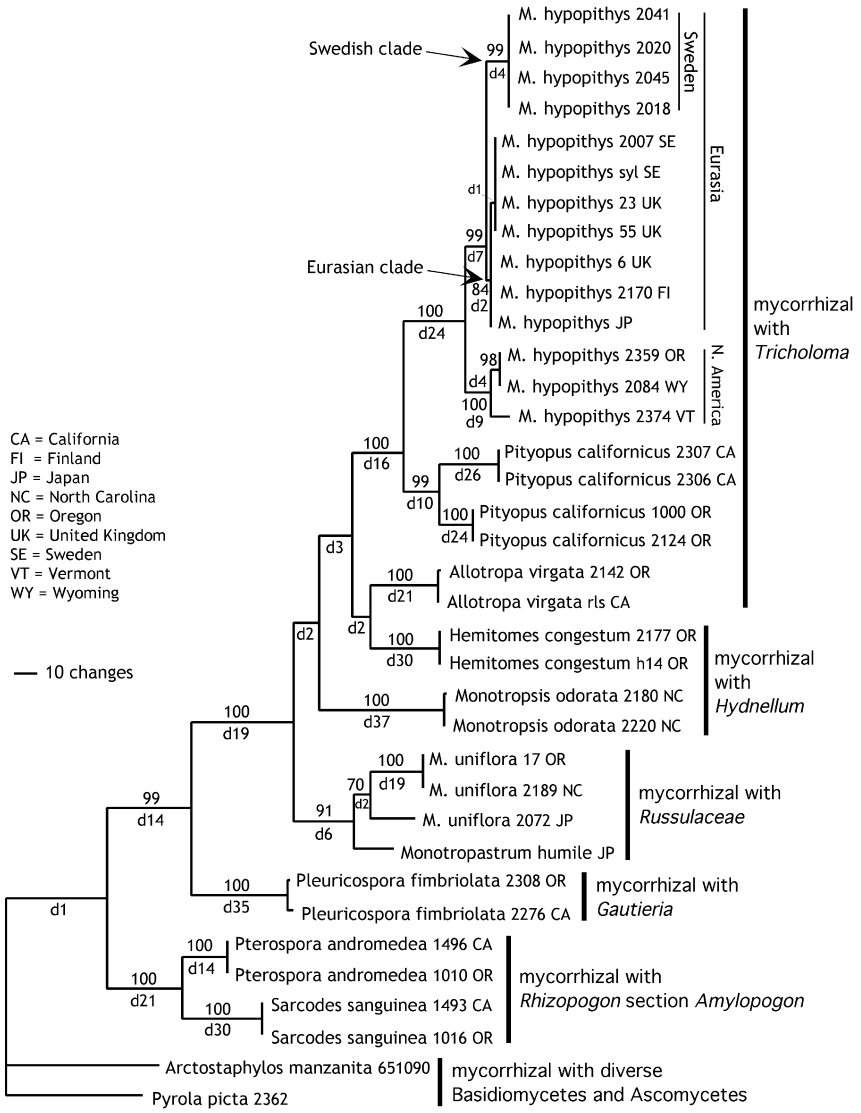

Fig. 3 Molecular phylogeny of the Monotropoideae based on parsimony analysis of nrDNA (ITS + 28s) sequence data. The mycorrhizal fungal associates of plants in each clade are indicated to the right (based on fungal mtLSU and nrITS sequence data). Only branches present in the strict consensus of the 160 most parsimonious trees are shown, all others were collapsed. Decay (dx) values and bootstrap values > 70% are near branches. *Arctostaphylos* and *Pyrola* were used as outgroups. Collection identifiers follow taxon names. Note the presence of two sister lineages within Eurasian *Monotropa hypopithys*, one lineage is exclusively Swedish and the other is pan-Eurasian. The topology within the Monotropoideae does not agree with the *rps*2 topology (Fig. 2). For instance, *Monotropa hypopithys* comprises two nonsister *rps*2 clades (the Eurasian lineage is highly divergent from the N. American) and two sister nrDNA clades.

ITS and 28s sequences within *M. uniflora*, *P. californicus* and *M. hypopithys*. These coincide with extant geographical disjunctions (Wallace 1975), except for one subclade of *M. hypopithys* which overlaps with a pan-Eurasian subclade in Skåne, southern Sweden (Fig. 3).

Plant rps2 *and nrDNA congruency*

Within the Monotropoideae various relationships involving *rps*2 long-branched taxa contradict the phylogenetic relationships of nrDNA (Figs 2 and 3). For instance, *M. hypopithys* comprises two nonsister *rps*2 clades (the Eurasian lineage is highly divergent from the N. American) and two sister nrDNA clades. The partition homogeneity test rejected congruence between *rps*2 and nrDNA ($P = 0.001$), indicating that combining the data would reduce phylogenetic accuracy relative to the uncombined data (Cunningham 1997). The sum of tree lengths for the uncombined data was 845 steps, which falls outside the distribution of replicate randomized partitions from combined data (875–890).

Discussion

Contrary to early predictions and patterns observed in photosynthetic plants (Harley & Smith 1983; Molina *et al*. 1992), plant lineages are specifically dependent on different lineages of fungi in the monotropoid mycorrhizal symbiosis. Furthermore, in the Monotropoideae epiparasitism leads to extensive phylogenetic tracking of fungi, 100% symbiotic fidelity in sympatry, and formation of complex geographical mosaics of specificity. These patterns of association are widespread not only in parasitic interactions (Price 1980; Thompson 1994), but now also in epiparasites of the ectomycorrhizal symbiosis.

Although the objective of this study is to evaluate systematically the mycorrhizal specificity in the Monotropoideae, some new phylogenetic results must be addressed. The relationships of the Monotropoideae within the Ericaceae are not resolved strongly. This may reflect a rapid radiation of the monotropoid, arbutoid and pyroloid groups. These three groups form mycorrhizas with diverse Basidiomycetes (exclusively in the case of the Monotropoideae, see below) and Ascomycetes, while all other Ericaceae form ericoid mycorrhizas with Ascomycetes (Smith & Read 1997). Conflicts between *rps*2 (Fig. 2) and nrDNA (Fig. 3) topologies may be attributed to rate variation in *rps*2 associated with decreased coding requirements (dePamphilis *et al*. 1997), and lateral plastid transfer (Kron *et al*. 1993). The possible existence of *rps*2 pseudogenes in the Monotropoideae requires further investigation. There are significant conflicts between nrDNA and prephylogenetic concepts (Copeland 1941; Furman & Trappe 1971), as well as with prior 28s phylogenetic analyses regarding the position of *Pityopus californicus*, *Monotropa hypopithys* and *Monotropsis odorata* (Cullings 1994; Cullings & Hileman 1997; Cullings 2000). Some of the latter conflicts may stem from the use in those studies of specimens that were misidentified, as some Monotropoideae species are difficult to distinguish from others (Wallace 1975). For instance, Cullings *et al*. (1996) reported *M. hypopithys* associated with a suilloid fungus from an area where it co-occurs with *Pterospora andromedea* (associated with *Rhizopogon* species). The suilloid clade includes *Rhizopogon*, *Suillus*, the Gomphidiaceae and others (Bruns *et al*. 1998). In fact, the phylogenetic placement of those *M. hypopithys* in the suilloid-associated *Sarcodes sanguinea–Pt. andromedea* clade suggests that the plants were actually *Pt. andromedea* and not *M. hypopithys* (which are distant from that clade in our analyses and which we find associated with *Tricholoma* species). *Monotropsis odorata* is supported within the Monotropoideae in both *rps*2 and nrDNA, a relationship with strong morphological support (Olson 1994). *M. hypopithys* and *P. californicus* are sister taxa in nrDNA, and they are considered morphologically most similar to each other compared to the rest of the Monotropoideae (Wallace 1975). The three nrDNA groups containing *M. hypopithys–Allotropa virgata–Hemitomes congestum–Monotropsis odorata*, *M. uniflora–Monotropastrum humile* and *Pt. andromedea–S. sanguinea* are consistent with pollen morphology (Takahashi 1987). The latter clade is basal in nrDNA, and it is known to differ from other Monotropoideae in a number of plesiomorphic features (Anderberg 1992). With respect to subclades within *M. hypopithys* (Fig. 3), the existence of distinct North American and Eurasian clades is also consistent with palynological evidence (Takahashi 1987). However, no other differences have been detected between populations from Wisconsin (USA), Nova Scotia (Canada), and Västergötland (Sweden) (Olson 1993), and the over 80 taxa previously segregated from *M. hypopithys* L. have been synonymized (Wallace 1975).

The identity of the fungal symbiotic partners of the Monotropoideae reported in this study agrees with studies that used limited samples for three species (Martin 1985, 1986; Cullings *et al*. 1996; Kretzer *et al*. 2000) and conflicts with all reports for five species (Reess 1885; Francke 1934; Björkman 1960; Singer 1965; Khan 1972; Trappe 1976; Kernan & Finocchio 1983; Castellano & Trappe 1985; Cullings *et al*. 1996). The present study has the largest sample, numerically, taxonomically and geographically. Yet, we find the highest levels of specificity ever reported (Table 1). Additionally, these specificity patterns are consistent with the phylogenetic patterns of host plants and indicate high levels of symbiotic conservatism (Figs 2 and 3). We attribute conflicts to erroneous prior identifications. A major obstacle is that in axenic conditions most ectomycorrhizal fungi grow slowly and few will produce sexual structures. Much caution needs to be exercised with fungal identification based on proximity of basidiocarps to monotropoid roots, or morphological identification of largely indistinct vegetative tissue either in symbiotic or axenic conditions. These methods are the basis for most prior reports (Reess 1885; Francke 1934; Björkman 1960; Singer 1965; Khan 1972; Trappe 1976; Kernan & Finocchio 1983; Castellano & Trappe 1985). However, it is noteworthy that our identifications agree for several taxa in *Russula* and *Tricholoma* with those of Martin (1985, 1986), who in some cases relied exclusively on dried root specimens for morphological identification. As discussed by Kretzer *et al*. (2000), the extent of fungal colonization and the age of the root tissue are particularly important in the avoidance of artefacts in molecular studies. Taking these precautions into account, we have expanded our previous sampling (Kretzer *et al*. 2000) to include the entire geographical range of *S. sanguinea* and we have found that specificity to *Rhizopogon ellenae* encompasses the Sierra Nevada and southern California ranges (*S. sanguinea* associates with *R. subpurpurascens*, sister taxon to *R. ellenae*, in southern Oregon; Bidartondo & Bruns, unpublished data).

Each Monotropoideae lineage appears restricted to a fungal genus or a set of closely related species (Table 1), a level of specificity that agrees with that observed previously between *S. sanguinea* and *R. ellenae* (Kretzer *et al*. 2000). This is particularly impressive because eight of the plant taxa are known to often grow a few metres from each other in western North America (Wallace 1975; M. I. Bidartondo, personal observation). Thus, specificity in the monotropoid symbiosis is apparently not determined by habitat or local availability of partners but rather by direct plant–fungal interactions. At least part of the specificity is established by specific germination cues derived from potential fungal associates. Bruns & Read (2000) have shown that under gnotobiotic conditions seeds of

S. sanguinea and *Pt. andromedea* are stimulated to germinate by a diffusible substance produced by some *Rhizopogon* species; no germination occurs with other fungi or on various nutrient media. However, the range of *Rhizopogon* species that stimulate germination (Bruns & Read 2000) in these two plants is broader than the range that is associated with mature plants (Table 1). In particular, *R. ellenae*, an exclusive associate of mature *S. sanguinea*, also stimulates *Pt. andromedea* seeds, and the exclusive associates of mature *Pt. andromedea*, *R. salebrosus* and *R. arctostaphyli*, stimulate *S. sanguinea* seeds. This pattern has also been detected in nature (Bidartondo & Bruns, unpublished data). This is a biologically relevant pattern because both plants and all three fungi are sympatric. Perhaps most remarkable is that these *Rhizopogon* species are each other's closest relatives (Kretzer *et al*. 2000; Bidartondo, unpublished data) and *Pt. andromedea* and *S. sanguinea* are sister species (Figs 2 and 3). Yet, a large sample of adult plants shows that there is no overlap in fungal associations (Table 1). This means that somewhere between seed germination and flowering, seeds that were stimulated to germinate by the 'wrong' fungi either switched to the correct one or died. These are fundamental modifications to Hadley's model of symbiotic development which emphasized nonspecificity in the symbiotic interactions of 'dust seeds' and fungi (Hadley 1970).

The *rps*2 and nrDNA phylogenies indicate high levels of specificity in all cases. In fact, we found no examples of fungi shared by two or more plant lineages (Table 1). The only exception may be *Tricholoma portentosum*, if this is indeed the same species in Europe and North America. This potentially widespread fungal species could have allowed the circumboreal expansion of *M. hypopithys*, the most widely distributed of all Monotropoideae. Clades within the Monotropoideae correspond with single clades of fungal associates. This is evident in the *P. californicus–M. hypopithys*, *Monotropastrum humile–M. uniflora*, and *Pt. andromedea–S. sanguinea* clades (Figs 2 and 3). Specificity is narrower at the subspecific level. For instance, a complex pattern of specificity emerged from the expanded nrDNA sampling of Eurasian *M. hypopithys*. Unlike other plant clades, the two terminal subclades detected (Fig. 3) do not correspond with an extant geographical disjunction since southern Swedish plants fall in both clades. The exclusively 'Swedish' clade is composed of plants from beech and spruce forests in Sweden and is associated with *T. columbetta*, *T. saponaceum*, or *T. portentosum*. The 'Eurasian' clade is composed of plants from pine forests or willow stands in Finland, Sweden, UK and Japan, and these are associated with *Tricholoma* section *Terrea*. The nrITS sequences of the fungi associated with the Swedish clade cluster apart from the fungi associated with the Eurasian clade in both distance and parsimony analyses (M. I. Bidartondo and T. D. Bruns, unpublished data). These patterns are also suggestive of geographical mosaics of specificity (Thompson 1994) in epiparasites among nearby forests of different ectomycorrhizal trees in northern Europe. A relatively simpler example of a geographical mosaic is found in *M. uniflora* N. Am. All plants from four populations encompassing an area ~9400 km^2 and spanning two mountain ranges in Oregon (western USA) shared *Russula brevipes* as symbiont. By contrast, plants from a single population < 0.5 km^2 in Vermont (eastern USA) were associated with *Russula brevipes*, *Lactarius theiogalus*, or two other *Russula* species groups. These patterns show the presence of much unexplored geographical variation in symbiotic specificity within the Monotropoideae, which we will evaluate in more detail elsewhere.

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The authors share an interest in the evolutionary ecology of ectomycorrhizal symbioses and epiparasitic mycorrhizal plants.
