Fine-level mycorrhizal specificity in the Monotropoideae (Ericaceae): specificity for fungal species groups

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Abstract

The Monotropoideae (Ericaceae) are nonphotosynthetic angiosperms that obtain fixed carbon from basidiomycete ectomycorrhizal fungi. In previous work, we showed that each plant species is associated with a single genus or a set of closely related genera of ecto-mycorrhizal fungi. Here we show that the level of specificity is much higher. We used a molecular phylogenetic approach to contrast specificity patterns among eight plant lineages and three fungal genera. We relied on fungal nuclear internal transcribed spacer (nrITS) sequence data obtained from 161 basidiocarps and 85 monotropoid roots representing 286 sampled plants screened using restriction length polymorphisms. From the phylogenetic placement of fungal symbionts in fungal phylograms, we found that three basal (*Sarcodes*, *Pterospora*, *Pleuricospora*) and one derived lineage (*Allotropa*) of plants target narrow clades of closely related species groups of fungi, and four derived lineages (*Monotropa hypopithys* species group, *Pityopus*) target more distant species groups. Within most plant lineages, geography and photobiont association constrain specificity. Specificity extended further in *Pterospora andromedea*, in which sequence haplotypes at the plastid *trn* L–F region of 73 plants were significantly associated with different fungal species groups even in sympatry. These results indicate that both the macro- and microevolution of the Monotro-poideae are tightly coupled to their mycorrhizal symbionts.

Keywords: coevolution, *Monotropa*, mycorrhizae, specialization, *Tricholoma*

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Introduction

Myco-heterotrophic plants acquire fixed carbon from their mycorrhizal fungi and they have arisen independently multiple times in angiosperm evolution (Leake 1994). Many of these plants are epiparasitic because they obtain fixed carbon indirectly from a neighbouring photosynthetic plant through a shared mycorrhizal fungus (Björkman 1960; Duddridge & Read 1982; Cullings *et al*. 1996; Taylor & Bruns 1997; McKendrick *et al*. 2000; Bidartondo & Bruns 2001). Thus, mycorrhizal epiparasites are exploiters of one of the most widespread and ancient interspecific mutualisms. All mutualisms are susceptible to exploiters or cheaters, species that can obtain rewards without providing service in return (Janzen 1975; Inouye 1983; *'aprovechados' sensu* Soberon & Martinez 1985; Bronstein 1994, 2001). Much doubt has been cast on the legitimacy of the most classic

example of cheating, nectar-robbing, because robbers can and do pollinate (Maloof & Inouye 2000). Other examples are legitimate, such as the seed-eating nonpollinating yucca moths and fig wasps, because the insects lack the morphological traits required for pollen manipulation (Pellmyr & Leebens-Mack 2000) or their contribution to pollination is negligible (Compton *et al*. 1991). Non-photosynthetic mycorrhizal exploiters are legitimate exploiters because, unlike green plants, they are unable to provide fixed carbon to their mycorrhizal fungi. Indeed, many lack chlorophyll *b* and have only traces of chloro-phyll *a* (Cummings & Welschmeyer 1998). Even though in symbiotic interactions the larger symbiont is convention-ally termed 'host' and in mycorrhizal interactions the plant is always the 'host', here we use this term to refer to the mycorrhizal fungi to imply that the fungi are the 'lineages being tracked' (*sensu* Page & Charleston 1998) by epiparasitic plants.

The Monotropoideae (Ericaceae) is a diverse subfamily of 10 genera of nonphotosynthetic mycorrhizal epiparasites

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that associate with five distant families of ectomycorrhizal basidiomycete fungi (Bidartondo & Bruns 2001). The centre of diversity of the Monotropoideae is western North America (eight genera), but they occur in evergreen and deciduous ectomycorrhizal forests throughout the northern hemisphere (dominated by Pinaceae, Fagaceae, Salicaceae, Dipterocarpaceae) (Wallace 1975). In a previous study, we showed that each plant lineage specializes on a single fungal genus, or in the case of *Monotropa uniflora* on two closely related genera (Bidartondo & Bruns 2001). In this study, we examine the pattern of symbiont affiliation at a finer phylogenetic scale for eight plant lineages, and show that specificity patterns are much more narrow than previously realized. We use this information to address basic questions about mycorrhizal cheating. How diverse is the range of mutualists with which cheaters interact (Bronstein 1994)? What is the pattern of specificity when sympatric related plants (e.g. sister taxa) target closely related fungi (e.g. within a single genus)? Is there exclusive symbiont fidelity or is there symbiont overlap? Is exploiter phylogeny congruent with fungal phylogeny at low phylogenetic levels? What biological features are associated with the patterns of cheater specificity and radiation (Pellmyr & Leebens-Mack 2000)?

Detailed knowledge of symbiont phylogenies is required to interpret the phylogenetic pattern of host association, and so phylogenetically explicit studies of the evolution of cheaters are still few (Pellmyr & Leebens-Mack 1999). The evolutionary relationships of the plants involved in the monotropoid mycorrhizal symbiosis have been re-examined recently (Bidartondo & Bruns 2001), but the evolutionary relationships of the fungi involved are poorly understood. To remove this problem, we built nuclear internal transcribed spacers (nrITS) phylograms for 161 members of *Gautieria* (Gomphaceae), *Rhizopogon* section *Amylopogon* (Rhizopogonaceae) and *Tricholoma* (Tricholomataceae). We then used them to place sequences representing the diversity of Monotropoideae mycorrhizal associates from a sample of 286 plants screened using molecular methods. For *Pterospora andromedea*, which associates sympatrically with two closely related fungal species groups (*Rhizopogon arctostaphyli* or *Rhizopogon salebrosus*), we also examined plant genotypes to test whether specialization occurs below the species level. We tested 73 *Pterospora andromedea* plants for association between the rapidly evolving plastid *trn* L–F region (Taberlet *et al*. 1991) and the identity of their fungal symbiont.

Materials and methods

Sampling of mycorrhizal tissue

We obtained monotropoid roots from a total of 88 populations and 286 plants. The rarity of some species precluded intensive sampling at many sites. Roots were obtained by removing one or more 2.5-cm soil cores near emerging inflorescences or by excavating part of the root mass. Samples were maintained at 4 °C until processed in the laboratory. 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. Monotropoid roots were lyophilized within a week after field collection. Plant lineage designations are based on plastid *rps* 2 and nuclear DNA (nrDNA) sequence data (Bidartondo & Bruns 2001). A list of sampling localities follows. *Monotropa hypopithys* N. Am: Albany County (WY), Mary's Pk., Benton Co. (OR), Chittenden Co. (VT), Perkins Cr., Siltcoos Lk., Lane Co. (OR), Crescent Lk., Klamath Co. (OR), Umpqua N.F., Douglas Co. (OR); *M. hypopithys* Sweden: Häckeberga, Örkelljunga, Janne, Skåne Prov. (SE), Uppland Prov. (SE); *M. hypopithys* Eurasia: Derbyshire (UK), Anglesey (UK), Iwate Prefecture (JP), Lappeenranta (FI), Dalby, North Yorkshire (UK), Sylvåkra, Skåne Prov. (SE); *Pityopus californicus*: Mary's Pk., Benton Co. (OR), Umpqua N.F., Douglas Co. (OR), Perkins Cr., Lane Co. (OR), Whitaker, Tulare Co. (CA); *Allotropa virgata*: Siskiyou N.F., Curry Co. (OR), Umpqua N.F., Douglas Co. (OR), Perkins Cr., Waxmyrtle, Lane Co. (OR), Stevenson S.P., Napa Co. (CA), Crescent Lk., Klamath Co. (OR); *Pleuricospora fimbriolata*: Umpqua N.F., Douglas Co. (OR), Gaddis Road., Stumpy Mds., Blodgett, Lk. Tahoe, El Dorado Co. (CA), Sierra N.F., Dinkey Cr., Fresno Co. (CA), Perkins Cr., Lane Co. (OR), Lk. Almanor, Plumas Co. (CA), Sequoia N.P., Tulare Co. (CA); *Sarcodes sanguinea*: Siskiyou N.F., Curry Co. (OR), Lk. Tahoe, El Dorado Co. (CA), Dinkey Cr., Sierra N.F., Fresno Co. (CA), Mt. Abel, Kern Co. (CA), Yuba Pass, Nevada Co. (CA), Quincy, Fredonyer Summit, Plumas Co. (CA), San Bernardino Co. (CA), Mt. Pinos, Ventura Co. (CA); Hart, Sequoia N.P., Tulare Co.; *Pterospora andromedea*: Chuska Mts., Apache Co. (AZ), Blodgett, El Dorado Co. (CA), Sierra N.F., Fresno Co. (CA), Siskiyou N.F., Josephine Co. (OR), Crescent Lk., Klamath Co. (OR), Round Valley, Quincy, Plumas Co. (CA), Whitaker, Tulare Co. (CA), Québec Prov. (Canada).

Identification of fungal lineages

We identified fungi using methods described by Gardes & Bruns (1996). In summary, we extracted genomic DNA from individual monotropoid root tips and amplified the ITS of the nuclear ribosomal repeat using a polymerase chain reaction (PCR) with the fungal-specific primer pair ITS1F/ITS4 (White *et al*. 1990; Gardes & Bruns 1993). We screened PCR products by restriction fragment length polymorphisms (RFLP) using the restriction endonucleases

*Alu*I, *Hin*fI and/or *Cfo*I (New England Biolabs Inc., Beverly, MA, USA). We estimated the molecular size of the restriction fragments using GELREADER Version 2.0.5 (National Center for Supercomputing Applications, Champaign, IL, USA). We then sequenced the ITS region for each unique ITS–RFLP type from most populations, and in some cases we sequenced multiple identical ITS–RFLP types from the same population.

Basidiocarps from the collections at several institutional and personal herbaria were selected to sample phylogenetic diversity within each genus or section and to maximize the number and geographical range of sampled species. In the case of *Rhizopogon* section *Amylopogon*, holotype and paratype specimens were available and they were used preferentially because preliminary analyses revealed that specific epithets were not applied consistently to other specimens. We extracted DNA from 136 basidiocarps and sequenced their nrITS region. We did not rely on ITS–RFLP comparisons (Kårén *et al.* 1997; Taylor & Bruns 1999) because these alone can be insufficient to discriminate closely related species groups (Kretzer *et al.* 2000). A list of herbaria and specimen vouchers follows. *Tricholoma*: San Francisco: DED5365, HDT54614, KMS304, KMS365, KMS285, KMS303, KMS286, KMS435, HDT38137, KMS344, KMS397, KMS281, HDT53510, KMS447, KMS246, DED sn, KMS324, HDT54945, HDT50916, HDT48249, KMS362, HDT54613, KMS436, KMS357, KMS296, HDT32084, KMS249, KMS428, KMS197, KMS248, KMS396, KMS232, HDT48319; Oslo: HO51033, HO51046, HO59602, HO70098; Uppsala: UP5021; Copenhagen: MC95187, MC95165, MC99197; Leiden: MEN95210, CB8966, MEN95213; Berkeley: ECV2379, MIB2027, TDB1490. The Accession nos for *Tricholoma* sequences obtained from GenBank are: AJ236081, AF062623–24, AF062617, AB036893, AF309531, TMU62964, UPSF013396, AB036901. *Rhizopogon*: San Francisco: HS2056, HDT46517, HDT30140, HDT52823, Bourell124; Ann Arbor: AHS70715, AHS71330, AHS74030, AHS71422, AHS66162, AHS66300, AHS66176, AHS71326, AHS68263, AHS70789, AHS69813, AHS69801, AHS65398, Kauffman sn, F2882, AHS69299, AHS66072, AHS69292, AHS69273, AHS66116, AHS65762, AHS59523, AHS68612, AHS69037, AHS68558, AHS68485, AHS58998, Sanders 78; New York: Tracy 5705; Corvallis: T8379, T4731, Garcia 4750, OSC44295, OSC56622, OSC55282, OSC49745, OSC58561, OSC49773, OSC59166; J. States personal herbarium: WYHF165, NMHF19, NMHF5, AHF419, AHF553, WYHF151, NMH38, AHF123, AHF133, AHF669; Berkeley: TDB1330, SNF210B, SNF32, SNF38, SNF260, SNF306. Accession nos for *Rhizopogon* sequences obtained from GenBank: AF224470–89, M91613. Sequences from T7624, T7899, AHS66137, T17476, T17562, AHS65699, T19168 are from Grubisha *et al.* (2002). *Gautieria*: Corvallis: T3401, OSC41323, CZA136, OSC48548, OSC38736, T4547, T7582, T8371, T11164, OSC48138, OSC48844; Berkeley: SNF213, SNF236, SNF180, SNF312,

SNF136, SNF334, SNF346, SNF95, SNF161; San Francisco: HS2634, HDT25707, Gerry 499, RH5818, HDT39337, HS2305, HDT53493, HDT45921; J. States personal herbarium: HS1831, NJS1948, HS1997, ORHF20, F4530, H479; Berne: HH2221, HH2222.

Plastid trn *L haplotypes*

We selected 73 *Pterospora andromedea* plants from populations in the Sierra Nevada of California where some plants associated with the *Rhizopogon salebrosus* species group and others with the *R. arctostaphyli* species group. The plastid *trn* L intron and *trn* L–F intergenic spacer regions were amplified from root DNA extracts using primers c and f (Taberlet *et al.* 1991). These were then sequenced. We tested for haplotype–fungal symbiont association with a G2 likelihood ratio χ^2 test using JMP (SAS Institute, Cary, NC, USA).

DNA sequencing

Sequencing of both strands was performed with an ABI model 377 Sequencer (Applied Biosystems Co., Foster City, CA, USA) using a BigDye™ Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems Co.) or a Thermo Sequenase™ Dye Terminator Cycle Sequencing Pre-Mix Kit (Amersham Pharmacia Biotech, Piscataway, NJ, USA). We processed the raw data using DNA SEQUENCING ANALYSIS Version 2.1.2 and SEQUENCE NAVIGATOR Version 1.0.1 (Applied Biosystems Co.). The 212 DNA sequences generated for this study have been deposited in GenBank under Accession nos AF377055–79, AF377082–252, AF384112–26, AF442136–8.

Phylogenetic analysis

Alignments were produced with CLUSTAL X (Thompson *et al.* 1997), adjusted manually using a colour font, analysed by neighbour joining and parsimony, and bootstrapped (1000 replicates) using PAUP Version 4.0b8 (Swofford 2001). Neighbour joining was used with the Kimura 2-parameter model. In the *Rhizopogon* section *Amylopogon* and in the *Allotropa–Pityopus–Monotropa hypopithys* sequence alignments, we coded informative indels as in Kretzer *et al.* (2000). Because of computational difficulties associated with parsimony analysis of the large sequence data sets on a Power Macintosh G4 (Apple Computer Inc., Cupertino, CA, USA), we used a stepwise approach to remove taxa until the analyses were feasible. First, from sets of identical sequences only one was chosen arbitrarily and retained. Second, from sets of sequences differing by 1 or 2 bp only one was chosen arbitrarily and retained. These steps sufficed for *Rhizopogon* section *Amylopogon* and *Gautieria*. For *Tricholoma*, the largest data

set, we used an additional third criterion: only one arbitrarily chosen sequence from each clade with > 95% distance bootstrap support (in the full data set) was retained for parsimony analysis. *Tricholoma* and *Rhizopogon* section *Amylopogon* were midpoint rooted. *Gautieria* was rooted with *Ramaria botrytis* based on results from nrLSU and mtSSU analyses (Humpert *et al.* 2001). The resulting phylograms were compared with the latest classification schemes such as those of Riva (1988), Noordeloos & Christensen (1999), and Shanks (1997) for *Tricholoma*, Smith & Zeller (1966) for *Rhizopogon* section *Amylopogon* and Stewart (1974) for *Gautieria*. Because we knew that there are multiple nrDNA lineages within *Monotropa hypopithys* and *Pityopus californicus* (Bidartondo & Bruns 2001), we tested congruency between plant ITS phylogeny and fungal ITS phylogeny with a partition homogeneity test (Farris *et al.* 1994) using 1000 random taxon addition sequences in PAUP excluding parsimony-uninformative characters. In this analysis we included *Allotropa virgata*, and excluded all *Tricholoma* species not associated with Monotropoideae lineages. We used 17 plant ITS sequences from Bidartondo & Bruns (2001) and 14 additional plant ITS sequences.

Results

All *Pleuricospora fimbriolata* symbionts were restricted to the *Gautieria monticola* species group in the Sierra Nevada of California and the southern Cascade Range in Oregon (Fig. 1). The full data set for *Gautieria* had 55 fungal taxa and 649 characters. The parsimony data set had 27 fungal taxa and 108 parsimony-informative characters, which produced 16 most parsimonious trees. The systematics of this false-truffle genus are notoriously difficult because of a paucity of morphological characters. Conflicting specific epithets (i.e. *G. chilensis*, *G. magnicellaris*) have been retained in the phylogram shown in Fig. 1. All sampled members of the *G. monticola* group share a ≈ 500 bp insertion between the 5′-end of the 18*S* and the ITS 1. This region was not included in the analysis. Deep relationships within the large-spored clade are largely unresolved. The three species from section *Trappea* that we sampled (*G. parksiana*, *G. gautierioides*, *G. vestita*) appear nested within section *Gautieria*, to which all other species sampled belong (Stewart 1974). The nrITS sequences of Australasian *Gautieria* species (*G. amara*, *G. novae-zelandiae*, *G. scabricrista*) and *Austrogautieria* species (*A. chlorospora*, *A. manjimupana*, *A. costata*) are distant from both North American and European *Gautieria* and this agrees with nr28*S* sequence data (Bidartondo, unpublished data); thus, they were not included in this analysis.

Sarcodes sanguinea symbionts were restricted to the *Rhizopogon ellenae* species group in the Sierra Nevada, Western Transverse Ranges and San Bernardino Mountains of California, and to the *R. subpurpurascens* species group in the Siskiyou Mountains of Oregon. *Pterospora andromedea* symbionts were restricted to the *R. salebrosus* and *R. arctostaphyli* species groups (Fig. 2) in western North America. An undescribed *Rhizopogon* section *Amylopogon* lineage was detected in one plant sampled from Québec (the rare and threatened north-eastern North American populations of *P. andromedea* are geographically disjunct). Both western fungal groups were often found on different plants growing only a few metres apart at sites in California and Oregon. The full data set for *Rhizopogon* section *Amylopogon* had 100 taxa and 595 characters. The parsimony data set had 33 taxa and 53 parsimony-informative characters, which produced 4820 most parsimonious trees. These different trees were primarily due to rearrangements within well-supported species groups. The strict consensus branches of the parsimony trees are indicated on the neighbour joining phylogram in Fig. 2. *Rhizopogon* is also a taxonomically challenging genus, and our preliminary analyses of specimen collections revealed that specific epithets applied to specimens often did not correspond to the phylogenetic placement of holotype specimens. Even paratypes were often found to significantly differ from holotypes. For this reason, in Fig. 2 specific epithets were removed from all specimens except type specimens.

All *Allotropa virgata* symbionts sampled were restricted to the *Tricholoma magnivelare* species group. *Pityopus californicus* and *Monotropa hypopithys* associate with several species groups of *Tricholoma* (Fig. 3). Three lineages of *Monotropa hypopithys* were identified by nrITS and 28*S* sequence data: North American, Swedish and Eurasian (Bidartondo & Bruns 2001). Two of these, Swedish and Eurasian, are sympatric in southern Sweden but associated with mutually exclusive clades of *Tricholoma*. The *T. portentosum*/*T. griseoviolaceum* species group is the only one used by more than one of the three *M. hypopithys* clades, but the two plant clades involved are allopatric. The plant ITS phylogeny is not congruent with the ITS phylogeny of Monotropoideae-associated *Tricholoma* species (partition homogeneity test, $P = 0.01$). The full nrITS sequence data set for *Tricholoma* had 93 taxa and 914 characters. The parsimony data set had 40 taxa and 200 parsimony-informative characters which produced 293 most parsimonious trees. There was poor support for deeper branching events. The phylogenetic distribution of fungal symbionts is represented in Fig. 3. The plant nrITS sequence data set for *Allotropa–Pityopus–Monotropa hypopithys* had 31 taxa, 699 characters and 154 parsimony-informative characters, which produced 1890 most parsimonious trees. The strict consensus topology mirrors that presented in Bidartondo & Bruns (2001), which we obtained from a narrower taxon sample. The *Tricholoma* phylogram is compared with the *Allotropa–Pityopus–Monotropa hypopithys* phylogram in Fig. 4. This shows multiple host-shifts in *Pityopus* and the *Monotropa hypopithys* group.
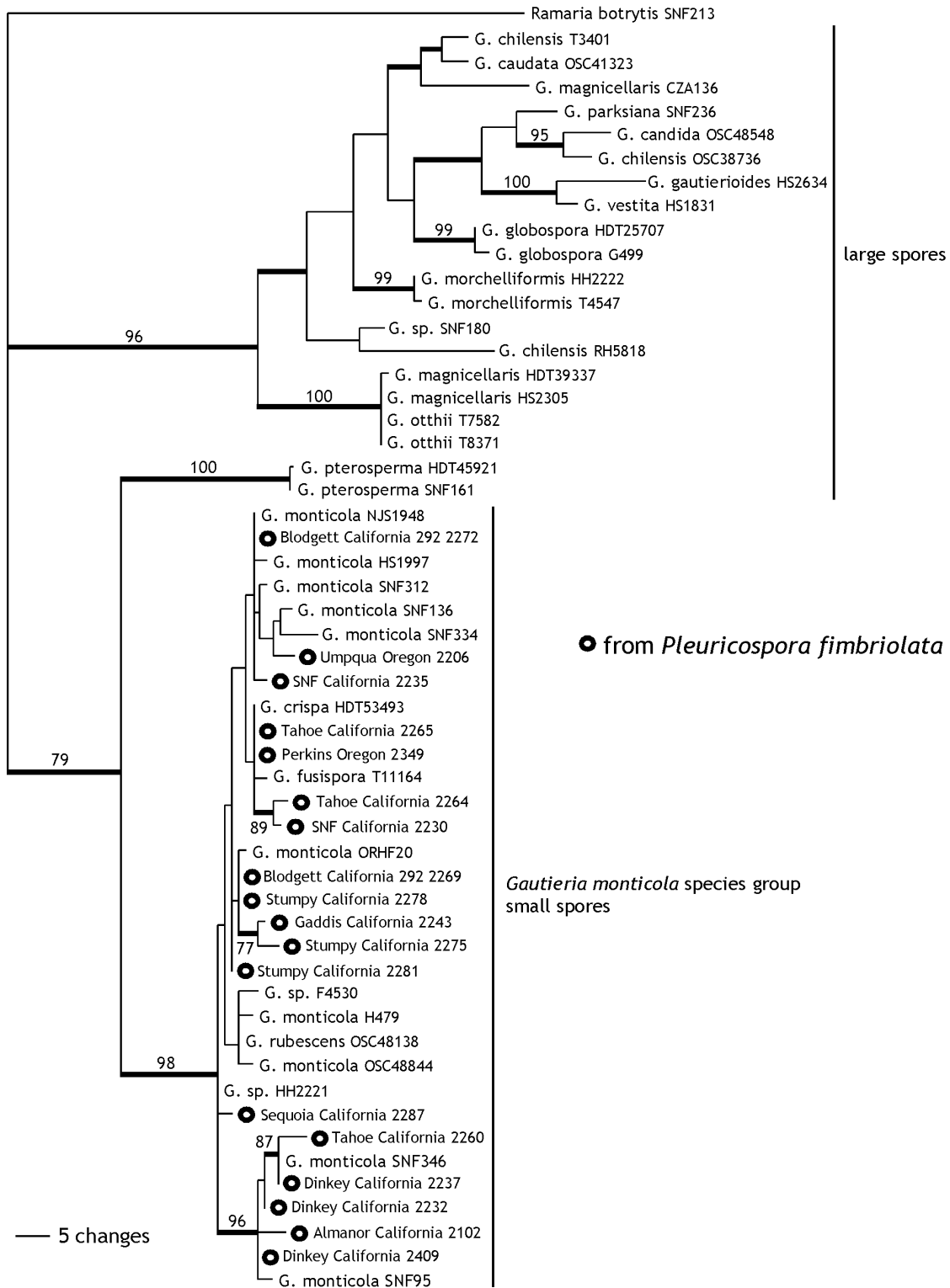
Fig. 1 Phylogenetic placement of mycorrhizal associates of *Pleuricospora fimbriolata* within the basidiomycete fungal genus *Gautieria*. This fungal nuclear internal transcribed spacer (nrITS) phylogram is based on neighbour joining (full data set), bootstrap (full data set, values > 70 are near branches), and parsimony analysis (restricted data set) of 36 basidiocarp sequences, and 18 sequences from monotropoid roots representing 13 populations and 42 plants. Symbols for sequences from monotropoid roots are followed by a locality and population/plant number. Branches present in the strict parsimony consensus are in bold. Bootstrap values are near branches. *Ramaria botrytis* was used as the outgroup.

Tahoe California TS2.2
Tahoe California T22.1
Dinkey California 8
SNF California 361
SNF California 355
Tahoe California 2246
SNF California 360
Fredonyer California 2114
HS2056
R. idahoensis AHS70715 PT
SNF210B
R. semireticulatus AHS71330 HT ←
Quincy 2116
AHS74030
R. idahoensis AHS71422 PT
R. subcaerulescens AHS66162 HT ←
R. idahoensis AHS66300 HT ←
R. ellenae AHS66137 HT ←
R. pedicellus AHS66176 PT
R. semireticulatus AHS71326 PT
T17476
HDT46517
SNF32
SNF California 358
Dinkey California 13
WYHF165
Hart California 2290
T17562
HDT30140
HDT52823
Yuba California 2112
B124
TDB1330
T8379
Abel California
Pinos California
Bernardino California
R. pinyonensis OSC44295 PT
NMHF19
NMHF5
AHF419
AHF553
G4750

Rhizopogon ellenae species group

71
78
100
86
93

R. pinyonensis species group

R. atroviolaceus AHS68263 PT
Siskiyou Oregon
T19168
R. milleri AHS70789 HT ←
R. kauffmanii AHS69813 HT ←
R. subpurpurascens AHS69801 PT
R. subpurpurascens AHS65669 PT
R. subpurpurascens AHS65398 HT ←
R. anomalus CHKsn HT (ITS1 only) ←

R. subpurpurascens species group

● from *Sarcodes sanguinea*
⊗ from *Pterospora andromedea*

⊗ Heney Québec 3000
F2882
R. salebrosus AHS69299 PT
⊗ SNF California symbiont 2B
⊗ Crescent Oregon 2380
R. subgelatinosus AHS66072 PT
WYHF151
R. salebrosus AHS69292 PT
R. salebrosus AHS69273 HT ←
R. fallax AHS66116 PT
R. fallax AHS65762 PT
R. subcaerulescens var. subcaerulescens AHS59523 PT
R. subcaerulescens var. subpannosus AHS68612 PT
⊗ Round California 2095
T4731
R. alkalivirens AHS69037 HT ←
R. subgelatinosus AHS68558 PT
SNF38
isolate from *Pinus muricata* 2272
⊗ SNF California 1496
⊗ Dinkey California 2238
⊗ Quincy California 2098
⊗ Blodgett California 2285
SS78
SNF260
OSC49773
NMH38
⊗ Chuska Arizona 2075
AHF123
AHF133
AHF669
OSC56622
OSC55282
T7624
T7899
⊗ Siskiyou Oregon
R. subbadius AHS68485 PT
R. subbadius AHS58998 HT ←
OSC49745

Rhizopogan salebrosus species group

92

OSC58561

99

100

R. arctostaphyli JPT5705 HT ←
⊗ Crescent Oregon 2398
⊗ SNF California 1448
⊗ Quincy California 2097
SNF306
⊗ Whitaker California 2299
⊗ SNF California isolate 378
OSC59166

R. arctostaphyli species group

— 0.5 changes
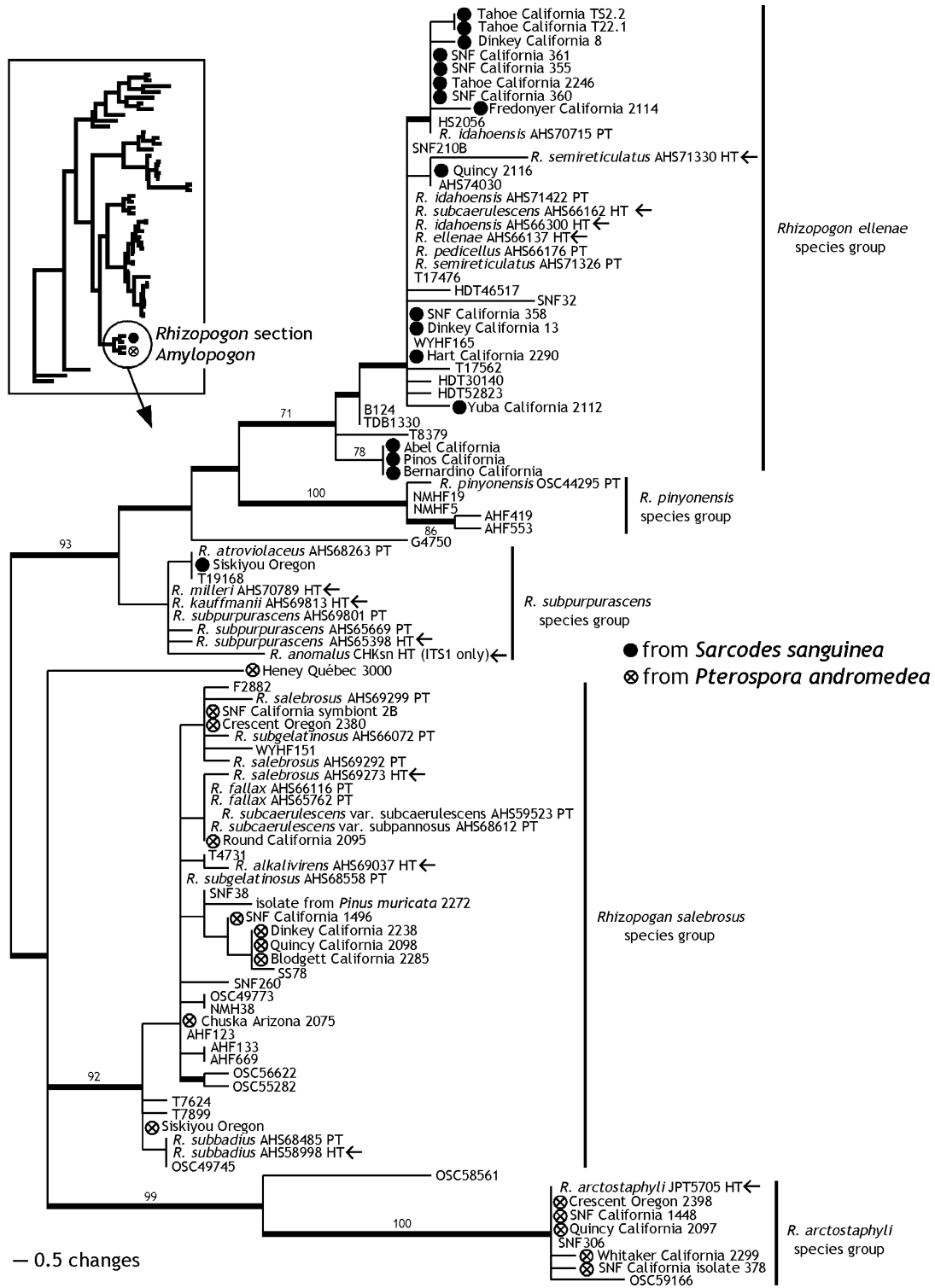
Inset:
Rhizopogon section *Amylopogon*

Fig. 2 Phylogenetic placement of mycorrhizal associates of *Sarcodes sanguinea* and *Pterospora andromedea* within the basidiomycete fungal genus *Rhizopogon* (the upper left small phylogram is from Grubisha *et al.* 2002), section *Amylopogon* (large phylogram). The large fungal nuclear internal transcribed spacer (nrITS) phylogram is based on neighbour joining (full data set), bootstrap (full data set, values > 70 are near branches), and parsimony analysis (restricted data set) of 51 new basidiocarp sequences, 15 basidiocarp sequences from Kretzer *et al.* (2000), 26 new sequences from monotropoid roots, seven monotropoid root sequences from Kretzer *et al.* (2000) and 1 sequence from a *Pinus muricata* root. Symbols for sequences from monotropoid roots are followed by a locality and population/plant number(s). Branches present in the strict parsimony consensus are in bold. Midpoint rooting was used. The monotropoid root sequences were selected by screening 42 populations and 171 plants via ITS–RFLP and sequencing all unique RFLP types. Species names have only been retained for sequences from type specimens (HT, arrow: holotype; PT: paratype).
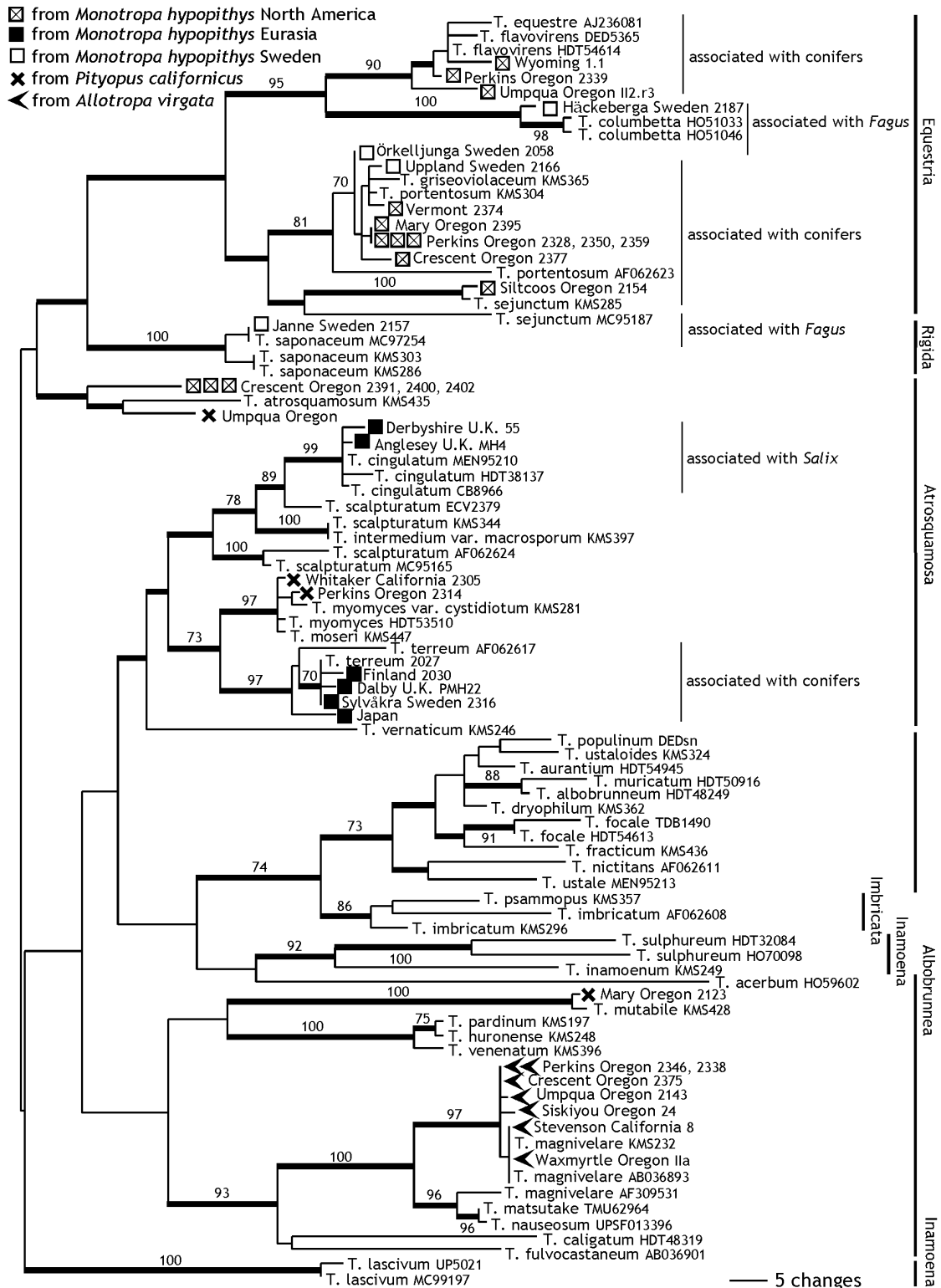
Fig. 3 Phylogenetic placement of mycorrhizal associates of *Monotropa hypopithys* N.Am., *M. hypopithys* Eurasia, *M. hypopithys* Sweden, *Pityopus californicus* and *Allotropa virgata* within the basidiomycete fungal genus *Tricholoma*. This fungal nuclear internal transcribed spacer (nrITS) phylogram is based on neighbour-joining (full data set), bootstrap (full data set, values > 70 are near branches) and parsimony analysis (restricted data set) of 10 basidiocarp sequences from GenBank, 49 new basidiocarp sequences, and 34 sequences from monotropoid roots representing 33 populations and 73 plants. Symbols for sequences from monotropoid roots are followed by a locality and population/plant number(s). Branches present in the strict parsimony consensus are in bold. Selected putative photobiont associations and Riva's (1988) sectional classification are to the right.
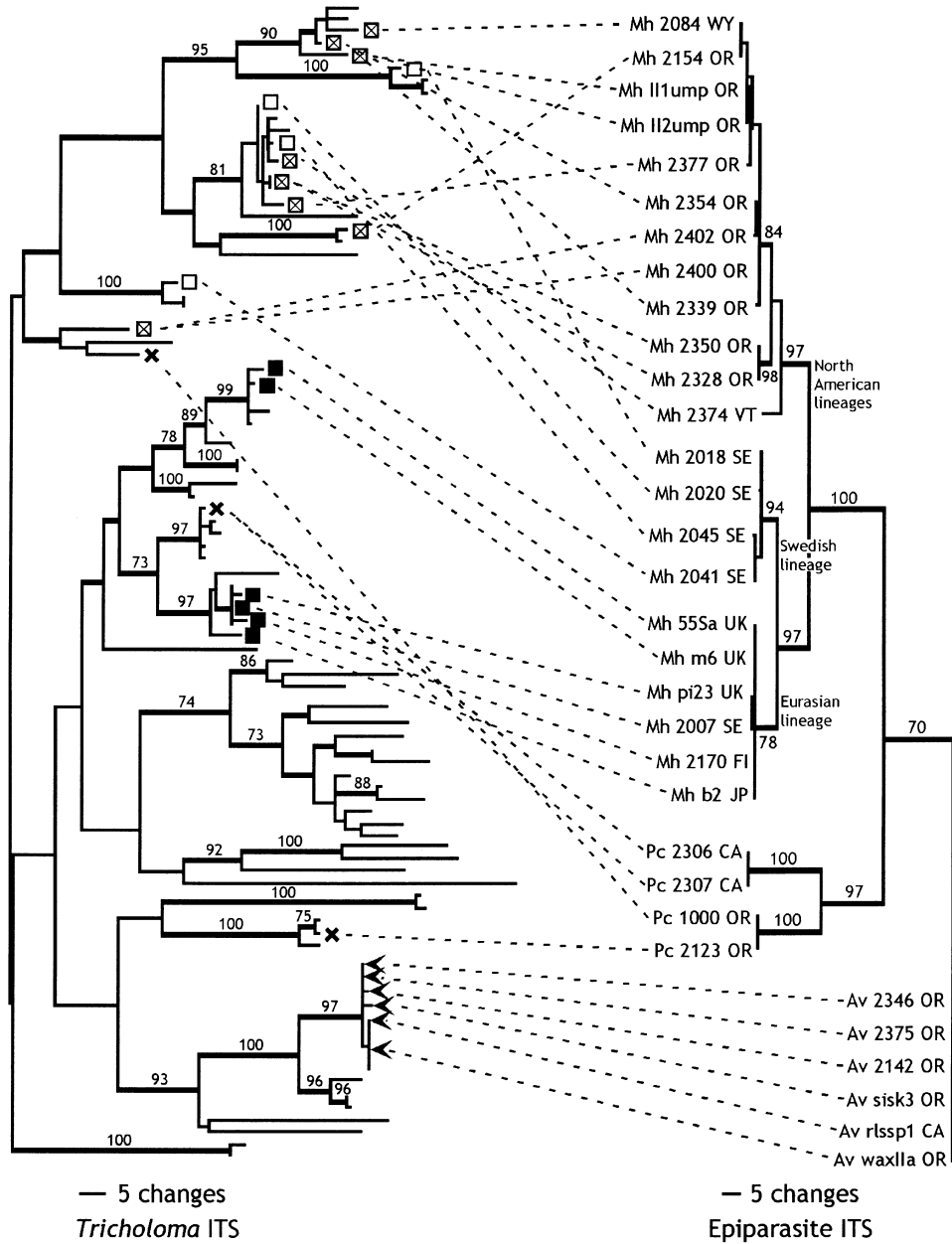
Fig. 4 Comparison of mycorrhizal fungi internal transcribed spacer (ITS) phylogeny (genus *Tricholoma*) (left) and epiparasitic plant ITS phylogeny (right). For fungal species names and collection identifiers refer to the phylogram in Fig. 3. The plant species are abbreviated Mh, Pc and Av, for *Monotropa hypopithys, Pityopus californicus* and *Allotropa virgata*, respectively. Bold branches were present in the strict parsimony consensus. Lineage designations for the *Monotropa hypopithys* species group correspond to those used by Bidartondo & Bruns (2001).

The contingency table for the *Pterospora andromedea* plastid *trn* L–F haplotypes and fungal symbiont identity were used for a G2 likelihood ratio χ^2 test (Table 1). The results of the test show a significant departure from expectations of random assortment between plant haplotypes and fungal species groups ($P < 0.0001$, likelihood ratio = 35.035, df_{error} = 64). Five distinct haplotypes were scored. Haplotypes 4 and 5 were characterized by conserved 14

and 7 bp insertions, respectively. Linked single indels and substitutions were also detected. Recombinant haplotypes were not observed. Every haplotype, except haplotype 5, was detected co-occurring with every other haplotype at least at one sampled site (data not shown). Haplotypes 2 and 5 were excluded from the statistical test because they were too rare. One DNA sequence per haplotype was submitted to GenBank.

Table 1 Contingency table for *Pterospora andromedea* plastid *trn* L–F haplotypes and fungal symbiont identity (*Rhizopogon salebrosus* or *R. arctostaphyli*). There is a significant association between plastid haplotype and mycorrhizal fungal associate ($P < 0.0001$). Every haplotype, except haplotype 5, was detected co-occurring with every other haplotype at least at one sampling site (data not shown)

	Pterospora andromedea haplotypes				
Fungal symbiont	Haplotype 1	Haplotype 2	Haplotype 3	Haplotype 4	Haplotype 5
Rhizopogon salebrosus	4	3	16	10	3
Rhizopogon arctostaphyli	30	0	6	1	0

Discussion

Mycorrhizal specificity

Our findings indicate that each lineage of the Monotropoideae exhibits specificity for select species groups within single fungal genera (Figs 1–3). We found that three basal (*Sarcodes*, *Pterospora*, *Pleuricospora*) and one derived plant lineage (*Allotropa*) target single or very closely related species groups of fungi, and four derived sister lineages (*Monotropa hypopithys* group, *Pityopus*) target more distant fungal species groups within a single genus. This is even narrower specificity than previously shown (Bidartondo & Bruns 2001) and indicates that recent evolutionary radiations have been tightly constrained to host-shifts within fungal genera. This is impressive because Monotropoideae species are massively prolific producers of dust-like seeds (each inflorescence of *P. andromedea* produces in excess of 2×10^5 seeds < 0.5 mm in diameter; Bakshi 1959), many are wind-dispersed (Wallace 1975), and their habitats are characterized by high mycorrhizal fungal diversity (Bidartondo *et al.* 2000). All of these factors should provide opportunities for frequent radical jumps (*sensu* Roy 2001). Instead, in the Monotropoideae the genetic basis of specificity is a stronger determinant of diversification than ecological opportunity. Furthermore, adaptations for dispersal are decoupled from fine-level mycorrhizal phylogenetic breadth: sister taxa with very different adaptations have similar breadth and sister taxa with very similar adaptations differ. *Sarcodes* seeds are contained in a sticky mass and *Pterospora* seeds are wind-dispersed, but both taxa have a similar phylogenetic breadth of associated *Rhizopogon* species (Fig. 2). *Allotropa* and *Monotropa hypopithys* are wind-dispersed and *Pityopus* seeds are contained in a sticky mass, but *Allotropa* uses one *Tricholoma* species, whereas *M. hypopithys* and *Pityopus* use several (Fig. 3). Remarkably, no fungal lineage is shared by sympatric plant lineages, even if the plants are sister taxa and they target the same fungal genus (*Pityopus*, *M. hypopithys* group), or even if the plants are sister taxa and they target the same section of one fungal genus (*Sarcodes* and *Pterospora*).

The *M. hypopithys* group has radiated extensively through the northern hemisphere, shifting among *Tricholoma* lineages

(Fig. 4). Some of these *Tricholoma* lineages are, in turn, specialized to different lineages of photobionts (Fig. 3). These patterns correspond to co-evolutionary tracking; i.e. shifts mediated by close phylogenetic relationship (Janz & Nylin 1998) rather than geographical availability (Roy 2001). The *M. hypopithys* group is circumboreal; it has the widest geographical distribution of any other in the Monotropoideae. The 'Eurasian' nrITS lineage (sampled in the UK, Finland, Sweden and Japan) specializes on two fungal clades in section *Atrosquamosa*, one associated with *Salix* spp. (*Tricholoma cingulatum*) and the other with *Pinus* spp. (*T. terreum*). The 'Swedish' nrITS lineage specializes on three fungal clades (*T. columbetta*, *T. saponaceum*, *T. portentosum*) in sections *Equestria/Rigida*, two associated with *Fagus sylvatica* and one associated with both *F. sylvatica* and *Picea abies* (Fig. 3). The radiation of *M. hypopithys* has occurred superimposed on this complex web of ectomycorrhizal specificities (Fig. 4). Although the shifts are constrained to occur within one genus they can occur between distantly related clades (e.g. *Pityopus*, *M. hypopithys* Eurasian clade). A two-step mechanism for this pattern can be hypothesized from what we know about *Sarcodes* and *Pterospora*. In these plants the first step in establishing the mycorrhizal interaction is taken by the seed, which germinates in response to a chemical signal from its fungal associate (*Rhizopogon* section *Amylopogon* species). This signal is specific; other fungal genera do not elicit seed germination. However, seeds germinate in response to *Rhizopogon* section *Amylopogon* species that are not found associated with mature plants (Bruns & Read 2000). This suggests that the first constraint on jumps may be host chemistry, which is probably shared among related hosts. The second constraint must involve physiological interactions between the germinating seed and the potential fungal host. Positive interactions between the plant and the fungus lead to a stable interaction. Negative interactions, in which the fungus recognizes the plant as a parasite, lead to rejection. The former fails if the genetic match is not adequate. The latter, essentially a co-evolutionary arms race (Thompson & Burdon 1992), fails if the fungus has been selected to recognize the plant. Either of these mechanisms might lead to shifts among related host taxa that were initially recognized by a positive germination cue. However, the arms

race model provides a mechanism for partitioning of fungal associates between Monotropoideae species when all partners occur in sympatry, as there would be strong selection for recognition. Nonetheless, the selective pressure to resist an exploiter is likely lower than that against a costly pathogen (Levin & Lenski 1985; Bronstein 2001). The arms race model also provides selection for races of the plants that are specialized on particular hosts, as may be the case in *Pterospora*. It predicts that host shifts might be more likely when seeds encounter naïve fungal populations, e.g. after rare dispersal events.

The Monotropoideae as a whole, and the *M. hypopithys* complex in particular, show that obligate exploiters of obligate mutualisms are not necessarily species-poor lineages, as documented in seed-eating nonpollinator (i.e. cheating) fig wasps and yucca moths; there is one described obligate cheater species in the wasps that pollinates 750–800 species of figs (Compton *et al.* 1991), and two narrow lineages of cheaters among the over 15 species of moths that pollinate yuccas (Pellmyr & Leebens-Mack 2000). To elucidate how common adaptive radiations of obligate mycorrhizal exploiters have been, it is necessary to examine the other nine vascular plant families in which nonphotosynthetic mycorrhizal lineages have evolved (Leake 1994) and also determine: (i) whether the plants target mycorrhizal fungi associated with green plants; (ii) whether they are derived from the breakdown of mutualism; (iii) what are the evolutionary relationships of the plants; and (iv) *idem* for the fungi. In return, these mycorrhizal systems can contribute to developing a general theory for the evolution of parasites amid mutualists. For instance, the obligate need for the coexistence of multiple species has been emphasized as a critical barrier to the stability of cheating (Compton *et al.* 1991; Pellmyr & Leebens-Mack 2000), but this barrier may be lowered for mycorrhizal cheaters because the mycorrhizal mutualism is diffuse (i.e. single fungi interact simultaneously with several green plants and single green plants interact simultaneously with several fungi), which leads to highly diverse mycorrhizal communities even at local spatial scales (e.g. 130–350 species/hectare; Bruns 1995).

The observation that mycorrhizal epiparasitic plants in the Ericaceae (Cullings *et al.* 1996; Kretzer *et al.* 2000; Bidartondo & Bruns 2001; this study) and Orchidaceae (Taylor & Bruns 1997, 1999) display high levels of mycorrhizal specificity provides an exception to the concept that mycorrhizal symbioses rest upon generality and redundancy (Perry *et al.* 1992; Perry 1998). In this study, *Sarcodes* and *Pterospora* did not associate with the vast majority of *Rhizopogon* species, *Pleuricospora* associated with only one *Gautieria* species group, and large clades of *Tricholoma* section *Albobrunnea* did not harbour any Monotropoideae associates. Indeed, numerous fungal species that are not targeted are often observed at the same locations and at the same time as targeted species (Bidartondo *et al.* 2000). Mycorrhizal specificity is notable on several grounds: (i) regardless of how much functional redundancy there may be within mycorrhizal communities, from an epiparasitic plant's perspective, mycorrhizal fungi are unique, which is striking because mycorrhizal fungal communities are impressively diverse; (ii) there are no known morphological or ecological traits shared by the fungi associated with the Monotropoideae, other than their obligate ectomycorrhizal habit; and (iii) epiparasites are one of the few known systems involving extreme specialization toward fungi, which have been considered to have exceptionally few specialized parasites (Jaenike 1978; Lacy 1984; Hanski 1989).

In addition, the *Pterospora andromedea* results show that additional specificity may exist at the plant genotype level. A similar observation comes from the nonphotosynthetic orchid *Corallorhiza maculata* in which individual plant genotypes only form mycorrhizae with narrow clades of the ectomycorrhizal basidiomycete family Russulaceae and their specificity is maintained regardless of co-occurrence with other genotypes (D.L. Taylor, personal communication). The latter suggests that crossing is limited. This raises the question of what factors select for the evolution and maintenance of mycorrhizal specificity in lineages of epiparasites, as it is difficult to envision how mycorrhizal interactions could lead directly to genetic isolation. Note that no nuclear sequence variation was detected among plants with different plastid *trn* L–F haplotypes in the nrITS from six *P. andromedea* plants (Bidartondo & Bruns 2001; Bidartondo, unpublished data), or in an intron-containing 444 bp region of the nuclear Calmodulin gene from 16 *P. andromedea* plants (Bidartondo, unpublished data).

The need for obligate manipulation of the host may select for extreme specificity in parasites and host manipulation may be explained by the need of the parasite to complete development on one host individual (Thompson 1994). There are two sources of evidence indicating that mycorrhizal exploiters are highly manipulative of their symbionts. First, the cell–to–cell interactions in the mycorrhizal roots of the Monotropoideae are tightly orchestrated and involve the development of unique fungal 'transfer' structures not observed in photobiont roots (Lutz & Sjolund 1973; Duddridge & Read 1982; Robertson & Robertson 1982). Second, *Sarcodes sanguinea* appears to locally stimulate both the mycorrhizal fungus and the photobiont roots (Bidartondo *et al.* 2000). Unlike other parasites, mycorrhizal exploiters do not colonize their symbionts or transmit vertically to them, instead mycorrhizal exploiters are actively colonized by the hyphae of their associated fungi. In addition, the evolution and maintenance of the biochemical mechanisms that allow mycorrhizal exploiters to actively sequester fungal carbon (whose direction of flow is reversed relative to green plant mycorrhizae) at the cellular level should also provide a source of selective pressures

toward specialization. If these mechanisms are homologous to those leading to cheating among green plants connected to a common mycorrhizal network (Simard *et al.* 1997), then nonphotosynthetic mycorrhizal exploiters are the plant parasitic extreme of the mycorrhizal symbiotic continuum.

Fungal phylogenies

Even though the objective of this study was to examine patterns of mycorrhizal specificity in the monotropoid symbiosis, new phylogenetic results emerged from our analyses of sequence data from basidiocarps of taxa related to the mycorrhizal symbionts of the Monotropoideae. The *Rhizopogon* section *Amylopogon* phylogram with type specimens from every species in the section indicates that: (i) Smith & Zeller's (1966) stirpes are polyphyletic; (ii) paratypes are often different to holotypes of the same species, so that para- and holotype specimens of those species are polyphyletic (as found for other species in *Rhizopogon* section *Villosuli*, A. Kretzer personal communication); (iii) there is no ITS variation within several sets of species, which may reflect lack of resolution in the ITS or description of developmental stages of a single species as different species (as inferred in the *R. villosulus* species group by Martin *et al.* 1998); and (iv) *R. rudus* does not belong within section *Amylopogon* but in section *Villosuli* (data not shown). Assuming that different ITS sequences represent different species (there is no evidence of paralogous or xenologous copies in *Rhizopogon*), we conclude that conflicts are due to the reduced morphology of *Rhizopogon* coupled to its high diversity in western North America (over 100 described species); these two factors led Smith, Zeller and subsequent taxonomists to admittedly have difficulties in distinguishing many *Rhizopogon* morphospecies. However, a single locus (e.g. ITS) may not distinguish closely related species (Avise & Wollenberg 1997; Maddison 1997; O'Donnell *et al.* 1998); therefore, some of the species groups we detected could be composed of more than one evolutionary lineage.

Similar taxonomic problems were encountered in *Gautieria*, another diverse false-truffle genus exhibiting reduced morphology. Many of the conflicts in the *Gautieria* phylogram probably stem from the inappropriate usage of specific epithets from one continent in another. For instance, *G. chilensis* from Mexico are polyphyletic and not closely related to *G. chilensis* from Chile. A small-spored clade (*G. monticola* species group) and a large-spored clade are strongly supported as reciprocally monophyletic. *G. monticola* has the smallest spores in the genus (Zeller & Dodge 1918; Stewart 1974; J. States, personal communication), but this character is not reflected in existing sectional divisions.

Various *Tricholoma* clades comprising several species groups were strongly supported and many of these are largely congruent with morphologically defined sections: *Tricholoma* and *Terrea* (Noordeloos & Christensen 1999), *Genuina* (Shanks 1997), *Equestria* and *Atrosquamosa* (Riva 1988). Nonetheless, sectional monophyly cannot be systematically evaluated here as many described *Tricholoma* species remain unsampled in this diverse genus (≈ 70 described species in Europe alone).

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