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RANGEWIDE ANALYSIS OF FUNGAL ASSOCIATIONS IN THE FULLY MYCOHETEROTROPHIC *CORALLORHIZA STRIATA* COMPLEX (ORCHIDACEAE) REVEALS EXTREME SPECIFICITY ON ECTOMYCORRHIZAL *TOMENTELLA* (THELEPHORACEAE) ACROSS NORTH AMERICA[1]

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Fully mycoheterotrophic plants offer a fascinating system for studying phylogenetic associations and dynamics of symbiotic specificity between hosts and parasites. These plants frequently parasitize mutualistic mycorrhizal symbioses between fungi and trees. *Corallorhiza striata* is a fully mycoheterotrophic, North American orchid distributed from Mexico to Canada, but the full extent of its fungal associations and specificity is unknown. Plastid DNA (orchids) and ITS (fungi) were sequenced for 107 individuals from 42 populations across North America to identify *C. striata* mycobionts and test hypotheses on fungal host specificity. Four largely allopatric orchid plastid clades were recovered, and all fungal sequences were most similar to ectomycorrhizal *Tomentella* (Thelephoraceae), nearly all to *T. fuscocinerea*. Orchid–fungal gene trees were incongruent but nonindependent; orchid clades associated with divergent sets of fungi, with a clade of Californian orchids subspecialized toward a narrow *Tomentella fuscocinerea* clade. Both geography and orchid clades were important determinants of fungal association, following a geographic mosaic model of specificity on *Tomentella* fungi. These findings corroborate patterns described in other fully mycoheterotrophic orchids and monotropes, represent one of the most extensive plant–fungal genetic investigations of fully mycoheterotrophic plants, and have conservation implications for the >400 plant species engaging in this trophic strategy worldwide.

Key words: cophylogeny; *Corallorhiza striata;* geographic mosaic; mycoheterotrophy; mycorrhiza; Orchidaceae; parasite; phylogenetics; plastid DNA; Thelephoraceae; *Tomentella*.

Studies of interactions between organisms often rely heavily on the concepts of specificity and specialization (Thompson, 1994, 2005). Few plant groups exemplify such diversity in specialized pollinator and mycorrhizal interactions as do the orchids (van der Pijl and Dodson, 1966; Dressler, 1981, 1993; Rasmussen, 1995; Taylor et al., 2002). The family Orchidaceae contains more species than any other plant family, with 19 128 described species as of Atwood's (1986) survey, and an estimated 25 000–35 000 species (Dressler, 1981, 1993). All terrestrial orchids are fully mycoheterotrophic (= holomycotrophic) during the life stage immediately following germination, the protocorm stage (Rasmussen and Rasmussen, 2007, 2009). Most orchid species eventually develop photosynthetic tissues, reducing their reliance on fungal-derived carbon (Rasmussen, 1995) and rendering them partially mycoheterotrophic (= mixotrophic). In essence, all orchids are parasites upon fungi during the protocorm stage. However, some orchids retain this juvenile characteristic of complete fungal dependence throughout their entire life cycle and thus remain obligate parasites on fungi. Fully mycoheterotrophic plants are of great interest to plant and fungal systematists, physiologists, and conservation biologists due to their highly modified morphology, unique nutritional mode, and rare status, respectively.

Transitions from autotrophy to heterotrophy are estimated to have occurred 30–31 times (Freudenstein and Barrett, in press). Orchids comprise over half of the approximately 400 fully mycoheterotrophic plant species (Leake, 1994; Freudenstein and Barrett, in press). Recent studies have addressed the question of whether fully mycoheterotrophic orchid taxa (which depend solely upon fungi) exhibit higher specificity toward fungi relative to photosynthetic orchids, which depend upon both photoassimilated and fungal-derived carbon (reviewed in Taylor et al., 2002; McCormick et al., 2004; reviewed in Waterman

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and Bidartondo, 2008). The logic behind this question is based on the hypothesis that fully mycoheterotrophic plants, to be effective parasites, must have evolved highly specialized machinery to maximize their physiological efficiency (Taylor, 1997; Taylor et al., 2002; Rasmussen and Rasmussen, 2007, 2009). Here we adapt Thompson's (1994) definition of specificity (sensu Taylor et al., 2002): relative phylogenetic diversity of fungi compared between orchid lineages. Photosynthetic orchid taxa display a range in specificity toward fungi, from generalists to extreme specialists (Taylor et al., 2002; McCormick et al., 2004; Shefferson et al., 2005, 2007; reviewed in Dearnaley, 2007; Otero et al., 2007; Waterman and Bidartondo, 2008). They typically associate with members of a polyphyletic, anamorphic group of *Rhizoctonia*-forming fungi (Roberts, 1999); their teleomorphs correspond largely to the fungal families Ceratobasidiaceae, Sebacinaceae, and Tulasnellaceae (Rasmussen, 1995, 2002; Taylor et al., 2002). *Rhizoctonia* are mostly saprotrophic or parasitic.

Many fully mycoheterotrophic orchids associate with fungal taxa that form ectomycorrhizae (ECM) with surrounding trees and shrubs (Taylor and Bruns, 1997, 1999; McKendrick et al., 2000; Selosse et al., 2002; Taylor et al., 2002, 2004; Girlanda et al., 2006; Roy et al., 2009a,b), a situation paralleled by the fully mycoheterotrophic monotropoid Ericaceae (Bidartondo et al., 2000; Bidartondo and Bruns, 2001, 2005). They are epiparasites on pre-existing mutualistic symbioses, depending upon ECM fungi directly and upon the associated photobionts indirectly. Fully mycoheterotrophic orchids most often show high specificity toward their mycorrhizal fungi (Taylor et al., 2002, 2004; Ogura-Tsujita and Yukawa, 2008; Waterman and Bidartondo, 2008; Roy et al., 2009a; but see Roy et al., 2009b). These fungi are thought to represent more stable, high-throughput carbon/nutrient resources as opposed to the potentially ephemeral resources represented by saprotrophic *Rhizoctonia* (Taylor, 1997; Taylor et al., 2002; Bidartondo, 2005; Waterman and Bidartondo, 2008).

The leafless orchid genus *Corallorhiza* Gagnebin depends upon ECM members of the Basidiomycete families Russulaceae and Thelephoraceae (Taylor, 1997; Taylor and Bruns, 1997, 1999; McKendrick et al., 2000; Taylor et al., 2002, 2004). Association with Thelephoraceae represents the ancestral condition in the genus [*C. striata, C. trifida* Chât., *C. odontorhiza* (Willd.) Poir., and *C. wisteriana* Conrad (in part)], with two independent shifts to Russulaceae: one in the *C. maculata* (Raf.) Raf. + *C. mertensiana* Bong. clade (Taylor and Bruns, 1997, 1999), and one within *C. wisteriana* (Taylor, 1997; C. Barrett et al., unpublished data). Evidence suggests that this genus contains both partially mycoheterotrophic and fully mycoheterotrophic species; *Corallorhiza trifida, C. odontorhiza*, *C. wisteriana*, *C. maculata* var. *mexicana*, and *C. bulbosa* A. Rich. and Gal. fall into the former category. All *C. striata* and US/Canadian lineages of *C. maculata* and *C. mertensiana* fall into the latter based on the pattern of pseudogene formation in critical photosynthesis genes like *rbcL* (Barrett and Freudenstein, 2008) and *psbA* (C. Barrett et al., unpublished data), and isotopic analysis (*C. trifida*; Zimmer et al., 2008; Cameron et al., 2009).

Restriction fragment analysis of the nuclear internal transcribed spacer (ITS) by Taylor et al. (2004) demonstrated that *Corallorhiza maculata* orchid genotypes consistently associated with specific ECM fungal clades spanning 22 species in the family Russulaceae, suggesting the existence of host races within *C. maculata* on these fungi. The latter represents one of very few studies on orchid–fungal associations to include DNA data from both partners. To date, the majority of orchid–fungal studies have not taken into account the often substantial variation near or below the species level on the side of the plants (but see Ogura-Tsujita and Yukawa, 2008). Besides recent studies on the photosynthetic genus *Cypripedium* L. (Shefferson et al., 2005, 2007), no orchid–mycorrhizal studies exist that employ plant gene trees for comparison to phylogenies of fungal symbionts, especially at lower taxonomic levels. Genus and species-level orchid phylogenies are certainly useful for understanding larger scale orchid–fungal coevolutionary patterns, but high-resolution infraspecific and population-level genealogies are crucial to more clearly elucidate basic evolutionary patterns of association and to assess the dynamics of specificity across potential geographic mosaics (Thompson, 1994, 2005). Furthermore, mycorrhizal associations appear to be evolving extremely rapidly in *Corallorhiza*, making the infraspecific level of analysis particularly compelling (Taylor, 1997; Taylor et al., 2004).

Corallorhiza striata is a fully mycoheterotrophic species complex composed of three varieties (*striata*, *vreelandii*, and *involuta*) and a recently described, endangered species, *C. bentleyi* Freudenstein (Freudenstein, 1997, 1999; Barrett and Freudenstein, 2009). The *C. striata* complex is an excellent candidate for comparison of orchid–mycorrhizal associations, due to its (1) widespread geographic distribution across North America (Mexico, USA, Canada), (2) local rarity and small population sizes in often highly fragmented habitats, (3) substantial morphological variation, and (4) status as an "ecological indicator" of mature, pristine forest ecosystems throughout its range. Thus, data on mycorrhizal fungi are important for knowledge on how best to conserve these orchids and the habitats in which they occur.

Barrett and Freudenstein (2009) found extensive variation within the *C. striata* complex and identified four geographically associated clades based on plastid DNA (ptDNA) sequencing of individuals from 42 populations across North America (clades A–D). Plastid DNA clade A is composed of two small-flowered taxa, *Corallorhiza striata* var. *involuta* (Greenm.) Freudenstein (Mexico), and the endangered *C. bentleyi* (eastern USA). Clades C and D correspond to the intermediate-size flowered *C. striata* var. *vreelandii* (Rydb.) L. O. Williams (southwestern USA and Mexico) and the large-flowered C. *striata* var. *striata* (northern USA and southern Canada), respectively. Last, populations of clade B are found exclusively in California, USA; their taxonomic rank is yet to be determined.

All members of the *C. striata* complex, including *C. bentleyi*, have characteristic black rhizome coloration due to dense mycorrhizal colonization of cortical cells by highly melanized fungi (Campbell, 1970; Freudenstein, 1992; Taylor, 1997; C. Barrett et al., personal observation), yet knowledge of these fungi is rudimentary. Anatomical examination of eight *C. striata* rhizomes from California yielded mycobionts with black-brown hyphae lacking clamp connections (Taylor, 1997), that were found to be closely related to ECM members of the *Thelephora* Ehrh.-*Tomentella* Pers. clade (Thelephoraceae) based on ITS sequences. However, this geographically limited sampling leaves open the question of whether *C. striata* associates exclusively with members of the *Thelephora-Tomentella* clade across its entire range or whether it associates with more phylogenetically divergent fungal partners.

The main objectives of this study were to (1) identify fungi associated with the *C. striata* complex from mature

(i.e., flowering) individuals and to identify potential patterns of association and specificity across North America, (2) assess relative diversity of *C. striata* fungal associates among orchid ptDNA clades, (3) address whether associations based on orchid and fungal gene trees are independent (i.e., to determine whether ptDNA clades within the *C. striata* complex consistently associate with specific fungal clades), and (4) ascertain the relative importance of geographic region and orchid plastid clade in patterns of fungal association.

MATERIALS AND METHODS

Sampling of plant and fungal tissues—Orchids were collected across North America maximizing geographic, habitat, and morphological diversity ($N = 42$ populations; Appendix 1). Due to small local population sizes in *C. striata*, individual orchids were harvested such that only one individual was collected for each 20 that were observed (in compliance with USDA Forest Service guidelines). For smaller populations, tissue samples were taken by collecting single flowers from a raceme and carefully excavating small samples of rhizome tissue (rather than harvesting entire individuals); this method was used for all collections of the endangered *C. bentleyi* with the goal of minimizing the effects of collection. Material for DNA isolation was either shipped fresh from the field and frozen at −80°C or silica dried. Rhizomes were freehand sectioned and examined with a compound light miscroscope to help characterize fungi based on hyphal anatomy. At least one plant per population (including rhizome) was specified as a voucher specimen and deposited at the Ohio State University Herbarium. Vouchers of the endangered *C. bentleyi* are represented by one pressed specimen (Bath Co., Virginia) and several FAA-fixed (5% formalin-5% acetic acid-50% ethanol) flowers stored in 70% ethanol.

DNA extraction, amplification, sequencing, and BLAST searches—Prior to DNA extraction, rhizomes were scrubbed with a brush and verified to be completely free of soil or fungal hyphae under a dissecting miscroscope as in Taylor et al. (2004). For each individual orchid, DNA was extracted separately from either 0.1 g or 1.0 g of rhizome (colonized by fungi) and floral tissue according to a modification of the CTAB method of Doyle and Doyle (1987). Culturing was not undertaken due to previous attempts documenting the difficulties associated with growing ECM fungi from *Corallorhiza* orchids in the absence of host photobionts (Taylor, 1997).

The fungal nuclear ITS region was amplified using fungal-specific primer ITS1-F (Gardes and Bruns, 1993) and broad spectrum primer ITS4 (White et al., 1990), following the PCR conditions of Taylor and Bruns (1999) and Taylor et al. (2004). Previous studies demonstrated that individual *Corallorhiza* orchids typically associate with single fungal genotypes (Taylor and Bruns, 1997, 1999; Taylor et al., 2004), so PCR products amplified from total rhizome DNA (plant + fungal) were directly sequenced using the same primers as for amplification. Sequencing reactions were performed using the BigDye Terminator reaction mix and run on an ABI 3100 Genetic Analyzer (Applied Biosystems, Foster City, California, USA) in 5 μL reactions (1/4 suggested volume) otherwise following the manufacturer's protocol. PCR products and cycle sequencing reactions were cleaned using the Agencourt AmPure and CleanSeq magnetic bead protocols, respectively (Agencourt Bioscience, Beverly, Massachusetts, USA). Sequence contigs were assembled using Sequencher (GeneCodes, Ann Arbor, Michigan, USA).

Nearly half of the fungal ITS electropherograms ($N = 52$) had at least one double peak (mean number of double peaks per polymorphic sequence = 1.9, range = 1–5 peaks per polymorphic sequence [excluding accession *HeshAR MB* with $N = 11$ dimorphisms]). Two of these samples (*JH1eR MN* and *HeshAR MB*) were cloned using the Invitrogen TOPO-TA kit (Invitrogen, Carlsbad, California, USA). Inserts were amplified with vector-specific primers M13F and M13R (supplied by manufacturer) and sequenced with fungal ITS primers ITS1-F and ITS4. Fungal ITS cloning was performed merely to gain insight into the nature of polymorphism in electropherograms, not as an exhaustive means to explore fungal diversity in all orchid rhizome samples. Both cloned accessions (GenBank accessions GU224039–GU224043, GU224045–GU224047 [*HeshAR MB*]; GU224048–GU224051 [*JH1eR MN*]) displayed two similar sequences, likely representing dikaryons. Ambiguity codes were applied to dimorphic positions, since 1–5 dimorphic base calls out of ca. 700 bases in an ITS sequence did not introduce significant uncertainty for sequence identification (i.e., BLAST searches), pairwise similarity/distance comparisons, or phylogenetic analyses. GenBank accession numbers for *C. striata* fungal ITS sequences are listed in Appendix 1.

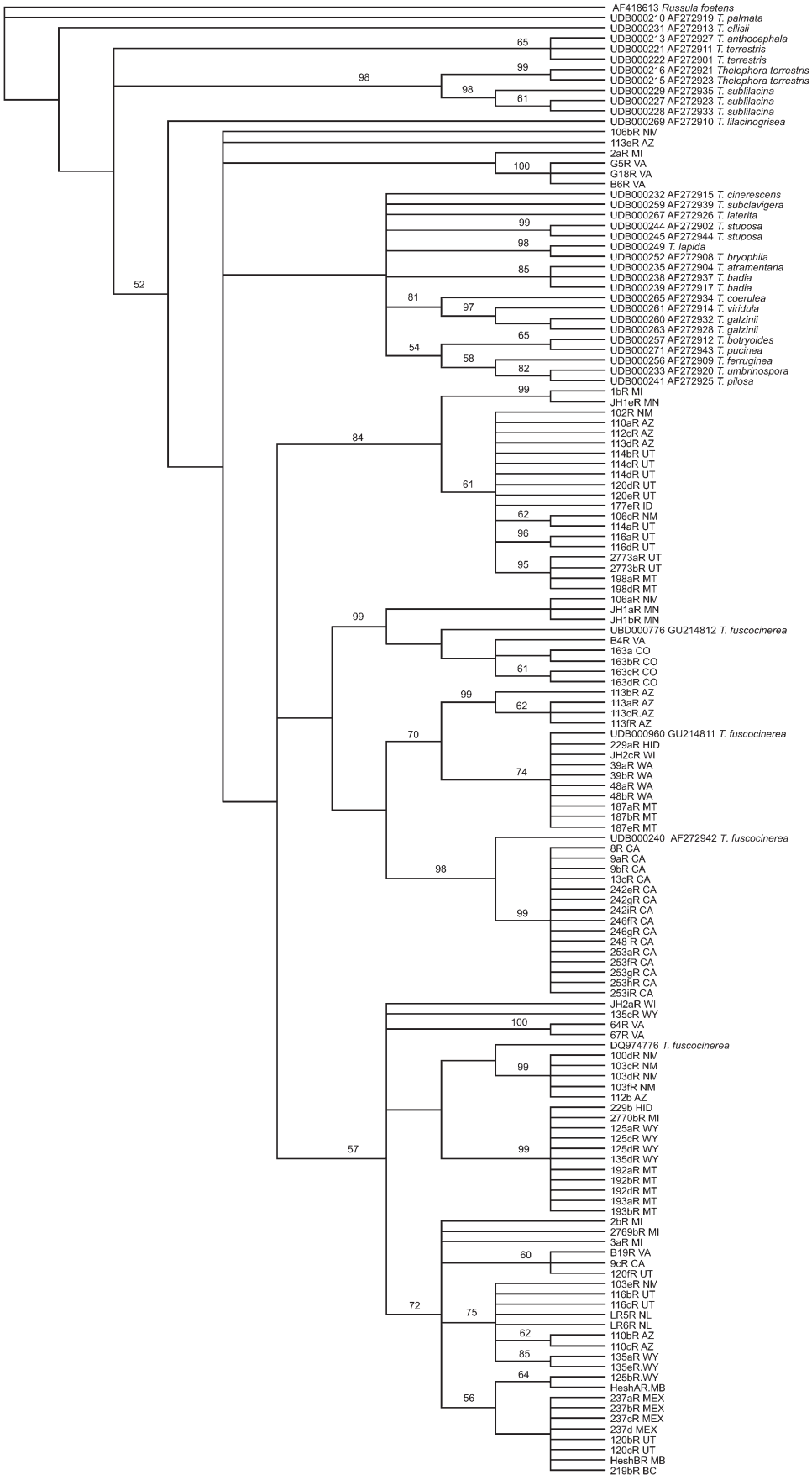
Sequences were initially subjected to BLAST searches in both GenBank (http://www.ncbi.nlm.nih.gov) and the UNITE fungal ITS sequence database (Kõljalg et al., 2005; http://unite.ut.ee). The latter includes a robust representation of the *Thelephora-Tomentella* group, derived largely from vouchered herbarium specimens identified from teleomorphs, reducing problems associated with BLAST comparisons to environmental samples and specimens of uncertain or erroneous taxonomy. The primary objective of these BLAST searches was to identify fungal taxa to which *C. striata*'s mycobionts have the closest affinities.

Fungal ITS alignment and percentage similarity analysis—For estimating the number of putative thelephoroid fungal species represented by *C. striata* mycobionts, all published fungal ITS sequences corresponding to *Tomentella* or *Thelephora* were downloaded from the International Sequence Database (INSD: GenBank, EMBL) for comparison. Additional sets of insufficiently identified sequences were obtained from Emerencia (Nilsson et al., 2005). These and DNA sequences from *C. striata* were merged in a data set composed of 3878 sequences. After excluding duplicates using the program BlastClust (Altschul et al., 1990) and short (i.e., containing only ITS1 or ITS2) or ambiguously aligned sequences, 1472 sequences remained for analysis. These were aligned with the program MAFFT v.6 (Katoh and Kuma, 2002), followed by manual corrections and removal of the flanking 18S and 28S rDNA using the program SeaView (Galtier et al., 1996).

The final data set consisted of 1472 sequences with 941 characters (Appendix S1, see Supplemental Data with the online version of this article). Clusters were generated using BlastClust with 90% sequence coverage, implementing both 97% and 97.5% similarity thresholds. To visualize the clusters, we created a neighbor-joining tree (NJ; Saitou and Nei, 1987) in the program PAUP* 4.0 (Swofford, 2002) under the SYM+I+Γ (Zharkikh, 1994) evolutionary model as suggested by the program MODELTEST 3.7 (Posada and Crandall, 1998). The goal of this analysis was not to generate a robust phylogenetic estimate for Thelephoraceae (which would not be possible based on ITS alone), but instead to gain a distance-based estimate of the number of putative fungal species (i.e., clusters) associated with *C. striata* across its geographic range.

Phylogenetic analyses—Two smaller fungal ITS matrices were analyzed to better characterize support for relationships of *C. striata* mycobionts with published Thelephoraceae sequences. All reference sequences for phylogenetic comparison were taken from the UNITE database (Kõljalg et al., 2005; http://unite.ut.ee), except for two from GenBank. Thirty-three basidiocarp-derived sequences representing 24 Thelephoraceae species were downloaded from UNITE, aligned with *C. striata* fungal sequences in the program Muscle v. 3.6 (Edgar, 2004), and manually checked in the program Mesquite v. 2.6 (Maddison and Maddison, 2009). *Russula foetens* (Pers.) Pers. (Russulaceae; GenBank accession number AF418613) was then added to the aforementioned alignment as an outgroup using the "-profile" alignment command in Muscle v.3.6. Indel characters were coded under the modified complex indel coding method (Simmons and Ochoterena, 2000; Müller, 2006) in the SeqState package (Müller, 2005), using the IndelCoder module. The resulting matrix consisted of 142 terminals and 768 characters (702 DNA + 66 indel characters) and is henceforth referred to as the UNITE matrix (Appendix S2, see online Supplemental). Next, a further reduced matrix excluding the database sequences (referred to as the striata-only matrix; Appendix S3, see online Supplemental Data) was analyzed for direct comparison with orchid ptDNA. This matrix consisted of 108 taxa, including a closely related outgroup taxon *Thelephora terrestris* (UNITE accession UDB000216, GenBank accession AF272921). Sequences were again aligned with Muscle v3.6 (Edgar 2004), and adjusted manually. Eight informative insertion/deletion characters in this matrix were coded according to the simple indel method of Simmons and Ochoterena (2000).

For the same individual orchids from which fungal ITS sequences were generated, two plastid DNA loci (*rbcL* and *rpl32-trnL* spacer) were sequenced from floral tissue. Details of amplification and sequencing are given in Barrett and Freudenstein (2008, 2009) for *rbcL* and Shaw et al. (2007) and Barrett and Freudenstein (2009) for *rpl32-trnL*. Sixty-three new *rbcL* and *rpl32-trnL* sequences were obtained in this study and used to construct a matrix (online Appendix S4) along with previously generated sequences in Barrett and Freudenstein (2009). GenBank accessions for plastid sequences are listed in Appendix 1. *Aplectrum hyemale* (Muhl.) Willd. was used for the outgroup (GenBank accessions FJ445516 [*rbcL*], FJ445599 [*rpl32-trnL*]), based on a close relationship to *Corallorhiza* (Freudenstein, 1992; Barrett and Freudenstein, 2008; Freudenstein and Senyo, 2008). Barrett and Freudenstein (2009)


AF418613 Russula foetens
UDB000210 AF272919 T. palmata
UDB000231 AF272913 T. ellisii
UDB000213 AF272927 T. anthocephala
UDB000221 AF272911 T. terrestris
UDB000222 AF272901 T. terrestris
UDB000216 AF272921 Thelephora terrestris
UDB000215 AF272923 Thelephora terrestris
UDB000229 AF272935 T. sublilacina
UDB000227 AF272923 T. sublilacina
UDB000228 AF272933 T. sublilacina
UDB000269 AF272910 T. lilacinogrisea
106bR NM
113eR AZ
2aR MI
G5R VA
G18R VA
B6R VA
UDB000232 AF272915 T. cinerescens
UDB000259 AF272939 T. subclavigera
UDB000267 AF272926 T. laterita
UDB000244 AF272902 T. stuposa
UDB000245 AF272944 T. stuposa
UDB000249 T. lapida
UDB000252 AF272908 T. bryophila
UDB000235 AF272904 T. atramentaria
UDB000238 AF272937 T. badia
UDB000239 AF272917 T. badia
UDB000265 AF272934 T. coerulea
UDB000261 AF272914 T. viridula
UDB000260 AF272932 T. galzinii
UDB000263 AF272928 T. galzinii
UDB000257 AF272912 T. botryoides
UDB000271 AF272943 T. pucinea
UDB000256 AF272909 T. ferruginea
UDB000233 AF272920 T. umbrinospora
UDB000241 AF272925 T. pilosa
1bR MI
JH1eR MN
102R NM
110aR AZ
112cR AZ
113dR AZ
114bR UT
114cR UT
114dR UT
120dR UT
120eR UT
177eR ID
106cR NM
114aR UT
116aR UT
116dR UT
2773aR UT
2773bR UT
198aR MT
198dR MT
106aR NM
JH1aR MN
JH1bR MN
UBD000776 GU214812 T. fuscocinerea
B4R VA
163a CO
163bR CO
163cR CO
163dR CO
113bR AZ
113aR AZ
113cR.AZ
113fR AZ
UDB000960 GU214811 T. fuscocinerea
229aR HID
JH2cR WI
39aR WA
39bR WA
48aR WA
48bR WA
187aR MT
187bR MT
187eR MT
UDB000240 AF272942 T. fuscocinerea
8R CA
9aR CA
9bR CA
13cR CA
242eR CA
242gR CA
242iR CA
246fR CA
246gR CA
248 R CA
253aR CA
253fR CA
253gR CA
253hR CA
253iR CA
JH2aR WI
135cR WY
64R VA
67R VA
DQ974776 T. fuscocinerea
100dR NM
103cR NM
103dR NM
103fR NM
112b AZ
229b HID
2770bR MI
125aR WY
125cR WY
125dR WY
135dR WY
192aR MT
192bR MT
192dR MT
193aR MT
193bR MT
2bR MI
2769bR MI
3aR MI
B19R VA
9cR CA
120fR UT
103eR NM
116bR UT
116cR UT
LR5R NL
LR6R NL
110bR AZ
110cR AZ
135aR WY
135eR.WY
125bR.WY
HeshAR.MB
237aR MEX
237bR MEX
237cR MEX
237d MEX
120bR UT
120cR UT
HeshBR MB
219bR BC

found that gene trees for each plastid locus were free of conflict, differing only in resolution, so plastid data were analyzed simultaneously in addition to 12 informative plastid indel characters (simple indel method; Simmons and Ochoterena, 2000).

Maximum parsimony (MP) searches were completed in the program TNT (Goloboff et al., 2008) for the plastid, UNITE, and striata-only fungal ITS matrices using 100 random addition sequences (RAS), 100 replicates (per RAS) of the parsimony ratchet (Nixon, 1999) upweighting 10% of the characters, and random sectorial searches (RSS; Goloboff, 1999) with a sector size of 35 taxa. Trees saved from these searches were subjected to tree bisection-reconnection (TBR) branch swapping, saving a total of 10 000 trees. Five thousand TNT jackknife pseudoreplicates (JK; Farris et al., 1996) were then implemented in TNT using the same heuristic search parameters as above and a 37% deletion probability. An incongruence length difference (ILD) test (Farris et al., 1994) was implemented in PAUP* v.4b10 (Swofford, 2003) to test congruence of the orchid plastid and fungal ITS data sets. Heuristic searches were run with 1000 replicates, each using 100 RAS, TBR, and holding 100 trees.

MODELTEST (Posada and Crandall, 1998) was used for fungal ITS (UNITE and striata-only matrices) and plastid model determination (excluding indel characters) implementing the Akaike information criterion (AIC; Akaike, 1974). This method indicated GTR+Γ (Tavaré, 1986) as the best fit model for the UNITE matrix, TrN+I+Γ (Tamura and Nei, 1993) for the striata-only matrix, and GTR+I for the plastid matrix. Maximum likelihood (ML) searches were conducted in RaxML (Stamatakis, 2006b), using the GTRMIX model, using the default number of rate categories (C) set to 25. GTRMIX uses the GTRCAT approximation to assign per-site rate parameters into a predefined number of categories (here, C = 25), then uses the GTRGAMMA model to evaluate the tree likelihood (Stamatakis, 2006a). Bootstrap searches (2000 replicates) were run using the GTRCAT approximation (Stamatakis, 2006a). Since RaxML does not use TrN models, additional analyses (UNITE and striata-only matrices) were run under this model in PhyML (Guindon and Gascuel, 2003; Guindon et al., 2005; http://atgc.lirmm.fr/phyml) using subtree pruning-regrafting branch swapping (SPR), starting from NJ trees.

Statistical analyses—The null hypothesis of independence between orchid and fungal clades was tested using r × c contingency tables, including: (1) χ^2 tests, (2) log-likelihood ratio G-tests, and (3) Fisher's exact tests with Monte Carlo simulation. The latter two are less sensitive to lower cell counts (Agresti, 1990; Zar, 1999). All contingency table tests were calculated in the R statistical package (R Development Core Team, 2006; script for G-test can be found at website http://www.psych.ualberta.ca/~phurd/cruft/g.test.r).

Choosing clades from gene trees for the purpose of implementing statistical tests of association represents a somewhat arbitrary means of data reduction from nested, hierarchical data to nonnested, categorical data. Furthermore, "clades as categories" may represent a form of nonindependent data, due to the fact that these categories are hierarchically related (unless they represent a hard polytomy). Taking this into consideration, we tested multiple scenarios of clade designation, with the objective of sequentially tracing the hierarchical structure of both orchid plastid and fungal ITS gene trees. In this way, it was possible to test multiple levels of association between both orchid and fungal clades. In addition, all plastid haplotypes for *C. striata* vars. *vreelandii* and *striata* were tested against fungal clades.

To test the null hypothesis of independent phylogenetic patterns between orchid plastid and fungal ITS gene tree topologies, statistical analyses were implemented in the program ParaFit (Legendre et al., 2002), spawned in the graphical interface CopyCat (Meier-Kolthoff et al., 2007). ParaFit tests statistical significance of cophylogenetic structure on both a "global" and individual basis. Three input matrices are required: one distance matrix for each interacting party and a matrix mapping their specific associations. Distance matrices can either be calculated beforehand or constructed from input trees. These distances are then transformed into matrices of principal coordinates used to calculate a global trace statistic, the significance of which is assessed by comparison to a distribution of randomly permuted associations (Legendre et al., 2002). For testing the significance of individual associations, ParaFit essentially removes each association and recomputes the matrix, quantifying the contribution of that association to the global tree statistic.

ParaFit has several advantages over other methods of cophylogenetic hypothesis testing at the infraspecific level; most importantly, it allows one to test the null hypothesis of independent phylogenies instead of a null hypothesis of strict codivergence/cospeciation (various methods are reviewed in Stevens, 2004). It is also (1) not restricted to species or higher-level cophylogenetic hypotheses, (2) able to handle polytomies, and (3) not based on component analysis (Page, 1993), reconciled trees analysis (Page, 1994), or assignment of costs to presumed occurrences of cospeciation, host switching, extinction, and duplication (Ronquist,1995, 1999; Charleston, 1998). These features are particularly relevant for infraspecific questions because in these cases one cannot assume that speciation is an appropriate model for all observed branching patterns.

Multiple ParaFit analyses were run. First, ML trees including branch length information were used as input. The second and third analyses were based on MP trees: two randomly chosen most-parsimonious trees were converted to patristic distance matrices, including branch lengths. MP strict consensus trees were not used for ParaFit analysis because they require that all branch lengths be assigned values of one in the input trees, thus discarding some useful information on clade divergence (Legendre et al., 2002). Associations were randomly permuted 9999 times, and significance was assessed at the $P = 0.05$ significance level.

The proportion of fungal ITS variation within and among orchid groupings was investigated using an analysis of molecular variance (AMOVA; Excoffier et al., 1992). The values π (nucleotide diversity), overall Φ_{ST} (fixation index), population-specific Φ_{ST} (Weir and Hill, 2002; Excoffier et al., 2005), and AMOVA variance components were estimated using the program Arlequin 3.1 (Excoffier et al., 2005), based on Tamura–Nei distances (as chosen by the AIC in MODELTEST). Nucleotide diversity (π) is defined as the average number of differences per site between two randomly chosen sequences (Nei and Li, 1979; Tajima, 1983); Φ_{ST} is an extension of F_{ST}, the proportion of variation due to subpopulation structure, taking into account relatedness of sequences (Excoffier et al., 1992). Population-specific Φ_{ST} gives an estimate of the relative contribution of that population to the overall genetic structure (Weir and Hill, 2002; Excoffier et al., 2005). Differences in π for fungal ITS sequences between orchid groupings would suggest differences in specificity, while differences in Φ_{ST} between orchid groupings would suggest associations with divergent sets of fungi (sensu Taylor et al., 2004).

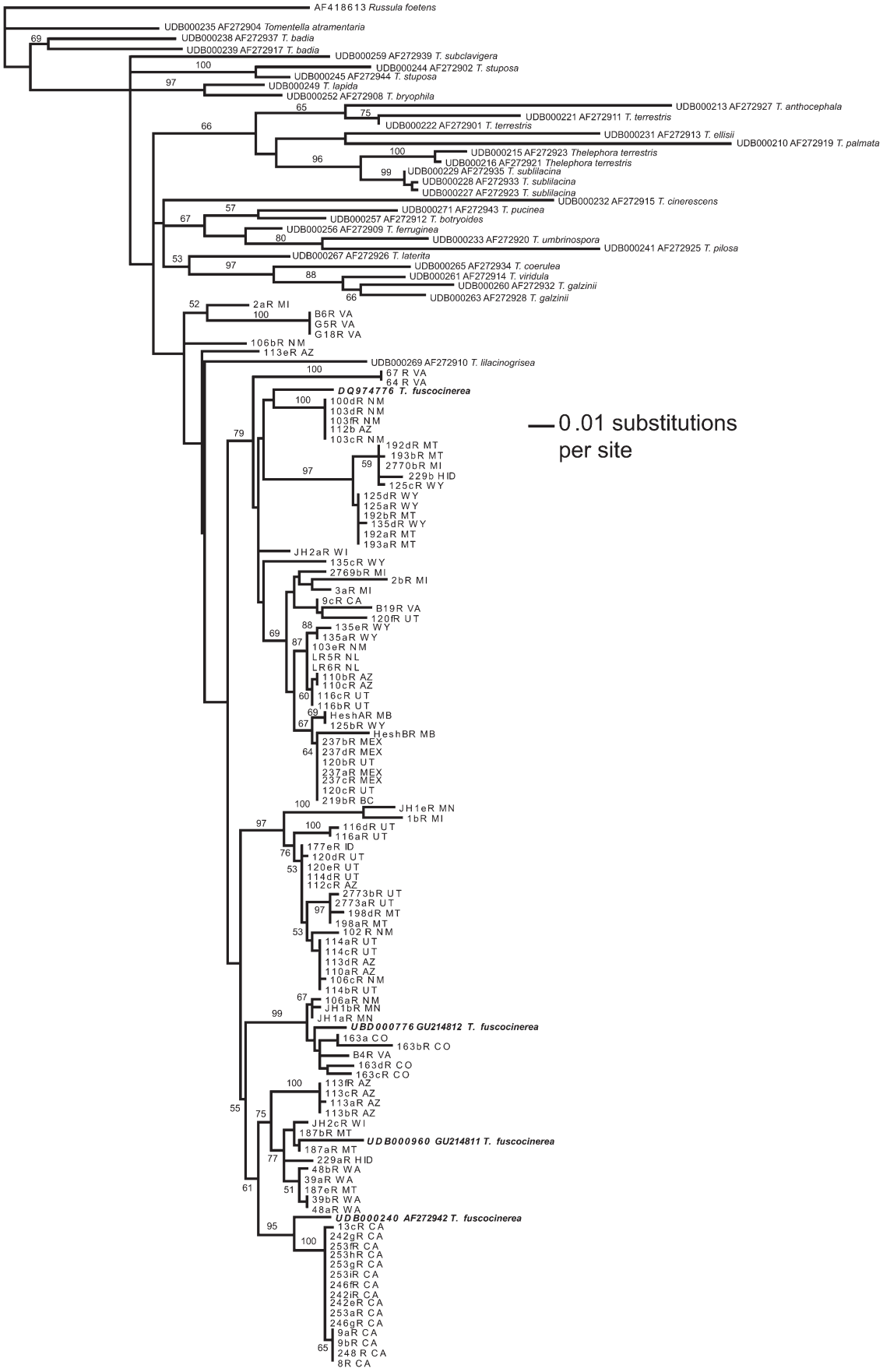
Multinomial logistic regression was used with the objective of testing potential predictive variables of fungal association within the *C. striata* complex. Plastid DNA clade, biogeographic region, and elevation category were used as independent variables (IVs) in the model, with fungal ITS clade as the dependent variable (DV). All four variables were nominal-categorical, with elevation divided into three categories: 0–1000 m, 1000–2000 m, and 2000–3000 m. Geography was represented by five regions of biogeographic relevance in North America: northern Rocky Mountains, southern Rocky Mountains, Mexican Sierras, California, and eastern North America (including the Great Lakes and Canadian Shield). 42° North latitude was used as a boundary between the northern and southern Rocky Mountain regions. Latitude and longitude were included as continuous covariates in addition to the four categorical variables. Analyses were run in the program SPSS v.17 (SPSS, Chicago, Illinois, USA) using simultaneous regression, analyzing main effects. The log-likelihood ratio χ^2 statistic was used to assess the fit of the final model to the data relative to an intercept-only (i.e., null) model. χ^2 distributed log-likelihood ratio statistics were also used to interpret the significance of contributions for each variable to the final model. Significant contribution to the model was assessed for each variable at the $P = 0.05$ significance level.

RESULTS

Morphological identification—All orchid rhizomes examined were densely colonized by fungi with highly melanized (i.e., black-brown) hyphae, showing no evidence of clamp connections, in accordance with the observations of Taylor (1997). Taylor (1997) described *C. striata* mycobionts as closely resembling *Tomentellastrum montanensis* (M. J. Larsen) M. J.

←

Fig. 1. Strict consensus of 10 000 most parsimonious trees (length = 1292 steps) for the UNITE fungal ITS data matrix (107 *Tomentella* sequences from *C. striata* + 35 reference sequences). Support values from 10 000 jackknife replicates are displayed above branches. Accessions beginning with "UDB" are from the UNITE database (left), all others are from GenBank (right). "R" in each *C. striata* accession name indicates that the fungal ITS sequence was obtained from rhizome DNA.


AF418613 Russula foetens
UDB000235 AF272904 Tomentella atramentaria
UDB000238 AF272937 T. badia
UDB000239 AF272917 T. badia
UDB000259 AF272939 T. subclavigera
UDB000244 AF272902 T. stuposa
UDB000245 AF272944 T. stuposa
UDB000249 T. lapida
UDB000252 AF272908 T. bryophila
UDB000213 AF272927 T. anthocephala
UDB000221 AF272911 T. terrestris
UDB000222 AF272901 T. terrestris
UDB000231 AF272913 T. ellisii
UDB000210 AF272919 T. palmata
UDB000215 AF272923 Thelephora terrestris
UDB000216 AF272921 Thelephora terrestris
UDB000229 AF272935 T. sublilacina
UDB000228 AF272933 T. sublilacina
UDB000227 AF272923 T. sublilacina
UDB000232 AF272915 T. cinerescens
UDB000271 AF272943 T. pucinea
UDB000257 AF272912 T. botryoides
UDB000256 AF272909 T. ferruginea
UDB000233 AF272920 T. umbrinospora
UDB000241 AF272925 T. pilosa
UDB000267 AF272926 T. laterita
UDB000265 AF272934 T. coerulea
UDB000261 AF272914 T. viridula
UDB000260 AF272932 T. galzinii
UDB000263 AF272928 T. galzinii
2aR MI
B6R VA
G5R VA
G18R VA
106bR NM
113eR AZ
UDB000269 AF272910 T. lilacinogrisea
67 R VA
64 R VA
DQ974776 T. fuscocinerea
100dR NM
103dR NM
103fR NM
112b AZ
103cR NM
192dR MT
193bR MT
2770bR MI
229b HID
125cR WY
125dR WY
125aR WY
192bR MT
135dR WY
192aR MT
193aR MT
JH2aR WI
135cR WY
2769bR MI
2bR MI
3aR MI
9cR CA
B19R VA
120fR UT
135eR WY
135aR WY
103eR NM
LR5R NL
LR6R NL
110bR AZ
110cR AZ
116cR UT
116bR UT
HeshAR MB
125bR WY
HeshBR MB
237bR MEX
237dR MEX
120bR UT
237aR MEX
237cR MEX
120cR UT
219bR BC
JH1eR MN
1bR MI
116dR UT
116aR UT
177eR ID
120dR UT
120eR UT
114dR UT
112cR AZ
2773bR UT
2773aR UT
198dR MT
198aR MT
102 R NM
114aR UT
114cR UT
113dR AZ
110aR AZ
106cR NM
114bR UT
106aR NM
JH1bR MN
JH1aR MN
UBD000776 GU214812 T. fuscocinerea
163a CO
163bR CO
B4R VA
163dR CO
163cR CO
113fR AZ
113cR AZ
113aR AZ
113bR AZ
JH2cR WI
187bR MT
UDB000960 GU214811 T. fuscocinerea
187aR MT
229aR HID
48bR WA
39aR WA
187eR MT
39bR WA
48aR WA
UDB000240 AF272942 T. fuscocinerea
13cR CA
242gR CA
253fR CA
253hR CA
253gR CA
253iR CA
246fR CA
242iR CA
242eR CA
253aR CA
246gR CA
9aR CA
9bR CA
248 R CA
8R CA
0.01 substitutions per site

Larsen, which is now a synonym of *Tomentella fuscocinerea* (Pers.) Donk.

Molecular identification—Initial GenBank BLAST searches indicated that all 107 *C. striata* fungal ITS sequences had 94–100% similarity to uncultured ectomycorrhizae listed as genus *Tomentella* (GenBank accession numbers FJ554055, EF218826, EU668208, AY702813, EF218835, AY702813, AY310863, or FJ210771). BLAST searches in UNITE indicated highest similarity in all but five cases to one of three *T. fuscocinerea* sequences (UNITE accession numbers UBD000240, UBD000776, or UBD001658). Three *C. bentleyi* accessions (*G5R VA, G18R VA*, and *B6R VA*) showed highest similarity to *Tomentella lilacinogrisea* (UDB003317), while *C. striata vreelandii* accessions *113eR AZ* and *106bR NM* most closely matched *T. cinerascens* (UDB003309) and *T. cinereoumbrina* (UDB003298), respectively. However, these accessions also showed very high similarity to *T. fuscocinerea*.

Phylogenetic analyses—Neighbor joining analysis under the SYM+I+Γ model with 3% and 2.5% ITS divergence criteria (Appendix S5, see online Supplemental Data) suggested that *C. striata* mycobionts comprise circa nine and 12 putative *Tomentella* species, respectively. Despite this finding, the majority of *C. striata* sequences grouped only with accessions of *T. fuscocinerea*.

The UNITE fungal ITS matrix—Parsimony and likelihood analyses of the smaller UNITE data matrix (Figs. 1, 2) yielded highly similar topologies to those of Kõljalg et al. (2000) and furthermore recovered the majority of the *C. striata* sequences as a single clade (including all *T. fuscocinerea* sequences from UNITE). However, this clade was not supported by JK or BS (Figs. 1, 2). Overall, JK and BS support values for *Tomentella* ITS clades were highest toward the tips, but both trees generally lacked support toward their bases (Figs. 1, 2). Nonetheless, the majority of *C. striata* fungal ITS sequences sampled in this study (101 of 107) showed supported relationships with a single ECM species, *Tomentella fuscocinerea*, and the remaining six were not exclusively resolved as sister to any other *Tomentella* species.

The striata-only fungal ITS matrix—The MP analysis of the striata-only fungal ITS data matrix in TNT returned 10 000 equally parsimonious trees of 386 steps (online Appendix S6). The ML analysis in RaxML under the GTRGAMMA model returned a topology with a score of −2929.16 (Fig. 3). The ML analysis under the TrN+I+Γ model in PhyML gave an identical topology (data not shown), indicating that for fungal ITS, the tree topology was not affected by selection of a more parameterized model (GTR). Thus, only the results from RaxML under GTR will be discussed hereafter for ML. Fungal ITS gene trees under MP and ML showed no major topological differences, but ML bootstrap scores were generally higher than MP jackknife scores (Fig. 3). Two deep clades were recovered (Fig. 3, clades 1a and 1b), but only clade 1b was supported in both ML and MP analyses. A number of moderate-high support clades fell within each of these two clades (designated 2a–f/3a–g, see Fig. 3). Fifteen of 16 sequences sampled from seven populations over a ca. 800 km transect through California formed a highly supported clade (3b: BS = 100, JK = 99).

The plastid matrix—Four most parsimonious trees of length 246 were recovered for plastid DNA (online Appendix S6), and the highest likelihood tree topology had a score of −5191.11 under the GTRMIX model in RaxML (Fig. 3). As with fungal ITS, there were no major topological differences between the MP and ML trees, except differences between ML bootstrap and MP jackknife values. The finding of four main plastid clades was identical to that from Barrett and Freudenstein (2009): clade A included *C. striata* var. *involuta* (Mexico) and *C. bentleyi* (eastern USA), clade B was exclusively composed of Californian sequences, clade C contained *C. striata* var. *vreelandii* (southwestern USA, Mexico, and Newfoundland), and clade D contained *C. striata* var. *striata* (northern USA and Canada).

Comparison of orchid and fungal gene trees—It is immediately obvious upon inspection of Fig. 3 that the *Tomentella* ITS and orchid plastid gene trees are not identical. An ILD test confirmed that orchid plastid and *Tomentella* ITS data sets were significantly incongruent ($P = 0.010$). However, the observed associations were far from being completely random. In particular, 15 of 16 Californian orchid accessions (all but accession *9c CA*) associated exclusively with a Californian clade of fungi. Also, three *C. bentleyi* accessions (part of clade A, which is sister to the remaining accessions of *C. striata*) associated with fungi at the base of the ITS tree (Fig. 3).

Analyses in ParaFit randomly permuting the orchid plastid tree on the *Tomentella* ITS tree indicated that the global null hypothesis of completely random associations between orchid and fungal gene trees was rejected, for both ML and MP input trees ($P = 0.002$ for each). For the sake of comprehensiveness, an analysis was also run randomly permuting the *Tomentella* ITS tree on the orchid plastid tree, which returned a globally similar result ($P = 0.001$ for both ML and MP trees). Tests of individual associations under the former permutational scenario showed that 34 (using ML trees) and 35 (using two different MP trees) of a possible 107 associations were significant, equating to 31.7% and 32.7% of all associations, respectively (Fig. 3; Appendix 2). Of these significant associations (i.e., significant for both MP and ML input trees), five occurred within clade A (*C. bentleyi* + *C. striata* var. *involuta*), 15 in clade B (Californian accessions), seven in clade C (*C. striata* var. *vreelandii*), and three in clade D (*C. striata* var. *striata*) (Fig. 3; Appendix 2).

Since 15 of 16 associations among Californian accessions were significant, the possibility exists that they alone were driving the globally significant results. To address this issue, we repeated the ParaFit analyses, excluding all Californian accessions, which returned a significant result ($P = 0.006$ for ML and MP trees). Next, members of *C. bentleyi* and *C. striata* var.

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Fig. 2. Highest-likelihood tree topology for the UNITE fungal ITS data matrix recovered under the GTRGAMMA model in RAxML (lnL = −6525.73). Bootstrap percentages from 2000 replicates are displayed above branches. Accessions beginning with "UDB" are from the UNITE database (left), all others are from GenBank (right). Scale bar = 0.01 substitutions per site. "R" in each *C. striata* accession name indicates that the fungal ITS sequence was obtained from rhizome DNA.

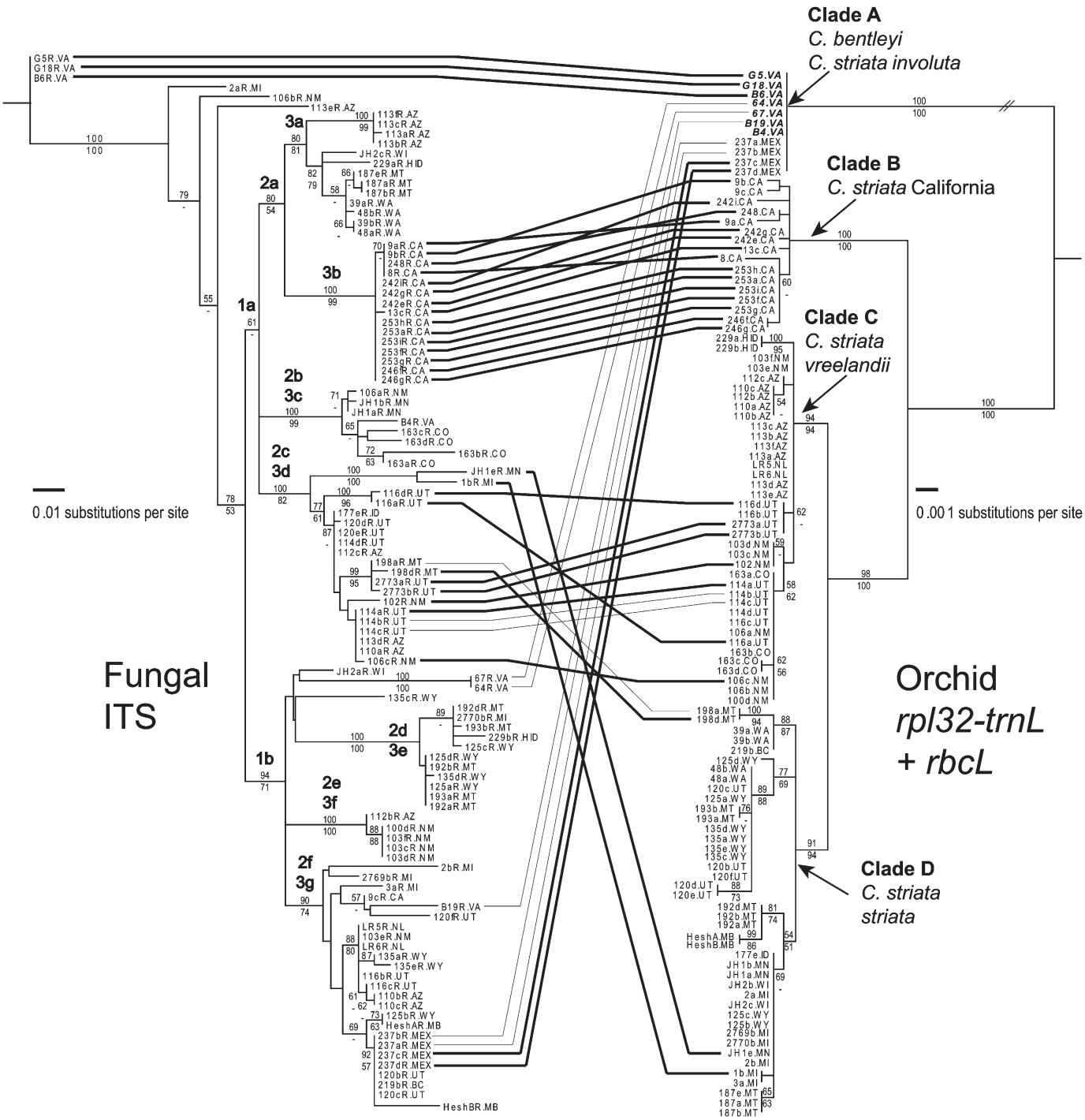

Fig. 3. Cophylogenetic analysis of fungal ITS (left; using the striata-only matrix) and orchid *rbcL+rpl32-trnL* (right) gene trees. Trees are represented by highest likelihood topologies under the GTRGAMMA model in RaxML. Two values are displayed for each branch: numbers above are ML bootstrap values based on 2000 replicates (above), and numbers below are results of 10000 maximum parsimony (MP) jackknife replicates from TNT (support values <50 are indicated by a "-" symbol). Larger numbers in boldface above support values in the fungal ITS gene tree are clade assignments for contingency tests, representing multiple levels (1a–b; 2a–f; 3a–g; see Appendix 3). Bold lines connecting accessions between the trees indicate significance for both MP and maximum likelihood (ML) input trees of that particular association at the $P = 0.05$ significance level based on 9999 ParaFit permutations. Thin lines indicate significance in either MP or ML (see Appendix 3). Left scale bar (fungi) = 0.01 substitutions per site and right scale bar (orchids) = 0.001 substitutions per site. "R" in each *C. striata* accession name indicates that the fungal ITS sequence was obtained from rhizome DNA.

involuta were removed in addition to the Californian accessions, to test their relative contribution to overall significance: this still returned a significant global value ($P = 0.019$). These findings suggest that there is some amount of cophylogenetic structure between orchid and fungal gene tree topologies, and that although it is not initially obvious, this is the case even when considering only vars. *vreelandii* and *striata* [clade (C,D)].

Statistical analyses—Results of all contingency table analyses are summarized in Appendix 3. Seven associations were excluded from contingency table analyses because they did not fall within well-defined clades (*2aR MI*, *106bR NM*, *113eR AZ*, *JH2aR WI*, *135cR WY*, *67R VA*, and *64R VA*). When only two deep *Tomentella* ITS clades were examined (1a, 1b; see Fig. 3), all comparisons with ptDNA clade groupings were significant. When six and seven *Tomentella* ITS clades (clade designations 2a–f and 3a–g, respectively) were considered, there was a general trend of higher significance as more ptDNA clades were sequentially considered (i.e., moving toward more terminal positions in the ptDNA tree), with the most significant test statistics in the case of seven *Tomentella* ITS clades and four ptDNA clades. Contingency tables including 18 ptDNA haplotypes (excluding clades A and B to remove their influence on overall significance) were significant for both two and six *Tomentella* ITS clades (Fisher's exact tests: $P = 0.0013$, $P < 0.001$, respectively). The 18 ptDNA haplotypes were not tested against seven *Tomentella* ITS clades because Californian sequences were not considered in this configuration (fungal clade 3b, plastid clade B). The majority of comparisons were highly significant, rejecting the null hypothesis of independence of associations between *Tomentella* ITS and plastid DNA clade associations at multiple levels. Using test statistic values (χ^2, log-likelihood ratio G) and their resultant *P*-values as rough measures with which to identify groupings that best explain patterns of association between clades from orchid and fungal trees, a configuration of seven *Tomentella* ITS and four ptDNA clades appeared to be optimal. This plastid-fungal clade configuration was chosen for incorporation into the logistic regression model (see below).

Fungal ITS diversity among four orchid groupings—Results from AMOVA, shown in Table 1, indicated that a greater proportion of *Tomentella* ITS variation was found within plastid clades than between them. This was expected, given that several orchid ptDNA clades (Fig. 3) associated with members of different *Tomentella* ITS clades. Nevertheless, *Tomentella* ITS variation between these groupings indicated significant differentiation overall ($\Phi_{ST} = 0.238$, $P < 0.001$). An additional AMOVA, partitioning among populations within clades (excluding all localities for which one individual was sampled; data not shown), showed that 33.48% of the variation was explained between populations within clades, while 47.91% was explained within populations.

Pairwise comparisons of Φ_{ST} estimates illustrated significant differentiation between all groupings (Table 2); these were highest for comparisons with plastid clade B. Estimates of *Tomentella* ITS nucleotide diversity (π, Table 3) for plastid clades A, C, and D were similar, but clade B (Californian accessions) had a value 5–6 times lower than the others. The population-specific estimate of Φ_{ST} was higher for Californian sequences in comparison to values for the other groupings. This difference increased when the *Tomentella* ITS sequence for accession *9cR CA* (GU220619, which fell outside the main Californian fungal clade; Fig. 3) was removed and the analysis recomputed. With the exclusion of this single accession, π decreased from 0.0073 to 0.0009, and population-specific Φ_{ST} increased from 0.263 to 0.289 (Table 3). These findings suggest that there is variation in specificity among orchid groupings: members of plastid clade B (Californian accessions) tend to associate with a narrow breadth of fungi relative to the other plastid clades.

TABLE 1. Analysis of molecular variance (AMOVA) for *Tomentella* ITS sequences, based on four plastid DNA clade groupings within the *Corallorhiza striata* complex.

Source of variation	df	SS	% Total variation	Φ_{ST}	*P*
Between groupings	3	275.58	23.84	0.238	<0.001
Within groupings	103	1091.59	76.16	—	—

Notes: df = degrees of freedom, SS = sum of squares, Φ_{ST} = between population variation coefficient, *P* = significance based on 10 000 permutations.

Logistic regression model—Overall, the multinomial logistic regression model with *Tomentella* ITS clade as the DV demonstrated adequate fit to the data relative to the intercept-only model (log-likelihood ratio $\chi^2 = 194.1$, df = 60, $P < 0.001$). Based on this model, 63.9% of all fungal associations were correctly classified. Table 4 illustrates that of the three potential explanatory IVs, geographic region had the most significant contribution ($P = 0.009$), followed by orchid plastid DNA clade ($P = 0.047$); elevation had a nonsignificant contribution ($P = 0.289$).

DISCUSSION

How does* C. striata *compare with other fully mycoheterotrophic plants in terms of specificity, and what model of specificity does it follow?—Before making any comparisons, specificity must be defined on a continuous scale, from phylogenetically broad to narrow associations: it is not a simple matter of specificity or no specificity (Thompson, 1994, 2005; Taylor et al., 2002, 2004; McCormick et al., 2004). The *C. striata* complex, considered as a whole, most certainly ranks high on the scale of specificity. Data on specificity of fully mycoheterotrophic plants toward their fungal associates come from two well-studied taxa: Orchidaceae (Taylor et al., 2002; Dearnaley, 2007; Waterman and Bidartondo, 2008) and monotropoid Ericaceae (Bidartondo and Bruns, 2002, 2005; Bidartondo et al., 2000). Of particular interest is comparison of *C. striata* with the congeneric *C. maculata-mertensiana* species complex. Members of the latter complex exhibit high specificity toward ECM Russulaceae and furthermore show no overlap in the russulaceous fungi upon which they depend (Taylor and Bruns, 1999). Taylor et al. (2004) demonstrated that *C. maculata* genotypes (based on restriction fragment analysis) associate with exclusive sets of fungi, spanning ca. 22 described species across the Russulaceae. *Corallorhiza maculata* and *C. striata* occupy similar geographic ranges across North America (Freudenstein, 1997), but *C. striata* (sensu lato) associates with a phylogenetically more narrow suite of mycobionts (i.e., fewer described species) than does *C. maculata* (sensu lato). This may be an overly simplistic comparison: these patterns become more complex when genetic variation within each orchid species complex is considered. It has been suggested that *C. maculata* is composed

TABLE 2. Pairwise Φ_{ST} estimates for *Tomentella* ITS sequences based on plastid DNA clades/taxonomic groupings within the *Corallorhiza striata* complex.

Plastid clade	Taxon	*C. bentleyi*+ *C. striata* var. *involuta*	*C. striata* (CA)	*C.striata* var. *vreelandii*
B	*C. striata* (California)	0.5776***		
C	*C. striata* var. *vreelandii*	0.1743***	0.4285***	
D	*C. striata* var. *striata*	0.1158**	0.4006***	0.0675***

***, $P < 0.001$; **, $P < 0.01$.

of several fungal host-associated races, since it was demonstrated that these associations consistently held even when different orchid genotypes were observed in the same population (Taylor et al., 2004). This was not testable for *C. striata* because no populations were found to harbor members of multiple plastid clades (considering clades A–D). Furthermore, there were no instances of complete exclusivity of *C. striata* plastid clades and associated *Tomentella* clades as was frequently observed in the *C. maculata-mertensiana* complex, although the high specificity of *C. striata* clade B from California on a single *Tomentella* ITS clade suggests a case of subspecialization (Taylor and Bruns, 1999; Taylor et al., 2004). A few additional sequences from the Californian coastal ranges (D. L. Taylor, unpublished data) included in the NJ analysis grouped outside of the 'Californian' fungal clade (online Appendix S5). However, no corresponding plastid DNA sequences were available for these fungal ITS sequences, so it remains to be determined whether Californian *C. striata* from outside the Sierra Nevada belong to plastid clade B.

The most likely interpretation of the findings for *C. striata* is that both geographic region and plastid DNA clade are strongly correlated (Table 4; Barrett and Freudenstein, 2009) and that the plastid clades comprising the *C. striata* complex tend to associate with divergent sets of fungi in different geographic regions. Furthermore, a substantial amount of variation (AMOVA, 33.48%) can be explained between populations within clades, suggesting even further geographic substructuring. There are some notable exceptions to geographic-plastid DNA clade correlation: the disjunctions of clade C (var. *vreelandii*) from the southwestern USA/Mexico to northeastern Canada and clade A from Mexico (var. *involuta*) to the eastern USA (*C. bentleyi*). Both are likely to be remnants of previously more continuous distributions (Barrett and Freudenstein, 2009).

The model of specificity that best describes the pattern observed in *C. striata* is a geographic mosaic (Thompson, 1994, 2005; Bidartondo, 2005). With reference to specificity/specialization, this model is based on numerous observations of geographic variation in associations between interacting organisms. In an extreme example, a symbiont taxon that might otherwise be described as a generalist or an opportunist is in fact a specialist on different host taxa or host genotypes/phenotypes when multiple populations are considered in a geographic context (Thompson, 1994). A growing body of research has demonstrated that geographic variation in associations between interacting organisms—including variation in specificity among closely related taxa or populations—has played a significant role in shaping the immense diversity of life on Earth (Thompson, 1994, 2005).

A particularly notable example of a geographic mosaic in specificity of associations comes from the fully mycoheterotrophic plant *Monotropa uniflora* L. (Ericaceae: Monotropoideae). This widespread North American plant was shown to specialize on various russulaceous species within *Russula* Pers., *Lactarius* Pers., and *Martellia* Mattir. (Bidartondo and Bruns, 2001; Bidartondo, 2005). Specifically, *M. uniflora* populations in Oregon associated exclusively with *Russula brevipes* Peck, while a single population in Vermont, USA associated with three *Russula* species groups and *Lactarius theiogalus* (Bull.) Gray, suggesting geographic variation in specificity toward target fungi.

If the pattern of specificity among *C. striata* plastid clades is interpreted in a phylogenetic context (and assuming that the orchid and fungal loci chosen are sufficient estimates of phylogenetic relationships in the *C. striata* complex), then an apparent pattern of divergence in specificity is observed with respect to clades A (*C. bentleyi* + *C. striata* var. *involuta*), B (California), and C + D (vars. *vreelandii* + *striata*). Clade B from California targets a much narrower suite of *Tomentella* fungi than do clades A or B + C (Fig. 3; Table 3). Unexpectedly, the geographically restricted, endangered *C. bentleyi* associated with members of multiple *Tomentella* clades. These findings were based on only seven accessions from three populations, suggesting that this species might not be as specific as some other members of the *C. striata* complex. Further sampling could reveal an even greater diversity of *Tomentella* fungi, but the extreme rarity of this species makes this difficult (ca. 15 populations, many of which consist of fewer than 100 individuals). It was not possible to assess the overall fungal diversity in the Mexican endemic *C. striata* var. *involuta* because only four individuals from one population were sampled due to difficulty in obtaining material.

A similar scenario of divergence in specificity was documented in the fully mycoheterotrophic orchid *Hexalectris spicata* (Walter) Barnhart (Taylor et al., 2003). In this species complex, the widespread *H. spicata* var. *spicata* associated with several *Rhizoctonia* ITS types (assessed via restriction fragment analysis and sequencing), while the more geographically restricted *H. spicata* var. *arizonica* (S. Watson) Catling and Engel and *H. revoluta* Correll each associated with unique ITS types. It will be important in future studies to incorporate the influence of geographic ranges of the plant taxa of interest into assessments of their relative specificities toward target fungi.

The finding of statistically significant associations between *C. striata* plastid clades and *Tomentella* ITS clades (Appendix 3) is evocative of the situation in *Pterospora andromedea* Nutt. (Ericaceae: Monotropoideae; Bidartondo and Bruns, 2002). Certain *P. andromedea* plastid *trnL-F* haplotypes from 73 individual plants consistently associated with one of two *Rhizopogon* Fr. species in the Sierra Nevada of California, even in sympatry. However, the geographic scope of this study was limited to only a portion of the North American range of *P. andromedea*. The finding of significant cophylogenetic structure between the *C. striata* plastid gene tree and the ITS tree of its fungal associates shares some features with the pattern observed between *Monotropa hypopithys* L. (Ericaceae: Monotropoideae) and its fungal associates in the genus *Tricholoma* (Tricholomataceae; Bidartondo and Bruns, 2002). Neither the present findings nor those from *M. hypopithys* represents a clear case for intraspecific phylogenetic tracking; "phylogeographic tendency" or "affinity" may be better terms to describe these patterns. Overall, *C. striata* appears to correlate well with several of the patterns observed in other fully mycoheterotrophic orchids and monotropes, indicating that these may be hallmark features for

TABLE 3. Population genetic parameter estimates for *Tomentella* ITS sequences, by ptDNA clade/taxonomic groupings within the *Corallorhiza striata* complex.

ptDNA clade	Taxonomic grouping	*N*	No. PM sites	π	SD	Population Φ_{ST}
A	*C. bentleyi* + *C. striata* var. *involuta*	11	76	0.0454	0.0243	0.232
B	*C. striata* CA	16	34	0.0073	0.0042	0.263
B*		15	3	0.0009	0.0001	0.289
C	*C. striata* var. *vreelandii*	38	116	0.0385	0.0192	0.236
D	*C. striata* var. *striata*	42	114	0.0419	0.0209	0.233

Notes: N, sample size; π, nucleotide diversity; SD, standard deviation; no. PM sites, number of polymorphic sites; population Φ_{ST}, population-specific fixation index estimate; B*, clade B excluding sample *9cR CA*.

the more than 400 species of fully mycoheterotrophic plants (Leake, 1994).

Why is* C. striata *so specific toward its fungal associates?—Obligate symbioses often result in extreme specificity of associations between interacting partners (Thompson, 1994). The causes for this phenomenon, however, are not always clear. One possible explanation involves selection for high specificity; evidence suggests that fully mycoheterotrophic plants (including orchids in the protocorm stage) manipulate their fungal associates (Rasmussen, 1995; Bidartondo, 2005; Rasmussen and Rasmussen, 2009). Thus, it would be expected that specialization on a narrow, closely related group of fungi might be the optimal situation for a fully mycoheterotrophic plant. Specialization may allow full mycoheterotrophs to circumvent challenges of associating with phylogenetically diverse fungi, because they may not have to deal with a potentially high diversity of defenses and food sources (Thompson, 1994). Furthermore, full mycoheterotrophs may target specific fungi due to their relative superiority in nutritional quality, their ability to compete effectively for soil nutrients/photobiotic carbon, or their high abundance in specific soil environments or microhabitats (Rasmussen and Rasmussen, 2009). Last, target fungi may display a form of ecological naïveté, having weak defenses (or none at all) relative to co-occurring fungi against manipulation by fully mycoheterotrophic plants.

An alternative explanation for the observation of high specificity in fully mycoheterotrophic plants is that they simply do not encounter certain fungal taxa (or variants thereof) due to non-overlapping geographic ranges or habitat preferences. This is not likely to be the case for the *C. striata* complex, for two reasons. First, there may be hundreds of fungal species within the direct vicinity of the orchids. In fact, other *Corallorhiza* species (*C. maculata*, *C. trifida*, *C. wisteriana*) that use different but sometimes closely related fungi are often found in sympatry (Taylor and Bruns, 1999; C. Barrett et al., personal observation). Second, in some instances, different *C. striata* individuals from the same population associated with fungi from different *Tomentella* clades (e.g., population 120 UT; Fig. 3), suggesting that multiple suitable *Tomentella* fungi often co-occur. Although the observation of the tendency for *C. striata* plastid clades to group with divergent sets of fungi in different geographic regions seems more logically congruent with the first hypothesis, discerning between these two alternative hypotheses deserves future attention.

TABLE 4. Multinomial logistic regression model, with fungal ITS clade as the dependent variable.

Variable	−2·lnL	χ^2	df	*P*
Intercept only	131.474	—	—	—
Plastid DNA clade	152.693	21.219	12	0.047
Biogeographic region	166.722	35.248	18	0.009
Elevation category	145.655	14.181	12	0.289

Notes: −2·lnL, log-likelihood ratio statistic; df, degrees of freedom; *P*, significance of likelihood ratio test in the regression model.

What are the implications of high specificity?—Living an extremely specialized lifestyle most likely has important implications for orchids and other full mycoheterotrophs with respect to their distributions, population sizes, and conservation (Rasmussen and Whigham, 1998; Otero and Flanagan, 2005; Tremblay et al., 2005; Waterman and Bidartondo, 2008; Rasmussen and Rasmussen, 2009). For example, this lifestyle may in part explain why *C. striata* has a widespread yet patchy distribution composed of small populations (Tremblay et al., 2005). The distribution and abundance of *Tomentella* fungi, including those that associate with *C. striata*, are poorly understood. The *Tomentella fuscocinerea* group is geographically widespread among forests of the northern hemisphere, but not locally abundant (U. Kõljalg, unpublished data). In a way, sampling of *C. striata* provides a window into the distribution, abundance, and diversity of these elusive fungi. The finding of up to 12 putative species (based on a 2.5% ITS divergence criterion for species boundaries) suggests the potential existence of several cryptic species within the currently circumscribed *T. fuscocinerea*.

In terms of conservation status, association with a narrow suite of *Tomentella* fungi (that are not particularly abundant in their respective habitats) can be viewed as a further restriction on the niche breadth of *C. striata*. Future research efforts should focus on whether the absence of *C. striata* at a particular locality is due strictly to the absence of suitable fungi or whether these *Tomentella* fungi are also commonly present at sites without *C. striata*. This issue may be addressed using in vivo "seed baiting" experiments (sensu Rasmussen and Whigham, 1993), but these experiments take into account neither the potential for selection to act upon the specific association following germination, nor the potential (on the contrary) for germination itself to act as a specificity bottleneck (sensu Bidartondo and Read, 2008). Germination and subsequent plant growth analyses (i.e., fitness comparisons), however difficult, may be the only way to increase knowledge on compatibilities of the observed orchid and fungal types.

One potentially important aspect not addressed in this study is whether there exists temporal variation in fungal associations throughout the life cycle of *C. striata*. Some orchids have been shown to associate with different fungi (or subsets of them) during different life stages or under different environmental stresses (McCormick et al., 2006; Bidartondo and Read, 2008),

particularly in the protocorm stage vs. the mature stage (Rasmussen, 2002; Dearnaley, 2007). For example, Xu and Mu (1990) demonstrated that the fully mycoheterotrophic orchid *Gastrodia elata* Blume requires an association with *Mycena* (Pers.) Roussel as a juvenile and with *Armillaria* (Fr.) Staude as a mature plant. Thus, a life history involving fungal switching could add another layer of complexity to conservation considerations (Zettler, 2005; Dearnaley, 2007); such host switching seems unlikely in *Corallorhiza* based on observations of both *C. maculata* and *C. odontorhiza* protocorms yielding similar mycobionts to mature plants (D. L. Taylor and M. McCormick [Smithsonian Environmental Research Center], unpublished data).

Conclusion—*Corallorhiza striata* is a fully mycoheterotrophic specialist plant targeting a narrow breadth of fungi in the genus *Tomentella*—specifically *T. fuscocinerea*—based on orchid plastid and fungal ITS sequence variation sampled over a broad geographic area in North America. Thus, extreme specificity of *C. striata* toward its mycobionts persists despite occurrence in a diversity of forest ecosystems throughout its geographic range from Mexico to Canada. There is further evidence for specificity within the *C. striata* complex based on findings of (1) significant cophylogenetic structure between orchid and fungal gene trees, (2) significant non-independence of associations between orchid and fungal clades, (3) association of *C. striata* clades with divergent sets of *Tomentella* fungi, (4) differences in fungal specificity among *C. striata* clades, and (5) significant relationships between *Tomentella* clades and both geography and orchid clades. The analyses presented here are among the most extensive of any orchid–fungal system in terms of incorporating genetic data from both orchids and their associated fungi and the first to take an explicitly phylogeographic approach. In addition, this study represents one of the most geographically comprehensive sampling schemes of any investigation of its kind (see also Taylor et al., 2004; Shefferson et al., 2005, 2007), making the case for high specificity of *C. striata* toward *Tomentella* fungi particularly convincing. Last, this study provides useful information for future conservation efforts regarding the consideration of both orchid and fungal variation for *C. striata* and the endangered *C. bentleyi*.

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APPENDIX 1. Collection information for voucher specimens used in this study. Each population number corresponds to at least one voucher in the Ohio State University Herbarium (e.g. 242 CA).

Taxon*, *collection code (population number, individual, state/province), collection locality, elevation (m a.s.l.), GenBank accessions: fungal ITS, *rbcL*, *rpl32-trnL*

Clade A: ***Corallorhiza bentleyi* Freudenst.** *CFB G5 VA*, Giles Co., Virginia, USA, 649, GU220605, GU223972, GU224033; *CFB G18 VA*, Giles Co., Virginia, USA, 649, GU220606, GU223971, GU224032; *CFB 64 VA*, Giles Co., Virginia, USA, 623, GU220607, GU223966, GU224027; *CFB 67 VA*, Giles Co., Virginia, USA, 655, GU220608, GU223967, GU224028; *CFB B4 VA*, Bath Co., Virginia, USA, 671, GU220609, FJ445529, FJ445612; *CFB B6 VA*, Bath Co., Virginia, USA, 671, GU220610, GU223970, GU224031; *CFB B19 VA*, Bath Co., Virginia, USA, 671, GU220611, FJ445530, FJ445613.

Clade A: ***C. striata* Lindl. var. *involuta* (Greenm.) Freudenst.** *CFB 237a MEX*, Toluca, Mexico, Mexico, 2831, GU220612, FJ445564, FJ445647; *CFB 237b MEX*, Toluca, Mexico, Mexico, 2831, GU220613, GU223953, GU224014; *CFB 237c MEX*, Toluca, Mexico, Mexico, 2831, GU220614, FJ445565, FJ445648; *CFB 237d MEX*, Toluca, Mexico, Mexico, 2831, GU220615, GU223954, GU224015.

Clade B: **C. *striata* Lindl. [Californian accessions]** *CFB 8 CA*, El Dorado Co, California, USA, 1676, GU220616, FJ445533, FJ445616; *CFB 9a CA*, Nevada Co., California, USA, 1594, GU220617, FJ445534, FJ445617; *CFB 9b CA*, Nevada Co., California, USA, 1594, GU220618, GU223968, GU224029; *CFB 9c CA*, Nevada Co., California, USA, 1594, GU220619, GU223969, GU224030; *CFB 13c CA*, Tehama Co., California, USA, 1410, GU220620, FJ445536, FJ445619; *CFB 242e CA*, Placer Co., California, USA, 1611, GU220621, GU223955, GU224016; *CFB 242g CA*, Placer Co., California, USA, 1611, GU220622, GU223956, GU224017; *CFB 242i CA*, Placer Co., California, USA, 1611, GU220623, GU223957, GU224018; *CFB 246f CA*, Tuolumne Co., California, USA, 1460, GU220624, GU223958, GU224019; *CFB 246g CA*, Tuolumne Co., California, USA, 1460, GU220625, GU223959, GU224020; *CFB 248 CA*, Madera Co., California, USA, 1420, GU220626, GU223960, GU224021; *CFB 253a CA*, Fresno Co., California, USA, 1933, GU220627, GU223961, GU224022; *CFB 253f CA*, Fresno Co., California, USA, 1933, GU220628, GU223962, GU224023; *CFB 253g CA*, Fresno Co., California, USA, 1933, GU220629, GU223963, GU224024; *CFB 253h CA*, Fresno Co., California, USA, 1933, GU220630, GU223964, GU224025; *CFB 253i CA*, Fresno Co., California, USA, 1933, GU220631, GU223965, GU224026.

Clade C: **C. *striata* Lindl. var. *vreelandii* (Rydb.) L. O. Williams** *100d NM*, Santa Fe Co., New Mexico, USA, 2652, GU220632, GU223916, GU223977; *CFB 102 NM*, Lincoln Co., New Mexico, USA, 2348, GU220633, GU223917, GU223978; *CFB 103c NM*, Otero Co., New Mexico, USA, 2654, GU220634, GU223918, GU223979; *CFB 103d* NM, Otero Co., New Mexico, USA, 2654, GU220635, GU223919, GU223980; *CFB 103e NM*, Otero Co., New Mexico, USA, 2654, GU220636, GU223920, GU223981; *CFB 103f NM*, Otero Co., New Mexico, USA, 2654, GU220637, FJ445575, FJ445658; *CFB 106a NM*, Catron Co., New Mexico, USA, 2740, GU220638, FJ445577, FJ445660; *CFB 106b NM*, Catron Co., New Mexico, USA, 2740, GU220639, FJ445578, FJ445661; *CFB 106c NM*, Catron Co., New Mexico, USA, 2740, GU220640, GU223921, GU223982; *CFB 110a AZ*, Graham Co., Arizona, USA, 2743, GU220641, FJ445519, FJ445602; *CFB 110b AZ*, Graham Co., Arizona, USA, 2743, GU220642, FJ445520, FJ445603; *CFB 110c AZ*,

Graham Co., Arizona, USA, 2743, GU220643, GU223922, GU223983. *112b AZ*, Pima Co., Arizona, USA, 2751, GU220644, FJ445522, FJ445605; *CFB 112c AZ*, Pima Co., Arizona, USA, 2751, GU220645, GU223923, GU223984; *CFB 113a AZ*, Gila Co., Arizona, USA, 2361, GU220646, FJ445523, FJ445606; *CFB 113b AZ*, Gila Co., Arizona, USA, 2361, GU220647, FJ445524, FJ445607; *CFB 113c AZ*, Gila Co., Arizona, USA, 2361, GU220648, GU223924, GU223985; *CFB 113d AZ*, Gila Co., Arizona, USA, 2361, GU220649, GU223925, GU223986; *CFB 113e AZ*, Gila Co., Arizona, USA, 2361, GU220650, GU223926, GU223987; *CFB 113f AZ*, Gila Co., Arizona, USA, 2361, GU220651, GU223927, GU223988; *CFB 114a UT*, Utah Co., Utah, USA, 2038, GU220652, FJ445582, FJ445665; *CFB 114b UT*, Utah Co., Utah, USA, 2038, GU220653, FJ445583, FJ445666; *CFB 114c UT*, Utah Co., Utah, USA, 2038, GU220654, GU223928, GU223989; *CFB 114d UT*, Utah Co., Utah, USA, 2038, GU220655, GU223929, GU223990; *CFB 116a UT*, Tooele Co., Utah, USA, 1982, GU220656, FJ445584, FJ445667; *CFB 116b UT*, Tooele Co., Utah, USA, 1982, GU220657, FJ445585, FJ445668; *CFB 116c UT*, Tooele Co., Utah, USA, 1982, GU220658, GU223930, GU223991; *CFB* i, Tooele Co., Utah, USA, 1982, GU220659, GU223931, GU223992; *CFB 163a CO*, Ouray Co., Colorado, USA, 2589, GU220660, GU223940, GU224001; *CFB 163b CO*, Ouray Co., Colorado, USA, 2589, GU220661, FJ445540, FJ445623; *CFB 163c CO*, Ouray Co., Colorado, USA, 2589, GU220662, FJ445541, FJ445624; *CFB 163d CO*, Ouray Co., Colorado, USA, 2589, GU220663, GU223941, GU224002; *JVF 2773a UT*, Salt Lake Co., Utah, USA, 2468, GU220664, FJ445580, FJ445663; *JVF 2773b UT*, Salt Lake Co., Utah, USA, 2468, GU220665, FJ445581, FJ445664; *CFB 229a HID*, El Chico, Hidalgo, Mexico, 2957, GU220666, GU223951, GU224012; *CFB 229b HID*, El Chico, Hidalgo, Mexico, 2957, GU220667, GU223952, GU224013; *CFB LR5 NL*, Lomond River, Newfoundland, Canada, 198, GU220668, FJ445568, FJ445651; *CFB LR6 NL*, Lomond River, Newfoundland, Canada, 198, GU220669, GU223976, GU224037.

Clade D: **C. *striata* Lindl. var. *striata*** *1b MI*, Presque Isle Co., Michigan, USA, 204, GU220670, FJ445547, FJ445630; *CFB 2a MI*, Schoolcraft Co., Michigan, USA, 213, GU220671, FJ445548, FJ445631; *CFB 2b MI*, Schoolcraft Co., Michigan, USA, 213, GU220672, FJ445549, FJ445549; *JVF 2769b MI*, Schoolcraft Co., Michigan, USA, 213, GU220673, FJ445553, FJ445636; *CFB 3a MI*, Mackinac Co., Michigan, USA, 230, GU220674, FJ445550, FJ445633; *JVF 2770b MI*, Mackinac Co., Michigan, USA, 230, GU220675, FJ445555, FJ445638; *Horky JH1a MN*, Crow Wing Co., Minnesota, USA, 376, GU220676, FJ445556, FJ445639; *Horky JH1b MN*, Crow Wing Co., Minnesota, USA, 376, GU220677, GU223973, GU224034; *Horky JH1e MN*, Crow Wing Co., Minnesota, USA, 376, GU220678, GU223974, GU224035; *Horky JH2a WI*, Douglas Co., Wisconsin, USA, 215, GU220679, GU223975, GU224036; *Horky JH2c WI*, Douglas Co., Wisconsin, USA, 215, GU220680, FJ445559, FJ445642; *CFB 39a WA*, Skamania Co., Washington, USA, 223, GU220681, FJ445589, FJ445672; *CFB 39b WA*, Skamania Co., Washington, USA, 223, GU220682, FJ445590, FJ445673; *CFB 48a WA*, Lewis Co., Washington, USA, 1149, GU220683, FJ445591, FJ445591; *CFB 48b WA*, Lewis Co., Washington, USA, 1149, GU220684, FJ445592, FJ445675; *CFB 120b UT*, Cache Co., Utah, USA, 2191, GU220685, GU223932, GU223993; *CFB 120c UT*, Cache Co., Utah, USA, 2191, GU220686, FJ445587, FJ445670; *CFB 120d UT*, Cache Co., Utah, USA, 2191, GU220687, GU223933, GU223994; *CFB 120e UT*, Cache Co., Utah, USA, 2191, GU220688, FJ445588, FJ445671; *CFB 120f UT*, Cache Co., Utah, USA, 2191, GU220689, GU223934, GU223995; *CFB 125a WY*, Lincoln Co., Wyoming, USA, 1810, GU220690, FJ445593, FJ445676; *CFB 125b WY*, Lincoln Co., Wyoming, USA, 1810, GU220691, FJ445594, FJ445677; *CFB 125c WY*, Lincoln Co., Wyoming, USA, 1810, GU220692, GU223935, GU223996; *CFB 125d WY*, Lincoln Co., Wyoming, USA, 1810, GU220693, GU223936, GU223997; *CFB 135a WY*, Natrona Co., Wyoming, USA, 2023, GU220694, FJ445595, FJ445678; *CFB 135c WY*, Natrona Co., Wyoming, USA, 2023, GU220695, GU223937, GU223998; *CFB 135d WY*, Natrona Co., Wyoming, USA, 2023, GU220696, GU223938, GU223999; *CFB 135e WY*, Natrona Co., Wyoming, USA, 2023, GU220697, GU223939, GU224000; *Heshka HeshA MB*, Whinnipeg, Manitoba, Canada, 238, GU220698, FJ445560, FJ445643; *Heshka HeshB MB*, Whinnipeg, Manitoba, Canada, 238, GU220699, FJ445561, FJ445644; *CFB 177e ID*, Idaho Co., Idaho, USA, 1600, GU220700, GU223942, GU224003; *CFB 187a MT*, Lewis and Clark Co., Montana, USA, 1573, GU220701, FJ445566, FJ445649; *CFB 187b MT*, Lewis and Clark Co., Montana, USA, 1573, GU220702, FJ445567, FJ445650; *CFB 187e MT*, Lewis and Clark Co., Montana, USA, 1573, GU220703, GU223943, GU224004; *CFB 192a MT*, Gallatin Co., Montana, USA, 1943, GU220704, GU223944, GU224005; *CFB 192b MT*, Gallatin Co., Montana, USA, 1943, GU220705, GU223945, GU224006; *CFB 192d MT*, Gallatin Co., Montana, USA, 1943, GU220706, GU223946, GU224007; *CFB 193a MT*, Sweet Grass Co., Montana, USA, 1962, GU220707, GU223947, GU224008; *CFB 193b MT*, Sweet Grass Co., Montana, USA, 1962, GU220708, GU223948, GU224009; *CFB 198a MT*, Fergus Co., Montana, USA, 1696, GU220709, GU223949, GU224010; *CFB 198d MT*, Fergus Co., Montana, USA, 1696, GU220710, GU223950, GU224011; *CFB* 219b *BC*, Thompson-Nicola, British Columbia, Canada, 841, GU220711, FJ445526, FJ445609.

APPENDIX 2. Significant cophylogenetic associations between orchids and *Tomentella* fungi based on 9999 permutations in ParaFit ($P = 0.05$), with significance of global (overall) cophylogenetic structure at bottom. *Note:* ML, maximum likelihood; MPT, most parsimonious trees.

(ML trees) accession	*P* -value	(Two MPT) accession	*P*-value
102 NM	0.018	*102 NM*	0.011
106c NM	0.034	*106c NM*	0.028
114a UT	0.048	*113d AZ*	0.030
116a UT	0.027	*114a UT*	0.039
116d UT	0.022	*114b UT*	0.040
13c CA	0.001	*114c UT*	0.049
198a MT	0.042	*116a UT*	0.035
198d MT	0.032	*116d UT*	0.032
1b MI	0.002	*13c CA*	0.001
237a MEX	0.048	*198d MT*	0.037
237c MEX	0.048	*1b MI*	0.013
237d MEX	0.043	*237b MEX*	0.040
242e CA	0.001	*237c MEX*	0.041
242g CA	0.001	*237d MEX*	0.038
242i CA	0.001	*242e CA*	0.001
246f CA	0.001	*242g CA*	0.001
246g CA	0.001	*242i CA*	0.002
248 CA	0.001	*246f CA*	0.001
253a CA	0.001	*246g CA*	0.001
253f CA	0.001	*248 CA*	0.001
253g CA	0.001	*253a CA*	0.001
253h CA	0.001	*253f CA*	0.001
253i CA	0.001	*253g CA*	0.001
2773a UT	0.019	*253h CA*	0.001
2773b UT	0.013	*253i CA*	0.001
64 VA	0.015	*2773a UT*	0.031
67 VA	0.012	*2773b UT*	0.021
8 CA	0.001	*8 CA*	0.001
9a CA	0.001	*9a CA*	0.001
9b CA	0.001	*9b CA*	0.001
B6 VA	0.001	*B19 VA*	0.043
G18 VA	0.006	*B6 VA*	0.008
G5 VA	0.003	*G18 VA*	0.006
JH1e MN	0.006	*G5 VA*	0.007
		JH1e MN	0.013
$P = 0.002$		$P = 0.002$	

APPENDIX 3. Tests for independence based on various r × c contingency table configurations between *Tomentella* ITS and orchid plastid clades. *Note:* The first column indicates the configuration of *Tomentella* ITS clades (refer to Fig. 3) used in that particular contingency table, while the heading row indicates the plastid clade configuration used (clades A–D; Fig. 3). The number of clades is indicated before the colon, with the clade designations immediately following. Values below are test statistics (except for in the case of Fisher's exact tests where only *P*-values are given), followed by significance level (***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$; NS, nonsignificant), and degrees of freedom. Failed, test could not be calculated due to computational limitations; N/A, configuration not tested.

	Plastid DNA clades			
Fungal clades	2: A,(B,C,D)	3: A,B,(C,D)	4: A,B,C,D	18 haplotypes (clades C+D only)
χ^2 test				
2: 1a, 1b	4.90*, 1	67.70***, 2	19.27***, 3	34.45***, 17
6: 2a-f	12.20*, 5	32.36***, 10	49.41***, 15	Failed, 85
7: 3a-g	12.20^{NS}, 6	101.00***, 12	121.85***, 18	N/A
Log-likelihood *G*-test				
2: 1a, 1b	7.16**, 1	79.82***, 2	22.04***, 3	42.53***, 17
6: 2a-f	12.39*, 5	40.12***, 10	62.10***, 15	Failed, 85
7: 3a-g	12.18^{NS}, 6	88.71***, 12	98.82***, 18	N/A
Fisher's exact test with Monte Carlo simulation				
2: 1a, 1b	$P = 0.020$	$P << 0.001$	$P << 0.001$	$P = 0.0013$
6: 2a-f	$P = 0.0072$	$P << 0.001$	$P << 0.001$	$P << 0.001$
7: 3a-g	$P = 0.0002$	$P << 0.001$	$P << 0.001$	N/A