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THE EVOLUTIONARY HISTORY OF MYCORRHIZAL SPECIFICITY AMONG LADY'S SLIPPER ORCHIDS

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Although coevolution is acknowledged to occur in nature, coevolutionary patterns in symbioses not involving species-to-species relationships are poorly understood. Mycorrhizal plants are thought to be too generalist to coevolve with their symbiotic fungi; yet some plants, including some orchids, exhibit strikingly narrow mycorrhizal specificity. Here, we assess the evolutionary history of mycorrhizal specificity in the lady's slipper orchid genus, *Cypripedium*. We sampled 90 populations of 15 taxa across three continents, using DNA methods to identify fungal symbionts and quantify mycorrhizal specificity. We assessed phylogenetic relationships among sampled *Cypripedium* taxa, onto which we mapped mycorrhizal specificity. *Cypripedium* taxa associated almost exclusively with fungi within family Tulasnellaceae. Ancestral specificity appears to have been narrow, followed by a broadening after the divergence of *C. debile*. Specificity then narrowed, resulting in strikingly narrow specificity in most of the taxa in this study, with

no taxon rewidening to the same extant as basal members of the genus. Sympatric taxa generally associated with different sets of fungi, and most clades of *Cypripedium*-mycorrhizal fungi were found throughout much of the northern hemisphere, suggesting that these evolutionary patterns in specificity are not the result of biogeographic lack of opportunity to associate with potential partners. Mycorrhizal specificity in genus *Cypripedium* appears to be an evolvable trait, and associations with particular fungi are phylogenetically conserved.

KEY WORDS **Cheating, coevolution, *ITS*, mean phylogenetic breadth, *mtLSU*, mutualism, *rbcL*, specificity, Tulasnellaceae.**

The mycorrhiza is a classic example of a widespread mutualism, in which a soil fungus contributes mineral nutrition to a plant, and the plant contributes photosynthetically fixed carbon back to the fungus via the root system (Smith and Read 1997). Most plants acquire the majority of their nutrients through this 500 million year old symbiosis, and mycorrhizal fungi acquire their key limiting nutrient, carbon, from their plant partner (Heckman et al. 2001). However, some plant groups, including the orchid family (Alexander and Hadley 1985; Rasmussen 1995; Bidartondo et al. 2004), the monotropes (Bidartondo 2005), some liverworts (Bidartondo et al. 2003), and nongreen plants from diverse lineages (Leake 1994), reverse the mutualistic nature of the mycorrhiza and procure carbon from their fungal partner for at least a portion of the life cycle. Because these plant groups include many nonphotosynthetic species that associate with ectomycorrhizal or arbuscular mycorrhizal fungi (Taylor and Bruns 1997; Bidartondo et al. 2002; Taylor et al. 2002), they have become increasingly viewed as "epi-parasites" of mutualistic symbioses (Furman and Trappe 1971; Hibbett 2002).

From Darwin's classic accounts of deceptive pollination traits in the orchids, this plant family has been thought to have become so species-rich primarily in response to specialization on and cheating of its pollinators (Darwin 1862; Cozzolino and Widmer 2005). However, speciation in the orchid family may have been influenced, if not partially driven, by mycorrhizal specialization and nutritional needs (Taylor et al. 2003; Otero and Flanagan 2006). Mycorrhizal infection is a requirement for the germination and/or growth of all orchid seeds in the wild (Bernard 1904), and carbon is transferred from fungus to orchid at this stage (Smith 1967; Alexander and Hadley 1985), a phenomenon known as "mycoheterotrophy" (Leake 1994). From germination, orchids may control the mycorrhizal relationship in such ways as to dictate even its morphology (Roberts 1999; Brundrett 2004). It is thus no surprise that in orchids, mycorrhizal specificity, defined as the phylogenetic breadth of fungi that a plant taxon associates with, is considered narrow (Taylor et al. 2002). In contrast, most other plants are mycorrhizal generalists, exhibiting phylogenetically broad associations (Molina et al. 1992; Hoeksema 1999; Massicotte et al. 1999).

Although overall mycorrhizal specificity in the orchid family is narrow, variation in specificity among orchid species is high, and ecological correlates have failed to account for this variation (McCormick et al. 2004). Genetic variation at some loci correlates with choice of mycorrhizal fungal host in some orchid species (Taylor et al. 2004), suggesting that this symbiosis should be capable of evolving via natural selection. It follows that mycorrhizal associations may be conserved phylogenetically, with closely related plant species likely to share fungal partners more than distantly related species, which have had more time to evolve since branching from their common ancestor. The result may be that specificity, measured as the phylogenetic breadth of fungal taxa that an orchid associates with, is conserved among closely related taxa.

Here, we present the first, definitive phylogenetic study of mycorrhizal evolution in one genus within the family Orchidaceae. This genus, *Cypripedium*, presents a number of appealing characteristics for the study of the orchid mycorrhiza. First, it is composed of species noted for their tendency to undergo adult whole-plant dormancy (Kull 2002), a condition in which the plant does not sprout for one or more years at a time, suggesting that mycorrhizae may remain important carbon sources in maturity (Gill 1989). Second, it is monophyletic (Cribb 1997; Cameron et al. 1999) and species-rich, composed of approximately 45 species throughout the temperate northern hemisphere, giving ample opportunity for evolution to have resulted in phenotypic variation among taxa. Last, though species are generally locally rare, they usually have large geographic ranges (Cribb 1997), offering many populations in which mycorrhizal associations may continue to evolve independently. We used a phylogenetic approach to assess evolutionary trends in mycorrhizal association and specificity. We asked which fungi form mycorrhizal associations with *Cypripedium* taxa. We then assessed phylogenetic relationships among *Cypripedium* taxa, and explored how associations with specific fungal hosts have evolved in the genus (hereafter, we refer to the fungus as the "host"). Because patterns in mycorrhizal association may be constrained by the geographic ranges and/or habitat preferences of the fungi, we also asked whether sympatric populations of *Cypripedium* taxa with similar habitat requirements associated

with the same or different mycorrhizal fungi. Last, we present the first reconstruction of the evolutionary history of mycorrhizal specificity as a quantifiable character.

Methods

SAMPLE COLLECTION

Fifteen *Cypripedium* taxa were chosen for this study: *C. acaule* Aiton (seven individuals sampled in four populations in Maryland and Massachusetts); *C. arietinum* R. Br. (10 individuals sampled in three populations in the Upper Peninsula of Michigan); *C. calceolus* L. (17 individuals sampled from 10 populations in Estonia and far-eastern Russia); *C. californicum* A. Gray (24 individuals sampled from 10 populations across northern California); *C. candidum* Mühl ex Willd. (seven individuals sampled from 3 populations in Illinois and Kentucky); *C. debile* Rchb. f. (13 individuals sampled from three populations in Japan and Taiwan); *C. fasciculatum* Kellogg ex S. Watson (26 individuals sampled from nine populations across northern California); *C. formosanum* Hayata (two individuals sampled from one population in central Taiwan); *C. guttatum* Sw. (21 individuals sampled from seven populations in central Alaska and northern Japan); *C. japonicum* Thunb. (18 individuals sampled from 11 populations in central Japan); *C. macranthon* var. *rebunense* (Kudo) Miyabe and Kudo (16 individuals sampled from three populations in far-northern Japan); *C. macranthon* var. *speciosum* Rolfe (Koidz.) (13 individuals sampled from four populations in Japan and far-eastern Russia); *C. montanum* Douglas ex Lindl. (27 individuals sampled from 11 populations across northern California); *C. parviflorum* Salisb. (13 individuals sampled from seven populations in Illinois and Kentucky); and *C. reginae* Walter (17 individuals sampled from four populations in Kentucky and West Virginia).

We attempted to sample multiple regions per species, populations per region, individuals per population, and roots per individual, though in some cases samples were limited due to conservation concerns. In most cases, sampling was extensive, as in *C. macranthon* var. *rebunense*, which was sampled in all three of its extant populations. Sampling was conducted between May and October every year from 2000 to 2006 with a total of 231 plants sampled from 90 populations in three continents. We sampled roots systematically in 1–2 cm intervals looking for morphological evidence of mycorrhizal colonization in the form of pelotons, or hyphal coils within plant root cortical cells (Rasmussen 1995). Several mycorrhizal root samples were taken per individual for DNA analysis. Only eight individuals yielded no mycorrhizal tissue, including five adult *C. japonicum* samples from one population in Ibaraki Prefecture, Japan, two *C. calceolus* seedlings from Estonia, and one adult *C. debile* from a population in Yamanashi Prefecture, Japan.

MOLECULAR METHODOLOGY

A total of 1030 DNA samples were extracted using the Qiagen DNeasy Plant Mini DNA kit (Qiagen, Inc., Valencia, CA). To assess candidate groups of mycorrhizal fungi, we polymerase chain reaction (PCR)-amplified rDNA regions widely used in constructing fungal phylogenies, restriction fragment length polymorphism (RFLP) analyzed the resulting PCR product, PCR-cloned samples showing RFLP patterns suggestive of multiple fungal endophytes, and sequenced all samples representative of the observed RFLP diversity within each population. We cast as wide a net as possible in PCR amplification by using the following primer sets for the internal transcribed spacer (hereafter, *ITS*): (1) ITS1F–ITS4 (Gardes and Bruns 1993; White et al. 1990), (2) ITS1F–ITS4B (Gardes and Bruns 1993), (3) ITS1F and ITS4-Tul (Taylor 1997a), (4) ITS1F-cNL2F (White et al. 1990), (5) ITS1OF-ITS4OF, and (6) ITS5OF-ITS4OF (primer sets 5 and 6 were developed by D. L. Taylor and will be described elsewhere; see http://mercury.bio.uaf.edu/∼lee_taylor/PCR_Primers_Orchid_Fungi.html for more details). We then PCR amplified the *mtLSU* in samples representative of the main fungal *ITS* clades with primers ML5 and ML6 (Bruns et al. 1998). To assess phylogenetic relationships among *Cypripedium* species, we PCR amplified and sequenced the plant *ITS* and *rbcL* regions for each sampled plant taxon with primers ITS1 and ITS4 (White et al. 1990) and *rbcL*-1F and *rbcL*-1360R (Kores et al. 1997), respectively. Several samples per taxon were amplified in case of within-taxon variation suggestive of cryptic species or hybrids, but no variation was found. PCR, RFLP, and PCR-cloning were conducted per Shefferson et al. (2005b). All samples in which mycorrhizal tissue was observed via compound microscope yielded fungal PCR product except for those corresponding to three *C. acaule*, one *C. debile*, four *C. montanum*, and two *C. reginae* individuals. No nonmycorrhizal samples yielded fungal PCR product.

PHYLOGENETIC RECONSTRUCTION

Sequences were aligned with ClustalX version 1.81 (Thompson et al. 1997). We excluded ambiguously aligned regions prior to analysis. Of note, we excluded both *ITS*1 and *ITS*2 from the fungal *ITS* alignment due to high divergence preventing alignment within the family Tulasnellaceae, instead focusing on the *5.8S* rDNA locus (Weiß and Oberwinkler 2001; Taylor et al. 2003; Suárez et al. 2006). This was the only locus consistently amplified for all tulasnelloid mycorrhizal samples in *Cyripedium*, and so the alignment consisted of an exhaustive sampling of mycorrhizal samples. All alignments also included reference taxa. In the fungal *5.8S* and *mtLSU* alignments, these included named fungal species in the Tulasnellaceae as well as tulasnelloid fungi associating with some *Cypripedium* species, as identified in Shefferson et al. (2005b) and Whitridge and Southworth (2004). Additionally, in the *mtLSU* alignment, we included any nontulasnelloid

mycorrhizal associates of *Cypripedium* taxa, and other basidiomycetes. In the plant *rbcL* and *ITS* alignments, we included *Mexipedium xerophyticum*, representing a genus sister to *Cypripedium* but poorly represented on GenBank. As outgroups, we included *Multiclavula* spp., *Dacrymyces chrysospermus*, and *Paphiopedilum* spp. in the fungal *5.8S*, fungal *mtLSU*, and plant *ITS* and *rbcL* alignments, respectively, per well-supported phylogenetic assessments of the Tulasnellaceae (Suárez et al. 2006), the Hymenomycetidae (Binder and Hibbett 2002; Weiß et al. 2004), and the Cypripedioideae (Cameron et al. 1999). The fungal *5.8S* and *mtLSU* alignments were 175 and 227 bp long, respectively, of which 88 and 84 bp were parsimony informative. The plant *rbcL* and *ITS* alignments were 1243 and 519 bp long, respectively, of which 29 and 180 bp were parsimony informative. We tested for incongruence between the plant *ITS* and *rbcL* phylogenies using a parsimony-based incongruence length difference (ILD) test in PAUP 4.0b10 (Swofford 2003), using 1000 test replicates.

We determined the best evolutionary model for each alignment as ranked by AIC in Modeltest version 3.7 (Posada and Crandall 1998), and conducted phylogenetic analyses as maximum-likelihood tree searches in PHYML version 2.4 (Guindon and Gascuel 2003; Guindon et al. 2005), using parameter settings as specified by Modeltest. In each analysis, a neighbor-joining tree was used as the starting tree, and we obtained one best tree and conducted 1000 maximum-likelihood bootstrap replicates to estimate support. In addition, we used fast site removal to test whether OTUs of named *Tulasnella* spp. clustered together due to long-branch attraction in our *5.8S* rDNA phylogeny (Dacks et al. 2002). Tree-Puzzle was used to assign rate categories to each site of the alignment assuming a gamma distribution with eight rate classes (Schmidt et al. 2002). Next, sites corresponding to class 8 were removed, yielding an alignment that was then analyzed phylogenetically via PHYML version 2.4 under the GTR model of evolution with four rate categories, no invariable sites, base frequencies and gamma distribution parameter estimated by the software, and 100 bootstrap replicates. We repeated this analysis three more times, but removing sites corresponding to rate classes 7 and 8, 6 through 8, and finally 5 through 8. We expected that if clade J in our fungal *5.8S* rDNA phylogeny had been an artifact of long-branch attraction (Fig. 2B), then this analysis should eventually have split these taxa up and placed them in different areas of the phylogeny, once enough rate variation had been removed.

Sequences generated in this study have been deposited in GenBank under accessions DQ925493–DQ925665 and EF370068–EF370114. Alignments and trees have been deposited in TreeBASE.

MYCORRHIZAL SPECIFICITY

To assess the evolution of mycorrhizal specificity among *Cypripedium* taxa, we first estimated mycorrhizal specificity per plant taxon as a quantitative character. Our measure, which we term "mean phylogenetic breadth" or simply "MPB," is the mean pairwise phylogenetic distance among all fungal strains identified via phylogenetic analysis as mycorrhizal hosts per plant taxon, weighted by the frequency of each association. Following Taylor et al. (2004), we first produced a matrix of pairwise phylogenetic distances among all *5.8S* fungal sequences in this study in PAUP 4.0b10 (Swofford 2003), under the same evolutionary model as used in the phylogenetic tree search in PHYML. We used these phylogenetic distances to produce pairwise distance matrices for each *Cypripedium* taxon in which each fungal haplotype was repeated per population in proportion to the numbers of individual plants associating with each corresponding fungal strain. We then repeated each proportionate set of haplotypes per population until each population had an equal number per plant taxon. Specificity per taxon was estimated as the mean of all phylogenetic distances in the lower diagonal of this matrix, resulting in a measure in which populations contributed equally to the taxon mean, while the fungal haplotypes in each population contributed according to the within-population frequency of each association. This estimate is roughly equivalent to the index of nucleotide diversity, π (Nei and Li 1979), but reflects the phylogenetic breadth of fungal associates per taxon rather than within-population genetic variation (Taylor et al. 2004). Under this metric, low MPB may be interpreted as narrow specificity, or high specialization, while high MPB may be interpreted as broad specificity, or low specialization. This measure of specificity was mapped as a continuous quantitative trait onto the best-supported *Cypripedium* phylogeny estimated in PHYML using the Ancestral State Reconstruction package in Mesquite (Maddison and Maddison 2005), using squared-change parsimony reconstruction (Maddison 1991). We repeated this same analysis with the *mtLSU* dataset, only that we substituted *5.8S* clades that did not yield *mtLSU* PCR product with their most closely related clades that yielded such product. No alternative reconstructions were considered. Standard errors for interior nodes were estimated by additive propagation of errors from the two descendants of each node, assuming independence of terms (Taylor 1997b).

TESTS OF ASSUMPTIONS

Seemingly specialized interactions may be observed in cases in which groups of symbionts are limited geographically relative to their partners. We assessed whether our results were confounded in this way by comparing the fungal groups mycorrhizal with each plant taxon in sympatric *Cypripedium* populations. We hypothesized that specialization should result in sympatric *Cypripedium* taxa associating with different sets or subsets of fungi, and compared associations via the fungal *5.8S* and *mtLSU* phylogenies. Conversely, if specificity is limited by the geographic range of the fungal symbionts, then we expected to find that sympatric *Cypripedium* populations would associate with similar fungal partners.

The use of phylogenetic distance as a measure of specificity may be biased if inadequate sampling is performed. We assessed bias due to limited sampling of both populations and individuals. To assess the former kind of bias, we performed a bootstrapping simulation using the *C. californicum* dataset, which was chosen because it was subject to the some of the most intensive sampling of any taxon and associated with a fairly wide breadth of tulasnelloid fungi. Using this dataset, we bootstrapped 100 iterations each of one through eight randomly sampled populations, and estimated MPB per iteration. Individuals per population were held constant. We then took the mean and standard error of each set of 100 samples to assess both accuracy and precision as a function of the number of populations sampled. To test bias due to limited sampling of individuals per population, we used data for two intensively sampled populations in which multiple *5.8S* strains of tulasnelloid fungi were found—one *C. reginae* population and one *C. marcanthon* var. *rebunense* population. The former population had one dominant and one minor fungal strain, while the other suggested a more or less even split in association between two fungal strains among individuals in the population. Because in both cases the number of individuals sampled was six, we enumerated all possible combinations of one through six individuals per populations and estimated population-level MPB using those combinations. We took the mean and standard error of each set of combinations to assess the potential for bias and imprecision.

Results

PLANT PHYLOGENY

The plant *rbcL* and *ITS* alignments were not incongruent (PAUP ILD test: $P = 0.896$), and so we analyzed them together. Modeltest suggested that the Tamura-Nei model of evolution with rate heterogeneity across sites and a proportion of sites invariable (TN93 + I + Γ) was the most appropriate evolutionary model for phylogenetic analysis of this alignment (Tamura and Nei 1993). Relationships among *Cypripedium* taxa were well resolved and supported by generally high bootstrap values in our combined *ITS* + *rbcL* gene-based phylogeny (Fig. 1). Our results contrast with previously published phylogenies that suggest that *C. californicum* diverged earliest (Cox 1995; Cox et al. 1997; Cribb 1997), instead supporting *C. debile* as the earliest diverging taxon.

FUNGAL ASSOCIATES

Modeltest selected the symmetrical model with rate heterogeneity across sites (SYM + Γ) as the most appropriate model of evolution for the fungal *5.8S* dataset (Zharkikh 1994). Because PHYML version 2.4 does not include this evolutionary model, we analyzed this dataset using the next best model: the general time-reversible model with rate heterogeneity across sites (GTR + Γ; Tavaré 1986). The best-supported evolutionary model in our fungal *mtLSU* dataset was the Hasegawa-Kishino-Yano model with both a proportion of sites invariable and rate heterogeneity across sites (HKY + I + Γ; Hasegawa et al. 1985).

Deeper nodes in the fungal *5.8S* tree were poorly supported, while clades at the tips had generally high support (Fig. S1). In contrast, our *mtLSU* tree supported a well-resolved pattern of relationships among *Cypripedium*-mycorrhizal fungi, members of the Tulasnellaceae, and other members of the Hymenomycetidae (Fig. S1). Two poorly supported *5.8S* clades corresponding to clades A–E and F–J correspond to the well-supported *28S* nuclear large subunit rDNA (*28S nucLSU*)-based clades snLT1 and snLT2, respectively, in a previous paper dealing with North American *Cypripedium* species (Shefferson et al. 2005b). These two large clades were also well supported and monophyletic in our *mtLSU* tree, with the exception of *5.8S* clade I, which appears on a long branch in *mtLSU* clade A (Fig. S1). *Tulasnella* spp. with *ITS* sequences available in GenBank at the time of writing all clustered within *5.8S* clade J, with almost all monophyletically clustering onto a long branch away from most *Cypripedium* associates (Fig. S1). Fast-site removal indicated that the OTUs found on this branch did not cluster together due to long-branch attraction. However, although *T. deliquescens* was supported as a member of *T. calospora* in our *5.8S* tree (Fig. S1), rendering the latter paraphyletic, it was sister to *Clavulina cristata* in our *mtLSU* tree (Fig. S1). Furthermore, both the *5.8S* and *mtLSU* trees suggested that symbionts in clades F–H may be *Tulasnella cystidiophora*. However, given that most of our generated sequences clustered away from named Tulasnellaceae accessions, we suggest that a great deal of unassessed phylogenetic diversity exists within this fungal family.

A few *Cypripedium* taxa exhibited unusual mycorrhizal patterns or occasional nontulasnelloid symbionts. *C. acaule* was the only species regularly associating with fungi other than the commonly encountered clades of fungi, yielding one *mtLSU* sequence sister to *Tulasnella tomaculum* (tulasnelloid clade J, Fig. S1) and two *mtLSU* sequences sister to *Russula laurocerasi* and (russuloid b clade, Fig. S1). One *C. formosanum* associated with *Russula* sp. as well, while one *C. reginae* individual associated with *Tulasnella deliquescens* (cantherelloid clade, Fig. S1) and another associated with *Hygrocybe cantherellus* (euagaric clade, Fig. S1). *Russula* spp. have been noted as occasional mycorrhizal partners of *C. fasciculatum* in Oregon and California (Shefferson et al. 2005b; Whitridge and Southworth 2004). Although these nontulasnelloid fungal species are rarely encountered, they nonetheless appear to be mycorrhizal associates of at least some *Cypripedium* species.

Several *Cypripedium* taxa varied ontogenetically in mycorrhizal colonization. *Cypripedium calceolus* lacked morphological evidence of mycorrhizal colonization in some seedlings but always exhibited pelotons in adults, a puzzling result since seedlings are more likely to need mycorrhizal nutrition than mature plants

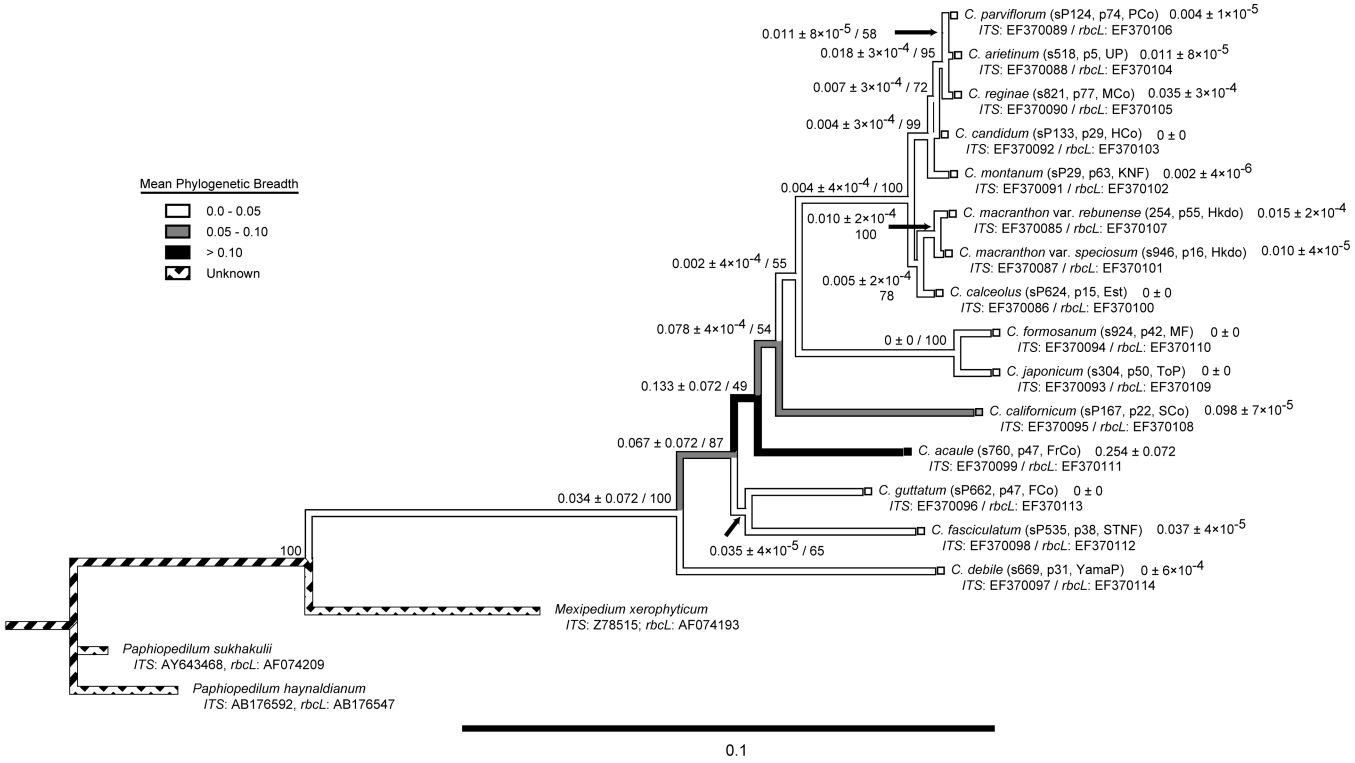

Figure 1. Combined *ITS* + *rbcL* phylogeny of sampled *Cypripedium* taxa showing the evolution of mycorrhizal specificity among *Cypripedium* taxa. Phylogeny constructed using 519 bp and 1243 bp alignments of the *ITS* (including *ITS1*, *5.8S*, and *ITS2*), and *rbcL* regions, respectively, for 18 taxa, and rooted with *Paphiopedilum haynaldianum* (*ITS*: AB176592, *rbcL*: AB176547) and *P. sukhakulii* (*ITS*: AY643468, *rbcL*: AF074209). We also included *Mexipedium xerophyticum* (*ITS*: Z78515; *rbcL*: AF074193) for greater resolution. The best tree resulting from heuristic maximum-likelihood analysis in PHYML is presented, with support values derived using 1000 bootstrap ML replicates (only values $\geq$ 50% shown). Specificity was estimated as the mean phylogenetic breadth (MPB) of fungal *ITS* sequences per plant taxon, with each sequence weighted by frequency within populations but equally among populations. Each taxon estimate was mapped onto the *Cypripedium* phylogeny as a continuous variable using the Ancestral State Reconstruction package in Mesquite (Maddison and Maddison 2005), under squared-change parsimony. Values at each node represent estimated ancestral MPB and standard error, followed by bootstrap strength as percentage. Shading in *Cypripedium* taxa corresponds to MPB value, with darker shades signifying higher values and hence wider specificity.

(Rasmussen 1995). *Cypripedium japonicum* exhibited the opposite pattern, although the rather unique tendency of this species to grow long, highly branched rhizomes may have prevented us from finding any points of colonization.

GEOGRAPHIC PATTERNS IN MYCORRHIZAL ASSOCIATIONS

Cypripedium mycorrhizal specificity does not appear to be limited by fungal distribution. Sympatric populations generally associated with different sets of mycorrhizal fungal partners, suggesting real partner choice and genetically controlled specialization. *Cypripedium candidum* and *C. parviflorum* were sampled in sympatric populations in Illinois and Kentucky. At the Hardin County, Kentucky, sites, the former associated with fungal *5.8S* clade G while the latter associated with clade A (Fig. S1), two groups likely to be different fungal species. Likewise, *C. candidum* in Lake County, Illinois., associated with clade G, while *C. parviflorum* associated with both clades E and G (Fig. S1). Similar patterns hold for species occurring in nearby sites. In the Klamath National Forest, *C. fasciculatum* associated only with *5.8S* clade C while *C. montanum* associated with clades C, D, E, and G (Fig. S1). In the Plumas National Forest, *C. fasciculatum* still associated only with clade C, but *C. montanum* associated with clades C and G.

Most *5.8S* clades of fungi occurred over multiple geographic regions, and many appeared holarctic. Clade A, for example, is found in North America, Europe, and Asia (Fig. S1). Clades C, D, F, and I appear to follow a northern hemisphere "Ring of Fire" distribution, although their closest relatives are also present outside of that range, most notably including the central and/or eastern United States. However, given that most geographic regions and potential substrates have not been sampled for *Tulasnella* spp., we cannot draw any conclusions about limits to these distributions of particular tulasnelloid clades.

EVOLUTION OF MYCORRHIZAL ASSOCIATIONS

Parsimony reconstruction of mycorrhizal specificity suggested that the most recent common ancestor of genus *Cypripedium* likely associated with a low-to-intermediate breadth of mycorrhizal fungi (i.e., narrow-to-intermediate specificity). Shortly after the divergence of *C. debile*, specificity broadened and led to the broadest associations in *C. acaule* and *C. californicum* (Fig. 1). However, after *C. californicum* diverged, specificity narrowed yet again, leading to far narrower associations in the remaining taxa than had yet evolved, with slight rebroadening occurring in *C. montanum*, *C. parviflorum*, and *C. reginae*. Because of high variability in the MPB of *C. acaule*, specificity reconstructions at the deepest nodes in the tree were most uncertain. Our *5.8S* and *mtLSU*-based metrics agreed on all these points with two exceptions: (1) *5.8S*-based specificity suggested that *C. californicum* associates with the broadest range of fungi, while *mtLSU*-based specificity suggested that *C. acaule* is the least specific, and (2) *5.8S*-based specificity suggested that *C. montanum* rebroadened to include a greater breadth of mycorrhizal fungi than *C. reginae*, while *mtLSU*-based specificity suggested the opposite.

Bootstrapping populations revealed that sampling few populations of a *Cypripedium* taxon does not lead to biased estimates of MPB. However, MPB may be estimated imprecisely in taxa in which fewer than three populations were sampled (Fig. 2). Furthermore, although MPB was not biased by limited sampling of individuals, standard errors of MPB were roughly equivalent to or higher than mean MPB in situations in which the number of individuals sampled was two or less in the case of *C. macranthon* var. *rebunense*, and three or less in the case of *C. reginae* (Fig. 3). Due to conservation concerns (e.g., population composed of five or fewer individuals), we sampled only one or two individuals in approximately 43% of the 90 populations in this study, and three individuals from a further 19%. We suggest that although limited sampling characterized most of the populations that we included in this study, this imprecision is most likely to affect taxon-level estimates of MPB for taxa in which few populations were sampled. Thus, our estimate of MPB is questionable only for *C. formosanum*, considering that we only sampled two individuals of one population.

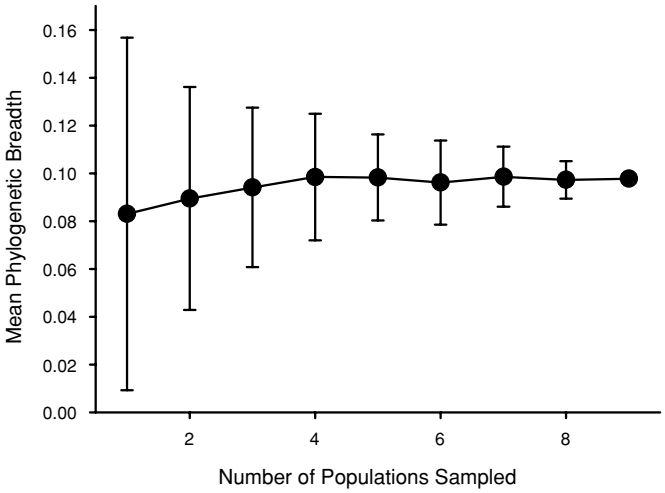

Figure 2. Accuracy and precision of mean phylogenetic breadth (MPB) estimates as a function of the number of populations sampled. Values represent overall means of 100 bootstrapped combinations of two through eight populations from the *C. californicum* dataset, with number of individuals held constant in each population. Error bars denote standard error, estimated as the standard deviation of sample means.

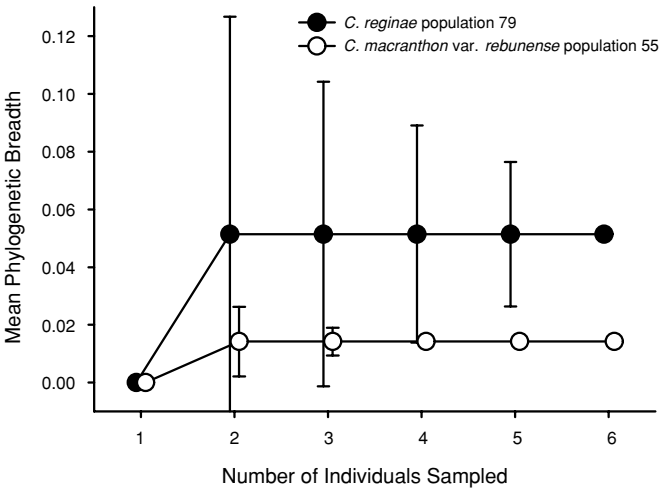

Figure 3. Accuracy and precision of population-level mean phylogenetic breadth (MPB) estimates as a function of the number of individuals sampled per population. Values represent overall means of all enumerated combinations of one through six individuals in two populations in which six individuals each were sampled. Error bars denote standard error, estimated as the standard deviation of sample means.

Discussion

Narrow mycorrhizal specificity in *Cypripedium* does not appear to be due to a lack of opportunity to associate with other fungal hosts. Specialization, and hence narrow specificity, may sometimes be inferred in cases in which potential symbiotic hosts are not encountered because they are absent from a certain geographic region (Euzet and Combes 1980). Alternatively, hosts may be present and encountered, but may not be biologically compatible. In our case, the presence of potential fungal hosts can be inferred because sympatric populations of *Cypripedium* taxa with similar ecologies generally associated with different fungal hosts. Also, the most widespread plant species in this study, *C. calceolus*, was also among the most narrowly specific (Fig. 1), in striking contrast to rare taxa such as *C. macranthon* var. *rebunense*

and *C. californicum*, which appeared less specialized. Furthermore, most of our *5.8S* clades of fungi were found in much of the Northern Hemisphere, rather than being constrained to any small region.

Mycorrhizal specificity in *Cypripedium* is similar to that in nonphotosynthetic plants. Nonphotosynthetic plant species commonly form mycorrhizae with only one fungal lineage each (Bidartondo 2005), but also occasionally expand their host breadth to associate with closely related fungal lineages. Genetic variation at key loci correlates closely with mycorrhizal fungal host in such plants (Bidartondo and Bruns 2002; Taylor et al. 2004), suggesting that choice of fungal hosts and mycorrhizal specificity are plant traits on which natural selection may be able to act (Bruns et al. 2002). Certainly, fungal host shifts have occurred among *Cypripedium* taxa, leading to differing sets of mycorrhizal tulasnelloid associates even among closely related taxa occurring in sympatry. If natural selection also acts on mycorrhizal specificity, then, as in nonphotosynthetic plants, specificity may be a function of need for resources that the mycorrhizal symbiosis can provide. However, this does not imply that changing mycorrhizal specificity may drive speciation in this genus, as specificity is generally similar among closely related taxa.

MYCORRHIZAL SPECIFICITY AND RESOURCE NEEDS

In most mature plants, growth is limited by either phosphorus or nitrogen, and mycorrhizal fungi greatly expand the plant's ability to forage these elements (Smith and Read 1997). However, in the case of *Cypripedium* mycorrhizae, carbon is likely to be provided by the fungus as well. Achlorophylous plants are thought to specialize on mycorrhizal fungi that provide them with the best flow of carbon (Bruns et al. 2002), undoubtedly their most limiting nutrient. Because *Cypripedium* species are, like other orchid species, generally mycotrophic as seedlings (Rasmussen 1995), narrow specificity in adult, photosynthetic plants may be an ontogenetic relic of early-life resource needs and specialization (McCormick et al. 2006). Orchid protocorms directly manipulate their host fungi when mycorrhizal contact is initiated (Rasmussen 1995). Such manipulation implies adaptation to the fungal host and suggests an adaptive advantage to narrow specificity (Thompson 1994), a similar rationale used to explain narrow specificity in mycorrhizal interactions in genus *Monotropa* (Bidartondo 2005).

The noted tendency of adult *Cypripedium* plants to forego sprouting and photosynthesis for years at a time suggests that fungal carbon may still be needed during adulthood (Gill 1989; Shefferson et al. 2001; Kull 2002;). Dormancy has been recorded more often in genus *Cypripedium* than in any other plant genus, and has been noted in *C. acaule* (Primack and Stacy 1998), *C. calceolus* (Kull 1995), *C. candidum* (Falb and Leopold 1993; Shefferson 2006), *C. macranthon* var. *rebunense* (T. Kawahara unpublished data), *C. parviflorum* (Shefferson et al. 2001), and *C. reginae* (Kéry and Gregg 2004). Recent evidence suggests that this phenomenon may adaptively benefit the plant by buffering survival against environmental stress (Shefferson et al. 2005a). How energy needs are met during times of dormancy is unknown, although recent evidence of ectomycorrhizal carbon and nitrogen isotope signatures in mature orchids indirectly supports the mycorrhiza as a potentially important carbon source (Gebauer and Meyer 2003; Bidartondo et al. 2004), as does recent experimental evidence showing both plant-to-fungus and fungus-to-plant carbon transfer in the photosynthetic orchid *Goodyera repens* (Cameron et al. 2006). Fungal carbon may be most necessary in taxa in which dormancy is most common. However, with few long-term demographic datasets available for much of this genus, it is not possible at present to tell whether any aspect of dormancy correlates with mycorrhizal association or specificity.

UNRESOLVED ISSUES

Genus *Cypripedium* is perhaps the most well-known orchid genus in subfamily Cypripedioideae, a monophyletic clade that diverged early in the evolution of the Orchidaceae (Cameron et al. 1999). The basalmost subfamily, Apostasioideae, composed of a small group of globally endangered plants, has been noted to associate with fungal families Tulasnellaceae and Ceratobasidiaceae (Kristiansen et al. 2004), but generally cannot be destructively sampled due to conservation concerns. Thus, the evolutionary history of the mycorrhiza in subfamily Cypripedioideae is important in understanding the evolutionary origins of the orchid mycorrhiza. In our study, we were not able to sample roughly two-thirds of the genus *Cypripedium*, of which many occur in difficult to access parts of Asia, particularly China and Siberia. Furthermore, few species exist of genera such as *Mexipedium* and *Selenipedium*, which are the closest relatives of *Cypripedium*, and these species are all rare (Cribb 1997). Genus *Paphiopedilum*, the only other major genus in the subfamily, is species-rich but is of extreme conservation concern due to overharvesting. We hope that further research on these groups can occur, but suggest that it may not be possible due to declining populations worldwide.

We also suggest a need to rigorously assess mycorrhizal specificity from the standpoint of the fungal partners, in this case orchid mycorrhizal members of the family Tulasnellaceae. Until recently, this family was thought to be almost entirely saprotrophic (Roberts 1999), but a recent discovery that some members of this family are ectomycorrhizal and also form jungermannioid mycorrhizae suggests that much of the ecology of this family is poorly understood (Bidartondo et al. 2003; Kottke et al. 2003). The cryptic nature of this fungus no doubt contributes to the relative lack of knowledge of its biology, and may explain why new species are still being discovered (Suárez et al. 2006).

Conclusions

In conclusion, we have shown that mycorrhizal specificity appears to be an evolving trait in one genus of plants, *Cypripedium*. Although specificity is narrow throughout much of the genus, a broadening has occurred in the past, and several species appear to be undergoing a broadening of their phylogenetic breadth of mycorrhizal associations, though not to the same extent as noted in basal taxa. Furthermore, host shifts appear to be relatively common. Last, our results do not appear to be the result of any bias caused by the absence of potential fungal partners, as sympatric *Cypripedium* taxa generally associated with differing sets of mycorrhizal fungi. We hope that further research will address whether mycorrhizal specificity is a trait subject to contemporary evolution by natural selection in the plants as well as the fungi.

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Supplementary Material

The following supplementary material is available for this article:

Figure S1. Phylogenetic relationships among fungi mycorrhizal with *Cypripedium* taxa and their relationships to other basidiomycetes.

This material is available as part of the online article from:
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