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Evolution of host breadth in broad interactions: mycorrhizal specificity in East Asian and North American rattlesnake plantains (*Goodyera* spp.) and their fungal hosts

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Abstract

Host breadth is often assumed to have no evolutionary significance in broad interactions because of the lack of cophylogenetic patterns between interacting species. Nonetheless, the breadth and suite of hosts utilized by one species may have adaptive value, particularly if it underlies a common ecological niche among hosts. Here, we present a preliminary assessment of the evolution of mycorrhizal specificity in 12 closely related orchid species (genera *Goodyera* and *Hetaeria*) using DNA-based methods. We mapped specificity onto a plant phylogeny that we estimated to infer the evolutionary history of the mycorrhiza from the plant perspective, and hypothesized that phylogeny would explain a significant portion of the variance in specificity of plants on their host fungi. Sampled plants overwhelmingly associated with genus *Ceratobasidium*, but also occasionally with some ascomycetes. Ancestral mycorrhizal specificity was narrow in the orchids, and broadened rarely as *Goodyera* speciated. Statistical tests of phylogenetic inertia suggested some support for specificity varying with increasing phylogenetic distance, though only when the phylogenetic distance between suites of fungi interacting with each plant taxon were taken into account. These patterns suggest a role for phylogenetic conservatism in maintaining suits of fungal hosts among plants. We stress the evolutionary importance of host breadth in these organisms, and suggest that even generalists are likely to be constrained evolutionarily to maintaining associations with their symbionts.

Keywords: Ceratobasidiaceae, *Ceratobasidium*, mycorrhiza, parasitic fungi, specificity

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Introduction

Phylogenetic patterns in the evolution of biological interactions are often studied in relation to whether they suggest cospeciation. In the simplest case, cospeciation is observed as cophylogeny between suites of interacting taxa, and can involve either a common evolutionary response to external factors, or a reciprocal evolutionary response (Brooks & McLennan 1991). Importantly, such analyses assume that the breadth of the interaction is only one species. For example, mammals may exhibit one-species-to-one-species relationships with body lice, leading to phylogenetic patterns in hosts and parasites (Hafner *et al*. 2003). Broad interactions cannot be studied readily from a cophylogenetic standpoint using contemporary methods, and so they have rarely been studied phylogenetically.

The evolutionary history of broad interactions may best be approached by quantifying specificity, or host

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breadth, in interacting clades. Phylogenetic approaches are often utilized to understand host specialization (Janz & Nylin 1998; Anderson 2006), and have recently been adapted to the study of communities (Lozupone *et al.* 2006; Hardy & Senterre 2007; Pommier *et al.* 2009). In broad interactions, the identities of interactors as well as the breadth of the interaction may change, resulting in the evolution of both parameters (Weiblen *et al.* 2006; Shefferson *et al.* 2007), the latter being quantitative rather than nominal. Such patterns may be influenced by different geographic and ecological distributions, resulting in geographic mosaics of interacting suites of species (Thompson 2009). When measured quantitatively, specificity may be mapped onto the phylogenies of all interactors and common patterns of evolution in specificity may be assessed.

This approach offers great promise in evolutionary studies of the mycorrhiza, a symbiosis based on nutrient exchange between terrestrial plants and soil fungi involving a polyphyletic group of taxa in both kingdoms (Smith & Read 2008), as well as in other horizontally transmitted microbial symbioses. The orchid mycorrhiza is a form of this interaction found in family Orchidaceae, the most species-rich family of flowering plants, and is unique because of its morphology and because it is thought to be typically parasitic—the plant obtains nutrients from the fungus, but little evidence exists of the reverse (Rasmussen 1995). The fungi forming these associations typically make their livings in other ways—many are saprotrophs living off organic matter in the soil, and others are typically ectomycorrhizal with other plants while others are plant parasites (Roberts 1999; Yamato *et al.* 2005). Many orchid species have evolved into purely parasitic, non-photosynthetic forms, living entirely off of fungal carbon (Taylor & Bruns 1997; Bidartondo 2005; Ogura-Tsujita *et al.* 2009), although recent evidence suggests that some *Goodyera* spp. may act more mutualistically (Cameron *et al.* 2008; Hynson *et al.* 2009). This purported family wide parasitism has generated much interest in the mycorrhizal specificity of orchids. DNA-based analyses of orchid mycorrhizae have revealed that orchids can be specialists or generalists, and all shades in between (Taylor & Bruns 1997; Weiß *et al.* 2004; Shefferson *et al.* 2005; Abadie *et al.* 2006). However, although the specificity of orchids for their fungi has been studied for many decades now, the macroevolutionary history of specificity has been ignored. The reasons are twofold: first, the fungi are unlikely to have evolved in response to the orchids because of the likely rarity of the interaction from the fungal standpoint, and second, both basal and derived orchids typically associate with at least three families of fungi, Ceratobasidiaceae, Sebacinaceae, and Tulasnellaceae (Yukawa *et al.* 2009). However, we argue that phylogenetic measures of specificity are far more informative than simple counts of host families (Taylor *et al.* 2004; Shefferson *et al.* 2007).

Here, we assess the evolution of mycorrhizal specificity in the rattlesnake plantain orchids (genus *Goodyera*, with genus *Hetaeria* used as an outgroup). This genus presents an interesting case study in the ecology of the orchid mycorrhiza because one species, *Goodyera repens*, has long been a subject of experimental research into the nature of the orchid mycorrhiza (Downie 1943; Hadley & Purves 1974; Alexander *et al.* 1984; Cameron *et al.* 2006). First, we identify the fungi mycorrhizal with these orchids. We then estimate mycorrhizal specificity as the mean pairwise phylogenetic distance among suites of fungi interacting with each orchid. Next, we map this quantity onto a phylogeny of the genus *Goodyera*, and assess whether specificity is partially determined by phylogeny.

Materials and methods

Study system

The genus *Goodyera* is a member of family Orchidaceae that includes approximately 80–100 species distributed primarily throughout the Northern Hemisphere, with highest diversity in tropical eastern Asia (Satake *et al.* 1985; Ormerod & Cribb 2003). These species are rhizomatous, terrestrial perennials, but can grow onto the bark of trees and rock faces in parts of East Asia. Ten *Goodyera* and two species of the closely related genus *Hetaeria* were sampled for this study (Table 1). Like most orchids, these species are typically rare, with small, disparate populations even in species with widespread distributions. Our choices in study species represents the suite of species within the genus that we could readily access geographically, and that were possible to access given conservation concern for rare orchids, and given the often difficult international politics governing work with rare and endangered plants. Our sampling thus reflects a balance between a need for study material, the need to preserve extant populations and species, and the difficulty of sampling the wide geographic range and taxonomic diversity of the group.

Goodyera foliosa (Lindl.) Benth. is found throughout Japan extending to Okinawa, and on the Korean Peninsula. *G. hachijoensis* Yatabe is found primarily in central Japan. *G. macrantha* Maxim. is found from central to southern Japan and on the Korean Peninsula. *G. oblongifolia* is found in western North America. *G. pendula* Maxim. is found in northern and central Japan. *G. procera* (Ker-Gawler) Hook. is found in southern Japan, China, India, and Malaysia. *G. repens* (L.) R.Br. is found throughout the Northern Hemisphere, even extending

Table 1 List of surveyed *Goodyera* species, regions and locales sampled, years harvested, and numbers of populations and individuals sampled at each locale. Numbers in parentheses refer to the number of plants yielding PCR product with fungal nucLSU or mtLSU primers. Asterisks (*) indicate species to which we added fungal haplotype data from other studies in order to compensate for low sampling in our study

Species	Country	Region	Year sampled	No. Pops sampled	No. plants sampled
G. foliosa var. *laevis*	Japan	Asahikawa, Hokkaido	2005	1	1
		Izu Archipelago	2005	4	12
		Kyoto City	2005	1	1
var. *maximowicziana*		Chiba Prefecture	2005	2	11
		Tochigi prefecture	2005	1	14
G. hachijoensis var. *hachijoensis*	Japan	Izu Archipelago	2005–2006	6	10
var. *izuhsmensis*		Izu Archipelago	2005–2006	1	2
var. *matsumurana*		Amami Oshima, Kyushu	2005	1	1
G. macrantha	Japan	Tochigi prefecture	2005	1	2
G. oblongifolia	USA	Columbia Gorge, Oregon	2003	2	2
		Klamath NF, California	2008	2	6
		Priest Lake, Idaho	1998	1	1
G. pendula	Japan	Kochi, Shikoku	2005	1	1
G. procera	Japan	Amami Oshima, Kyushu	2005	5	8
*G. repens**	USA	Nelson County, Virginia	2001	1	2
G. schlechtendaliana	Japan	Chiba prefecture	2005	1	1
		Izu Archipelago	2005	1	1
		Mt. Tsukuba, Ibaraki	2005	1	1
G. tesselata	USA	Massachusetts	2002	3	5
G. velutina	Japan	Izu Archipelago	2005	3	8
Hetaeria cristata	Japan	Izu Archipelago	2005	2	2
H. agyokuna	Japan	Izu Archipelago	2005	1	2
Totals				42	94

to northern tropical Africa. *G. schlechtendaliana* Reichb. fil. is found throughout Japan, the Korean Peninsula, and in eastern China. *G. tesselata* is found in eastern North America. *G. velutina* Maxim. is found in southern Japan and the Korean Peninsula. *Hetaeria cristata* Blume is found in central and southern Japan, Indonesia, and Taiwan. *Hetaeria agyokuna* (Fukuyama) Nackejima is in southern Japan and in Taiwan.

Field methods

Sampling occurred from spring 2002 until summer 2007. We obtained locations of target populations from local experts, landowners, and land managers, and visited sites throughout Japan and the USA. At each site, we chose plants representing a range of life stages, from small, vegetative sprouts to large, flowering individuals. Between two and six roots were sampled per plant, including 408 root samples from 94 individuals in 42 populations (Table 1). The total number of plants sampled was kept at no more than 10% of each sampled population due to conservation concern. All root samples were kept on ice in the field, and were transported to the laboratory for microscopy and DNA extraction within four days of field sampling.

Laboratory methods

All roots were surface-sterilized using 20% bleach solution (Taylor & Bruns 1997). Light microscopy was used to identify mycorrhizal samples, and four to five samples of roughly 0.5–1.0 cm in length were chosen per plant. Characterization of mycorrhizal fungi involved: (i) extraction of fungal and plant DNA from mycorrhizal plant tissue; (ii) amplification of fungal genomic regions useful in determining fungal identity; (iii) assessment of basic patterns in fungal diversity within roots, individuals, populations, and species; (iv) DNA sequencing of unique strains; and (v) phylogenetic analysis for identification of mycorrhizal fungi and assessment of specificity. Details of laboratory methods are provided in Shefferson *et al.* (2005, 2007). We included root tissue samples not colonized by mycorrhizal fungi to provide negative controls. We tested each sample with each of the following sets of primers targeting the internal transcribed spacer (ITS): ITS1F-ITS4 (White *et al.* 1990; Gardes & Bruns 1993), ITS1F-cNL2F (White *et al.* 1990), ITS1-ITS4B (Gardes & Bruns 1993), and ITS1OF-ITS4OF (Taylor & McCormick 2008). Some samples were also tested with ITS1–ITS4Tul (Taylor 1997), although this was limited to samples that failed to

amplify via other primers and a few others, and did not yield any PCR product not reported for other primer sets. PCR involved 35 cycles with an annealing temperature of 55 °C using an Eppendorf Mastercycler epGradient S Thermocycler (Eppendorf AG, Hamburg, Germany), and all species yielded fungal PCR product except *G. macrantha*. Although we attempted to amplify the mitochondrial large subunit (mtLSU) using primers ML5–ML6 (White *et al.* 1990), these PCRs were unsuccessful. Representative samples were chosen for each plant via RFLP analysis of ITS PCR product using the restriction enzymes *Dde*I, *Hin*fI, and either *Mbo*I or *Nla*III (Gardes & Bruns 1996). The ITS and rbcL regions from each plant species were also amplified via the primers ITS1P–ITS4 (White *et al.* 1990; Taylor & Bruns 1997) and rbcL1F–rbcL1367R (Kores *et al.* 1997), respectively. PCR cloning was performed with Stratagene XL-10 Gold Ultracompetent cells (Stratagene Inc., La Jolla, CA, USA) and the pDrive cloning vector (Qiagen Inc.) when RFLP analysis suggested the presence of multiple fungi. Clones representative of the major RFLP-types were chosen for sequencing. We cycle sequenced unique PCR samples with BigDye v. 3.1 chemistry (Applied Biosystems Inc., Foster City, CA, USA), and electrophoresed each sample on an ABI 3730 Genetic Analyzer (Applied Biosystems Inc.) at the DNA Synthesis and Sequencing Facility (University of Georgia).

Phylogenetic analysis

Sequences were edited in ChromasPro 1.5 for Windows (Technelysium Pty. Ltd, Tewantin, Queensland, Australia) and analyzed with BLAST (Altschul *et al.* 1997) against the NCBI sequence database (National Center for Biotechnology Information, GenBank: http://www.ncbi.nlm.nih.gov) to detect similar sequences of known phylogenetic placement. We then confirmed BLAST designation via phylogenetic analysis in a fungal ITS alignment representing the major groups of basidiomycetes and ascomycetes (Taylor & Bruns 1997). Further analyses involved adding sequences to alignments representing narrower phylogenetic breadth, with reference sequences imported from GenBank. Sequences were aligned using ClustalX 2.0.11 for Windows XP (Thompson *et al.* 1997; Larkin *et al.* 2007). The appropriate model of DNA evolution was determined using FindModel (http://www.hiv.lanl.gov/content/sequence/findmodel/findmodel.html; Posada & Crandall 1998). Phylogenetic analysis involved maximum likelihood searches in PhyML for Windows XP (Guindon & Gascuel 2003; Guindon *et al.* 2005; Ansimova & Gascuel 2006), using the best model of DNA evolution as chosen by FindModel. Branch support was estimated via 1000 maximum likelihood replicates. Rarely encountered fungi with strong BLAST support were not phylogenetically analyzed, though they are presented with BLAST results in this paper. Plant ITS and rbcL sequences were also analyzed as above, with phylogenetic analysis proceeding on both loci together. Sequences generated in this study have been deposited in GenBank under accessions HM140988–HM141077, and HM151401–HM151402. Phylogenetic trees and alignments have been deposited on TreeBASE.

Analysis of specificity

Per Taylor *et al.* (2004), we quantified specificity as the mean pairwise phylogenetic distance, π (Nei & Tajima 1981), among fungal haplotypes corresponding to unique species or major clades identified in phylogenetic analysis, using Arlequin 3.11 for Windows (Excoffier *et al.* 2005). All fungal haplotypes found within each orchid taxon were pooled to estimate π, and we did not treat haplotypes originating from the same sample differently than we treated haplotypes from other samples within the same taxon. We used only the fungal 5.8S region in order to include the broadest assemblage of fungi for each plant species, including ascomycetes and basidiomycetes. We added π for *G. pubescens* from fungal data taken from McCormick *et al.* (2004). We also added a *Ceratobasidium cornigerum* haplotype to *G. repens*, based on previous reports suggesting it to be a common symbiont of that orchid species (Alexander & Hadley 1985; Cameron *et al.* 2006). We mapped these quantities via least squares onto the plant phylogeny using the *ape* package in R (Paradis 2006; R Development Core Team 2007). We ran this analysis twice, with π for *G. macrantha* equalling 1 (narrow specificity) or 15 (broad specificity) to compensate for the lack of successful PCR from this species, but found no difference in evolutionary patterns so only present the former result. We assessed whether plant phylogeny determines mycorrhizal specificity in two ways. First, we tested for phylogenetic autocorrelation in specificity using Geary's randomization approach to Moran's autocorrelation index using the *ape* package in R (Gittleman & Kot 1990; Thioulouse *et al.* 1995; Paradis 2006). Second, we regressed the mean pairwise phylogenetic distance between suites of fungi associating with each plant taxon as a function of the plant phylogenetic distance, with phylogenetic distances estimated in Arlequin 3.11 for Windows (Excoffier *et al.* 2005). The latter analysis differed from the former in that the former tested whether the quantitative value of specificity itself varied with plant phylogenetic distance, while the latter tested the degree to which the phylogenetic distance between the suites of hosts associating with each plant taxon varied with plant phylogenetic distance.

The number of sampled plants per population and populations per taxon varied in this study (Table 1). We first assessed whether these inequalities may have affected our results via regression analyses of mean fungal π per orchid taxon as a function of the number of populations per species and mean individuals per population. All analyses were conducted as general linear models in PASW Statistics 17.0 for Windows (SPSS Inc., Chicago, IL, USA). Because a number of taxa could only be sampled in low quantities (e.g., < 3 individuals), we also tested whether this low sampling may have biased our specificity towards narrow host breadth. To do so, we characterized the frequencies of the number of fungal haplotypes found per individual of the most widely sampled taxon that exhibited wide specificity, *G. foliosa* var. *maximowicziana*. We then created bootstrapped datasets representing random draws from the fungal haplotypes found in this taxon, with the number of haplotypes per individual chosen according to the frequencies of fungal haplotypes per individual in this taxon. Each dataset corresponding to each number of sampled individuals included 100 replicates. This bootstrapped dataset was created in C++ and was used as input in Arlequin 3.11 for Windows (Excoffier *et al*. 2005). Specificity for each replicate in each dataset was estimated as before, and we estimated the mean π and associated standard error for each dataset. We then assessed the minimum number of individuals needed to accurately assess specificity in that taxon as the point at which mean π no longer increased with increasing number of sampled individuals.

Results

Fungal identification

FindModel suggested that the most appropriate model of DNA evolution in our phylogeny of the largest clade of *Goodyera* mycorrhizal fungi (Ceratobasidiaceae) was the HKY + Γ model. In our phylogeny of the next most common fungal associates, within the Ascomycota, it was the GTR + Γ model. FindModel further suggested that the most appropriate model of DNA evolution in our phylogeny of *Goodyera* and *Hetaeria* species was the HKY model. Bootstrap support was low deep within our main phylogenies, but fairly strong closer to the tips (Figs 1 and 3).

Goodyera species associated overwhelmingly with species in the fungal family Ceratobasidiaceae, but also with occasional fungi in other families (Table S1, Supporting information). *G. foliosa* associated with *Ceratobasidium papillatum* or a close relative, as well as unnamed *Ceratobasidium* taxa sister to *C. angustisporum* (Fig. 1). *G. hachijoensis* associated with *C. cornigerum*, a fungus potentially identified as *C. albasitensis*, and a fungus near *C. angustisporum* (Fig. 1). *G. oblongifoilia* associated with fungi near *C. albasitensis*, *C. bicorne*, and *C. angustisporum* (Fig. 1). *G. pendula* associated with *C. cornigerum* and fungi near *C. angustisporum* (Fig. 1). *G. procera* associated with *C. cornigerum*. *G. repens*, *G. tesselata*, and *G. velutina* associated with fungi falling near *C. angustisporum*. *G. schlechtendaliana* associated with these same groups, as well as fungi falling near *C. papillatum* and *C. oryzae-sativae* (Fig. 1). Of these three, *G. repens*' associate was surprising given its occurrence away from *C. cornigerum*, which was previously noted to be its main symbiont. *Hetaeria cristata* and *H. agyokuna* both associated only with *Ceratobasidium cornigerum* (Fig. 1). Additionally, *G. foliosa*, *G. hachijoensis*, *G. procera*, and *G. velutina* had sporadic associations with potentially mycorrhizal ascomycetous endophytes falling near *Phialophora finlandia* and *Chalara dualis* (Fig. S1, Supporting information; Table S2, Supporting information). *G. velutina* also rarely associated with potentially ectomycorrhizal associates falling into genera *Russula* and *Clavulina* (Table S2, Supporting information).

Mycorrhizal specificity

Assessed as the mean pairwise phylogenetic distance among all fungal haplotypes, including Ceratobasidiaceae, ascomycetes, and all other potentially mycorrhizal fungi, specificity did not vary with sampling effort. A general linear model of π as a function of the number of populations and plants per population sampled suggested that both factors did not account for a significant share of the variation in π (populations: $F_{3,3} = 0.421$, $P = 0.752$; plants per population: $F_{6,3} = 1.759$, $P = 0.344$). Further, bootstrap analysis of the *G. foliosa* var. *maximowicziana* dataset suggested that samples of two individuals were the minimum needed to maximize estimated specificity in this broadly associating orchid (Fig. 2), most likely due to the tendency for this species to be colonized by multiple mycorrhizal fungi (mean number of mycorrhizal fungi per individual = 1.85 ± 0.15 haplotypes).

Assessment of the phylogenetic contribution to mycorrhizal specificity was equivocal but suggestive. Geary's randomization test yielded a low Moran's *I*, and was not statistically significant ($I = -0.094$, $P = 0.318$). Mean pairwise phylogenetic distance between the suites of fungi associating with each plant species was significantly determined by phylogenetic distance among plant taxa ($F_{68,35} = 791.1$, $P < 0.001$). The ancestral condition appears to have been narrow specificity (Fig. 3). A broadening of host breadth occurred after the speciation of *G. oblongifolia*, with extremely broad specificity observed in *G. procera* and

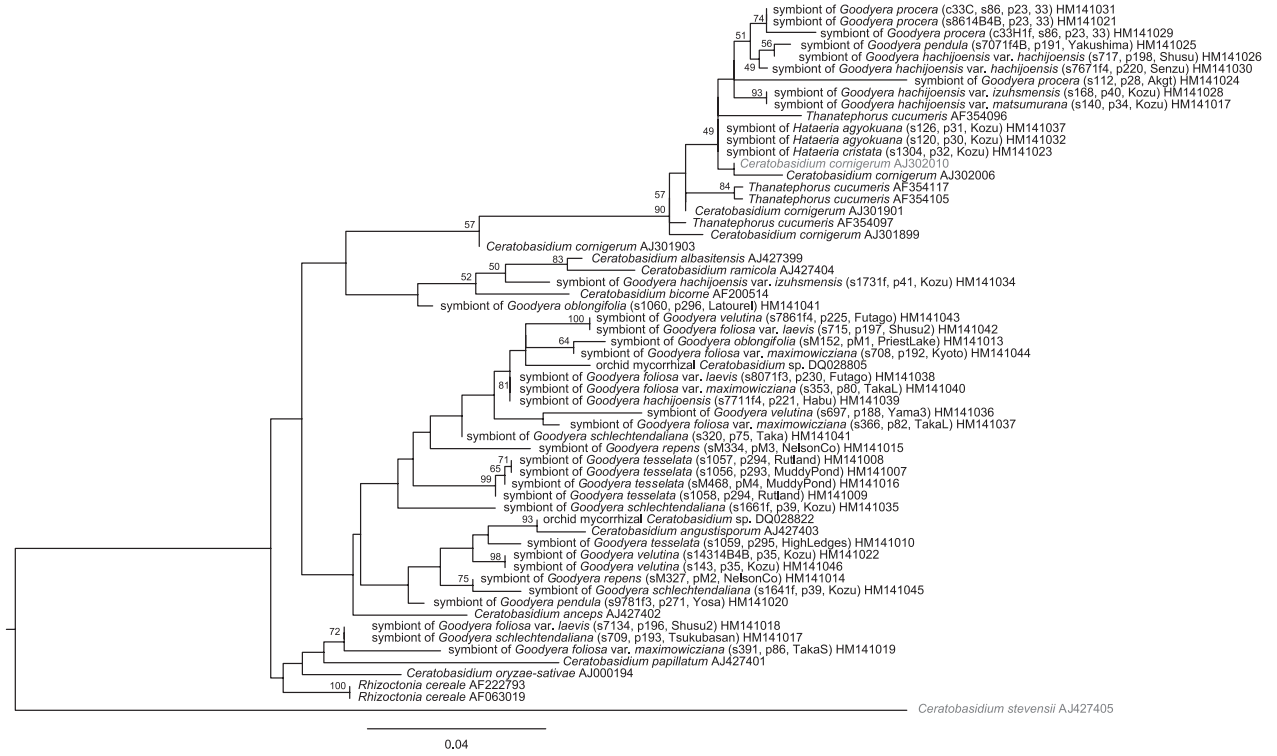

Fig. 1 Phylogenetic placement of fungal taxa in the family Ceratobasidiaceae mycorrhizal with *Goodyera* species. Phylogeny determined with sequences from the fungal internal transcribed spacer (ITS) region, and includes reference sequences from NCBI GenBank. Analysis was via maximum likelihood in PHYML for Windows (Guindon & Gascuel 2003; Guindon *et al.* 2005), and involved 1000 bootstrap replicates. Phylogeny is midpoint-rooted, due to the lack of agreement on the evolution of the members of the family Ceratobasidiaceae.

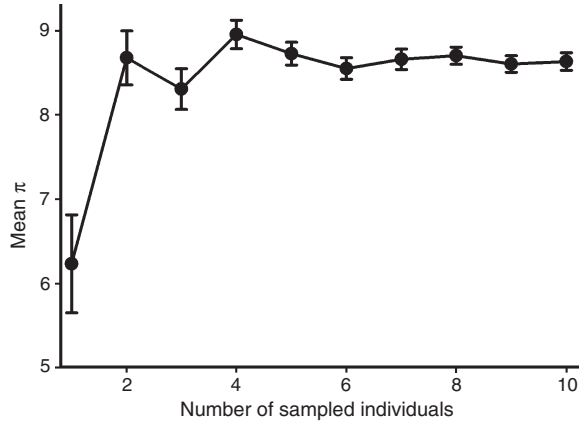

Fig. 2 Assessment of bias in specificity estimates as a function of the number of *Goodyera* individuals sampled. Data from sampled *G. foliosa* var. *maximowicziana* were used. We bootstrapped 'individuals'' of this taxon using a probabilistic assessment of the number of fungal haplotypes per individual (0.30 probability of one fungal haplotype, 0.55 of two, and 0.15 of three), and random draws with replacement from the pool of all sampled fungal haplotypes discovered in this taxon. One hundred such replicates were bootstrapped for each number of sampled individuals, and the mean π and associated standard error for each group of 100 replicates was estimated in Arlequin 3.11 for Windows (Excoffier *et al.* 2005).

the *G. foliosa* clade also evolving relative generalization. Specificity then renarrowed in the *G. hachijoensis* group (Fig. 3).

Discussion

Mycorrhizal specificity appears correlated with phylogeny in this system, and so macroevolutionary history is an important consideration determining the observed pairing of plant and fungus in the orchid mycorrhiza. This study is among the first to suggest that suites of symbiotic hosts evolve to differ more with increasing phylogenetic distance. These patterns indicate a role for phylogenetic conservatism in determining which fungal species form mycorrhizas with plants, as it does in determining food webs (Cattin *et al.* 2004). Theoretically, this may stem from the fact that symbiotic hosts often form a kind of habitat or niche for their partner taxa, and phylogenetic conservatism is typically thought of in the determination of niche (Wiens & Graham 2005; Lovette & Hochachka 2006).

Quantitatively, the mycorrhizal specificity we observed in the plant hosts appears typical of orchid mycorrhizal associations. For example, previous assess-

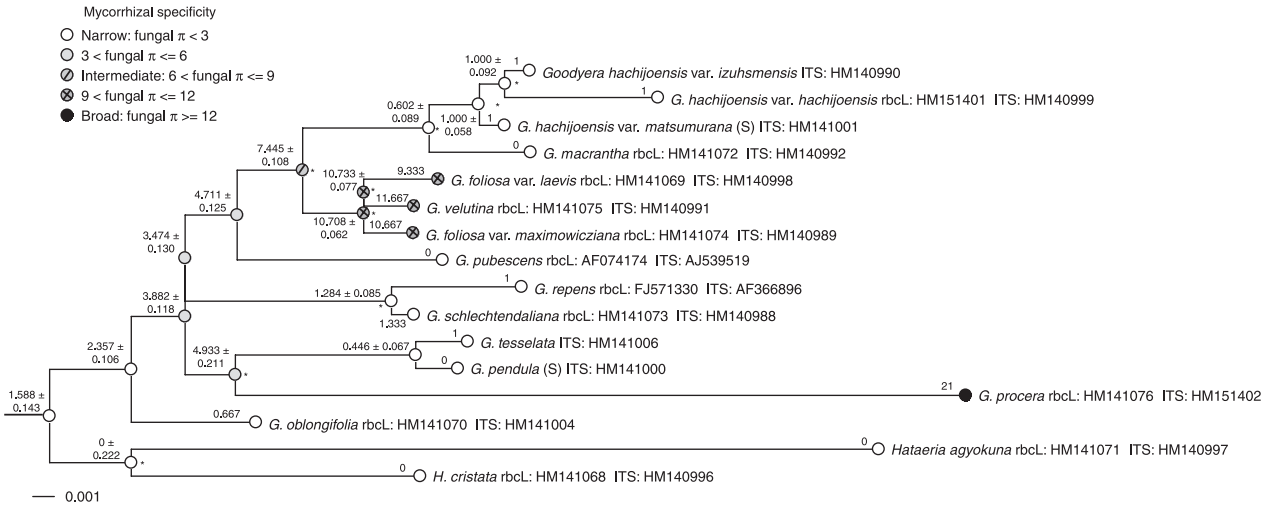

Fig. 3 Phylogeny of *Goodyera* and *Hetaeria* species sampled, showing the evolution of mycorrhizal specificity. Here, specificity was quantified as the mean pairwise phylogenetic distance, π, among fungal 5.8S haplotypes found mycorrhizal with sampled plants. Values at nodes include the estimated specificity ± 1 SE above the node. Clades with bootstrap support ≥ 50% are noted with asterisks. Phylogenetic analysis was via maximum likelihood analysis in PHYML for Windows Windows (Guindon & Gascuel 2003; Guindon *et al.* 2005), and involved 1000 bootstrap replicates. Phylogeny is rooted with *Hetaeria cristata* and *H. agyokuna* as the outgroup. No rbcL sequences were obtained for *G. hachojoensis* var. *izuhsmensis* and var. *matsumurana*, and for *G. pendula* and *G. tesselata*. Character evolution was inferred via least squares in the *ape* package in R (Paradis 2006; R Development Core Team 2007). Taxon names are followed by (S) if only one individual of that taxon was sampled.

ments of mycorrhizal specificity from the plant standpoint in terrestrial and tropical orchid systems have identified typically narrow suites of hosts, with some species exhibiting fairly broad associations (Otero *et al.* 2002, 2004; McCormick *et al.* 2004). Ecological determinants have rarely explained these trends, although sometimes host shifts occur with ontogeny or stress (McCormick *et al.* 2006), and tropical species may be more generalist than temperate species (Roy *et al.* 2009). A phylogenetic assessment of specificity in another orchid system, the lady's slipper genus *Cypripedium*, revealed that phylogeny is an important determinant of mycorrhizal specificity in plants (Shefferson *et al.* 2007). These orchids typically exhibit narrow host breadth, with expansions known to have evolved only twice in the genus, each time leading to one species (Shefferson *et al.* 2007). Such patterns were repeated here: a narrow interaction with fungi within the genus *Ceratobasidium* appears to be ancestral in this group, supporting other evidence that interactions with this fungal genus may be as old as the orchid mycorrhiza itself (Yukawa *et al.* 2009). However, low bootstrap support in our phylogenies reinforces the need for further work on this system in order to strengthen inference about ancestral states.

The distribution and life history of genus *Goodyera* may be determined in part by the combined ecology of its mycorrhizal fungal hosts. *Goodyera* forms mycorrhizae overwhelmingly with the basidiomycete genus *Ceratobasidium* and occasionally associates with other fungi, including other basidiomycetes such as *Clavulina* sp. and *Russula* sp., and some ascomycetes, such as the ectomycorrhizal *Phialophora finlandia*. Although *Tulasnella* spp. have been noted to form mycorrhizas with this orchid group, this association was phylogenetically rare in our dataset, occurring commonly only in *G. pubescens* (McCormick *et al.* 2004). An expanded sampling may find more.

Fungi in the genus *Ceratobasidium* are basal hymenomycetes that live saprotrophically in the environment, parasitize plant tissues, and sometimes form ectomycorrhizae (Downie 1943; Roberts 1999; Yagame *et al.* 2008). They are often economically and ecologically important pathogenic fungi. *Ceratobasidium cornigerum* is a major pathogen of grasses and cereal crops (Roberts 1999), although it is mycorrhizal with some other orchids (Otero *et al.* 2002). *C. anceps* parasitizes fern leaves (Gregor 1935), *C. bicorne* is a root parasite of *Pinus* spp., and *C. calosporum* is a free-living saprotroph (Roberts 1999). Some previous studies have suggested that *Goodyera* may commonly parasitize carbon resources from its mycorrhiza (Hadley & Purves 1974; Alexander & Hadley 1985), although more recently carbon donation has also been observed (Cameron *et al.* 2006, 2008). Although ectomycorrhizal fungi are typically thought to be better carbon donors for parasitic plants than saprotrophic fungi (Bruns *et al.* 2001; but see Ogura-Tsujita *et al.* 2009), the potentially pathogenic nature of *Ceratobasidium* species likely makes them excellent sources of energy for

plants that can tap into their nutrient flows. If orchids sometimes specialize on good sources of organic carbon, as can be said of myco-heterotrophs (Bruns *et al.* 2002), then specialization on these parasitic fungi may also create a stable source of carbon, water, and potentially other nutrients in times when the habitat is harsh. Even in this case, carbon flow from orchid to fungus has been observed often enough in *Goodyera* to warrant suspicion that it may not be a parasitic group (Cameron *et al.* 2006, 2008; Hynson *et al.* 2009).

In summary, we have shown evidence supporting phylogenetic conservatism in the evolution of host breadth in a broad interaction, that of the orchid mycorrhiza in the rattlesnake plantains (*Goodyera* spp.). Although our results corroborate existing patterns observed in genus *Cypripedium*, we argue that a broader sampling within the genus, in particular extending to species in more difficult to access portions of the Earth, will be essential to generalizing our inferences to further systems. We also argue that the most beneficial future direction for research on the evolutionary ecology of broad interactions focus on the expansion of the theory and quantitative framework for assessing these patterns, particularly in situations where species designations are unclear [e.g. certain other fungal groups, including family Tulasnellaceae, per Shefferson *et al.* (2007)]. Further research should also focus on whether evolution in these orchids and their mycorrhizal fungi occurs as in ways predicted by the geographic mosaic theory of coevolution, in which coevolution may be initially rare in an interaction and yet eventually dominate it due to chance events and the dynamics of interacting populations.

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Supporting Information

Additional supporting information may be found in the online version of this article:

Table S1 ITS haplotypes of basidiomycete root endophytes in sampled *Goodyera* plants, likely to function as mycorrhizal fungi. Here, the number of endophytes refers to the likely number of species found per the *Ceratobasidium* phylogeny (Fig. 1) or via BLAST results

Table S2 BLAST search results of ITS sequences of fungi outside of the Ceratobasidiaceae encountered in sampled *Goodyera* roots

Fig. S1 Phylogenetic placement of ascomycetous taxa mycorrhizal with *Goodyera* species. Phylogeny determined with sequences from the fungal ITS region, and includes references sequences from NCBI GenBank. Analysis was via maximum likelihood in PHYML for Windows (Guindon & Gascuel 2003; Guindon *et al*. 2005), and involved 1000 bootstrap replicates. Phylogeny is midpoint-rooted.

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