**Rhizoctonia fungi enhance the growth of the endangered orchid Cymbidium goeringii**

Jianrong Wu, Huancheng Ma, Mei Lü, Sufen Han, Youyong Zhu, Hui Jin, Junfeng Liang, Li Liu, and Jianping Xu

**Abstract:** Orchids are among the most prized ornamental plants in many societies throughout the world. As a result, consumer demands have created a significant pressure on wild populations of many species, including *Cymbidium goeringii* Rchb. f., a rare terrestrial orchid endemic in China, Korea, and Japan. To help conserve natural populations of *C. goeringii*, we recently started investigating methods to cultivate these orchids. Here we fulfilled Koch’s postulates and demonstrated that fungal strains isolated from the roots of natural *Cymbidium* plants increased fresh mass, plant height, number of leaves, and root length of *C. goeringii*, and that the two fungal strains originally isolated from *C. goeringii* showed overall greater effects on growth than two other strains from other *Cymbidium* species. Internal transcribed spacer sequence analyses revealed that the four fungal strains likely represented at least two new taxonomic groups, both belonging to the family Ceratobasidiaceae of the *Rhizoctonia* fungi. Our study demonstrated that these fungal strains could potentially help the commercial cultivation of the increasingly rare and endangered orchid *C. goeringii*.

**Key words:** orchids, mycorrhizal fungi, symbiosis, molecular marker.

**Introduction**

The Orchidaceae is the world’s largest plant family and contains over 25 000 species (Arditti 1992; Berg-Pana 2005). Of the 1000 or so genera in Orchidaceae, the genus *Cymbidium*, or “boat orchids”, is among the best known and most widely grown of all orchids and orchid hybrids (DuPuy and Cribb 1988; Berg-Pana 2005). *Cymbidium* includes over 50 species of terrestrial, lithophytic, and epiphytic orchids from mountainous regions of China, Korea, Japan, Southeast Asia, and India (DuPuy and Cribb 1988; Llamas 2003). Their beautiful flowers and their ability to survive and grow in cold and temperate environments have made them among the most sought-after ornamental plants that have long attracted horticultural enthusiasts. Indeed, *Cymbidium* orchids have been cultivated for thousands of years, starting in China and gradually expanding to other
parts of the world, including Europe and North America. The popularity of Cymbidium in North America is reflected by a society and an annual conference specifically dedicated to this genus (www.cymbidium.org).

At present, most of the >50 species in the genus Cymbidium can be found in the wild, and limited artificial propagation is available for most species within this genus, including the terrestrial orchid Cymbidium goeringii Rchb. f. However, artificial propagation is generally slow and cannot meet the consumer demand. Cymbidium goeringii is native to southwestern and northeastern China, the Korean peninsula, and Japan (Chung and Chung 2000; Lee 2002). This orchid is among the most highly prized ornamental plants in the world, with some plants sold for thousands of US dollars apiece (Light 2007). In recent years, the consumer demand for orchids, especially for C. goeringii, has increased dramatically both within and outside of China, for private homes, businesses, and large-scale public displays. The increasing demands have put significant pressures on natural populations of C. goeringii, especially in southwestern China (Anonymous 2004). How best to meet the consumer demand and conserve the natural populations of this and other orchids are two significant challenges facing conservation biologists and horticulturists.

One approach to meet the challenges described above is to develop effective cultivation methods. Several methods to germinate orchid seeds have been established (e.g., Thompson 1977; Liu et al. 2002). However, plantlets of terrestrial orchids from germinated seeds typically have very low survival rates after transplanting, grow slowly, with delayed or no flowering (e.g., Liu et al. 2002; Shimura and Koda 2005; Betty et al. 2006; Stewart and Kane 2006). In contrast, orchid seeds inoculated with mycorrhizal fungi have shown enhanced germination and greater growth than those not inoculated with any fungi (e.g., Peterson et al. 1998; Dearnaley 2007; Otero et al. 2007). Indeed, the importance of mycorrhiza in orchid seed germination was first discovered about a hundred years ago by a French botanist, Noel Bernard (Bernard 1909), Arditti (1984) provided a detailed account of the historical research activities associated with the roles of fungi in orchid seed germination. In addition, it has been known since the 1930s that mycorrhizal fungi are broadly distributed in orchid roots and are important for the growth of orchids (Burgeff 1936; Warcup and Talbot 1967; Arditti 1984 and references therein; Dearnaley 2007). Though the predominant view of this relationship is that nutrient flow is unidirectional from the fungi to the orchids, recent research also suggested that fungi could potentially benefit from this relationship by obtaining nutrients from orchids (e.g., Richardson and Currah 1995; Cameron et al. 2006).

In this study, we analyzed the relationship between fungi and the terrestrial orchid C. goeringii. Specifically, we first isolated fungi from the roots of four plants from three different Cymbidium species. These strains were analyzed by morphological and molecular techniques. Four strains were then inoculated to the seedlings of C. goeringii. The growth characteristics of these plants were measured and compared. In addition, fungal strains were re-isolated from the inoculated plants and compared with those originally inoculated. Two hypotheses were tested in the analyses. The first is that fungal strains isolated from the roots of natural Cymbidium plants should enhance the growth of C. goeringii. The second is that fungi isolated from C. goeringii roots should show greater enhancements in C. goeringii plants than those isolated from other Cymbidium orchids. The phylogenetic affiliations of the four strains within the broad Rhizoctonia alliances were analyzed and discussed.

Materials and methods

Seeds and seedlings

The ripe seed capsules of Cymbidium goeringii were collected in a natural forest in the Baoshan region, Yunnan Province, southwestern China. The dominant trees in this forest were Quercus acutissima Carr., a broad-leaf evergreen species, and Pinus yunnanensis Franch., a coniferous tree endemic to Yunnan. The sampled site was at 1980 m above sea level, with an average annual temperature about 16 °C, and a range between 10 and 30 °C. To identify an appropriate medium to germinate C. goeringii seeds, we tested a total of 48 media formulations. The starting culture medium was that established for germinating seeds of other orchids by Liu et al. (2002) and Xu and Guo (1989). The medium contained the half-strength Murashige and Skoog (MS) basal medium (Murashige and Skoog 1962; product M5519, Sigma-Aldrich, Oakville, Ontario, Canada). However, we further experimented with a range of concentrations for three ingredients: α-naphthalene acetic acid (NAA: 0.2, 0.3, 0.4, and 0.5 mg/L), coconut milk (10%, 15%, 20%), and activated carbon (2, 3, 4, and 5 g/L). Seeds were incubated at 25 °C with a 12 h light–12 h dark cycle. The medium with 1/2MS + 0.5 mg/L NAA + 15% (v/v) coconut milk + 3 g of activated carbon/L was found most suitable for germinating C. goeringii seeds, yielding 96% germination rate for the ripe seeds.

Orchid collection and fungal strain isolation

To obtain natural mycorrhizal fungal strains from Cymbidium plants for testing, we collected four Cymbidium plants representing three species native to Yunnan province. Two C. goeringii plants were collected from the Baoshan region in southwestern Yunnan; one Cymbidium sinense plant from the Xishuangbanna region in southern Yunnan; and a hybrid orchid (Cymbidium × cattleya) between Cymbidium faberi and Cymbidium hookerianum from Kunming, the provincial capital in Yunnan.

Mycorrhizal fungi were isolated from the collected roots according to the method described in Warcup and Talbot (1967), with slight modifications. Briefly, the orchid root surfaces were first washed with tap water to get rid of soil particles and organic debris. The roots were then sterilized by immersing them in 70% ethanol for 1 min, followed by submerging them in a sodium hypochlorite solution containing 1% chlorine for 1 min, and finally by immersing them in mercuric chloride (1%) for 6 min. A piece of the sterilized root from each plant, approximately 5–6 mm in length, was put into 1 mL of sterilized distilled water in a Petri dish (9 cm in diameter) and crushed with a sterilized glass rod to disperse intracellular fungal hyphal coils (pelotons). About 20 mL of sterilized potato dextrose agar (PDA, pH 5.6) medium was cooled to 40–45 °C, poured into the Petri
dish, and mixed with the crushed roots through gentle shaking. The mixture was incubated at 25 ºC in the dark for 3–4 d. To prevent bacterial growth, the antibiotic chloramphenicol (final concentration 50 µg/mL) was added to the culture medium.

Fungal colonies of consistent appearance that grew from a peloton were transferred onto fresh PDA medium using a sterilized scalpel and cultured individually. To further purify the cultures, small pieces of agar containing hyphal tips from each isolate were subcultured two more times. These cultures were then observed under a light microscope (400×) and putatively identified based on criteria established for other orchid mycorrhizal fungi using hyphal morphology in culture (Sneh et al. 1991; Currah et al. 1997).

Molecular identification

Four *Rhizoctonia*-like fungal isolates (one from each of the four orchids) were selected for further molecular identification, and inoculation experiments. Isolates CLB111 and CLB113 were purified from the two *C. goeringii* plants; isolate MLX102 was from a *C. sinense* plant; and isolate KW214 was from the hybrid orchid *Cymbidium × cattleya*. Their molecular identifications were based on their DNA sequences at the internal transcribed spacer (ITS) regions of the nuclear ribosomal rRNA gene cluster. Briefly, the four strains were first cultured on YEPD agar medium (per litre of medium: 10 g yeast extract; 20 g peptone; 20 g dextrose; 20 g agar; in 1 L of water) for 7 d. Mycelia from the top of the agar were harvested and ground into a fine powder in liquid nitrogen using a micropipette tip in a 1.5 mL microcentrifuge tube. Subsequent steps of the DNA extraction followed those described by Xu et al. (2000). The ITS regions were amplified from the extracted DNA by the polymerase chain reaction (PCR) with primers ITS1F (5'-CTTGGGTCATTTAGAGGAAGTAA-3', Gardes and Bruns 1993) and ITS4 (5'-TCTTCCGCTATTGATATGC-3', White et al. 1990). A typical PCR reaction contained 5 µL of template DNA solution (~20 ng), 0.75 U of the *Tag* DNA polymerase, 0.25 µmol/L of each primer, 200 µmol/L of each deoxyribonucleotide triphosphate, and 3 µL of PCR buffer in a total volume of 30 µL. The reaction was performed using the following conditions: an initial denaturation step at 94 ºC for 5 min, a subsequent step of 35 cycles at 94 ºC for 30 s, 50 ºC for 30 s, and 72 ºC for 2 min, and a final elongation step at 72 ºC for 5 min. PCR products were purified using the MicroCLEAN kit 2MCL-10 following the manufacturer’s instructions (DiaMed, Mississauga, Ontario, Canada). The purified DNA fragments were sequenced with an ABI 3700 DNA analyzer and an ABI BigDye3.1 terminator cycle sequencing kit (Mobix Laboratory, McMaster University, Hamilton, Ont.). The same ITS1F and ITS4 primers described above for PCR were used for the sequencing reactions.

The sequences from the four strains were compared with those in the GenBank through BLAST searches. Our BLAST searches identified that their close matches were all from the broad *Rhizoctonia* fungi (see details below in Results). To determine the potential identity of our four isolates and their relationships among each other and between them and other species within the broad *Rhizoctonia* fungi, we retrieved representative sequences (at least two per group) from the following taxonomic groups of the *Rhizoctonia* fungi (Sharon et al. 2008): (i) *Rhizoctonia solani* anastomosis groups (AG) 1–12; (ii) *Ceratobasidium* sp. AG-A–AG-S; (iii) *Waitea circinata*; (iv) *Thanatephorus cucumeris*; (v) uninucleated *Rhizoctonia* (UNR) 1 and 2; and (vi) *Tulasnellla* spp. Also downloaded were GenBank sequences highly similar to our four sequences as well as all ITS sequences of *Rhizoctonias* isolated from orchids. Following that by Sharon et al. (2008), the ITS sequence from an isolate of *Athelia* (=*Sclerotium* rolfsii) (GenBank accession AY684917) was retrieved and used as an outgroup. In total, 203 ITS sequences were downloaded from GenBank. The 203 ITS sequences and our own four sequences were aligned by the ClustalW2 program, using the program default setting (www.ebi.ac.uk/Tools/clustalw2/index.html).

The aligned sequences were then imported into the phylogenetic software program PAUP4.0b10 (Swofford 2002) and analyzed using three methods: maximum parsimony (MP), neighbor-joining (NJ), and maximum-likelihood (ML). In our analyses, gaps were treated as missing data, and the Kimura-2-Parameter distance measure was used for tree construction.

Establishing a symbiotic relationship between *Cymbidium goeringii* seedlings and fungi

To determine whether the isolated fungi would establish symbiotic relationships with *C. goeringii* plants, we inoculated seedlings of *C. goeringii* with mycelial cultures of the four specific fungal isolates, CLB111, CLB113, MLX102, and KW214. To prepare for the inoculation, the germinated *C. goeringii* seedlings were first transplanted to pots containing moss, vermiculite, and sand in a ratio of 1:1:1 (v/v/v). Prior to transplantation, the pots containing the substrates were autoclaved at 121 ºC for 60 min to eliminate any microorganisms in the substrate. The transplanted *C. goeringii* seedlings were grown in the greenhouse for 1 year. The greenhouse was maintained at 25 ºC, 70%–80% humidity, with natural light–dark cycles typical of Kunming city.

After 10 months of growth in the greenhouse, the above four fungal strains were prepared for inoculations. These strains were first grown in sterilized *Quercus acutissima* leaf medium at 25 ºC in the dark for 2 months. The *Q. acutissima* leaves containing fungal mycelia were then buried as inocula near the *C. goeringii* seedlings, one leaf per seedling. The inoculated seedlings were grown in the greenhouse and examined for evidence of symbiosis at 4.5 months and 9.5 months after the inoculation. Thirty seedlings were inoculated for each fungal strain. For the negative control, 30 seedlings were inoculated with sterile *Q. acutissima* leaf medium without any fungal strains, also buried near the *C. goeringii* seedlings.

After 4.5 months and 9.5 months, the growth responses of inoculated and uninoculated *C. goeringii* seedlings were measured in their total fresh mass, plant height, leaf number, and root length. Fifteen random seedlings were examined for each growth characteristic for each fungal strain at each of the two time points. The statistical significance of the potential differences among the treatments (including and excluding the negative control) was determined using the analysis of variance (ANOVA) approach for each of the four measured traits. In addition, for each trait, we determined the
statistical significance for the difference between all individual pairs of the five treatments using t tests (Sokal and Rohlf 1981).

To confirm that the inoculated roots of *C. goeringii* do indeed contain genetic materials from both the orchids and the inoculated fungal strains, total genomic DNA samples were extracted from the roots of inoculated orchids, uninoculated orchids, and pure fungal cultures. In addition, the pure fungal cultures from the inoculated orchids were re-isolated, and DNA was obtained following the procedures described above. DNA from orchid roots was isolated following the protocol described in a commercial extraction kit from Shanghai HuaShun Biotechnology & Services Ltd. (Shanghai, China). The samples were then cleaned using the commercial GeneClean kit (Qbiogene, Carlsbad, California, USA). The purified nucleic acids were digested by the RNase A enzyme to eliminate RNA molecules. The remaining nucleic acids (DNA) were then precipitated following the protocol described in Xu et al. (2000), and the DNA samples were stored in a –20 °C freezer for the following two types of analyses.

The first type of analysis relied on random amplified polymorphic DNA (RAPD) markers. DNA samples from the original and re-isolated fungal strains by themselves, from the roots of *C. goeringii* uninoculated with any fungi, and from the roots of *C. goeringii* inoculated with the fungal strains were analyzed using nonspecific primers for PCR amplification. Three primers with 10 nucleotides each were screened. The sequences of these three primers were S19 (5'-ACCCCCGGAAG-3'), S57 (5'-TTTCCCCAGG-3'), and S65 (5'-GATGACCGCC-3'). PCR amplification conditions followed those described by Liew and Irwin (1994). The amplified products were analyzed using agarose gel electrophoreses (Xu et al. 2000).

In the second analysis, ITS sequences of the re-isolated fungal strains from the infected roots of *C. goeringii* were obtained and compared with those of the originally inoculated strains. The protocols for obtaining the fungal strains and their ITS sequences followed those described above.

### Results

Based on colony morphology and microscopic characteristics, one isolate from the root of each of the four orchid plants was purified and identified as belonging to the broad *Rhizoctonia* fungi. The identities of these strains were further investigated by sequencing and by comparing their ITS sequences to those in the GenBank. These strains were then used to test their roles on the growth of *C. goeringii*. Below we describe the analyses of these sequences and the effects of fungal inoculations on *C. goeringii* growth.

#### Phylogenetic analyses

Based on BLASTn search comparisons with sequences from representatives of the broad *Rhizoctonia* fungi in the GenBank, we identified that these four fungal strains had three unique ITS sequences and none of them had an ITS sequence identical to any of the sequences deposited in the GenBank (see supplementary data, Fig. S1).3 Because of the large number of taxa included for the comprehensive analyses shown in supplementary data,3 Fig. S1, for better visualization, we selected a subset of the taxa for further analyses using three different phylogeny construction algorithms: MP, NJ, and ML. The results of the analyses are presented in Fig. 1, and the supplementary data, Figs. S2 and S3, for MP, NJ, and ML, respectively. Though minor differences were found among the phylogenetic trees generated by the three different methods, all the basal branches and the majority of the terminal branches showed consistent clustering patterns among the sequences (Fig. 1, and see supplementary data, Figs. S2 and S3). Specifically, for this study, all three phylogenetic analyses consistently clustered our four strains with the same groups of sequences, as described below.

The analyses and comparisons identified that strains CLB111 and KW214 shared an identical ITS sequence, and phylogenetic analysis placed them basal to the AG-D group of the genus *Ceratobasidium* (Fig. 1 and see supplementary data, Figs. S1, S2, and S3). Their sequence identities to the most closely related AG-D group of sequences ranged between 78.1% (to AG-D-III) and 83.4% (to AG-D-II). Similarly, the ITS sequences of strains MLX102 and CLB113 were distinctly different from all of the published GenBank sequences, and these two strains were most closely clustered with representative sequences of the AG-G group, also within *Ceratobasidium* (Fig. 1). The ITS sequence identities of strains MLX102 and CLB113 to the most closely related AG-G group of sequences were 93.2% (between CLB113 and GenBank accession AB196647) and 85.5% (between MLX102 and GenBank accession DQ102410), respectively. While our analyses confirmed that these four strains belonged to the broad *Rhizoctonia* fungi, the high relative sequence divergence between our strains and the sequenced taxa of *Rhizoctonia* fungi in the GenBank suggest that these four strains may represent two or three new taxonomic groups. The GenBank accession numbers for our four ITS sequences are FJ211862–FJ211865.

#### The effects of fungal inoculation on orchid growth

Our analyses of plant fresh mass, plant height, leaf number, and root length at 4.5 months after inoculation showed no statistically significant difference between the inoculated and uninoculated orchids (data not shown). In contrast, orchids inoculated with the four fungal isolates all showed significantly enhanced growth at 9.5 months after inoculation (Fig. 2; Table 1). Our ANOVA results confirmed that the treatments had statistically significant effects on all four plant-growth indicators examined here (Table 2). Specifically, seedlings inoculated with the four fungal strains had greater biomass, height, leaf number, and root length than those not inoculated with any fungi (*t* > 60 in all four pairwise comparisons, *df* = 28, *P* < 0.0001; Table 1).

Interestingly, when the negative control (i.e., the no fun-
Fig. 1. A maximum parsimonious tree of ITS sequences of the four isolates analyzed here and the representatives of *Rhizoctonia* fungi from the GenBank. The details of the GenBank sequences selected to represent various groups of *Rhizoctonia* fungi were presented in the main text. Each entry begins with a GenBank accession number, followed by a “|”, its taxonomic identification as provided in the GenBank, and for those isolated from orchids, with the label “Orchid” attached to it, and when available, followed by strain name. Strains with the JTO prefix were from orchid mycorrhiza from Central America and the Caribbean islands (Otero et al. 2002 and 2007). Arrows indicate the two groups of strains isolated and tested in our study. Taxonomic abbreviations: R.sp., *Rhizoctonia* spp.; C.sp., *Ceratobasidium* spp.; R.s., *Rhizoctonia solani*; Th.c., *Thanatephorus cucumeris*; Cr.o-s, *Ceratorhiza oryzae-sativae*; C.c., *Ceratobasidium cornigerum*; c.a.,*Ceratobasidium angustisporum*. Tree length, 2125; consistency index, 0.440, retention index, 0.752.
gal inoculation treatment) was excluded, our ANOVA results still showed statistically significant differences among the remaining four treatments for three of the four traits: fresh biomass, plant height, and leaf number (Table 2). Specifically, the two fungal strains (CLB111 and CLB113) originally isolated from *C. goeringii* showed greater growth enhancement on *C. goeringii* seedlings than the other two strains (MLX102 and KW214) isolated from other *Cymbidium* species in fresh biomass, plant height, and leaf number (Table 1). The only exception was root length, where strains CLB113 and MLX102 had similar effects (Table 1). Other

trait-specific effects were also observed between strains CLB111 and CLB113, and between strains MLX102 and KW214 (Table 1). Our combined phylogenetic and plant growth results suggest that the effects of these strains on *C. goeringii* seedlings seemed independent of their ITS sequence relationships (Fig. 1; Table 1). Overall, these results are consistent with the existence of strain-specific and trait-specific fungal–orchid interactions.

Our analyses using RAPD markers confirmed that the roots inoculated with fungal strains contained signature bands of both the orchids and the inoculated fungi (Fig. 3).
PCR with each of the three RAPD primers showed unambiguous fingerprinting patterns for the plant, the fungi, and the plants inoculated with the four fungal strains.

Re-isolation of fungal strains from inoculated orchid roots and their molecular identification

Fungal strains from the roots of inoculated greenhouse orchids were obtained following the protocol described initially for isolating fungi from wild orchid roots. As expected, no fungus was isolated from the noninoculated orchids. The re-isolated fungal strains from inoculated plants were compared with the original inoculated fungal strains using RAPD markers and ITS sequencing. Our results identified that the re-isolated strains were genetically identical to the originally inoculated strains (data not shown).

Discussion

We identified that four strains of *Rhizoctonia* fungi enhanced the growth of the endangered terrestrial orchid *C. goeringii*. Our study fulfilled Koch’s postulates originally proposed for identifying the causes of human infectious diseases (Koch 1876). Specifically, in this study, we isolated pure cultures of *Rhizoctonia* fungi from the roots of wild *C. goeringii* plants, we inoculated these strains into *C. goeringii* seedlings, and the inoculated seedlings showed increased growth, and we confirmed that re-isolated strains from these plants were identical to those originally inoculated. Below we discuss the relevance of our results in orchid mycorrhizal research and orchid conservation.

Experimental approaches

Many studies have demonstrated the positive effects of fungal inoculation on orchid seeds and plants (e.g., Otero et al. 2002, 2007; Chang and Chou 2007). Most of such studies have only relied on microscopic observations to confirm the formation of pelotons in the cortex of plant roots (see recent review by Dearnaley 2007). In this study, we presented a complementary approach using molecular typing to confirm that only the inoculating fungal strains were present in the roots of inoculated orchids and that the inoculated fungi were responsible for the enhanced growths of the plants. This approach may help speed up future identifications of fungal strain – orchid associations in inoculation experiments.

Another novelty of our study is in the orchid developmental stage when fungal inoculation was performed. As far as we know, previous investigations examining the effects of mycorrhizal fungi on orchids all inoculated orchid seeds with mycorrhizal fungi (e.g., Lee 2002; Dearnaley 2007; Otero et al. 2007), not orchid seedlings. Our experimental results demonstrated that *C. goeringii* seeds can be germinated readily without any fungal inoculants and that the inoculation of fungi to orchid seedlings could be effective at establishing symbiotic relationships between these two partners.

Diversity and taxonomy of orchid mycorrhizal fungi

Most recent studies of orchid fungi focused on the survey of the diversity of fungi from orchid roots (e.g., Shan et al. 2002; Brundrett et al. 2003; Ma et al. 2003; Bonnardeaux et al. 2007; Dearnaley 2007; Otero et al. 2007). Both culture based and non-culture based (i.e., direct analyses of orchid root DNA) analyses have been carried out. These studies have yielded a large amount of information about the diversity of potential contributors to the growth and reproduction of a variety of orchids. The following fungal Families have been identified commonly associated with

Table 2. Results from the analysis of variance (ANOVA) on the effects of fungal inoculations on orchid seedlings of *Cymbidium goeringii*.

<table>
<thead>
<tr>
<th>Plant traits</th>
<th>Treatment groups included</th>
<th>Source of variation</th>
<th>Degrees of freedom</th>
<th>Sum of squares (SS)</th>
<th>Mean squares (MS)</th>
<th>F_s</th>
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<tr>
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<td>All five treatments</td>
<td>Among groups</td>
<td>4</td>
<td>415.61</td>
<td>103.90</td>
<td>2526.18***</td>
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<td></td>
<td>Four fungi-inoculated</td>
<td>Within groups</td>
<td>70</td>
<td>2.87</td>
<td>0.04</td>
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<tr>
<td></td>
<td>treatments</td>
<td>Among groups</td>
<td>3</td>
<td>3.56</td>
<td>1.19</td>
<td>23.24***</td>
</tr>
<tr>
<td>Plant height</td>
<td>All five treatments</td>
<td>Within groups</td>
<td>56</td>
<td>2.86</td>
<td>0.051</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Four fungi-inoculated</td>
<td>Among groups</td>
<td>4</td>
<td>1054.1</td>
<td>263.5</td>
<td>693.48***</td>
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<td></td>
<td>treatments</td>
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<tr>
<td>Leaf number</td>
<td>All five treatments</td>
<td>Among groups</td>
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<td>27.11</td>
<td>9.04</td>
<td>19.29***</td>
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<td>Root length</td>
<td>All five treatments</td>
<td>Among groups</td>
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<td>26.23</td>
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<td>Four fungi-inoculated</td>
<td>Within groups</td>
<td>4</td>
<td>212.88</td>
<td>53.22</td>
<td>227.16***</td>
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<td>treatments</td>
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<td>16.41</td>
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<td>Among groups</td>
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<td>3.49</td>
<td>1.16</td>
<td>6.82***</td>
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<tr>
<td></td>
<td>Four fungi-inoculated</td>
<td>Within groups</td>
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<td>980.98***</td>
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<td>Within groups</td>
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<tr>
<td></td>
<td>Among groups</td>
<td>3</td>
<td>0.47</td>
<td>0.156</td>
<td>1.24ns</td>
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</tbody>
</table>

Note: F_s, sample variance ratio in an F distribution; ***, Statistically significant difference among treatments at P < 0.001; ns, no significant difference among treatments.
orchids: Russulaceae, Tulasnellaceae, Hydnangiaceae, Sebacinaeae, Ceratobasidiaceae, Thelephoraceae, Hymenogastraceae, Pyronemataceae, and Cortinariaceae (Dearnaley 2007). However, in most of these cases, the exact roles of these fungi in the growth of orchids remain to be experimentally demonstrated.

The four strains isolated and examined here belong to the broad Rhizoctonia fungi. The teleomorphic form (i.e., sexual form) of Rhizoctonia fungi include three families of basidiomycetes mentioned above: Tulasnellaceae, Sebacinaeae, and Ceratobasidiaceae (Ogoshi 1987; Peterson et al. 1998; Shan et al. 2002; Sharon et al. 2008). The anamorphic form (i.e., asexual form) of Rhizoctonia fungi is classified into the following four genera Ceratorhiza, Epulorhiza, Moniliopsis, and Rhizoctonia (Moore 1988). Because the sexual stages are infrequently observed for Rhizoctonia fungi, most orchid-associated fungi are initially classified into the Rhizoctonia group that also includes saprophytes, symbionts, and pathogens of other plants (Ogoshi 1987; Brundrett et al. 2003; Bonnardeaux et al. 2007; Yagame et al. 2008).

Despite the lack of sexual features for identification of orchid-associated mycorrhizal fungi, the advances in DNA sequencing and phylogenetic analyses are allowing relatively simple inferences of the relationships between the anamorphic Rhizoctonia fungi and their known sexual counterparts and (or) relatives. For example, based on ITS sequences, several lineages of Ceratobasidium (family Ceratobasidiaceae) were found from the neotropic orchid Ionopsis utricularioides in the Caribbean Islands and Central America (Otero et al. 2002; 2007). Most of the strains were clustered within a large clade that included representative sequences of the AG-B, AG-S, and AG-Q groups in Ceratobasidium (Fig. 1, and see supplementary data, Fig. S1; GenBank sequences with strain names prefixed with JTO). In contrast, the four strains from our study were basal to the AG-D and AG-G groups and similar to ITS sequences from several other phylogenetically related AGs, including AG-C, AG-H, AG-I, AG-L, and AG-O (Fig. 1). Interestingly, these groups of sequences included several Rhizoctonia fungi obtained from orchids in geographically dispersed areas, such as India (EU605732), Singapore (AJ318437), Hungary (AM040889), Australia (AJ427403), Italy (DQ061931), and Panama (DQ084001). In contrast, other AGs of the Rhizoctonia fungi seemed to lack orchid symbionts (Fig. 1, and see supplementary data, Fig. S1). While this biased distribution of orchid mycorrhizal symbionts might be due to limited sampling and (or) our limited understanding on this subject, it is tempting to speculate that Rhizoctonia fungi symbiotic to orchids might be phylogenetically constrained.

It should be noted that the taxonomy of Rhizoctonia fungi is in a state of flux with continued developments. As a result, it is difficult to assign the four strains isolated here to specific species or even genera. Based on the ITS sequences, these four strains seemed significantly divergent from all the known taxonomic groups (Fig. 1, and see supplementary data, Fig. S1). Specifically, the identities between our sequences and their closest known representatives were much lower than those within most of the known species or anastomosis groups (typically >93% for the ITS region; Sharon et al. 2008) within the broad Rhizoctonia group. The analyses of DNA sequences from additional gene fragments might reveal whether these strains represent reproductively isolated groups different from other groups.

**Host- and strain-specific effects**

Our results support the hypothesis that there are plant trait specific and fungal strain specific effects on the growth of C. goeringii seedlings. These results are consistent with several recent studies on the effect of Rhizoctonia fungi on the growth of other orchids. For example, Chang and Chou (2007) identified that Rhizoctonia spp. enhanced the growth of orchid Anoectochilus formsanosis Hayatal. Shan et al. (2002) showed that the germination and development of three orchid species (Arundina chinensis, Spathoglottis pubescens, and Spiranthes hongkongensis) were strongly stimulated by the Epulorhiza isolates, Bonnardeaux et al. (2007) analyzed the symbiotic relationships between six terrestrial orchids and 12 fungi from Australia and South Africa. Their symbiotic germination assays uncovered complex webs of fungal strain – orchid compatibility. Two weed-like orchids Disa bracteata and Microtis media were found to have the broadest webs of mycorrhizal fungi, followed by Diuris magnifica and Thelymitra crinita, and the remaining two Caladenia falcata and Pterostylis sanguinea.
germinated only with their own fungus. Some of these relationships were also developmental stage specific (Bonardeaux et al. 2007). Similarly, Otero et al. (2007) identified that germination and seedling growth of the tropical epiphyte orchid *Itonopsis urticarioroides* were differentially enhanced by strains of *Ceratobasidium* (anamorph *Cerato- rhiza*) widely distributed in the roots of wild populations of these plants.

In general, while certain groups of fungi are found broadly distributed across many orchid species and geographic areas, most studies also indicate certain degrees of host specificity, at least during some of the hosts’ developmental stages (Shan et al. 2002; Shefferson et al. 2005; Bonardeaux et al. 2007; Chang and Chou 2007; Dearnaley 2007; Otero et al. 2007). Our results here are largely consistent with these earlier observations.

Conclusions and perspectives

In conclusion, we isolated four isolates of *Rhizoctonia* fungi from three species of *Cymbidium* orchids from southwestern China’s Yunnan province. These isolates showed significant divergence in ITS sequences from other known fungi. Our phylogenetic analysis suggests that they likely represent new species and (or) lineages within the broad *Rhizoctonia* group. The laboratory inoculation experiments demonstrated that all four fungal isolates were capable of enhancing the growth of *C. goeringii* seedlings. As it was shown here, the seeds and seedlings of *C. goeringii* could germinate and grow without any fungal symbionts. However, *C. goeringii* seedlings inoculated with the *Rhizoctonia* fungi grew significantly faster than those without any fungal inoculations. The strains and knowledge obtained here should help us increase the commercial propagation of *C. goeringii* and potentially other closely related orchids. The increased artificial propagation should help alleviate the pressure on natural populations of this rare orchid.

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