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Full Length Research Paper

Species composition and molecular analysis of symbiotic fungi in roots of *Changnienia amoena* (Orchidaceae)

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The diversity and population structure of symbiotic fungi in *Changnienia amoena* (Orchidaceae) were carried out. A total of 67 isolates were recovered from root samples, all isolates were identified based on morphological criteria and rDNA ITS phylogeny. Overall, 18 taxa were detected. Ascomycetes were the major fungal group, the remaining three isolates were genetically close to *Mortierella* (Zygomycetes) and *Tulasnella* (Basidiomycetes), respectively. However, taxa composition of four regions showed only little overlap. These findings provided additional evidence that the non-mycorrhizal endophytes constituted an important component of fungal community in orchids. The potential roles of some species to host were discussed.

Key words: Endophytic fungi, population structure, molecular phylogeny.

INTRODUCTION

Mycorrhizal fungi are indispensable for the orchids to plant development, which can stimulate seed germination, protocorm development, and seedling growth (Stewart et al., 2007; Peterson et al., 1998). Apart from mycorrhizal fungi, endophytic fungi are also omnipresent colonizers in orchids both in below- and above-ground tissues. It is likely that all orchids contain fungal endophytes which may be a largely overlooked component of fungal biodiversity (Bayman et al., 1997). However, the ecological roles of endophytic fungi in orchids are largely unknown. *Changnienia amoena* is a monotypic and endemic orchid in China (Zhang et al., 2009), which only distributes in the downstream of the Yangtze River and the southern Shanxi, it is an excellent wild flowers and famous medicinal plant (Xiong et al., 2003). Unfortunately, *C. amoena* is threatened with extinction due to anthropic and climatic influences (Fu, 1992). Recently, some work has addressed mycorrhizal fungi in C. *amoena*. Yan et al. (2006) had observed mycorrhizal microscopic structural features and preliminary identified mycorrhizal fungi through morphological characteristics, but collection sites and fungal diversity are not clear.

Zhang et al. (2009) had studied mycorrhizal fungi of C. *amoena* in only one site (Tianmu mountain, Zhejiang province), and identified a mycorrhizal fungus belonging to *Tulasnella* genus. In this study, we aim to examine the geographic patterns of symbiotic fungal diversity in *C. amoena*. We collected the samples from four sites including Dajingwu (Zhejiang province), Kaishanlaodian (Zhejiang province), Tiangtanzhai (Anhui province) and Shennongjia (Hubei province). We expect that it has a referential value to utilize the symbiotic fungi and protect rare and endemic orchid plants.

MATERIALS AND METHODS

Sample collection

C. amoena were sampled in 2009 from four habitats, we collected

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Table 1. Detailed information of the sampling sites.

Site	Longitude(°)	Latitude (°)	Elevation(m)	Sampling date
Dajingwu	30.22 N	119.26 E	800	6 April, 2009
Kaishanlaodian	30.22 N	119.26 E	1000	6 April, 2009
Shennongjia	31.32624 N	110.49387 E	1370	22 April, 2009
Tiantangzhai	31.13438 N	115.78233 E	1036	18 April, 2009

two samples in the Dajingwu, Tianmu mountain Zhejiang and Kaishanlaodian, Tianmu mountain Zhejiang, the third site is Shennongjia, Hubei province, the last site is Tiangtanzhai, Anhui province (Table 1). *C. amoena* all habitated under forests in four sites, the difference is that *C. amoena* grow in kaishanlaodian under jndia cedar forest, the rest all under broad leaved forest.

Fungal endophytes isolation, purification and storage

When sampling, we took special care not to damage their root systems. Healthy roots were cut from the plants and rinsed with tap water slightly, surface-sterilized using ethanol (75%) for 30 s, immersed in NaClO (0.5%) for 3~5 min and finally rinsed in sterile distilled water with three times. Roots were cut into 0.5 to 1 cm pieces and cultured in plate with potato dextrose agar (PDA) medium supplemented with 50 mg/L streptomycin, tetracycline and penicillin respectively, to avoid bacteria growth. Plates were sealed with parafilm to prevent desiccation and incubated at 25℃ with darkness. The emerging hyphae from segments were transferred to new PDA medium for purification. The recovered pure cultures were inoculated at PDA slant, cultured for 7 days sealed with sterile liquid paraffin.

Fungal identification

The isolated strains were initially identified to genus or species level according to morphological appearance. Isolates were inoculated on PDA at 25℃ for at least one week, and then observed colonial morphology, picked hyphae were observed hyphal morphology, spores and sporulating structures under the optical microscope, the remaining non-sporulating cultures were identified using molecular tool.

DNA extraction, PCR

For extraction of fungal DNA, mycelium was scraped directly from the margin of a colony of all isolates, Genomic DNA was extracted using the Multisource Genomic DNA Miniprep Kit (Axygen Incorporation, China), the internal transcribed spacer (ITS) region of fungi were amplified using the fungal specific primers ITS-1 (5′-TCCGTAGGTGAACCTGCGG-3′) or ITS-5 (5′-GGAAGTAAAAGTCGTAACAAGG-3′) and ITS-4 (5′-TCCTCCGCTTATTGATATGC-3′). The PCR reactions (50μL):

10 x PCR buffer 5μL, dNTPs 0.4μL, DNA template 5μL, primers 0.5μL, Taq enzyme 1μL, ddH_2O 37.6μL.

PCR reaction cycle parameters as follows:

Pre-denaturation 96℃ for 1 min; denaturation 94℃ for 30 S; annealing 55℃ for 40 s; extension at 72℃ for 60 s; 35 reaction cycles; extension at 72℃ for 10 min.

The PCR products were purified by PCR product kit (Axygen Incorporation, China), after purification, sequenced using ABI 3730 sequencer (Applied Biosystems, USA) with the same primers as the PCR amplification.

Sequencing and phylogenetic analysis

All DNA sequences were compiled and deposited in NCBI GenBank under the accession numbers (GU166437-GU166503). Analysis of sequences was performed using BLAST sequence similarity searches against the National Center for Biotechnology Information database (http://www.ncbi.nlm.nih.gov). The sequences were aligned using Clustal X (version 1.8) and manually edited using GENDOC 2.6. The phylogenetic trees were constructed using PAUP*4.0 b 10. The alignment was analysed using Maximum parsimony and trees were inferred using the heuristic search option with TBR branch swapping; using 1,000 bootstrap replications tested the stability of clades. Gaps were coded as missing data.

RESULTS

Endophytic fungal population structure

Totally, 27, 6, 22 and 12 isolates were obtained from *C. amoena* located in Dajingwu, Kaishanlaodian, Tiangtanzhai and Shennongjia, respectively. The ITS sequences were submitted to the GenBank, relying on BLAST matches with the GenBank database, the putative taxonomic identification of endophytic fungi was represented as shown in Table 2. In total, 18 distinct taxa were recorded. Fungi belonging to *Podospora*, *Chaetomium*, Xylariaceae and *Bionectria* were frequently found in roots of C. *amoena*. The genetic diversity of Xylariaceous fungi is also determined inferring from ITS phylogeny (Figure 2). *Tulasnella calospora*, appearing to form mycorrhizae with a number of orchids, was recorded only in one site (Figure 1). In addition, an isolate of Zygomycete, *Mortierella*, was also identified. However, taxa composition of four regions showed only little overlap.

Blast search reveals that ITS sequences of some taxa are relatively divergent to currently described known species (similarity below 95%), representing potential species new to science (Table 1).

Phylogenetic analysis of fungal endophytes in Xylariaceae and Helotiales

In this study, we examined the genetic diversity of orchid

Table 2. Taxonomic placement of sporulating and sterile morphotypes inferred from BLAST searches and morphological descriptions. Isolates and accession numbers for each taxon are also included. Areas shaded in grey indicated the potentially novel fungal lineages; areas shaded in yellow indicated the orchid mycorrhizal fungus.

Location	Isolate name	Accession numbers	Taxa	Classification	Nearest match	Max identity (%)
Dajingwu	C1R2	GU166437	*Podospora* sp.1	Sordariomycetes, Sordariales	*Podospora setosa* (AF486637)	98
	C1R3	GU166438	*Podospora* sp.1	Sordariomycetes, Sordariales	*Podospora setosa* (AF486637)	99
	C1R44	GU166442	*Podospora* sp.1	Sordariomycetes, Sordariales	*Podospora setosa* (AF486637)	98
	C1R5	GU166440	*Podospora* sp.1	Sordariomycetes, Sordariales	*Podospora setosa* (AF486637)	98
	C2R1	GU166443	*Podospora* sp.1	Sordariomycetes, Sordariales	*Podospora setosa* (AF486637)	98
	C2R11	GU166446	*Podospora* sp.1	Sordariomycetes, Sordariales	*Podospora setosa* (AF486637)	98
	C2R2	GU166444	*Podospora* sp.1	Sordariomycetes, Sordariales	*Podospora setosa* (AF486637)	98
	C2R3	GU166445	*Podospora* sp.1	Sordariomycetes, Sordariales	*Podospora setosa* (AF486637)	99
	C3R1	GU166450	*Podospora* sp.1	Sordariomycetes, Sordariales	*Podospora setosa* (AF486637)	98
	C3R3	GU166452	*Podospora* sp.1	Sordariomycetes, Sordariales	*Podospora setosa* (AF486637)	98
	C3R4	GU166453	*Podospora* sp.1	Sordariomycetes, Sordariales	*Podospora setosa* (AF486637)	98
	C3R5	GU166454	*Podospora* sp.1	Sordariomycetes, Sordariales	*Podospora setosa* (AF486637)	99
	DR1	GU166455	*Podospora* sp.1	Sordariomycetes, Sordariales	*Podospora setosa* (AF486637)	98
	DR3	GU166457	*Podospora* sp.1	Sordariomycetes, Sordariales	*Podospora setosa* (AF486637)	98
	DR4	GU166458	*Podospora* sp.1	Sordariomycetes, Sordariales	*Podospora setosa* (GU391421)	99
	DR5	GU166459	*Podospora* sp.1	Sordariomycetes, Sordariales	*Podospora setosa* (AF486637)	99
	DR6	GU166460	*Podospora* sp.1	Sordariomycetes, Sordariales	*Podospora setosa* (AF486637)	99
	DR7	GU166461	*Podospora* sp.1	Sordariomycetes, Sordariales	*Podospora setosa* (AF486637)	99
	DR9	GU166463	*Podospora* sp.1	Sordariomycetes, Sordariales	*Podospora setosa* (AF486637)	99
	C2R22	GU166447	*Xylaria* sp.1	Sordariomycetes; Xylariales	*Xylaria curta* (EU715684)	99
	DR2	GU166456	*Chaetomium* sp.1	Sordariomycetes; Sordariales	*Chaetomium nigricolor* (GQ922570)	99
	C1R22	GU166441	*Eupenicillium* sp.1	Eurotiomycetes; Eurotiales	*Eupenicillium reticulisporum* (AF033437)	98
	C2R33	GU166448	*Eupenicillium* sp.1	Eurotiomycetes; Eurotiales	*Eupenicillium reticulisporum* (AF033437)	100
	C2R44	GU166449	*Eupenicillium* sp.1	Eurotiomycetes; Eurotiales	*Eupenicillium reticulisporum* (AF033437)	100
	C3R2	GU166451	*Eupenicillium* sp.1	Eurotiomycetes; Eurotiales	*Eupenicillium reticulisporum* (AF033437)	100
	DR8	GU166462	*Eupenicillium* sp.1	Eurotiomycetes; Eurotiales	*Eupenicillium reticulisporum* (AF033437)	100
	C1R4	GU166439	*Eupenicillium* sp.2	Eurotiomycetes; Eurotiales ,	*Eupenicillium brefeldianum* (AF033435)	99
Kaishanlaodian	LR3	GU166466	*Hypoxylon* sp.2	Sordariomycetes; Xylariales	*Hypoxylon fragiforme*(EU715619)	93
	LR6	GU166469	*Hypoxylon* sp.2	Sordariomycetes; Xylariales	*Hypoxylon fragiforme*(EU715619)	94
	LR4	GU166467	Helotiales sp.2	Leotiomycetes; Helotiales	Mycorrhizal fungal sp. (EU880593)	100
	LR5	GU166468	Helotiales sp.2	Leotiomycetes; Helotiales	Mycorrhizal fungal sp.(EU880593)	99
	LR1	GU166464	*Tulasnella calospora*	Agaricomycetes;Cantharellales	*Tulasnella calospora* (FJ613255)	99
	LR2	GU166465	*Tulasnella calospora*	Agaricomycetes;Cantharellales	*Tulasnella calospora* (FJ613255)	99

Table 2. Continued.

Tiantangzhai	AR1	GU166470	*Geomyces* sp.1	Eurotiomycetes; Onygenales	*Pseudogymnoascus roseus* (AY608924)	100
	AR11	GU166479	*Geomyces* sp.1	Eurotiomycetes; Onygenales	*Pseudogymnoascus roseus* (AY608924)	100
	AR12	GU166480	*Geomyces* sp.1	Eurotiomycetes; Onygenales	*Geomyces pannorum* (FJ590611)	100
	AR18	GU166486	*Geomyces* sp.1	Eurotiomycetes; Onygenales	*Geomyces pannorum* (FJ590611)	100
	AR21	GU166489	*Geomyces* sp.1	Eurotiomycetes; Onygenales	*Geomyces vinaceus* (AJ608972)	99
	AR22	GU166490	*Geomyces* sp.1	Eurotiomycetes; Onygenales	*Geomyces vinaceus* (AJ608972)	99
	AR3	GU166471	*Geomyces* sp.1	Eurotiomycetes; Onygenales	*Geomyces* sp. (FJ379797)	97
	AR4	GU166472	Phialophora hyalina	Sordariomycetes Magnaporthales	Uncultured fungus clone (FJ528688)	96
	AR5	GU166473	Helotiales sp.1	Leotiomycetes; Helotiales	Uncultured fungus(EU113204)	99
	AR6	GU166474	Helotiales sp.1	Leotiomycetes; Helotiales	Uncultured fungus (FN298704)	97
	AR13	GU166481	Helotiales sp.1	Leotiomycetes; Helotiales	Uncultured fungus(EU113204)	99
	AR10	GU166478	*Xylaria* sp.1	Sordariomycetes; Xylariales	*Xylaria curta* (EU715684)	99
	AR16	GU166484	*Xylaria* sp.1	Sordariomycetes; Xylariales	*Xylaria curta* (EU715684)	99
	AR7	GU166475	*Xylaria* sp.1	Sordariomycetes; Xylariales	*Xylaria curta* (EU715684)	99
	AR9	GU166477	*Xylaria* sp.1	Sordariomycetes; Xylariales	*Xylaria curta* (EU715684)	99
	AR8	GU166476	*Hypoxylon* sp.2	Sordariomycetes; Xylariales	*Hypoxylon fragiforme*(EU715619)	94
	AR14	GU166482	*Nemania*	Sordariomycetes; Xylariales	Uncultured fungus (EU113197)	95
	AR19	GU166487	*Ascobolus* sp.1	Pezizomycetes; Pezizales	*Ascobolus crenulatus* (DQ491504)	91
	AR20	GU166488	*Ascobolus* sp.1	Pezizomycetes; Pezizales	*Ascobolus crenulatus* (DQ491504)	92
	AR23	GU166491	*Ascobolus* sp.1	Pezizomycetes; Pezizales	*Ascobolus crenulatus* (DQ491504)	92
	AR17	GU166485	*Lecythophora* sp.1	Sordariomycetes; Coniochaetales	*Aureobasidium* sp. (GQ906942)	99
	AR15	GU166483	*Mortierella* sp.	Mucoromycotina; Mortierellales	*Mortierella* sp. (FJ810149)	99
Shennongjia	DJL3	GU166492	*Chaetomium* sp.1	Sordariomycetes; Sordariales	*Chaetomium nigricolor* (GQ922570)	100
	DJL10	GU166495	*Chaetomium* sp.2	Sordariomycetes; Sordariales	*Chaetomium* sp. (DQ093661)	99
	DJL15	GU166496	*Chaetomium* sp.2	Sordariomycetes; Sordariales	*Chaetomium* sp. (DQ093661)	98
	DJL22	GU166501	*Chaetomium* sp.2	Sordariomycetes; Sordariales	*Chaetomium* sp. (DQ093661)	99
	DJL19	GU166499	*Bionectria* sp.1	Sordariomycetes; Hypocreales	*Bionectria ochroleuca* (FJ238113)	100
	DJL16	GU166497	*Bionectria* sp.1	Sordariomycetes; Hypocreales	*Bionectria ochroleuca* (FJ238113)	98
	DJL18	GU166498	*Bionectria* sp.1	Sordariomycetes; Hypocreales	*Bionectria ochroleuca* (AY669327)	98
	DJL21	GU166500	*Bionectria* sp.1	Sordariomycetes; Hypocreales	*Bionectria ochroleuca* (FJ238113)	98
	DJL23	GU166502	*Bionectria* sp.1	Sordariomycetes; Hypocreales	*Bionectria ochroleuca* (AY669327)	98
	DJL25	GU166503	*Bionectria* sp.1	Sordariomycetes; Hypocreales	*Bionectria ochroleuca* (FJ238113)	98
	DJL9	GU166494	*Neonectria* sp.1	Sordariomycetes; Hypocreales	*Neonectria radicicola* (AY295331)	100
	DJL6	GU166493	*Ascobolus* sp.1	Pezizomycetes; Pezizales	*Ascobolus crenulatus* (DQ491504)	90

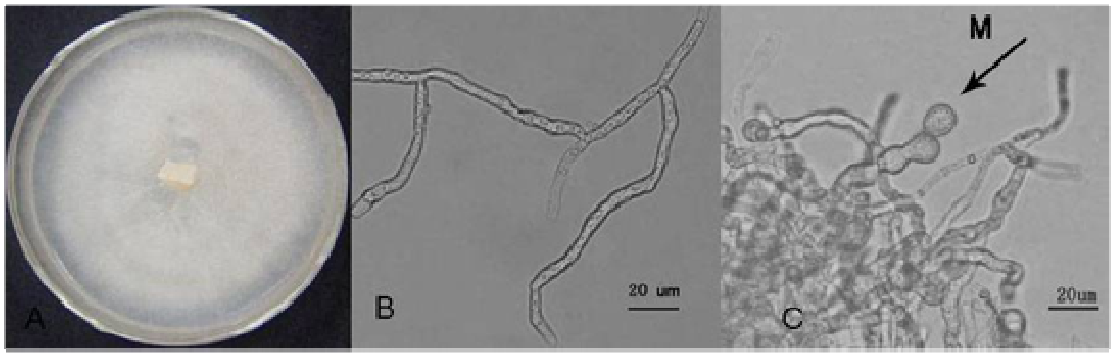

Figure 1. The colony morphology of *Tulasnella calospora* on potato dextrose agar for 14 days (A), mycelium morphology (B) and chain of monilioid cells (C).

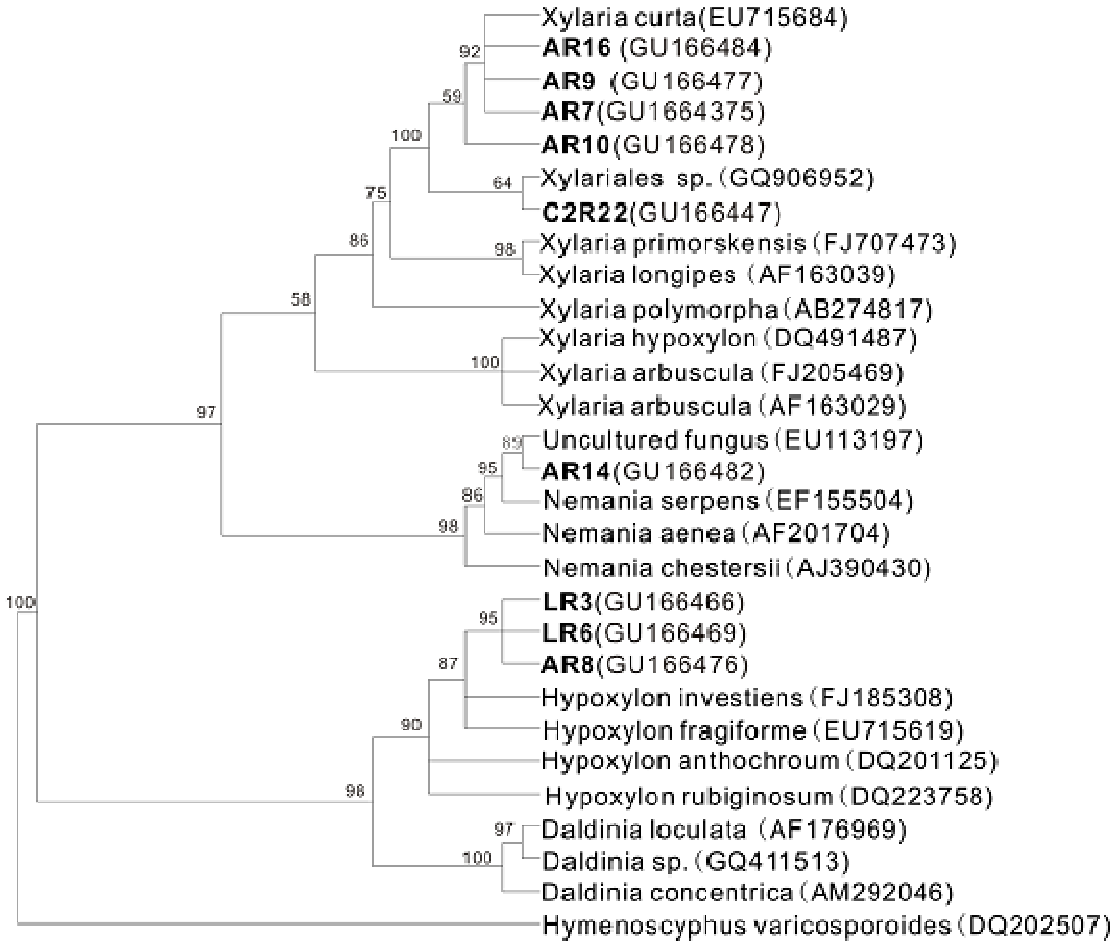

Figure 2. Phylogenetic tree of endophytic Xylariaceae fungi as inferred based on ITS1-5.8S-ITS2 sequence (CI = 0.6957, RI = 0.8611, RC = 0.5991, HI = 0.3043). MP (Maximum Parsimony) bootstrap values>50% are indicated above branch nodes. Tree length = 562. Number of bootstrap replicates = 1,000. *Hymenoscyphus varicosporoides* was used as outgroup taxa to root the tree.

endophytes in Xylariaceae and Helotiales according to ITS phylogenetic analysis. As shown in Figure 2, all Xylariaceae fungi can be divided into three clades with high bootstrap values. Isolates AR7, AR9, AR10, AR16 and C2R22 clustered within *Xylaria* group, AR14 was placed into *Nemania* clade, and LR3, LR8 and AR8 fell

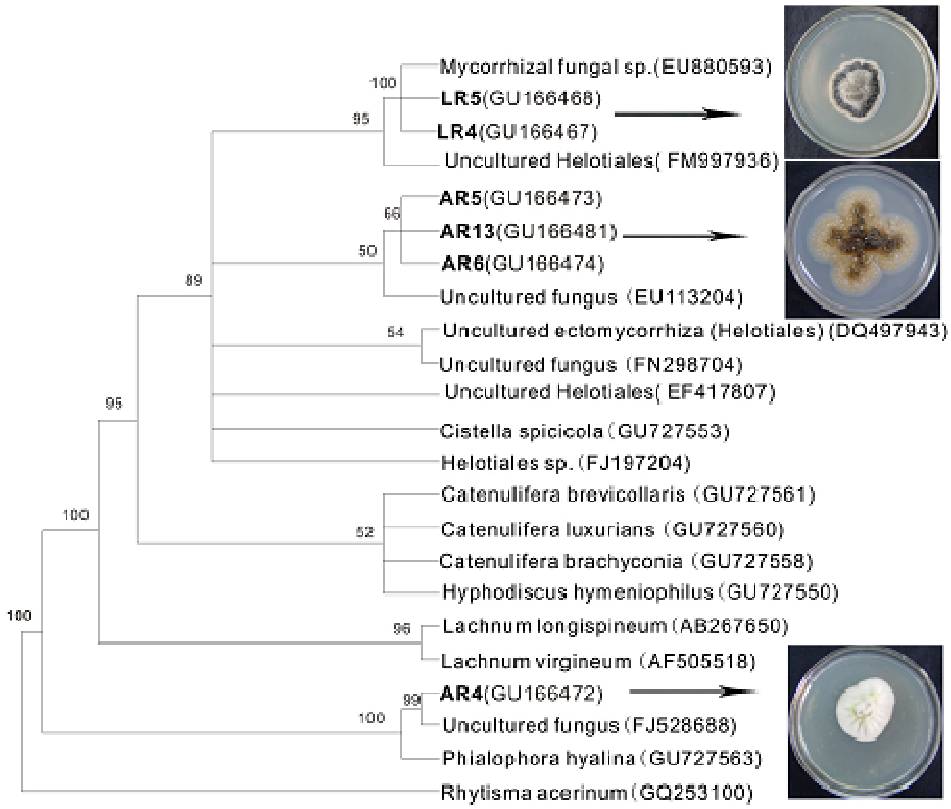

Figure 3. Phylogenetic relationships of taxa belonging to Helotiales as inferred based on ITS1-5.8S-ITS2 sequence (CI = 0.6494, RI = 0.6889, RC = 0.4474, HI = 0.3506). MP (Maximum Parsimony) bootstrap Values>50% are indicated above branch nodes. Tree length = 425.Number of bootstrap replicates = 1,000. *Rhytisma acerinum* was used as outgroup taxa to root the tree.

into *Hypoxylon* clade. With regards to Helotiales fungi, phylogentic analysis also placed five isolates into two distinctive clades (Figure 3). LR5 and LR4 were genetically close to an unidentified mycorrhizal fungus. AR5, AR13, and AR6 were sister to an uncultured fungus. However, we still can not define the six isolates into genera, because taxonomic assignment of Helotiales sequences in the GenBank is little reflecting difficulties with taxonomy and limited information of this group (Wang et al., 2006 a, b), we only know AR4 is high relative to *Phialophora*. Due to their absence of conidia production, accurate taxonomic appeared to be difficulty.

DISCUSSION

In this study, we performed a systematic investigation of symbiotic fungi in roots of *C. amoena*, a rare and endangered orchid plant. Expectedly, many Ascomycetous fungi were recovered and some of them were rarely reported as endophytes in Orchidaceae. However, *Tulasnella calospora*, a universal orchid symbiont, was only found in one site with low frequency (Figure 1). In general, non-mycorrhizal endophytes have been isolated from various orchids both in above-ground and below-ground tissues (Currah et al., 1997; Bayman et al., 1997; Bayman and Otero, 2006; Tao et al., 2008; Gezgin and Eltem, 2009; Pellegrino and Bellusci, 2009; Yuan et al., 2009). Although, the ecological roles played by these fungal groups are largely unknown, their ubiquity and diversity drive us to re-evaluate their ecological significances. It has been proposed that endophytic fungi would be a limiting factor for affecting the establishment and population sizes of orchids (Bayman et al., 1997). It should be noted that *Fusarium* species are common endophytes in orchid roots. *F. semitectum*, a red pigment producer, could induce protocorm formation and seed germination in *Cypripedium reginae* (Vujanovic et al., 2000; Vujanovic and Vujanovic, 2007).

In vitro co-culture system also indicates that *Fusarium* sp. and *Pyrenochaeta* sp. enhance the seedling growth of *Dendrobium loddigesii* (Chen et al., 2010). We assume that production of hormones such as gibberellins (GAs) by *Fusarium* spp. may be responsible for breaking the dormancy of orchid seeds and triggering a set of signaling events of plant growth and development (Tsavkelova et al., 2008). A novel hypothesis, named as 'mycovitalism', has been presented to address the roles

of non-mycorrhizal endophytes on orchid seed viability (Vujanovic and Vujanovic, 2007). In addition, some imperfect fungi including *Cytosporella*, *Gilmaniella* and *Mortierella* have been confimed to form symbiosis with germinating orchids (Ochora et al., 2001). In this study, one *Mortierella* isolate was identified in *C. amoena* roots. Recently, an endophytic isolate, isolated from an achlorophyllous orchid, *Epipogium roseum*, was also identified as *M. alpine* (unpublished data). It seems likely that some soil saprophytic fungi exhibit a degree of host-specificity and their ecological roles in orchid development and nutrition should not be underestimated. The Pezizales and Helotiales, two ascomycetous orders, have been shown to comprise taxa interacting as mycorrhizas (Julou et al., 2005).

In this study, we had also identified some taxa belonging to the two orders. To our knowledge, this is the first report that *Ascobolus* sp. serving as an orchid endophyte. In Helotiales, two taxa showed affinities to 'uncultured fungus' or unidentified 'mycorrhizal fungus' (Figure 3). Conidia and sporulating structure are not observed in pure cultures, therefore, their accurate taxonomy are still not resolved. Previous work also revealed fungi in the Pezizales and Helotiales usually encountered orchid roots (Stark et al., 2009). These ascomycetous taxa possibly form potential mycorrhizas for *C .amoena*, but experimentalevidences are needed. Unquestionably, orchid mycorrhizal fungi are very important for growth and survival of orchid plants, however, little is known about the roles of endophytic fungi for orchid performance, they may have been greatly underestimated as the importance of mycorrhizal fungi (Stark et al., 2009). In the future work, great attentions should be paid to endophyte-mediated growth promotion on this orchid species. Also, examination of interspecific interaction between mycorrhizal fungi and endophytes can yield more information concerning the complex fungal community associated with orchids.

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