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Isolation and identification of endophytic and mycorrhizal fungi from seeds and roots of *Dendrobium* (Orchidaceae)

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Abstract The seed germination of orchids under natural conditions requires association with mycorrhizal fungi. *Dendrobium nobile* and *Dendrobium chrysanthum* are threatened orchid species in China where they are considered medicinal plants. For conservation and application of *Dendrobium* using symbiosis technology, we isolated culturable endophytic and mycorrhizal fungi colonized in the protocorms and adult roots of two species plants and identified them by morphological and molecular analyses (5.8S and nrLSU). Of the 127 endophytic fungi isolated, 11 *Rhizoctonia*-like strains were identified as Tulasnellales (three strains from protocorms of *D. nobile*), Sebacinales (three strains from roots of *D. nobile* and two strains from protocorms of *D. chrysanthum*) and Cantharellales (three strains from roots of *D. nobile*), respectively. In addition, species of *Xylaria*, *Fusarium*, *Trichoderma*, *Colletotrichum*, *Pestalotiopsis*, and *Phomopsis* were the predominant non-mycorrhizal fungi isolated, and their probable ecological roles in the *Dendrobium* plants are discussed. These fungal resources will be of great importance for the large-scale cultivation of *Dendrobium* plants using symbiotic germination technology and for the screening of bioactive metabolites from them in the future.

Keywords Taxonomy · *Rhizoctonia*-like fungi · Seed packet · *D. nobile* · *D. chrysanthum*

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Introduction

Orchidaceae is one of the most species-rich plant families. Their commercial and horticultural values and ethnobotanical uses (in medicine or food) have long fascinated mankind. However, overharvesting and habitat destruction have driven many species in the wild towards extinction. Currently, all orchid species are listed on the Convention on International Trade in Endangered Species (Cribb et al. 2003).

Orchids have unique modes of acquiring nutrients (myco-heterotrophy) such that seed germination depends on carbon supplied from mycorrhizal fungi (Leake 1994; Smith and Read 2008). As the seeds of orchids are minute and contain limited energy reserves, their colonization by a compatible fungus is essential for germination under natural conditions (Dearnaley 2007). The potential applications of mycorrhizal association have recently drawn great attention from both horticulturists and conservationists (Nontachaiyapoom et al. 2010; Zettler et al. 2007; Rasmussen 2002). However, there still remain major issues regarding how to select the adaptable mycorrhizal fungi to ensure successful commercial cultivation.

Symbiotic germination experiments *in vitro* with fungi from orchid roots and taxonomic comparisons of fungi colonized in orchid roots have suggested that the mycorrhizal specificity of orchid is very complex (Dearnaley 2007). Photosynthetic mycorrhizal plants are associated with a wider range of mycorrhizal fungi than myco-heterotrophic plants (Smith and Read 2008), but some photosynthetic orchids are associated with only a single dominant mycorrhizal fungus (Shefferson et al. 2005). Epiphytic orchids, for example, usually establish symbiotic associations with only a few clades of fungi, particularly members of the Tulasnellaceae and Ceratobasidiaceae

(Suárez et al. 2006). In the early developmental stages of orchids (seed germination), the extent of mycorrhizal fungi specificity is not clear. Limited study has indicated that the seedling growth stage requires a more specific mycorrhizal association than the germination or mature stages of the forest orchids' life cycle (Bidartondo and Read 2008). Other studies have shown a succession of fungal species over the life cycle of the orchid (Hennigan 2009). For example, *Gastrodia elata*, an achlorophyllous orchid, needs only one fungus for germination (*Mycena osmendicula*), but secondary colonization by *Armillaria* sp. is necessary for full orchid development (Xu and Mu 1990). This may explain why some symbiotic germination experiments have high failure rates when using fungi collected from the roots of adult orchids (Sharma et al. 2003; Stewart and Kane 2006).

Dendrobium is a large genus of tropical epiphytic orchids. Some members of this genus, such as *Dendrobium candidum*, *Dendrobium nobile*, and *Dendrobium chrysanthum*, produce special pharmacological effects on gastritis infection, cancer, and aging, which makes them highly valued in Chinese traditional herbal medicine (Pharmacopoeia Committee of the P. R. China 2005). Currently, artificial cultivation using non-symbiotic tissue culture does not meet market demand because of slow growth and low survival rates. Therefore, the development of an effective method for propagating these endangered species for both conservation and commercial production is needed.

A number of studies regarding the role of the non-mycorrhizal endophytic fungi in *Dendrobium* have shown that endophytic fungi colonized in orchid roots could stimulate host plant growth (Hou and Guo 2009; Yuan et al. 2009), increase concentrations of total alkaloids and polysaccharides (Chen and Guo 2005), and even exhibit strong antibacterial activity (Xing et al. 2011). Additionally, it has been reported that fungal endophytes might produce metabolites similar to or more active than that of their hosts (Strobel 2002). Thus, collecting the endophytic fungi associated with *Dendrobium* sp. and screening their bioactive metabolites could, to some extent, assist in the search for novel compounds that is directed towards plants and help preserve wild plant resources from further destruction.

Symbiotic germination utilizing mycorrhizal fungi may be a way to overcome the difficulties of orchid seed germination and enhance the growth of seedlings (McKendrick et al. 2002; Johnson et al. 2007). Most of the previous symbiotic germination studies used mycorrhizal fungi isolated from only the roots of mature orchids, not from seeding stages. Although molecular approaches have identified some previously uncultured mycorrhizal fungi from orchid roots (Yukawa et al. 2009), the artificial propagation of orchids through symbiotic germination will require the isolation of the fungus.

With this knowledge, the present investigation was carried out to study the mycorrhizal and non-mycorrhizal endophytic fungi isolated from protocorms and from the roots of the two medicinal species of *Dendrobium* using *in situ* seed bait experiments. The study aims to address the diversity of *Dendrobium* seed/root-associated fungi and to obtain some culturable fungi that may be valuable for orchid propagation and conservation.

Materials and methods

Sample collection

Plants and seeds of *D. nobile* and *D. chrysanthum* were collected from Chishui, Guizhou Province and Xishuangbanna, Yunnan Province in China. The methods of seed collection and *in situ* seed germination experimentation were carried out according to Rasmussen and Whigham (1993). Seeds were collected from mature capsules that were about to release seeds and were examined under a dissecting microscope to ensure the presence of mature proembryos. Approximately 500 seeds were placed in each seed packet. A total of 100 seed packets of *D. nobile* and 50 seed packets of *D. chrysanthum* were placed in Guizhou province under humus and moss near the roots of adult populations of *D. nobile* located on either cliffs or tree trunks (Table 1). All seed packets were harvested after 7 months and then stored at 4°C until they were processed for 3 days in the laboratory. For examination, the packets were rinsed with tap water and opened with a mini-knife under a dissecting microscope; the protocorms (mycorrhizal seedlings) were then selected for isolating endophytic fungi.

Fungal isolation

Although single peloton isolation is the preferred method for isolating orchid symbionts (Warcup and Talbot 1967; Kristiansen et al. 2001), the isolation of pelotons from many epiphytic orchids is difficult because the orchid roots do not have massive mycorrhizal infections (Bayman et al. 2002). Based on our former study (unpublished data), fewer mycorrhizal fungi (*Rhizoctonia*-like fungi) were isolated from *Dendrobium* root using the traditional isolation method (plant tissues surface sterilize). Thus, in the present study, we followed the methods described by Porras-Alfaro and Bayman (2007) and Otero et al. (2002) for fungal isolation. Mycorrhizal areas identified as brown zones along the length of the roots were selected (Fig. 1). Brown and non-brown roots were washed with tap water and surface-sterilized in 75% ethanol for 1 min, in 3% NaClO for 3 min and then rinsed in sterile water three times; next,

Table 1 Details of the locations where studied plant materials collected and packets containing seeds of orchids were buried

Location	Plant name and label	Plant individual number	Orchid seeds buried	Seed packets	Burial (months)
Chishui town, Guizhou Province	*Dendrobium nobile*	2	*Dendrobium nobile*	100	7
	Dendrobium chrysanthum	2	*Dendrobium chrysanthum*	50	7
Xishuangbanna, Yunnan Province	*Dendrobium nobile*	2			
	Dendrobium chrysanthum	2			

Seeds were collected at the same site where they were buried

0.5–1-cm pieces were cut from each root. Five root pieces were plated on potato dextrose agar (PDA) medium. In total, 270 root fragments of *D. nobile* and 280 root fragments of *D. chrysanthum* were used for isolation. Protocorms were rinsed in distilled water and surface-sterilized in 75% ethanol for 10–20 s, 2.5% NaClO solution for 20–60 s, and finally rinsed in sterile distilled water four times. The sterilized seeds were put onto a glass slide and cut into small pieces with a scalpel to disperse the intracellular hyphal coils (pelotons). The isolation medium used was PDA supplemented with 50 μg ml^{-1} streptomycin and 50 μg ml^{-1} chloramphenicol. After isolation, the fungi were subcultured on PDA medium for identification.

Morphological and molecular identification

In order to maximize the morphological diversity of samples to be screened and to avoid repeated analyses of cultures that, at least in some cases, are derived from the same root, we selected some strains for subculture from each primary inoculation petri dish (containing 8–10 root segments) based on recognizably distinct morphology. At least two to three cultures were characterized from each petri dish, and no attempts were made to equate colony morphologies from different plates of the same plants (Cannon and Simmons 2002). Endophytic fungi were preliminarily grouped into genus or species level groups based on spore and culture characteristics (colony shape, height and color of aerial hyphae, base color, growth rate, margin, surface texture, and depth of growth into medium).

Fig. 1 Mycorrhizal zone of root of *Dendrobium nobile* (brown zone)

Sequence-based methods were also conducted to assist in specimen identification. Cultures of mycelia sterilia (failed to sporulate) were selected for DNA isolation. Genomic DNA was extracted from pure mycelium with the E.Z.N.A. Fungal DNA kit (Omega Bio-Tek, Doraville, GA, USA) according to the manufacturer's protocol. Internal transcribed spacer (ITS) regions and the 5.8S and large subunit rRNA (nrLSU) were amplified with primers pairs ITS1 and ITS4 and primers pairs LROR and LR7 (White et al. 1990), respectively. PCR amplification and sequencing were carried out in aqueous volumes of 25 μl. The reactions contained 12.5 μl of 2× Master PCR Mix [0.1 U Taq Polymerase/μl, 500 μM dNTP each, 20 mM Tris–HCl, 100 mM KCl, and 3 mM $MgCl_2$ (TIANGEN Biotech, Beijing, China)], 0.5 μl of each primer (10 μM), 1.0 μl of template DNA (50 ng/μl), and 10.5 μl of ddH_2O. The cycling parameters were an initial denaturation at 95°C for 3 min, followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 53°C for 30 s, and extension at 72°C for 30 s, with a final extension at 72°C for 7 min. Negative controls (without DNA template) were prepared for every group of amplifications. PCR products were purified using Watson's PCR purification kit (Watson, China). Sequencing was performed with a BigDye Terminator sequencing kit (Applied Biosystems, USA) and analyzed with an ABI 3730 automated sequencer (Applied Biosystems, USA).

All of the DNA sequences from each culture were compared to those in the GenBank database. For preliminary non-mycorrhizal endophytic fungi identification, we

adopted a BLAST method. Taxa that had more than 99% ITS sequence similarity were identified as reference taxa species; taxa that had less than 99% but more than 95% ITS sequence similarity with previously described species were identified at the genus level (Sánchez et al. 2008). Some species could not be identified on the basis of ITS sequence similarity.

Phylogenetic analyses

For mycorrhizal fungi (*Rhizoctonia*-like fungi), the well-characterized, closely related taxa obtained from BLAST searches were included in the phylogenetic analysis. For comparative analyses of the mycorrhizal fungi sequences from *D. chrysanthum* and *D. nobile*, 5.8S and nrLSU alignments, including the closest BLAST matches together with the fungal sequences from mature plants of tropical epiphytes (Suárez et al. 2006, 2008; Nontachaiyapoom et al. 2010), were analyzed. Sequences were aligned using Clustal X 1.83 (Thompson et al. 1997). Maximum parsimony analyses were performed by PAUP* version 4.0 beta 10 (Swofford 2002) using a heuristic search comprising of 1,000 replicates of random stepwise addition using tree bisection and reconnection (TBR) branch swapping with MulTrees option in effect and zero length branches collapsed. Bayesian analyses were implemented with MrBayes 3.1.2. The best-fitted evolutionary model was estimated using MrModeltest v. 1.01 (Nylander 2002). Bayesian analyses were run for 5 million generations, with trees sampled every 1,000 generations. The first 20, 000 trees, representing the burn-in phase, were discarded. A probability of 95% was considered significant. *Auricularia* sp. was set as the outgroup based on previous studies (Suárez et al. 2008; Shefferson et al. 2005) and the primary BLAST results.

Results

Fungi isolation rates of from roots and seed packets of two *Dendrobium* species

Few brown roots (mycorrhizal roots) were among the *Dendrobium* materials collected. Of the seven roots of *D. nobile*, only one root was brown, and yet four strains of mycorrhizal fungi were isolated from this single 10-cm brown root. In contrast, no brown roots were found among the six roots of *D. chrysanthum* that were collected (see Fig. 1).

The isolation rate of endophytic fungi was 48.1% (130 segments growing fungi/total 270 root segments) from the roots of *D. nobile* and 58.5% (164 segments growing fungi/280 root segments) from the roots of *D. chrysanthum*. The diversity of the mycorrhizal fungi isolated from *D. nobile* was low, with only five strains isolated from 270 root segments. In contrast, isolation rates of mycorrhizal fungi from protocorms were relatively high. In the 100 seed packets of *D. nobile*, only six protocorms were found, and three strains of mycorrhizal fungi were isolated. Similarly, in the 50 seeds packets of *D. chrysanthum*, a total of 29 protocorms were found, but only two strains of mycorrhizal fungi were isolated. A possible explanation is that some of the mycorrhizal fungi may not be readily culturable.

Fungal identification and morphological characteristics of *Rhizoctonia*-like fungi

We analyzed six protocorms and 270 root segments of *D. nobile* and obtained 62 endophytic fungi, including nine strains of *Rhizoctonia*-like fungi (three strains from protocorms and six strains from roots). In contrast, 29 protocorms and 280 root segments of *D. chrysanthum* were analyzed, and 65 endophytic fungi were isolated. Among the 65 fungi, two strains of *Rhizoctonia*-like fungi were isolated from protocorms. A great variety of non-mycorrhizal endophytic fungi were isolated from orchid roots and germinating seeds, and these species were distributed among three defined classes within Ascomycota (Sordariomycetes, Dothideomycetes, and Eurotiomycetes) as well as among the *incertae sedis*. Taxon affinities are listed the Table 2. Species of *Xylaria, Fusarium*, *Trichoderma*, *Colletotrichum*, *Pestalotiopsis*, and *Phomopsis* were the dominant fungal endophytes.

In total, 11 strains of *Rhizoctonia*-like fungi were preliminarily identified and grouped based on colony and hyphal characteristics. Samples 3563, 3564, and 3565 (isolated from protocorms of *D. nobile*) displayed nearly identical morphological characteristics and may represent isolates of the same species (only 3564 is shown in Fig. 2). The isolates grew slowly (0.5–0.6 mm/day), and 2-week-old colonies were white to light beige with irregular margins on PDA (Fig. 2a). Aerial mycelia were sebaceous with submerged margins. Monilioid cells were not detected on PDA (Fig. 2b).

Colony 3597, from protocorms of *D. chrysanthum*, was white and grew more rapidly than 3564. Three-week-old colonies were up to 7–8 cm in diameter. Aerial mycelia were sebaceous with submerged margins and grew radially from the center to the margin (Fig. 2c). Hyphae were hyaline, very fine (1–3 μm in diameter), and did not have clamp connections. Monilioid cells were global or subglobal (2.5–5×2.7–6 μm) in branched or unbranched short chains (Fig. 2d). Colony 3601, also from protocorms of *D. chrysanthum*, was white with regular margins. Three-week-old colonies were up to 8 cm in diameter. Aerial mycelia were sebaceous with submerged margins (Fig. 2e). Hyphae were 2–4 μm in diameter. Monilioid cells were irregular global, ellipsoidal, or sausage (Fig. 2f). Samples 5173 and 5390 from *D. nobile* root had similar colony characteristics

Table 2 127 strains of endophytic fungi and their closest relatives from GenBank

Fungal taxonomy	Isolate label	GenBank match (ITS-5.8S)			*Dendrobium nobile*		*Dendrobium chrysanthum*		Total
		Reference taxa (RT)	Accession no. of RT	Identity (%)	Germination seeds	Root	Germination seeds	Root	
Sordariomycetes	3554, 3566, 3567, 3568, 3573, 3574, 3576, 3577, 3580	*Trichoderma* sp.			2		7		9
	3556, 3584-3589	*Pestalotiopsis* sp.	FJ947050	99%	1		2		3
	3558	*Verticillium* sp.	FJ948142	99%	1				1
	3570	*Verticillium leptobactrum*	EF641871	99%			1		1
	3559	*Mycoleptodiscus* sp.	GU220382	93%	1				1
	3562	*Arthrinium euphorbiae*	AB220241	99%	1				1
Sordariomycetes	3578	*Glomerella truncata*	EU326180	99%			1		1
	5070, 5073, 5074, 5075	*Fusarium soloni*						4	4
	3586	*Fusarium oxysporum*					1		1
	3590	*Fusarium proliferatum*	GQ924905	99%			1		1
	5135, 5134, 5139, 5155, 5171	*Fusarium* sp.				5			5
	3579	Apiosporaceae sp.	DQ117959	99%			1		1
	3581, 3594, 5060, 5064, 5067, 5130	*Colletotrichum gloeosporioides*	GQ120492	99%		1	2	3	6
	3588, 3591	*Pochonia bulbillosa*	AB378554	98%			2		2
	3596	*Chloridium virescens* var. *chlamydosporum*	EF029220	98%			1		1
	3723, 3724, 3725, 3726, 3786, 5062	*Xylaria* sp1	FJ205470			4		2	6
	5053, 5056, 5058, 5063, 5065, 5072, 5082, 5132, 5138, 5157, 5164, 5166	*Xylaria* sp2				5		7	12
	5054	*Xylaria* sp3						1	1
	5071,5077	*Xylaria* sp4						2	2
	5144, 5145, 5147, 5151	*Xylaria* sp5				4			4
	5131, 5156, 5163, 5165	*Xylaria venosula*		96%		4			4
	5055, 5128, 5129, 5146	*Xylaria grammica*	GU300097	97%		3		1	4
	5133	*Xylaria castorea*	GU324751	94%		1			1
	5162	*Xylaria coccophora*	GU300093	95%		1			1
	5052, 5059	*Phialophora* sp.						2	2
	5057, 5068, 5076	*Phomopsis* sp.						3	3
	5079	*Cylindrocarpon destructans*						1	1
	5167	*Muscodor albus*	AY927993	99%		1			1
Dothideomycetes	3592	*Alternaria* sp.	FJ233188	99%			1		1
	3593	*Epicoccum nigrum*	FM991735	99%			1		1
Incertae sedis	3595	*Evlachovaea* sp.	FJ177456	99%			1		1
Eurotiomycetes	3600	*Penicillium funiculosum*					1		1
Agaricomycetes	*5168*	*Ceratobasidium* sp.	AB286935	97%		2			2
	5170	*Rhizoctonia* sp.	AJ242904	98%		1			1
	5173	Uncultured *Sebacina*	EU910926	94%		1			1
	5390	Uncultured *Sebacina*	EU910901	88%		1			1
	4035	Uncultured Sebacinales	FJ788822	89%		1			
	3597	Uncultured *Sebacina*	EU910901	88%			1		1
	3601	Uncultured *Sebacina*	EU910926	94%			1		1
	3563, *3564*, *3565*	*Epulorhiza* sp.	FJ613194	97%	3				3
	5169	*Rhizoctonia*-like fungi				1			
		Unidentified fungi			4	13	5	9	37
Total					13	49	30	35	127

Italicized fonts showed the *Rhizoctonia*-like fungi isolated from our study

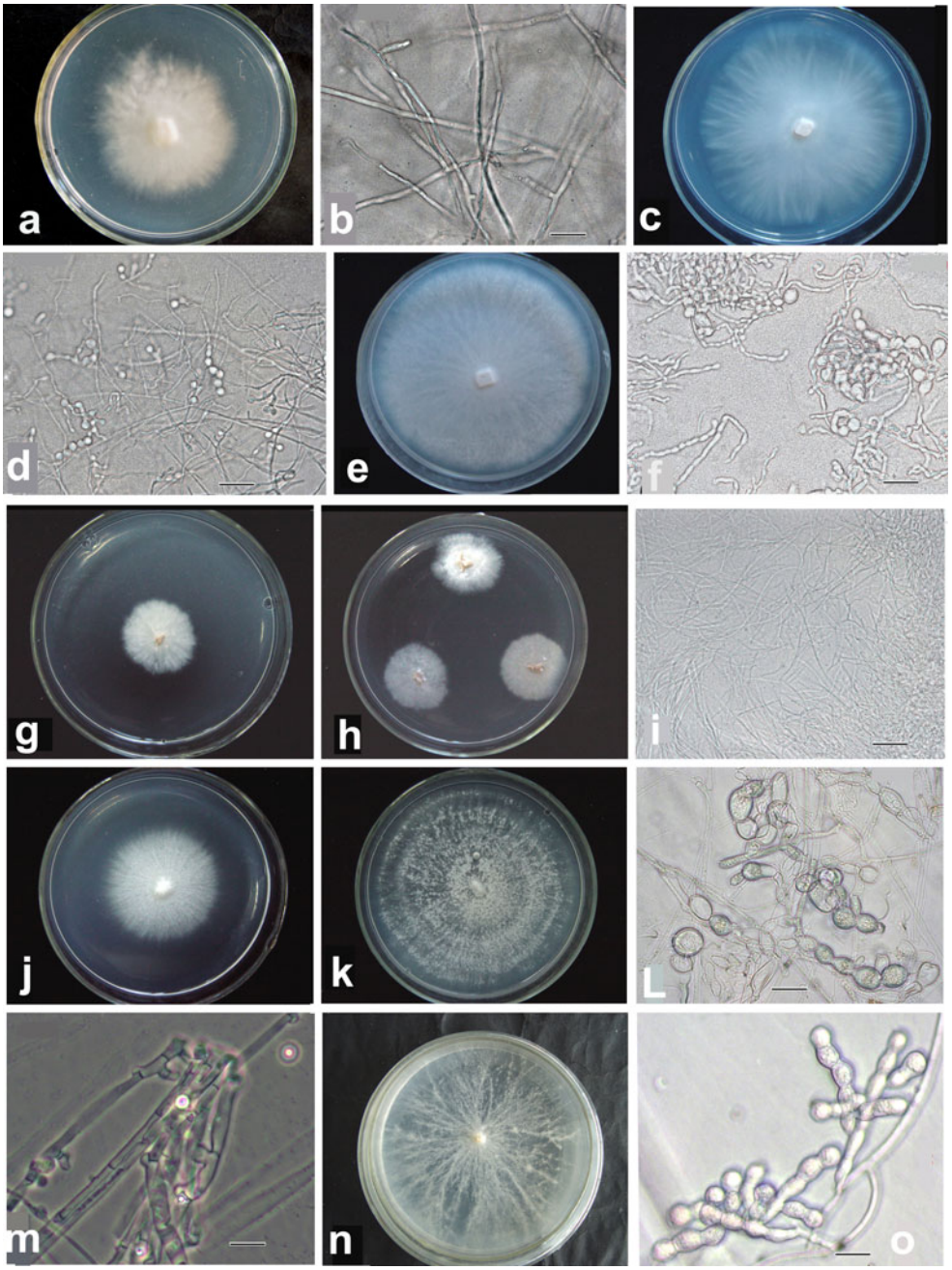

Fig. 2 Cultural and morphological characteristics of *Rhizoctonia*-like isolates. **a** Colony of 3564 at day 14. **b** Hyphal branching of 3564. **c** Colony of 3597 at day 21. **d** Monilioid cells of 3597. **e** Colony of 3601 at day 21. **f** Monilioid cells of 3601. **g**, **h** Colony of 5173 at day 15. **i** Hyphae of 5173. **j** Colony of 5168 at day 7. **k** Colony of 5168 at day 23. **l** Monilioid cells of 5168. **m** Hyphal branching with clamp connections of 5168. **n** Colony of 5169 at day 23. **o** Monilioid cells of 5169. *Scale bars*= 20 μm (except in **m**=10 μm)

(only 5173 is shown in Fig. 2). The colony was white and grew slowly. One-week-old colonies were up to 2.5–3 cm (Fig. 2g, h). Aerial mycelia were sebaceous with regular margins. Monilioid cells were not detected on PDA (Fig. 2i).

Samples 5168 and 5170, also isolated from the roots of *D. nobile*, had colony characteristics (only 5168 is shown in Fig. 2) similar to each other that were distinct from the other fungi. The color was light buff, and they had concentric zonation on PDA (Fig. 2j, k). Three-week-old colonies were up to 6–7 cm in diameter on PDA. Monilioid cells were ellipsoidal or global, hyaline in short branch or unbranched chains 6–14×9–27 μm long (Fig. 2l). Hyphae were hyphine and had clamp connections (Fig. 2m). Sample 5169 from the roots of *D. nobile* formed white, arachnoid colonies and were 6–7 cm in diameter after 3 weeks (Fig. 2n). Aerial mycelia were white and sparse. Monilioid cells were ellipsoidal or global, hyphine in short branch or unbranched chains 6–14×9–27 μm long (Fig. 2o).

Phylogenetic analyses of *Rhizoctonia*-like fungal specimens isolated from seeds and roots of *Dendrobium* plants

The ITS sequence of sample 3564 was preliminarily identified by BLAST searches as closest to those of

Epulorhiza sp. (anamorphic *Tulasnella*), with 97% similarity (FJ613194). The ITS sequences of the isolates 5168 and 5170 showed the highest similarity to those of the *Ceratobasidium* sp. and their anamorph, *Rhizoctonia*, with 97% and 98% identity, respectively. The ITS sequences of samples 5390 and 3597 did not show significant homology to any known cultured fungal species. Samples 5390 and 3597 had ITS sequences showing 88% similarity with a sequence from *Sebacina* sp. in GenBank (accession number EU910901). ITS sequences of isolates 3601 and 5173 had 94% similarity with another sequence from *Sebacina* sp. (accession number EU910926).

Phylogenetic tree constructed using 5.8S and 28S sequences and root with *Auricularia* sp. Sequence alignments of 5.8S rDNA contained 91 sequences and 151 characters, of which 70 were parsimony-informative. A maximum parsimony analysis on 5.8S produced 47 trees (TL=484, CI=0.7054, RI=0.9738).The strict consensus tree is shown in Fig. 3. The phylogenetic analysis of the 5.8S sequences divided the eight strains of *Rhizoctonia*-like fungi into three major clades with high bootstraps support (BP=100%) (Fig. 3). Clade I was comprised of sequences belonging to the Sebacinales and contained isolates 3601, 3597, 5173, 5390, and 4035. Clade II contained the sequences identified as *Epulorhiza* spp. and its teleomorph, *Tulasnella* spp., and the sequence from sample 3564. Clade III was composed of the sequences from samples 5168 and 5170, *Sistotrema* spp., *Ceratobasidium* spp., and their anamorph, *Rhizoctonia* spp.

The nrLSU data set contained 64 taxa and 1,150 total characters, of which 620 were parsimony-informative. A maximum parsimony analysis (22 trees, TL=911, CI=0.487, RI=0.812) produced topologies very similar to those obtained by the Bayesian analysis (Fig. 4). Seven of the *Rhizoctonia*-like fungi that were isolated were present in two large clades (sequencing of nrLSU from 3564 failed due to low quantity and quality of DNA): Clade I contained 4035, 3597, 5390, 5173, 3601, *Sebacina* spp., and other species of the Sebacinales (100% BP, 99% PP). Clade II was composed of 5168, 5170, *Botryobasidium* spp., *Sistotrema* spp., *Ceratobasidium* spp., and other species of the Cantharellales (100% BP). From the phylogenetic tree of constructed from nrLSU sequences, it can be inferred that isolates 5168 and 5170 are closely related to *Sistotrema* sp. Isolates 5390, 5173, 3597, and 4035 formed a small group, while strain 3601 grouped with some *Sebacina* species.

Discussion

Mycorrhizal symbionts of *Dendrobium* spp. were recorded as mostly belonging to the tulasnelloid fungi (Goh et al. 1992; Roberts 1999; Nontachaiyapoom et al. 2010). Morphological and phylogenetic analyses in our study indicated that the 11 *Rhizoctonia*-like isolates belong to three different phylogenetic groups. They were isolated from different orchid plants at different developmental stages: group I (tulasnelloid) from protocorms of *D. nobile*, group II (Sebacinales) from adult roots of *D. nobile* and protocorms of *D. chrysanthum*, and group III (Cantharellales) from adult roots of *D. nobile*. Furthermore, two fungal strains isolated from protocorms of *D. chrysanthum* belong to two species of Sebacinaceae. Failure to isolate mycorrhizal fungi from the roots of *D. chrysanthum* may be explained either by the fact that some of the mycorrhizal fungi are not readily culturable (fungi require specific media) or that fast-growing fungal species may have covered slower-growing *Rhizoctonia* strains, such as *Tulasnella* sp. It is also possible that there were potentially more isolates which were not recovered due to limited samples.

Previous studies regarding the specificity of orchid mycorrhizal relationships have demonstrated that the degree of specificity is variable, depending on the plant species and, possibly, on the developmental stage of individual plants (Otero et al. 2002; Xu and Mu 1990). Our results also suggest that plant–fungus specificity may be determined by both developmental stages of the plants and the plant species, although the data is very limited. As shown in Figs. 3 and 4, fungi isolated from the protocorm phase of *D. nobile* belonged to *Epulorhiza* spp. (anamorphic *Tulasnella*), whereas fungi isolated from adult root tissue were identified as anamorphic Sebacinales and Cantharellales. Similarly, fungi from the protocorm phase of *D. chrysanthum* were mainly anamorphic *Sebacinaceae*, which differ from those isolated from the protocorms of *D. nobile*. Moreover, preliminary symbiosis germination experiments have demonstrated that two strains (3597 and 3601), originating from the protocorms of *D. chrysanthum* that were found in the seeds packets, can promote seed germination and seedling development *in vitro* of *D. candidum* and *D. nobile* (Wang et al. 2011), while isolate 3564 has not had any conspicuous effects on the germination of *D. nobile*. This implies that *D. nobile* may not be a suitable mycorrhizal host for fungi from *D. nobile* protocorms or that the seed germination of *Dendrobium* species requires the presence of specific fungal species (Sebacinales but not *Tulasnella* spp.).

Since Rasmussen and Whigham (1993) developed a suitable seed baiting method in the hopes of gaining insight into the role of fungi in germination of orchid seed, this technique has been successfully used by McKendrick et al. (2000), Brundett et al. (2003), and Bidartondo and Read (2008) as an effective means for investigating *in situ* orchid seed germination and seedling development. Little has been reported in the literature concerning the isolation of mycorrhizal fungi from protocorms of *Dendrobium* using

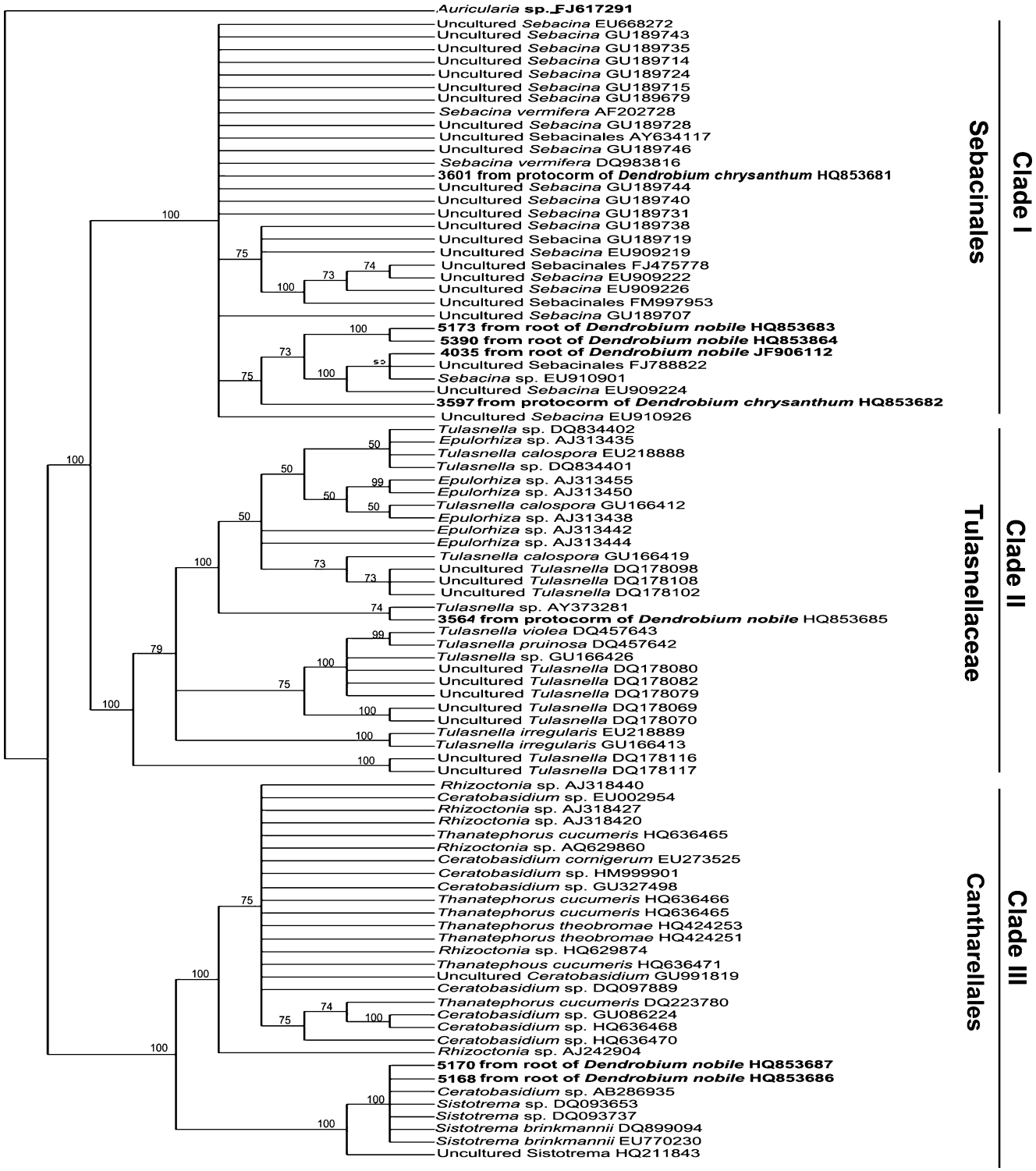

Fig. 3 Maximum-parsimony tree for *Rhizoctonia*-like isolates as inferred from 5.8S ribosomal RNA gene sequences. *Numbers above branches* are bootstrap values (≥50%) from 1,000 replicates

the baiting technique *in situ*. The present study is the first report about culturable mycorrhizal fungi isolated from germinating seed of *Dendrobium*. These culturable fungi have potentially valuable applications for the artificial cultivation of *Dendrobium* sp.

Furthermore, previous studies on non-mycorrhizal endophytic fungi in tropical orchid reported that *Xyaria* species were the most frequently observed symbiont in the roots of *Lepanthes* (Bayman et al. 1997). However, *Fusarium*, *Colletotrichum*, and *Phomopsis* species were also frequent-

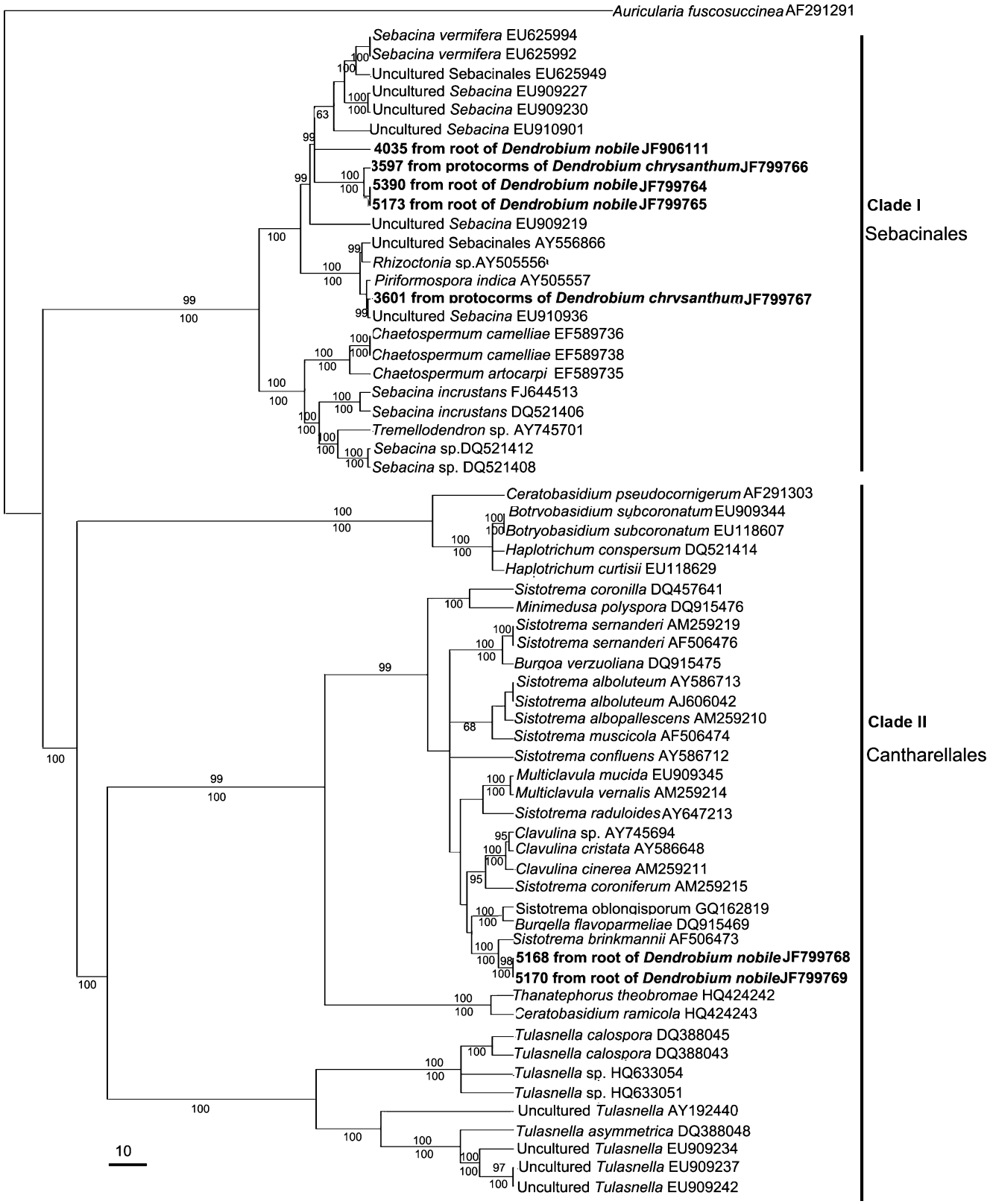

Fig. 4 Maximum-parsimony tree for *Rhizoctonia*-like isolates based on an alignment of nrLSU. *Numbers above branches* indicate posterior probabilities (>95%) and *numbers below branches* are bootstrap values (≥50%) from 1,000 replicates. The *bar* represents 10 substitutions per nucleotide position

ly observed in the root of medicinal plants of the *Dendrobium* genus (Yuan et al. 2009). In the current study, species of *Xylaria*, *Fusarium*, *Colletotrichum*, *Phomopsis*, and *Pestalotiopsis* were ubiquitous in the roots of *D. nobile* and *D. chrysanthum*, similar to the previous reports. Although *Xylaria* species were the predominant endophytic fungi found in the root of *Dendrobium*, their role as mycorrhizal fungi has not been illustrated and their ecological function remains unclear.

Endophytic fungi have been described as mutualists that protect host plants against insect herbivory, and many of those fungi produce biologically active secondary metabolites (Stone et al. 2000). Chen et al. (2010) and Xing et al. (2011) reported that endophytic fungi (e.g., *Fusraium* and *Phoma*) isolated from *Dendrobium thyrsiflorum*, *Dendrobium devonianum*, and *Dendrobium loddigesii* exhibited strong antibacterial or antifungal activities as well as plant-growth-promoting activities. These results imply that endophytic fungi isolated from different *Dendrobium* species could have potentially diverse applications.

Although the number of plants used to explore fungal diversity was limited (the two *Dendrobium* plants species are endangered in the wild, and thus, out of concerns for conservation, we only collected two individual plants per species), the fact that the culturable fungi isolated from *Dendrobium* plants in the current study is an important extension of previous work. However, the data from fungi that could not be cultured may differ from the current study. Thus, to obtain a better understanding of the diversity of mycorrhizal fungi associated with medicinal *Dendrobium* plants, the direct PCR amplification of fungi from the roots or protocorms, combined with more extensive sampling, should be performed in the future.

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