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ORIGINAL PAPER

Isolation and identification of *Rhizoctonia*-like fungi from roots of three orchid genera, *Paphiopedilum*, *Dendrobium*, and *Cymbidium*, collected in Chiang Rai and Chiang Mai provinces of Thailand

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Abstract Three orchid genera, *Paphiopedilum*, *Cymbidium*, and *Dendrobium*, are among the most heavily traded ornamental plants in Thailand. In this study, 27 isolates of *Rhizoctonia*-like fungi were isolated from root sections of mature orchids in the three orchid genera, collected from diverse horticultural settings in Chiang Mai and Chiang Rai provinces of Thailand. Fungal identification was done by the morphological characterization, the comparison of the internal transcribed spacer and 5.8S ribosomal DNA sequences, and the phylogenetic analysis. *Epulorhiza repens* was found to be the most common species found in the roots of various species of all three orchid genera, whereas *Epulorhiza calendulina*-like isolates were strictly found in the roots of *Paphiopedilum* species. We have also isolated and described an anamorph of *Tulasnella irregularis*, four new anamorphic species in the genus *Tulasnella*, and a new anamorphic species in the family Tulasnellaceae. Our study provides information on diversity of root-associated fungi of the orchid genera and at the sampling sites that were rarely addressed in the previous studies.

Keywords Orchid mycorrhia · *Rhizoctonia* · *Tulasnella* · *Epulorhiza* · *Paphiopedilum* · *Cymbidium* · *Dendrobium*

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Introduction

Mycorrhizal association is known to be beneficial to orchids (Dearnaley 2007; Rasmussen 2002). In nature, orchids rely on the infection of mycorrhizal fungi to provide the carbon sources for seed germination and seedling establishment (Yam and Arditti 2009). In some green and achlorophyllous orchid species, the dependency on mycorrhizal fungi prolong into adulthood (Abadie et al. 2006; Gebauer and Meyer 2003; Julou et al. 2005; Leake 1994; Rasmussen and Rasmussen 2007). Moreover, the applications of the mycorrhizal association for horticultural and conservation purposes have recently gained considerable attention (Rasmussen 2002; Rasmussen and Rasmussen 2007; Swarts and Dixon 2009; Zettler et al. 2007).

With a few exceptions (Bidartondo et al. 2004; Selosse et al. 2004), the majority of orchid mycobionts are basidiomycetes (Dearnaley 2007; Rasmussen 2002). Many fungi isolated from mycorrhizal orchid roots have been classified as *Rhizoctonia* or *Rhizoctonia*-like fungi (Otero et al. 2002; Shimura et al. 2009). *Rhizoctonia*-like fungi include the anamorphic genera, e.g., *Ceratorhiza*, *Epulorhiza*, *Moniliopsis*, and *Rhizoctonia*, of a variety of teleomorphs,

e.g., *Ceratobasidium*, *Sebacina*, *Tulasnella*, and *Thanatephorus* (Dearnaley 2007; Rasmussen 2002). The members in this form-genus are recognized by the cultural morphology such as colony color, hyphal branching, constriction of hyphae, and formation of septa a short distance from the point of origin of hyphal branches (Sneh et al. 1991). The approaches that have been used to identify the *Rhizoctonia*-like fungi include (1) cytomorphological characterization of anamorph and teleomorph (Sneh et al. 1991), and (2) anastomosis group (Sneh et al. 1991), characterization of ultrastructure of cell wall and septal pore (Currah and Sherburne 1992; Suarez et al. 2006; Wells and Bandoni 2001), and molecular characterization (e.g., Ma et al. 2003; Otero et al. 2002; Suarez et al. 2006; Taylor and McCormick 2008). Among the mentioned methods, the molecular analysis is one of the most efficient because (1) a finer distinction between strains is possible (Rasmussen 2002), (2) induction of teleomorphic stage is not necessary, and (3) it is rapid and needs no expensive instrument. Recently, Taylor and McCormick (2008) have published an effective primer pair that allows the amplification of the internal transcribed spacer (ITS) and 5.8S ribosomal DNA (rDNA) region of all tested Basidiomycota and does not exclude *Tulasnella* species, and thus should give a less biased view of orchid associations within the Basidiomycota.

Thailand is home to approximately 170 genera and 1,230 species of wild orchids (Nanakorn and Indharamusika 1998). The country is also famous for the diversity of orchid hybrids and is one of the world largest orchid exporters. Some of the most heavily traded orchid genera as recorded in the Convention on International Trade in Endangered Species of Wild Fauna and Flora statistics include *Dendrobium*, *Cymbidium*, and *Paphiopedilum*. Despite the rich orchid diversity, the number of scientific studies of orchid mycorrhizal diversity in Thailand is limited (Athipunyakom et al. 2004). Northern Thailand is rich in orchid diversity. More than 600 species of orchids including some species in the genera *Dendrobium*, *Cymbidium*, and *Paphiopedilum* are native to this region (Nanakorn and Indharamusika 1998). Additionally, this region is an important center of orchid cultivation. A large number of orchid tissue culture laboratories and nurseries are located in Chiang Rai and Chiang Mai provinces. In this study, we isolated 27 isolates of *Rhizoctonia*-like fungi from roots of orchids collected from diverse horticultural settings in the two provinces. Fungal identification was done using both morphological and molecular characterization. The results obtained from this study provide information on diversity of root-associated fungi of the orchid genera and at the sampling sites that were rarely addressed in the previous studies, and are potentially useful for orchid propagation and conservation.

Materials and methods

Plant materials

Roots of three commercial orchid genera, *Paphiopedilum*, *Dendrobium*, and *Cymbidium* collected from diverse sites in Chiang Mai and Chiang Rai provinces of Thailand were investigated for root-associated fungi. Orchid species and hybrids, locations of the orchid plants, and the original sources of the plants are presented in Table 1. The sampling locations and the sites of the orchid original sources are shown in Fig. 1.

Fungal isolation

Collected roots packed in zip-lock bags were transferred to the Microbiology Laboratory of Mae Fah Luang University in Chiang Rai province on ice. Hand sections of roots were examined under a compound light microscope for the presence of pelotons. Colonized roots were surface-sterilized in 3% (*v*/*v*) H_2O_2 and 70% (*v*/*v*) ethanol for 10 min, and subsequently rinsed three times in sterile distilled water. Root sections of 1–2 mm thickness were obtained by cutting the middle part of the root using a thin blade and forceps under a dissecting microscope. After the removal of velamen, root sections were placed on potato dextrose agar (PDA) containing 50 µg/ml oxytetracycline, 50 µg/ml streptomycin, and 50 µg/ml penicillin (Otero et al. 2002). Samples were incubated at 30°C. They were observed for fungi growing from the cortical cells of the root sections every 2 days for at least 2 weeks. Hyphal tips of the right-angle branching fungi were transferred to fresh PDA. Fungal isolates that have *Rhizoctonia*-like characteristics: (1) branching near the distal septum of cells in young vegetative hyphae, (2) constriction of hyphae at the branch points, and (3) formation of septum a short distance from the branch point (Sneh et al. 1991), were selected for further characterization and DNA extraction.

Morphological characterization

All fungal isolates were cultured on PDA at 30°C in the dark, with the exception that the isolates Cl-QS-0-1 and Cl-QS-0-2 were grown on PDA supplemented with 1× Murashige and Skoog (MS) vitamins (Murashige and Skoog 1962). For fast-growing fungal isolates, diameters of three colonies were measured every 2–3 days until it reached 9 cm. For slow-growing fungal isolates, diameters of three colonies were measured every week for at least 3 weeks. Growth rate was calculated by plotting the colony diameters against times. Cultural characteristics (e.g., colony color, colony zonation, and types of hyphae) were observed over a 1-month period. For microscopic examination, hyphae

Table 1 Orchid species and hybrids studied, locations of the orchid plants, the original sources of the plants, and the corresponding fungal isolates

Code for fungal isolate[a]	Orchid species/hybrid[b]	Location[c]	Original source[d]
Cl-QS-0-1[e], Cl-QS-0-2[e]	*C. lowianum* (Cl)	QS	0
Cs-QS-0-1	*C. sinense* (Cs)	QS	0
Ct-PC-1-1	*C. tracyanum* (Ct)	PC	1
C1-DT-TC-1	*C.* Golden Elf (C1)	DT	TC
C2-DT-TC-1	*C.* Miniature (C2)	DT	TC
C3-DT-TC-1, C3-DT-TC-2	*C.* Golden Elf× *Grammatophyllum measuresianum* (C3)	DT	TC
Da-KP-0-1	*D. anosmum* (Da)	KP	0
Dcr-QS-0-1, Dcr-QS-0-2	*D. crystallinum* (Dcr)	QS	0
Df-QS-3-1	*D. friedericksianum* (Df)	QS	3
D1-KT-TC-1	*Dendrobium* Salaya Red (D1)	KT	TC
Pca-QS-0-1	*P. callosum* (Pca)	QS	0
Pch-SM-TC-1	*P. charlesworthii* (Pch)	SM	TC
Pch-QS-0-1[e], Pch-QS-0-2[e], Pch-QS-0-3	*P. charlesworthii* (Pch)	QS	0
Pe-QS-0-1	*P. exul* (Pe)	QS	0
Ps-KT-0-1	*P. sukhakulii* (Ps)	KT	0
Ps-AT-0-1[e], Ps-AT-0-2[e]	*P. sukhakulii* (Ps)	AT	0
Pv-PC-2-1, Pv-PC-2-2	*P. villosum* (Pv)	PC	2
Pv-PC-1-1	*P. villosum* (Pv)	PC	1
Pv-QS-0-1[e], Pv-QS-0-2[e]	*P. villosum* (Pv)	QS	0

[a] Code for fungal isolate is composed of the code for corresponding orchid species/hybrid, the code for location, the code for original source of the orchid plant, and a number corresponding to the order in which the fungus has been isolated

[b] Letters in parentheses represent codes for corresponding orchid species/hybrids

[c] Locations are indicated by codes: *QS* represents Queen Sirikit Botanic Garden, Mae Rim District, Chiang Mai; *PC* represents *P. villosum* conservation project, Doi Inthanon, Chom Thong District, Chiang Mai; *DT* represents Doi Tung Development Project, Doi Tung Royal Villa, Mae Fah Luang District, Chiang Rai; *KP* represents a private collection of Mr. Kittchai Phulek, Mae Fah Luang University, Muang District, Chiang Rai; *KT* represents Khamtiang Market, Muang District, Chiang Mai; *SM* represents Suanbua Maesa Orchid Nursery, Mae Rim District, Chiang Mai; *AT* represents a private collection of Mr. Auttapon Taluengjit, Nang Lae, Muang District, Chiang Rai

[d] Original sources are represented by codes: *0* indicates that the original sources were unknown or uncertain; *1* represents Doi Inthanon National Park, Chiang Mai province; *2* represents Khun Yuam District, Mae Hong Son province; *3* represents Mae Sariang District, Mae Hong Son province. All plant tissue culture laboratories are given the same code, *TC*

[e] Fungal isolates obtained from the same root

mounted in either distilled water or a solution consisting of one drop of 0.5% (*w*/*v*) Safranin O and one drop of 3% KOH were examined under a compound light microscope (Axiotech, Carl Zeiss, Jena, Germany) attached with a digital camera (DSC-S85, Sony, Tokyo, Japan).

DNA extraction, PCR, and sequencing

Mycorrhizal tissue was collected from the fungal colony growing on PDA by melting the medium on a hot plate. All tissues were kept in a −80°C freezer before use. DNA was extracted using a GF-1 Plant Extraction Kit (Vivantis Technologies, Selangor DE, Malaysia) with the modification that the frozen tissue was homogenized in Buffer PL using a tube pestle. Amplification reactions of 15 μl containing 0.75 unit of *Taq* DNA polymerase (Invitrogen, Carlsbad, CA, USA), 1X polymerase chain reaction (PCR) Buffer (20 mM Tris–HCl, 50 mM KCl, pH8.4), 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, and 400 nM forward and reverse primers were carried out to test the pair of primers and annealing temperature that gave large quantity of single PCR product. The PCR conditions were an initial denaturation at 95°C for 2 min, followed by 35 cycles of denaturation at 94°C for 1 min, annealing at various temperatures (between 52°C and 58°C) for 30 s, and elongation at 72°C for 30 s, with a final extension at 72°C for 5 min. Three sets of primer pairs used in this study were (1) ITS1-OF-1 (5′-AACTCGGCCATT TAGAGGAAGT-3′)/ITS4-OF, (2) ITS1-OF-2 (5′-AACT GGTCATTTAGAGGAAGT-3′)/ITS4-OF, and (2) ITS1-OF-1/ITS4 (Taylor and McCormick 2008; White et al. 1990). For sequencing, amplification was carried out in a 100-μl reaction using components and conditions described above. The reaction product was purified with a Gel/PCR DNA Fragments Extraction Kit (RBC Bioscience, Taipei, Taiwan). Purified samples were then sent to first Base Pte Ltd (Singapore) for sequencing using two sequencing

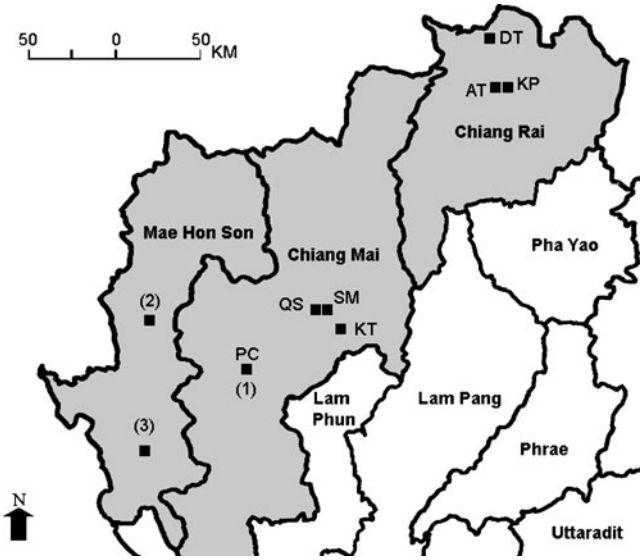


Fig. 1 Map of the upper northern region of Thailand showing the sampling locations and the sites of the original sources of the orchid plants. Names of provinces are written in *bold*. *QS* Queen Sirikit Botanic Garden; *PC Paphiopedilum villosum* conservation project, Doi Inthanon; *DT* Doi Tung Development Project, Doi Tung Royal Villa; *KP* a private collection of Mr. Kittchai Phulek, Mae Fah Luang University; *KT* Khamtiang Market; *SM* Suanbua Maesa Orchid Nursery; *AT* a private collection of Mr. Auttapon Taluengjit; *1* Doi Inthanon National Park; *2* Khun Yuam District; *3* Mae Sariang District

primers, ITS1 and ITS4 (White et al. 1990). Two sequences obtained for each sample were manually trimmed and subsequently assembled into one contig using CAP3 (Huang and Madan 1999). All sequences obtained in this study were deposited at GenBank under accession number GU166403-GU1664029.

Sequence analysis

BLAST searches (www.ncbi.nlm.nih.gov/BLAST, Altschul et al. 1997), using two types of query sequences: ITS1-5.8S rDNA-ITS2 or 5.8S rDNA, were conducted on all sequences to determine their close known relatives. Multiple sequence alignments and calculation of percent identity were performed with CLUSTALW2 (http://www.ebi.ac.uk/Tools/clustalw2/index.html). For further sequence analyses, sequences were handled with BIOEDIT (Hall 1999), and sequence alignments were performed using CLUSTALX version 2.0 (Larkin et al. 2007) with minor manual editing. Due to the reportedly highly diverse ITS sequences of orchid mycorrhizas (Shefferson et al. 2007; Suarez et al. 2006; Taylor and McCormick 2008), an alignment of 5.8S rDNA sequences of all fungal isolates was initially performed to calculate a maximum parsimony tree showing position of major clades, then the phylogenetic relationship among the fungal isolates in the major clade was analyzed based on the ITS1-5.8S rDNA-ITS2 sequences. The well-characterized, closely related taxa obtained from BLAST searches were also included in the phylogenetic analyses. The maximum parsimony analysis was performed by the program PAUP* version 4.0 beta 10 (Swofford 2002) using heuristic search comprising 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. Bootstrap analysis was performed with 1,000 replicates, each comprising 100 random addition heuristic search cycles, TBR branch swapping, and MulTrees on. Tree rearrangements were limited to 5,000 per bootstrap replicate.

Results

Fungal isolation and morphological characterization

Although the peloton isolation is the preferable method for isolation of orchid symbionts (e.g., Rasmussen 2002; Taylor and Bruns 1997; Warcup and Talbot 1967), isolation of pelotons from many orchids is difficult because the orchid roots do not have massive mycorrhizal infections (Bayman et al. 2002; Otero et al. 2002, 2007). Additionally, under a dissecting microscope, we observed that it was not possible to distinguish between live and dead pelotons and between pelotons and other contaminants after the root tissues were teased apart. Therefore, we used a method described by Otero et al. (2002) for the fungal isolation.

Fungal pelotons could be observed in nearly all root sections of terrestrial orchids (i.e., *Paphiopedilum* spp., *Cymbidium sinense*, and all *Cymbidium* hybrids) used in this study, whereas only roots of epiphytic orchids (i.e., *Dendrobium* spp., *Cymbidium lowianum*, and *Cymbidium tracyanum*) that were in contact with the growth substrate contained pelotons. This finding is consistent with the reports of previous investigators (Goh et al. 1992; Porras-Alfaro and Bayman 2007). From each individual orchid sample (i.e., individual species collected at a given location), only the *Rhizoctonia*-like isolate(s) that showed unique cultural characteristics were given codes and subjected to further morphological characterization and collection of mycelium for DNA extraction. Of the 19 individual orchid samples, each of 12 samples gave rise to one *Rhizoctonia*-like isolate, whereas each of the six samples gave rise to two *Rhizoctonia*-like isolates, and only one sample gave rise to three *Rhizoctonia*-like isolates (Table 1). Because isolates Cl-QS-0-1 and Cl-QS-0-2 grew very slowly on PDA (less than 0.6 mm/day), MS vitamins were added to the culture medium. Amino acids and vitamins, especially thiamine, were reported to be essential for the growth of some orchid endophytes and *Rhizoctonia* isolates (Hadley and Ong 1978; Hijner and Arditti 1973).

Colonies of isolates Cl-QS-0-1 and Cl-QS-0-2 cultured on PDA supplemented with MS vitamin grew at the rate of 1.1 and 1.2 mm/day, respectively. Some of the *Rhizoctonia*-like isolates can be grouped based on the colony and hyphal characteristics. These isolates are (1) Pca-QS-0-1, Pch-QS-0-3, and Pe-QS-0-1; (2) D1-KT-TC-1 and C3-DT-TC-2; (3) Pv-QS-0-2 and Df-QS-3-1; and (4) 13 *Rhizoctonia*-like isolates (i.e., Cs-QS-0-1, Da-KP-0-1, Dcr-QS-0-1, DCr-QS-0-2, Pch-SM-TC-1, Pch-QS-0-1, Pch-QS-0-2, Ps-KT-0-1, Ps-AT-0-1, Ps-AT-0-2, Pv-PC-2-1, Pv-PC-2-2, and Pv-PC-1-1), tentatively identified as *Epulorhiza repens*.

Pca-QS-0-1, Pch-QS-0-3, and Pe-QS-0-1 isolates were distinct from the other isolates based on the color of light buff to orange on PDA (Fig. 2a) and obovoid, fusiform or irregular monilioid cells, produced in short, branched or unbranched chains, 5–11×12–27 μm (Fig. 2b). Hyphae were 3–4.5 μm in diameter. Because the phenotypes were similar to those described for *Epulorhiza calendulina*-like isolates (Ma et al. 2003), the Pca-QS-0-1, Pch-QS-0-3, and Pe-QS-0-1 isolates were tentatively identified as *E. calendulina*-like *Rhizoctonia*.

Colonies of D1-KT-TC-1 and C3-DT-TC-2 grew slowly (2–3 mm/day). Three-week-old colonies were white to light beige with irregular margin (Fig. 2c). Aerial hyphae were 2–4 μm in diameter. Monilioid cells are ellipsoidal, 6–10×8–12 μm, in branched or unbranched chains (Fig. 2d).

Pv-QS-0-2 and Df-QS-3-1 isolates formed appressed colony with submerged margin (Fig. 2e). Colonies, white to beige, grew at 1.8–1.9 mm/day. Hyphae were 2–4 μm in diameter and frequently contained oil globules. Monilioid cells were irregular (Fig. 2f).

Thirteen isolates tentatively identified as *E. repens* isolates showed different cultural characteristics (data not shown). Colonies grew at the rate 3–9 mm/day. Colony colors were white or beige with aerial and submerged hyphae ranging from 2 to 9 μm in diameter. Colony of two isolates (i.e., Ps-KT-0-1 and Dcr-QS-0-2) showed concentric zonation. Monilioid cells were ellipsoidal or globose, 6–14×10–25 μm. Sclerotia were observed in seven isolates.

Other eight isolates, Cl-QS-0-1, Cl-QS-0-2, C1-DT-TC-1, C2-DT-TC-1, C3-DT-TC-1, Ct-PC-1-1, and Pv-QS-0-1, could neither be grouped nor identified because they did not yield many distinguishing characters or their morphology did not match any known species. The isolate Cl-QS-0-1 (Fig. 2g, h) showed no detectable monilioid cells. Hyphae of this isolate were 1.2–4 μm in diameter. The isolate Cl-QS-0-2 (Fig. 2i) showed very fine hyphae, 1.7–2.4 μm in diameter, and irregular monilioid cells formed at the tip cells of main hyphae (Fig. 2j). The isolates C1-DT-TC-1 (Fig. 2k), C2-DT-TC-1(Fig. 2m), and C3-DT-TC-1 showed hyphae of 3–4 μm in diameter. Only the monilioid cells of C1-DT-TC-1 were observed. They were obovate or elliposoidal in shape, usually formed in a short chain (Fig. 2l). The isolate Ct-PC-1-1 did not show detectable monilioid cells in the 18-day-old culture. The isolate Pv-QS-0-1 (Fig 2n) grew at 6 mm/day. Hyphae were 2.5–4 μm in diameter. Monilioid cells were ellipsoidal to globose, 9–13×9–18 μm (Fig. 2o). Alkaline safranin O staining revealed that all of the fungal isolates were binucleate (e.g., Fig 2b, d, l). No teleomorphic state of any *Rhizoctonia*-like isolate was observed throughout the study.

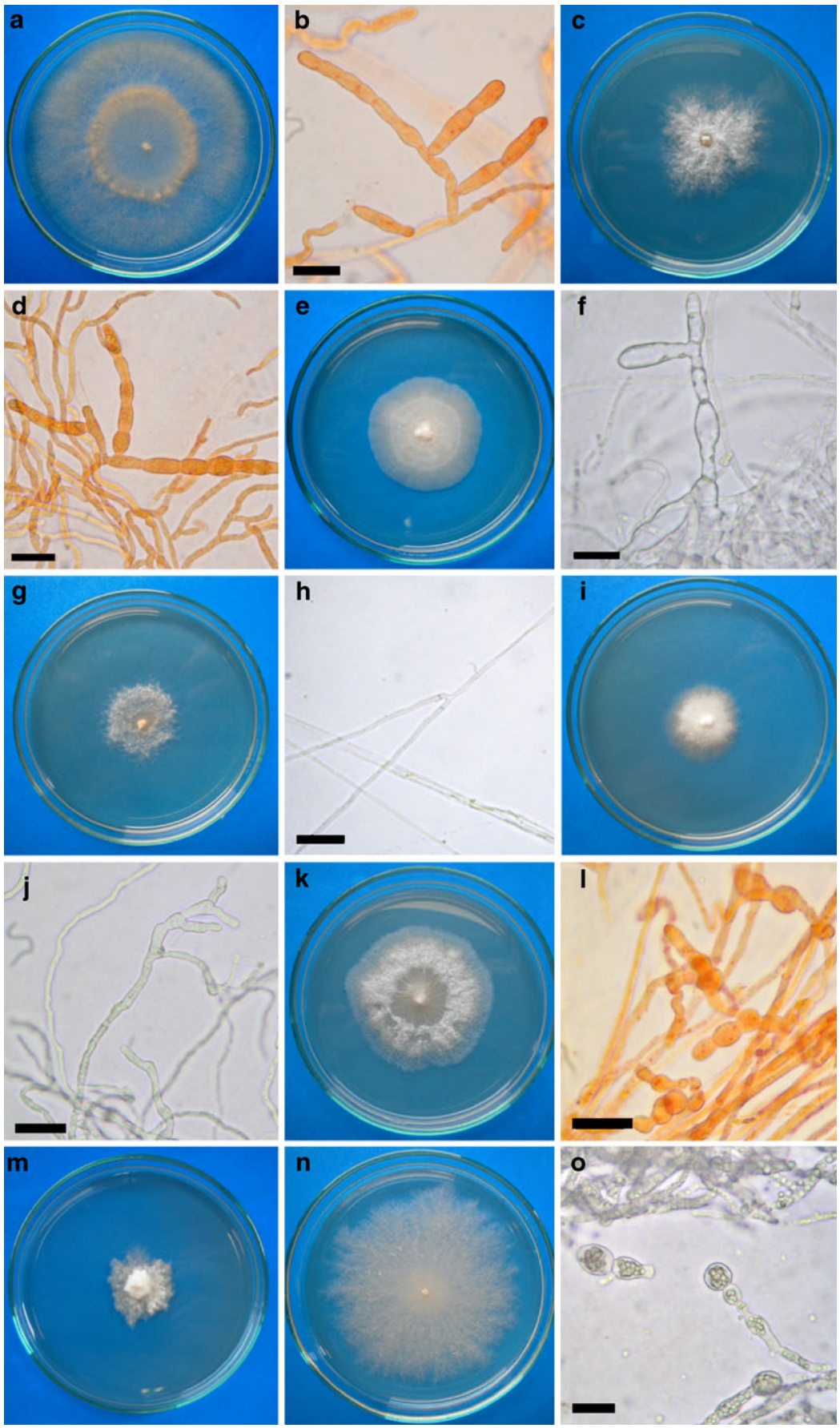
Molecular characterization

Because an initial attempt to culture some *Rhizoctonia*-like isolates in potato dextrose broth was not successful, we collected the fungal mycelia on PDA by melting the medium on a hot plate and have found that the material was suitable for the DNA extraction method used in this study. The primers ITS1-OF (the mixture of ITS1-OF-1 and ITS1-OF-2) and ITS4-OF have been shown to be effective across all Basidiomycota and was recommended to be used for initial characterization of the unknown orchid symbionts (Taylor and McCormick 2008). However, we found that in many cases: (1) using either the primer ITS1-OF-1 or ITS1-OF-2 alone and the primer ITS4-OF or (2) using the primer ITS1-OF-1 and the primer ITS4 could increase the specificity and amplification efficiency (data not shown). Large quantity of a PCR product is essential when the purified PCR product is subjected to direct DNA sequencing without being cloned into a vector.

Results of the BLAST searches are shown in Table 2. The ITS-5.8S rDNA sequences of the isolates Pca-QS-0-1, Pch-QS-0-3, and Pe-QS-0-1 shared high homology with those of *E. calendulina*-like isolates (Ma et al. 2003). The sequence identity of the ITS-5.8S rDNA ranged from 94% to 96% and that of 5.8S rDNA ranged from 99% to 100%. According to the morphological and molecular data, these three isolates were identified as *E. calendulina*-like *Rhizoctonia*. The 5.8S rDNA sequence of the isolate Pv-QS-0-1 also showed high sequence identity (92–93%) to those of *E. calendulina*-like isolates; however, its ITS-5.8S rDNA sequence showed only 65% identity (Table 3). Unlike the isolates Pca-QS-0-1, Pch-QS-0-3, and Pe-QS-0-1, the colony color of the isolate Pv-QS-0-1 was light beige (Fig. 2n), and monilioid cells were elliposoidal to globose (Fig. 2o).

The ITS and 5.8S rDNA sequences of the isolates D1-KT-TC-1 and C3-DT-TC-2 showed 99% and 100% identity with the ITS and 5.8S rDNA sequences of *Tulasnella irregularis* (Taylor and McCormick 2008), respectively. According to the molecular data, these two isolates were identified as anamorphs of *T. irregularis*.

The ITS-5.8S rDNA sequences of the isolates Pv-QS-0-2 and Df-QS-3-1 showed highest identity to those of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◀ **Fig. 2** Cultural and morphological characters of *Rhizoctonia*-like isolates. **a** Culture of isolate Pe-QS-0-1 at day 14. **b** Monilioid cells of isolate Pe-QS-0-1 stained with alkaline safranin O. **c** Culture of isolate D1-KT-TC-1 at day 12. **d** Monilioid cells of isolate C3-DT-TC-2 stained with alkaline safranin O. **e** Culture of isolate Df-QS-3-1 at day 22. **f** Monilioid cells of Df-QS-3-1. **g** Culture of isolate Cl-QS-0-1 at day 18. **h** Hyphal branching of isolate Cl-QS-0-1. **i** Culture of isolate Cl-QS-0-2 at day 18. **j** Monilioid cells of isolate Cl-QS-0-2. **k** Culture of isolate C1-DT-TC-1 at day 13. **l** Monilioid cells of isolate C1-DT-TC-1 stained with alkaline safranin O. **m** Culture of isolate C2-DT-TC-1 at day 29. **n** Culture of isolate Pv-QS-0-1 at day 14. **o** Monilioid cells of isolate Pv-QS-0-1. *Bars* represent 20 μm

Tulasnella violea isolates in GenBank (accession numbers DQ457643 and AY373303), but with only 88% identity. The isolates C1-DT-TC-1, C2-DT-TC-1, and C3-DT-TC-1 also have their ITS-5.8S rDNA sequences showing 85%, 87%, and 84% with the two sequences of *T. violea*, respectively. It is important to note that the ITS-5.8S rDNA sequences of the two *T. violea* isolated by different investigators (Matheny et al. 2006; McCormick et al. 2004) showed sequence identity as high as 100%.

The sequence comparison confirmed that the 13 isolates, tentatively identified as *E. repens* based on morphological characteristics, and the isolate Ct-PC-1-1 were *E. repens* isolates. Ma et al. (2003) reported that the sequence identity of the ITS region of their *E. repens* isolates was 84–99% (76–100% for ITS1 and 84–99% for ITS2) and 97–100% for 5.8S rDNA. Similarly, the fungal isolates obtained in this study shared 79–98% sequence identity for the ITS region and 96–100% sequence identity for the 5.8S rDNA among each other and showed 81–95% and 95–99% sequence identity for the ITS region and the 5.8S rDNA, respectively, with those of the *E. repens* isolates of Ma et al. (2003).

The ITS-5.8S rDNA sequences of the isolates Cl-QS-0-1 and Cl-QS-0-2 did not show high homology with high sequence coverage to any known cultured fungal species. The ITS-5.8S rDNA sequence of the isolate Cl-QS-0-1 showed 81% identity with uncultured member of Tulasnellaceae (GenBank accession number DQ925494), whereas the 5.8S rDNA sequence showed 98% identity with the *Tulasnella* sp. (GenBank accession number AY373316). The ITS-5.8S rDNA sequence of the isolate Cl-QS-0-2 showed 83% identity with the uncultured *Tulasnella* sp. (GenBank accession number DQ178072), whereas the 5.8S rDNA sequence showed 99% and 98% identity with the uncultured *Tulasnella* sp. (GenBank accession number DQ178081) and *Tulasnella pruinosa* (GenBank accession number DQ457642), respectively. These two fungal isolates are potentially new species in the genus *Tulasnella*.

The maximum parsimony (MP) analysis using 5.8S rDNA sequences divided the fungal isolates into two major groupings with high bootstrap values (Fig. 3) and five subclades with bootstrap support of greater than 70% (Fig. 3). The first grouping consisted of four subclades (Clade I to IV). Clade I contained six sequences obtained from our isolates, the sequences of three *Tulasnella* species (i.e., *Tulasnella asymmetrica*, *T. violea*, and *T. pruinosa*), and a sequence of uncultured *Tulasnella*. Clade II comprised 14 sequences of the isolates identified as *E. repens* and the sequences of *E. repens* and its teleopmorph, *Tulasnella calospora*, obtained from the GenBank database. The sequences of *Epulorhiza anaticula* and *Tulasnella danica* included in the analysis also belonged to this clade. Clade III contained the sequences from isolates C3-DT-TC-2, D1-KT-TC-1, and that of *T. irregularis*. The sequence of Cl-QS-0-1 belonged to Clade IV together with the sequences of uncultured Tulasnellaceae and unidentified *Tulasnella* sp. The second major grouping consisted of the sequences of *E. calendulina*-like isolates reported in this study and by Ma et al. (2003) and the sequence of isolate Pv-QS-0-1. All our isolates in Clade V were obtained from *Paphiopedilum* species collected at Queen Sirikit Botanic Garden (QS).

The MP analysis of the ITS-5.8S rDNA data set of Clade I yielded one most parsimonious tree (length=367, CI=0.777, RI=0.771, data not shown). The groupings in general agreed with what was observed in the MP analysis based on the 5.8S rDNA data (Fig. 3), that is, the sequences obtained from our study, with the exception of Cl-QS-0-2, were clustered together in one subclade. Furthermore, the MP analysis using the ITS-5.8S rDNA data divided this subclade into two subgroups with high bootstrap values and placed this subclade as a sister taxon to the subclade composed of *T. asymmetrica*, *T. violea*, *T. pruinosa*, and isolate Cl-QS-0-2 with low bootstrap values (Fig. 4).

The MP analysis of the ITS-5.8S rDNA data of members of Clade II generated a strict consensus tree of 67 most parsimonious trees (length=477, CI=0.727, RI=0.719, data not shown). According to the MP analysis, the *E. anaticula* and *T. danica* were clustered in one subclade, whereas *E. repens* and *T. calospora* were clustered in a second subclade. These groupings were strongly supported by the bootstrap analysis (Fig. 5). The MP analysis using the ITS-5.8S rDNA data set also divided the subclade comprising *E. repens* and *T. calospora* into numerous subgroups in which three subgroups containing our isolates were strongly supported by bootstrap values (Fig. 5). Subgroup 1 contained two *E. repens* isolates obtained from the roots of *Paphiopedilum charlesworthii* collected from QS and *Paphiopedilum villosum* collected from a nursery of the *P. villosum* conservation project in the Doi Inthanon National Park (PC), and one *E. repens* isolate reported by Ma et al. (2003). Subgroup 2 contained isolates obtained from two epiphytic orchids (*C. tracyanum* collected from PC and *Dendrobium anosmum* collected from a nursery in Chiang

Table 2 Twenty-seven *Rhizoctonia*-like isolates and their closest relatives from GenBank

Fungal isolate	Query[a]	Close relative	Percentage identity, gaps	Reference
Cl-QS-0-1	(1)	DQ925494 uncultured Tulasnellaceae	81% (516/633), gaps 7% (48/633)	Shefferson et al. 2007
	(2)	AY373316 *Tulasnella* sp.	98% (158/160), gaps 0% (0/160)	McCormick et al. 2004
Cl-QS-0-2	(1)	DQ178072 uncultured *Tulasnella*	83% (504/605), gaps 6% (39/605)	Suarez et al. 2006
	(2)	DQ178081 uncultured *Tulasnella*	99% (168/169), gaps 0% (0/169)	Suarez et al. 2006
	(2)	DQ457642 *Tulasnella pruinosa*	98% (167/169), gaps 0% (0/169)	Matheny et al. 2006
Cs-QS-0-1	(1)	AJ313446 *Epulorhiza* sp. Nq	96% (604/628), gaps 0% (6/628)	Ma et al. 2003
	(2)	EU218888 *Tulasnella calospora*	100% (160/162), gaps 0% (0/160)	Taylor and McCormick 2008
Ct-PC-1-1	(1)	DQ388045 *Tulasnella calospora*	95% (587/615), gaps 2% (16/615)	Suarez et al. 2006
	(2)	EU218888 *Tulasnella calospora*	100% (160/160), gaps 0% (0/160)	Taylor and McCormick 2008
C1-DT-TC-1	(1)	DQ457642 *Tulasnella pruinosa*	85% (516/607), gaps 8% (52/607)	Matheny et al. 2006
	(2)	DQ457642 *Tulasnella pruinosa*	98% (166/168), gaps 0% (0/168)	Matheny et al. 2006
C2-DT-TC-1	(1)	DQ388047 *Tulasnella asymmetrica*	87% (482/552), gaps 5% (29/552)	Suarez et al. 2006
	(2)	DQ457642 *Tulasnella pruinosa*	98% (166/168), gaps 0% (0/168)	Matheny et al. 2006
C3-DT-TC-1	(1)	DQ457642 *Tulasnella pruinosa*	84% (511/603), gaps 8% (52/603)	Matheny et al. 2006
	(2)	DQ457642 *Tulasnella pruinosa*	98% (165/167), gaps 0% (0/167)	Matheny et al. 2006
C3-DT-TC-2	(1)	EU218889 *Tulasnella irregularis*	99% (470/471), gaps 0% (0/471)	Taylor and McCormick 2008
	(2)	EU218889 *Tulasnella irregularis*	100% (156/156), gaps 0% (0/156)	Taylor and McCormick 2008
Da-KP-0-1	(1)	AJ313446 *Epulorhiza* sp. Nq	96% (621/642), gaps 0% (6/642)	Ma et al. 2003
	(2)	EU218888 *Tulasnella calospora*	100% (159/159), gaps 0% (0/159)	Taylor and McCormick 2008
Dcr-QS-0-1	(1)	AB369940 *Tulasnella calospora*	87% (590/675), gaps 5% (40/675)	Shimura et al. 2009
	(2)	AB369940 *Tulasnella calospora*	99% (159/160), gaps 0% (0/160)	Shimura et al. 2009
Dcr-QS-0-2	(1)	DQ388041 *Tulasnella calospora*	96% (552/574), gaps 1% (11/574)	Suarez et al. 2006
	(2)	DQ388041 *Tulasnella calospora*	99% (159/160), gaps 0% (0/160)	Suarez et al. 2006
Df-QS-3-1	(1)	AY373303 *Tulasnella violea*	88% (438/496), gaps 6% (31/496)	McCormick et al. 2004
	(2)	DQ457642 *Tulasnella pruinosa*	98% (167/169), gaps 0% (0/169)	Matheny et al. 2006
D1-KT-TC-1	(1)	EU218889 *Tulasnella irregularis*	99% (509/512), gaps 0% (2/512)	Taylor and McCormick 2008
	(2)	EU218889 *Tulasnella irregularis*	100% (156/156), gaps 0% (0/156)	Taylor and McCormick 2008
Pca-QS-0-1	(1)	AJ313446 *Epulorhiza* sp. Nq	96% (604/628), gaps 0% (6/628)	Ma et al. 2003
	(2)	AJ313459 *Epulorhiza* sp. H21	99% (169/170), gaps 0% (0/170)	Ma et al. 2003
Pch-SM-TC-1	(1)	AJ313446 *Epulorhiza* sp. Nq	97% (573/586), gaps 0% (4/586)	Ma et al. 2003
	(2)	DQ388041 *Tulasnella calospora*	100% (159/159), gaps 0% (0/159)	Suarez et al. 2006
Pch-QS-0-1	(1)	AJ313445 *Epulorhiza* sp. B1	98% (629/637), gaps 0% (0/637)	Ma et al. 2003
	(2)	EU218888 *Tulasnella calospora*	100% (160/160), gaps 0% (0/160)	Taylor and McCormick 2008
Pch-QS-0-2	(1)	AJ313446 *Epulorhiza* sp. Nq	98% (607/614), gaps 0% (1/614)	Ma et al. 2003
	(2)	EU218888 *Tulasnella calospora*	100% (159/159), gaps 0% (0/159)	Taylor and McCormick 2008
Pch-QS-0-3	(1)	AJ313458 *Epulorhiza* sp. Hm1	94% (544/573), gaps 1% (11/573)	Ma et al. 2003
	(2)	AJ313459 *Epulorhiza* sp. H21	100% (170/170), gaps 0% (0/170)	Ma et al. 2003
Pe-QS-0-1	(1)	AJ313459 *Epulorhiza* sp. H21	95% (407/427), gaps 1% (8/427)	Ma et al. 2003
	(2)	AJ313459 *Epulorhiza* sp. H21	100% (170/170), gaps 0% (0/170)	Ma et al. 2003
Ps-KT-0-1	(1)	AJ313446 *Epulorhiza* sp. Nq	97% (628/642), gaps 0% (4/642)	Ma et al. 2003
	(2)	EU218888 *Tulasnella calospora*	100% (159/159), gaps 0% (0/153)	Taylor and McCormick 2008
Ps-AT-0-1	(1)	AY643804.3 *Tulasnella calospora*	96% (566/586), gaps 1% (7/586)	Bougoure et al. 2005
	(2)	AB369939 *Tulasnella calospora*	98% (158/160), gaps 0% (0/160)	Shimura et al. 2009
Ps-AT-0-2	(1)	AY643804.3 *Tulasnella calospora*	96% (511/527), gaps 0% (2/527)	Bougoure et al. 2005
	(2)	AB369939 *Tulasnella calospora*	98% (157/160), gaps 0% (0/160)	Shimura et al. 2009
Pv-PC-2-1	(1)	AB369939 *Tulasnella calospora*	98% (634/646), gaps 1% (8/646)	Shimura et al. 2009
	(2)	AB369939 *Tulasnella calospora*	100% (160/160), gaps 0% (0/160)	Shimura et al. 2009
Pv-PC-2-2	(1)	AJ313445 *Epulorhiza* sp. B1	98% (628/638), gaps 0% (1/638)	Ma et al. 2003
	(2)	DQ388041 *Tulasnella calospora*	100% (160/160), gaps 0% (0/160)	Suarez et al. 2006

Table 2 (continued)

Fungal isolate	Query[a]	Close relative	Percentage identity, gaps	Reference
Pv-PC-1-1	(1)	AB369939 *Tulasnella calospora*	97% (629/645), gaps 1% (8/645)	Shimura et al. 2009
	(2)	AB369939 *Tulasnella calospora*	100% (160/160), gaps 0% (0/160)	Shimura et al. 2009
Pv-QS-0-1	(1)	DQ925507 uncultured Tulasnellaceae	94% (552/584), gaps 2% (14/584)	Shefferson et al. 2007
	(2)	DQ925507 uncultured Tulasnellaceae	100% (169/169), gaps 0% (0/169)	Shefferson et al. 2007
	(2)	AJ313459 *Epulorhiza* sp. H21	94% (148/157), gaps 0% (0/157)	Ma et al. 2003
Pv-QS-0-2	(1)	AY373303 *Tulasnella violea*	88% (438/497), gaps 6% (33/497)	McCormick et al. 2004
	(2)	DQ457642 *Tulasnella pruinosa*	98% (167/169), gaps 0% (0/169)	Matheny et al. 2006

[a] Query type: *(1)* ITS-5.8S rDNA and *(2)* 5.8S rDNA

Rai province) and a terrestrial orchid (*C. sinense*) collected from QS. Subgroups 1 and 2 were closely related to each other and also related to four other isolates obtained in this study and the isolate Nq reported by Ma et al. (2003). Subgroup 3 contained isolates obtained from *P. villosum* collected from PC, *Paphiopedilum sukhakulii* collected from a nursery in Chiang Rai province, and the *T. calospora* sequence used in the study of Suarez et al. (2006).

The MP analysis of the ITS-5.8S rDNA data set of Clade V yielded one most parsimonious tree (Fig. 6) in which the topology was consistent with that obtained from the MP analysis of the 5.8S rDNA data set (Fig. 3). Furthermore, the MP analysis using the ITS-5.8S rDNA data showed that the isolates Pch-QS-0-3 and Pe-QS-0-1 were closely related, and the cluster comprising these two isolates and the *E. calendulina*-like isolates reported by Ma et al. (2003) was a sister taxon to the isolate Pca-QS-0-1. Ma et al. (2003), based on the ITS-5.8S rDNA and 5.8S rDNA sequence comparison, suggested that their *E. calendulina*-like and *E. repens* isolates were from different genera, and the *E. calendulina*-like isolates were more related to *Sebacina* than to *Tulasnella*. Our sequence comparison (Table 3) and phylogenetic studies (Figs. 3 and 6) supported the notion. However, the monophyletic relationship of *Sebacina vermifera* and the *E. calendulina*-like isolates was supported by only 64% of the bootstrap replicates.

Discussion

Twenty-seven isolates of *Rhizoctonia*-like fungi were isolated from root sections of mature orchids in three genera, *Paphiopedilum*, *Cymbidium*, and *Dendrobium*. Since the mycelia of *Rhizoctonia*-like fungi do not yield many distinguishing characters that enable identification below generic level (Rasmussen 2002) and several investigators repeatedly reported the unsuccessful attempts to induce sexual structures of *Rhizoctonia*-like fungi from tropical orchids (Athipunyakom et al. 2004; Ma et al. 2003; Otero et al. 2002), we decided not to induce the sexual stage but instead used the molecular analysis in addition to the morphological characterization for the identification of our *Rhizoctonia*-like isolates; and because of the availability of the sequences in the National Center for Biotechnology Information database and effective primers (Taylor and McCormick 2008), the ITS-5.8S rDNA region was chosen for the analysis.

The morphological characters, and later confirmed by the ITS-5.8S rDNA sequences, indicated that our 14 isolates were *E. repens*. This fungal species was the most common *Rhizoctonia* found in roots of both epiphytic and terrestrial orchids collected from diverse sites (e.g., a botanic garden, a nursery in a national park, and several private nurseries—one obtained the orchid plant from a tissue culture laboratory). Unlike the *E. repens* isolates, the *E. calendulina*-like isolates were strictly found in the roots

Table 3 Percent sequence homology of ITS-5.8S rDNA (above diagonal) and 5.8S rDNA (below diagonal) for *Epulorhiza calendulina* isolates, Pv-QS-0-1, *Sebacina vermifera* (GenBank accession number AF202728), *Ceratorhiza* sp. (GenBank accession number EU218895), and *Epulorhiza repens* isolates

	E. calendulina	Pv-QS-0-1	*S. vermifera*	*Ceratorhiza*	*E. repens*
E. calendulina	–	65	59–64	47	21–23
Pv-QS-0-1	92–93	–	45	50	23–25
S. vermifera	85–87	84	–	37	23–28
Ceratorhiza	83	79	88	–	17–18
E. repens	67–69	67–70	68–69	58–60	–

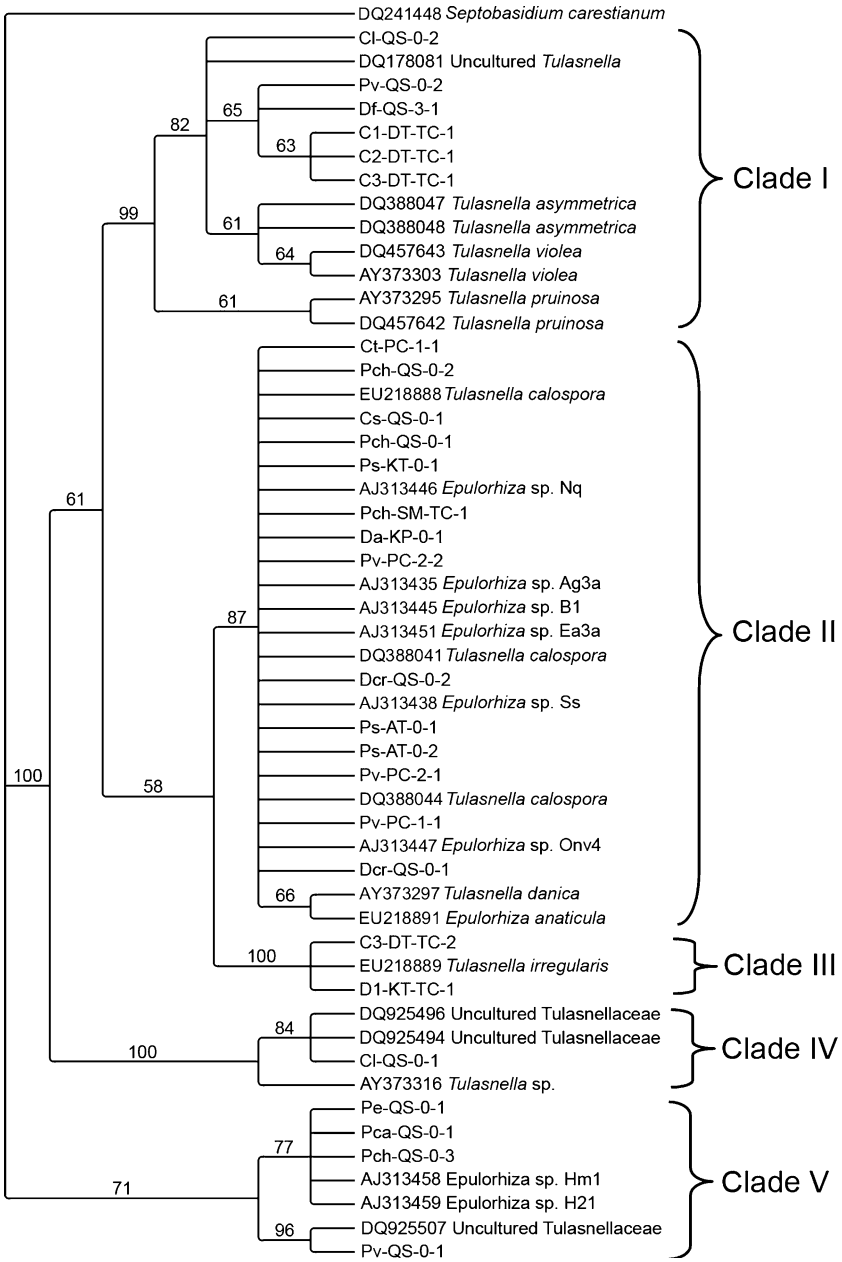

Fig. 3 Strict consensus maximum parsimony tree of 541 trees (length=154, CI=0.708, RI=0.943) based on the 5.8S rDNA data. *Septobasidium carestianum* (GenBank accession number DQ241448) was used as an outgroup taxon. *Numbers above the branches* indicate nodes supported in >50% of 1,000 bootstrap replicates. Sequences obtained from GenBank are shown with accession numbers

of three *Paphiopedilum* species. These findings are consistent with the reports that *E. repens* has a wide host range and *E. calendulina* is the mycorrhiza of terrestrial orchids (Athipunyakom et al. 2004; Ma et al. 2003; Zelmer and Currah 1995). Based on the morphological characters, however, ten *Rhizoctonia*-like isolates could not be designated to any described taxonomic groups. *T. irregularis* was isolated from the roots of *Dendrobium dicuphum* in Northern Territory of Australia by Warcup and Talbot (1980). As far as we know, the anamorph of this fungal species has not been isolated and described. Using the molecular data, we were able to determine that our two *Rhizoctonia*-like isolates (i.e., D1-KT-TC-1 and C3-DT-TC-2) obtained from the roots of the terrestrial *Cymbidium* hybrid and the epiphytic *Dendrobium* hybrid were anamorphs of *T. irregularis*. Since the two orchid plants were commercially propagated through tissue culture, they likely started the association with the fungi at the mature stage. Bayman et al. 2002, by treating seedlings and juveniles of *Lepanthes rupestris* Stimson with fungicides, reported that mycorrhizal fungi have both positive and negative effects on growth and survival of *L. rupestris* plants. However, interpretation of results was complicated by the presence of a range of non-mycorrhizal fungi and the effect of fungicides (Dearnaley 2007). Fang et al. (2008) inoculated tissue culture seedlings of the hybrid *Cymbidium* with six unidentified fungal strains isolated from *Cymbidium faberi* and *Cymbidium sinensis*. They reported that three of the six strains were beneficial for the orchid

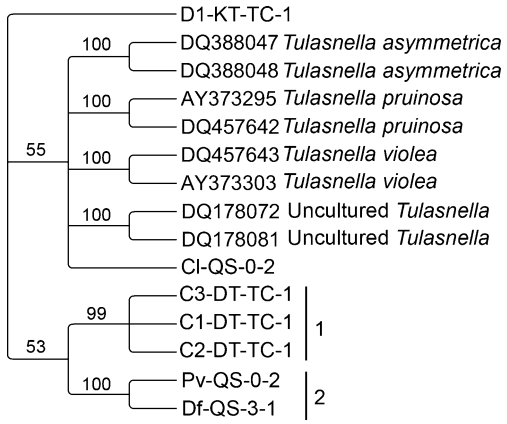

Fig. 4 Fifty percent majority-rule consensus tree of Clade I based on the ITS-5.8S rDNA data. The isolate, D1-KT-TC-1, identified as *Tulasnella irregularis*, was used as an outgroup taxon. *Numbers above the branches* indicate nodes supported in >50% of 1,000 bootstrap replicates

seedlings. These studies have shed light on the use of orchid mycorrhizal fungi as plant growth promoter microorganisms. Nevertheless, the effect of mycorrhizal fungi on growth and survival of adult, photosynthetic orchids is not well understood and, therefore, further investigation is necessary.

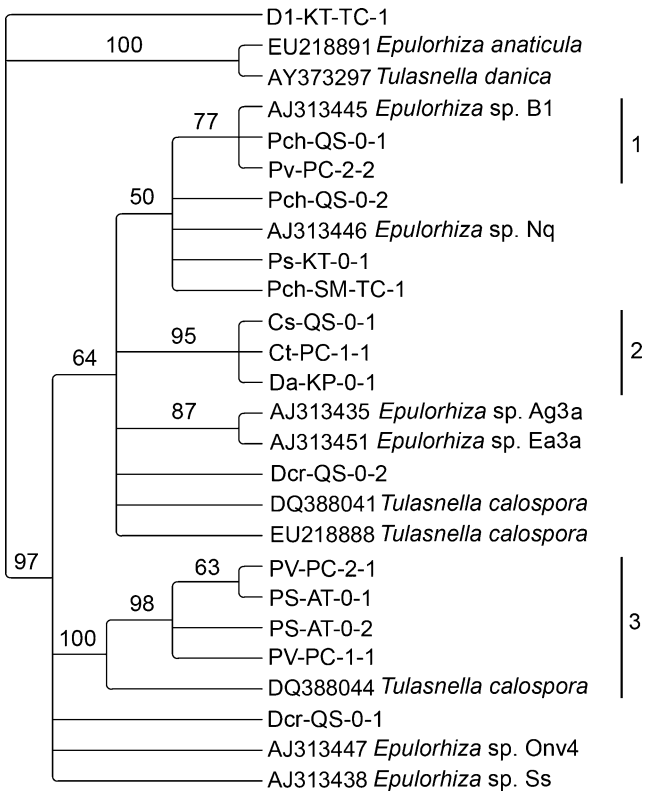

Fig. 5 Fifty percent majority-rule consensus tree of Clade II based on the ITS-5.8S rDNA data. The isolate, D1-KT-TC-1, identified as *Tulasnella irregularis*, was used as an outgroup taxon. *Numbers above the branches* indicate nodes supported in >50% of 1,000 bootstrap replicates

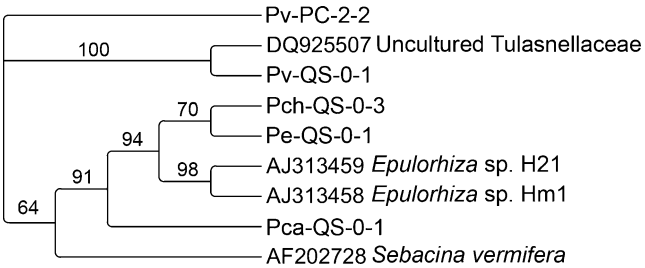

Fig. 6 Strict consensus maximum parsimony tree (length=455, CI=0.879, RI=0.751) of Clade V based on the ITS-5.8S rDNA data. *Numbers above the branches* indicate nodes supported in >50% of 1,000 bootstrap replicates. The isolate, Pv-PC-2-2, identified as *Epulorhiza repens*, was used as an outgroup taxon

We have also reported five new ITS-5.8S rDNA sequence types: (1) the sequence type of Cl-QS-0-1; (2) the sequence type of Cl-QS-0-2; (3) the sequence type of the members in Clade I, subgroup 1; (4) the sequence type of the members in Clade I, subgroup 2; and (5) Pv-QS-0–1. Based on the sequence comparison and the phylogenetic analysis, the isolate Cl-QS-0-1 is a new anamorph species in the genus *Tulasnella* and the most distant relative to all the orchid-associated *Tulasnella* (Fig. 3). The isolate Cl-QS-0-2 is potentially another new anamorph species in the genus *Tulasnella*. The ITS and 5.8S rDNA sequence of this isolate is closely related to those of *T. asymmetrica*, *T. violea*, and *T. pruinosa*; however, the taxonomic position of this fungus has remained unresolved (Fig. 4). Subgroups 1 and 2 in Clade I represent two anamorphic species in the genus *Tulasnella* that are closely related (Fig. 4). Their closest known relatives are possibly *T. asymmetrica*, *T. violea*, and *T. pruinosa*. It is important to note that the major nodes in the MP tree of Clade I did not obtain firm statistical support (Fig. 4). The inclusion of more genes in the phylogenetic analysis of members of this clade is probably necessary to increase the phylogenetic signal and consequently the resolution of the phylogenetic tree. The last new sequence type, the ITS-5.8S rDNA sequence of the isolate Pv-QS-0-1, did not show high homology with high sequence coverage to any known cultured mycorrhizal species (Table 2). According to our sequence comparison (Table 3) and phylogenetic analysis, this isolate is closer to *E. calendulina*-like isolates and *S. vermifera* than the *Tulasnella* clades (Figs. 3 and 6). The closest known relative of the isolate Pv-QS-0-1 is the uncultured Tulasnellaceae reported by Shefferson et al. (2007; GenBank accession number DQ925507). Therefore, this fungus is potentially a new anamorph species in a new genus of the Tulasnellaceae. Representative fungal isolates from each subclade have been deposited at the BIOTEC Culture Collection, BIOTEC Central Research Unit, Thailand.

Specific mycorrhizal associations have been observed in some green orchids (reviewed by Dearnaley 2007). Unfortu-

nately, the limited number of the experimental samples did not allow us to draw conclusions on the host/fungal specificity. It is also important to note that the orchid roots used in this study were obtained from the horticultural settings, and thus the fungal colonization found in this study might not reflect that of naturally occurring orchids.

Even though our fungal isolates were not obtained from pelotons and their mycorrhizal status is arguable, the fungal isolates are potential orchid symbionts because (1) the fungi grew from the cortical cells containing live pelotons and (2) the morphological and molecular characteristics of these isolates match the characteristics of known orchid symbionts. Moreover, in our preliminary experiments, the isolates Pv-PC-1-1 and Da-KP-0-1 were able to stimulate the symbiotic germination of the *Cymbidium finlaysonianum* seeds, and the isolates Da-KP-0-1, Ps-KT-0-1, and Pch-SM-TC-1 could infect and form pelotons inside the cortical cells of *Cymbidium* plantlets obtained from in vitro culture (unpublished data).

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