CHAPTER 3

ISOLATION AND IDENTIFICATION OF ORCHID MYCORRHIZAL FUNGI

3.1 Introduction

Orchid mycorrhizal fungi typically consist of coils of hyphae, called pelotons, within the cortical cells of plant roots (Brundrett *et al.* 2001). The fungi can be isolated from roots of many adult orchids on non-specialized media and can be grown easily in pure culture. The techniques for isolation include plating fragments of surface sterilized roots on solid media and careful separation and plating of individual fungal pelotons (Yamato *et al.* 2005; Smith and Read, 2008; Zhu *et al.* 2008).

A diversity of fungi has been found to associate with orchids. Many orchid mycorrhizal fungi have proved to be compatible with seed *in vitro*. Species are distinguished by hyphal characteristics and molecular approaches due to sexual spores not being present (Robert, 1999; Kristiansen *et al.* 2001; 2004). The main group of orchid mycorrhizal fungi belongs to the Basidiomycetes and has been placed in the form genus *Rhizoctonia* or Rhizoctonia-forming fungi (Moore, 1987; Andersoon1991; Rasmussen *et al.* 1995; Robert, 1999; Weber and Webster, 2001; Sangthong, 2002; Chou and Chang, 2004; Smith and Read, 2008).

The number of Thai terrestrial native orchids is estimated to be up to 600 species (Nanakorn and Indharamusika, 1998). However, most of the forests are secondary or degraded, and have high levels of human impact in many parts due to slash and burn agriculture. Therefore, the conservation of terrestrial orchids in Thailand depends on research including on orchid biology and mycorhizal symbionts.

The research described in this thesis involved study of the diversity of orchid mycorrhizal fungi isolated from roots of six Thai native terrestrial orchids. The isolated fungi were classified into genera level base on morphological characterization and molecular analysis.

3.2 Materials and methods

3.2.1 Orchid source and root sampling

Rhizosphere soil and whole plants of six terrestrial orchids namely, *Doritis pulcherrima* Lindl, *Eulophia spectabilis* (Dennst.) Suresh, *Paphiopedilum bellatulum* (Rchb. f.) Stein, *Pecteilis susannae* (L.) Raffin, *Phiaus tankervilleae* (Banks ex l' Heliter) Bl, and *Spathoglottis affinis* de Vries, were collected from natural habitats and local markets in Chiang Mai province, Thailand (Figure 3.2). There were four collection sites as follows:-

1. Mae-Sa, Chiang Mai province (Figure 3.1a). The elevation is about 600 m above sea level. It is dry dipterocarp forest. The soil is a sandy loam.

2. Sa-Luang, Chiang Mai province (Figure 3.1b). The elevation is about 850 m above sea level. It is dry dipterocarp forest. The soil is a sandy loam.

3. Queen Sirikit Botanic Garden, Chiang Mai (Figure 3.1c). The elevation is about 700 m above sea level. It is deciduous forest. The soil is loamy with high organic matter content.

4. Doi Suthep, Doi Sutheep-Pui National Park, Chiang Mai province (Figure 3.1d). The elevation is about 1,200 m above sea level. It is a primary evergreen forest. The soil is loamy with high organic matter content.

The orchid samples were kept in plastic bags and were used within 48 hours to check for mycorrhizal colonization and to isolate fungal endophytes.

3.2.2 Orchid roots observation and mycorrhizal colonization

Three selected healthy roots of each terrestrial orchid sample were washed with tap water to remove debris. Then, the root samples were placed into 10% (w/v) KOH solution and autoclaved at 121°C, 15 P/inch^2 for 15 min to clear the root samples. The cleared roots were stained with 0.05% (w/v) trypan blue in lactoglycerol (1:1:1 lactic acid, glycerol and water) and autoclaved at 121°C, 15 P/inch^2 for 15 min. The stained roots were observed under a compound microscope. Peloton structure and colonization by mycorrhizal fungi were assessed (McGonigle *et al*. 1990; Brundrett *et al.* 1996).

3.2.3 Fungal isolation

Endophytic fungi were isolated using a modified procedure of Yamato *et al*. (2005) and Zhu *et al*. (2008). Orchid root segments were rinsed using running tap water for 2 h to remove debris and cut into 1 and 5 cm segments. The segments were surface sterilized in a solution containing 95% ethanol: 5.25%

NaOCl: sterile distilled water (1:1:1 v/v/v) for 2 min, followed by 3 rinses in sterile distilled water.

Half of the 1 cm segments were cut into 3 segments in a sterile Petri dish and the pieces were placed on plates containing ¼ Potato dextrose agar (PDA) supplemented with 100 µg/ml of streptomycin and 50 µg/ml of chloramphenicol using aseptic techniques. The inoculated plates were incubated in the dark at 25°C for 30 days and observed every 2 days during the incubation period. Each of the remaining 1 cm segments was crushed in a sterile Petri dish using a sterile glass rod. Approximately 20 ml of cooled (45°C) autoclaved Czapek Dox modified agar was poured into each Petri dish and mixed before solidification. The plates were incubated in the dark at 25°C for 21 days. The incubated plates were observed under a stereomicroscope every 2 days during the incubation period.

The root hairs, epidermis, and velamen of the 5 cm segments were removed by peeling and scraping using sterile equipments (needle, sharp knife, and forceps). The peeled root segments were immersed in sterile water supplemented with 150 µg/ml of streptomycin for 10 min and washed 1 time with sterile water. Then, one peeled root segment was teased using a sterile needle and forceps in a sterile Petri dish containing 10 ml of sterile water to obtain a suspension of cortical cells. A 50 µl of cortical cell suspension was dropped into a 1 cm^3 block of ¼ PDA containing 100 µg/ml of streptomycin and 50 µg/ml of chloramphenicol in a sterile Petri dish and incubated in the dark at 25°C for 30 days. The incubated plates were observed under stereomicroscope every 2 days during the incubation period.

Fungal colonies emerging from root tissues and cortical cells containing peloton structure were transferred to new PDA plates and assigned the CMU numbers. The pure cultures were kept on PDA slants at 4°C for further use and also placed in sterile distilled water at 4°C and 20% glycerol at -20°C for long-term preservation.

Figure 3.1 Collection sites of terrestrial orchid samples: (a) site 1, Mae-Sa, (b) site 2, Sa-Luang, (c) site 3, Queen Sirikit Botanic garden and (d) site 4, Huay-Khog-Ma, Doi Suthep. All sites located in Chiang Mai province, Thailand

Figure 3.2 Location of Chiang Mai province, in northern part of Thailand (Ezilon Maps, 2011)

3.2.4 Fungal identification

3.2.4.1 Morphological observation

Fungal morphology (e.g. fungal colony growth pattern, pigment production, spore formation, conidia formation, sclerotium formation, clamp connection formation, and septate or non-septate hypha) was examined using keys and methods outlined by Barnett and Hunter (1987), Currah and Zelmer (1992), Currah *et al.* (1997), Robert (1999), Watanabe (2001) and Athipunyakom *et al.* (2004a).

3.2.4.2 Molecular analysis

The molecular approach used was modified from the procedure of Promputtha *et al.* (2005) and Yamato *et al.* (2005). Mycelium of selected fungal isolates was scraped directly off culture plates, transferred into 1.5 ml centrifuge tubes, and freeze dried using a lyophilizer (Dura-Dry^{TM}, USA). The freeze-dried mycelium was mixed with sterile white quartz sand and 600 µl of preheated (60°C) 2x CTAB buffer (2% (v/w) CTAB, 100 mM Tris-HCl, 1.4 M NaCl, 20 mM EDTA, pH 8.0). It was then ground with a pestle for 5-10 min and incubated at 60°C for 60 min with occasional swirling every 15 min. Then 600 µl of phenol: chloroform (1:1) was added into each tube and mixed. The mixture was centrifuged at 13,000 rpm for 30 min and the aqueous extraction layer was transferred into a new 1.5 ml centrifuge tube. The phenol: chloroform (1:1) extraction was repeated 2 times or until no interface was visible. Two volumes of cold absolute ethanol were added into each tube and the tube was inverted gently.

The tube was stored overnight at -20°C to precipitate DNA, centrifuged at 14,000 rpm for 15 min at 4°C and the DNA pellet was washed twice with 70% cold ethanol and dried at room temperature for 2-4 h. The dried pellet was suspended in 50 µl of TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8) containing 20 µg/ml RNase and incubated at 37°C for 60 min. The DNA samples were checked for purity by electrophoresis in 1% (w/v) agarose gel stained with ethidium bromide under UV light.

For fungal ITS sequencing and phylogenetic analysis, the internal transcribed spacer (ITS) regions of nuclear rDNA were amplified by polymerase chain reaction (PCR) with primers ITS4 and ITS5 (White *et al.* 1990) under the following thermal conditions: 95°C for 2 min, 30 cycles of 95°C for 30 s, 50°C for 30 s, 72°C for 1 min, and a cycle of 72°C for 10 min. Amplicons were checked on 1% agarose gels stained with ethidium bromide under UV. PCR products were purified using PCR clean up Gel extraction NucleoSpin® Extract II purification Kit (Macherey-Nagel, Germany) following the manufacturer's protocol. The purified the PCR products were directly sequenced. Sequencing reactions were preformed and the sequences were automatically determined in a genetic analyzer (1^{ST} Base, Malaysia) using PCR primers mentioned above. Sequences were used to query GenBank via BLAST (http://blast.ddbj.nig.ac.jp/top-e.html) and a phylogenetic tree was constructed using the Molecular Evolution Genetics Analysis (MEGA) software version 4.0 (Tamura *et al.* 2007).

3.3 Results

3.3.1 Orchid roots observation and mycorrhizal colonization

Six terrestrial orchid samples (*Doritis pulcherrima, Eulophia spectabilis*, *Paphiopedilum bellatullum*, *Pecteilis susannae*, *Phaius tankervilleae*, and *Spathoglottis affinis*) were collected from their natural habitats and local markets in Chiang Mai province, Northern Thailand during the wet seasons (Table 3.1, Figure 3.3 - 3.8). Peloton structures, coils of hyphae, of orchid mycorrhizal fungi were found in cortical cells of all orchid roots after staining with 0.05% trypan blue in lactoglycerol (Figure 3.3c - 3.8c). All orchid root samples taken were heavily colonized (100%).

Table 3.1 Collection site and time of collection of six terrestrial orchid samples

Orchid sample	Collection time	Location
Doritis pulcherrima	July-09	Chiang Mai local market
Eulophia spectabilis	October-08	site 4; Doi Suthep
Paphiopedilum bellatullum	May-07	Chiang Mai local market
Pecteilis susannae	July-05	site 1; Mae-Sa
	August-07	site 2; Sa-Luang
	July-07 and September-07	site 3; Queen Sirikit Botanic Garden
Phaius tankervilleae	July-09	site 3; Queen Sirikit Botanic Garden
Spathoglottis affinis	August-05	Chiang Mai local market

All sites located in Chiang Mai province, Thailand.

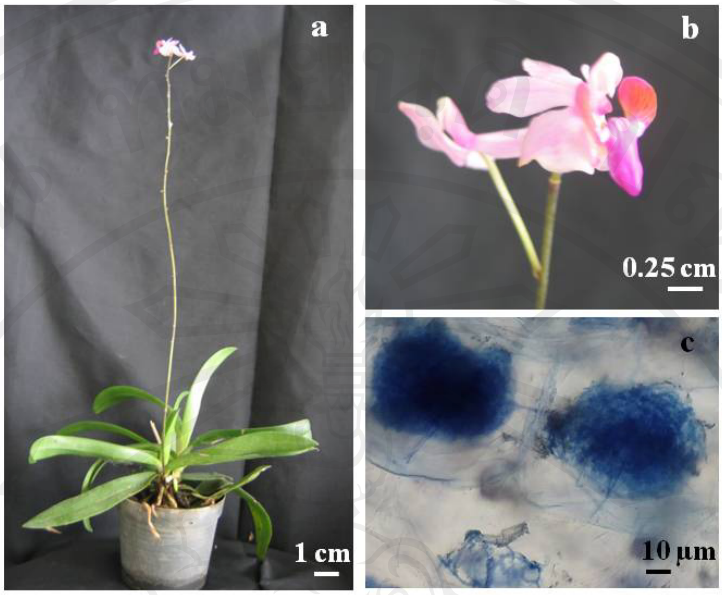

Figure 3.3 *Doritis pulcherrima*: orchid plant (a), flower (b) and peloton structures, coiled hyphae, in cortical cells of roots stained with 0.05% (w/v) trypan blue in lactoglycerol (c)

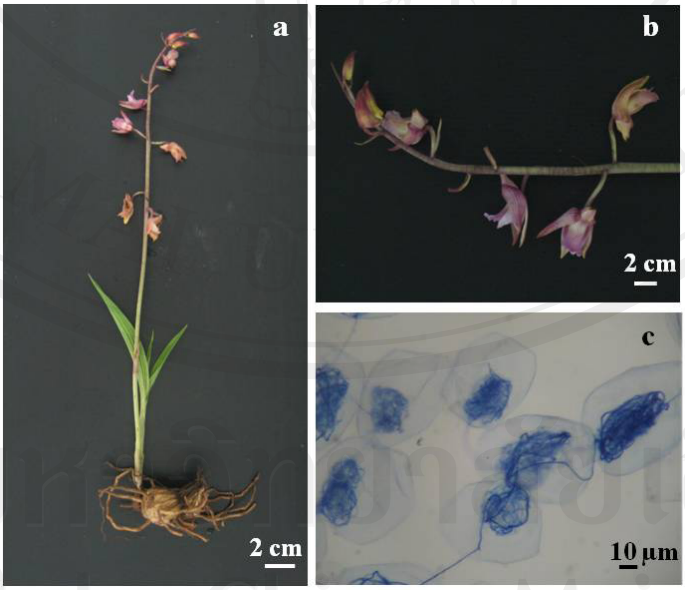

Figure 3.4 *Eulophia spectabilis*: orchid plant (a), flower (b) and peloton structures, coiled hyphae, in cortical cells of roots stained with 0.05% (w/v) trypan blue in lactoglycerol (c)

Figure 3.5 *Paphiopedilum bellatulum*: orchid plant (a), root system (b) and peloton structures, coiled hyphae, in cortical cells of roots stained with 0.05% (w/v) trypan blue in lactoglycerol (c)

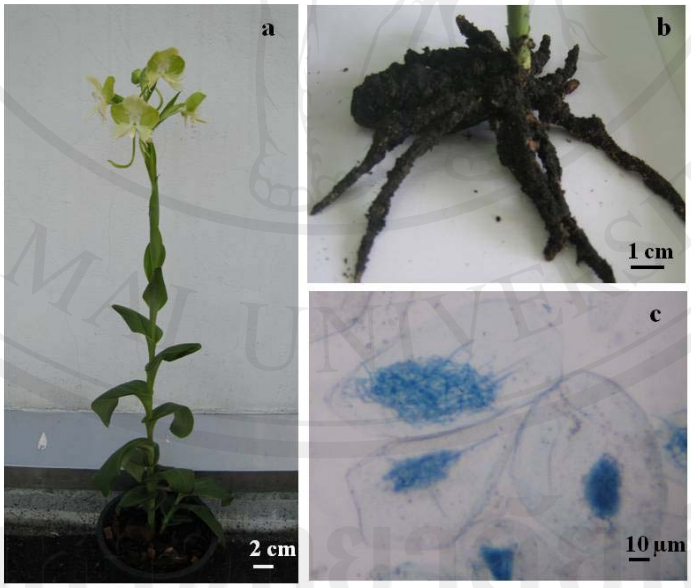

Figure 3.6 *Pecteilis susannae*: orchid plant (a), root system (b) and peloton structures, coiled hyphae, in cortical cells of roots stained with 0.05% (w/v) trypan blue in lactoglycerol (c)

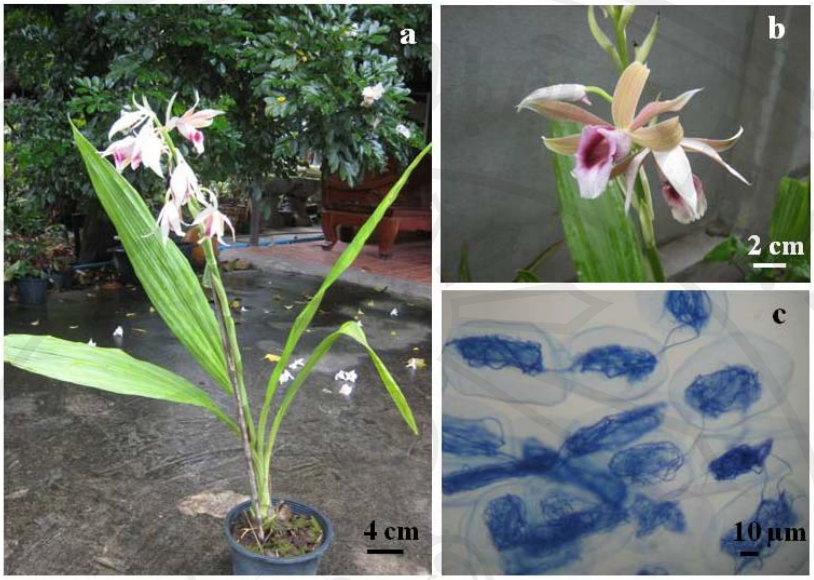

Figure 3.7 *Phaius tankervilleae*: orchid plant (a), flower (b) and peloton structures, coiled hyphae, in cortical cells of roots stained with 0.05% (w/v) trypan blue in lactoglycerol (c)

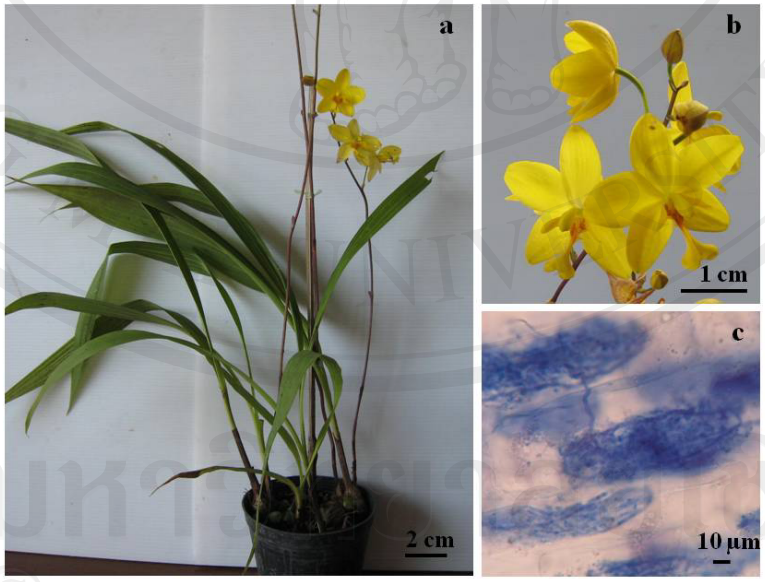

Figure 3.8 *Spathoglottis affinis*: orchid plant (a), flower (b) and peloton structures, coiled hyphae, in cortical cells of roots stained with 0.05% (w/v) trypan blue in lactoglycerol (c)

3.3.2 Fungal isolation and identification

Sixty-six endophytic fungal isolates were recovered from the roots of six terrestrial orchid samples (*P. susannae*, *P. bellatulum*, *P. tankervilleae*, *E. spectabilis*, *D. pulcherrima* and *S. affinis*) (Figure 3.9 and Table 3.2). Based on morphology and sequences of ITS regions of nuclear rDNA, 23% of the fungal isolates were assigned to the genus *Epulorhiza*, 21% to the genus *Fusarium*, 14% to the genus *Cladosporium*, 8% to the genus *Xylaria*, 5% to the genus *Colletotrichum*, 5% to the genus *Tulasnella*, 5% to the genus *Trichocladium*, 3% to the genus *Dothiodeomycete*, 3% to the genus *Pestalotiopsis*, 2% to the genus *Gloeotulasnella*, 2% to the genus *Phomopsis*, 1% to the genus *Chaetomium*, 1% to the genus *Cladophilophora*, 1% to the genus *Eupenicillium*, 1% to the genus *Gibberella*, 1% to the genus *Nodulis*, 1% to the genus *Phoma* and the remaining 3% were mycelia sterilia (Figure 3.10).

Features of *Rhizoctonia*-like fungi, monilioid hyphae or chains of short swollen compartments (Figure 3.11), were present in the *Epulorhiza* isolates after culturing on PDA for 21 days. In order to confirm this preliminary classification, the Fungal ITS sequences were analyzed. Parsimony and distance phylogenetic analyses showed that all 19 fungal endophytes identified as members of the genus *Epulorhiza*, *Tulasnella* and *Gloeotulasnella* belonged to the related group of orchid mycorrhizal fungi, containing *Epulorhiza*, *Tulasnella* and *Gloeotulasnella*, with high bootstrap support of 99% (Figure 3.12). Eight fungal endophyte isolates (CMU-AUG 002, CMU-AUG 025, CMU-AUG 031, CMU-AUG 040, CMU-SLP 007, CMU-SLP 008, CMU-NUT 012 and CMU-AU 211) formed a branch that was closely related to the genus *Epulorhiza* and *Tulasnellaceae* sp., eight fungal

endophyte isolates (CMU-AUG 007, CMU-AUG 013, CMU-AUG 028, CMU-STE 004, CMU-STE 014, CMU-DP 506, CMU-NUT 013 and CMU-AU 212) were clustered with *Epulorhiza* and *Tulasnella*, two fungal isolates (CMU-STE 011 and CMU-DP 514) were clustered with *Tulasnella* and fungal isolate CMU-STE 003 formed a branch that was closely related to the genus *Gloeotulasnella*.

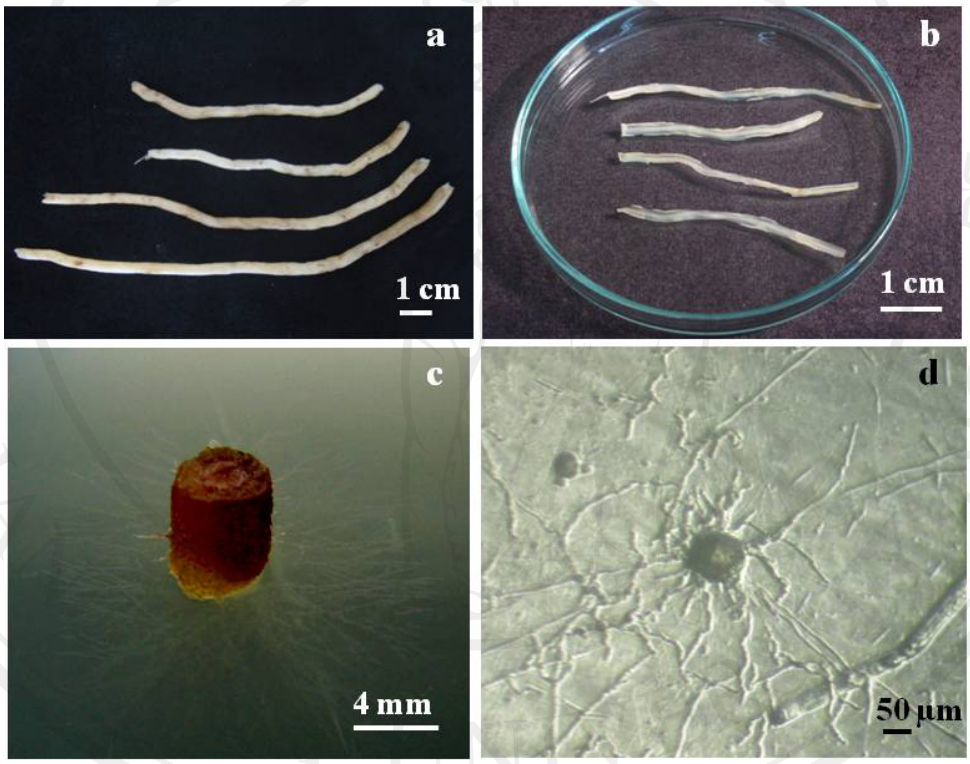

Figure 3.9 Isolation of fungal endophytes from roots of *Pecteilis susannae*: (a) roots of terrestrial orchid, (b) peeled roots after removal of the velamen and root hairs, (c) fungal mycelium emerging from root segment and (d) fungal colony emerging from root cell containing peloton structure, single peloton, on agar medium

Table 3.2 Endophytic fungi isolated from roots of six terrestrial orchids (*Doritis pulcherrima*, *Eulophia spectabilis*, *Paphiopedilum bellatullum*, *Pecteilis susannae*, *Phaius tankervilleae*, and *Spathoglottis affinis*)

Orchid	Fungal taxa	Fungal isolates	Location
Doritis pulcherrima	*Cladosporium cladosporiodes*	CMU-DP 504	local market
	Cladosporium oxysporium	CMU-DP 519	local market
	Cladosporium sp.	CMU-DP 518	local market
	Epulorhiza sp.	CMU-DP 506	local market
	Fusarium solani	CMU-DP 508	local market
	Trichocladium sp.	CMU-DP 501	local market
	Tulasnella sp.	CMU-DP 514	local market
	Xylaria sp.	CMU-DP 503	local market
Eulophia spectabilis	*Dothiodeomycete* sp.	CMU-STE 012	site 4 Doi Suthep
	Epulorhiza sp.	CMU-STE 004	site 4 Doi Suthep
	Epulorhiza sp.	CMU-STE 014	site 4 Doi Suthep
	Fusarium solani	CMU-STE 018	site 4 Doi Suthep
	Gibberilla fujikuroi	CMU-STE 009	site 4 Doi Suthep
	Gloeotulasnella sp.	CMU-STE 003	site 4 Doi Suthep
	Tulasnella sp.	CMU-STE 011	site 4 Doi Suthep
Paphiopedilum bellatulum	*Cladosporium* sp.	CMU-SLP 020	local market
	Epulorhiza sp.	CMU-SLP 007	local market
	Epulorhiza sp.	CMU-SLP 008	local market
	Fusarium sp.	CMU-SLP 005	local market
	Fusarium sp.	CMU-SLP 040	local market
Pecteilis susannae	*Cladosporium* sp.	CMU-AUG 035	site 1 July 2005
	Cladosporium sp.	CMU-AUG 038	site 1 July 2005

Table 3.2 (continued)

Orchid	Fungal taxa	Fungal isolates	Location
Pecteilis susannae	*Colletotrichum gloeosporioides*	CMU-AUG 001	site 1 July 2005
	Epulorhiza sp.	CMU-AUG 002	site 1 July 2005
	Epulorhiza sp.	CMU-AUG 028	site 1 July 2005
	Fusarium sp.	CMU-AUG 050	site 1 July 2005
	Fusarium sp.	CMU-AUG 055	site 1 July 2005
	Pestalotiopsis sp.	CMU-AUG 044	site 1 July 2005
	Xylaria sp.	CMU-AUG 022	site 1 July 2005
	Mycelia sterilia	CMU-AUG 036	site 1 July 2005
	Cladosporium sp.	CMU-AUG 078	site 2 August 2007
	Cladophialophora sp.	CMU-AUG 077	site 2 August 2007
	Colletotrichum sp.	CMU-AUG 070	site 2 August 2007
	Epulorhiza sp.	CMU-AUG 007	site 2 August 2007
	Epulorhiza sp.	CMU-AUG 013	site 2 August 2007
	Epulorhiza sp.	CMU-AUG 040	site 2 August 2007
	Fusarium sp.	CMU-AUG 021	site 2 August 2007
	Nodulis sporium	CMU-AUG 006	site 2 August 2007
	Trichocladium pyriforme	CMU-AUG 024	site 2 August 2007
	Xylaria sp.	CMU-AUG 019	site 2 August 2007
	Dothiodeomycete sp.	CMU-AUG 003	site 3 July 2007
	Epulorhiza sp.	CMU-AUG 031	site 3 July 2007
	Fusarium sp.	CMU-AUG 005	site 3 July 2007
	Mycelia sterilia	CMU-AUG 020	site 3 July 2007
	Epulorhiza sp.	CMU-AUG 025	site 3 September 2007
	Fusarium sp.	CMU-AUG 008	site 3 September 2007
	Pestalotiopsis sp.	CMU-AUG 042	site 3 September 2007

Table 3.2 (continued)

Orchid	Fungal taxa	Fungal isolates	Location
Pecteilis susannae	*Phoma* sp.	CMU-AUG 033	site 3 September 2007
Phaius tankervilleae	*Cladosporium* sp.	CMU-NUT 015	site 3 July 2009
	Cladosporium sp.	CMU-NUT 016	site 3 July 2009
	Chaetomium sp.	CMU-NUT 003	site 3 July 2009
	Epulorhiza sp.	CMU-NUT 012	site 3 July 2009
	Epulorhiza sp.	CMU-NUT 013	site 3 July 2009
	Fusarium sp.	CMU-NUT 001	site 3 July 2009
	Fusarium sp.	CMU-NUT 010	site 3 July 2009
	Phomopsis sp.	CMU-NUT 018	site 3 July 2009
	Xylaria sp.	CMU-NUT 009	site 3 July 2009
Spathoglottis affinis	*Colletotrichum gloeosporioides*	CMU-AU 006	local market
	Epulorhiza sp.	CMU-AU 211	local market
	Eupenicillium sp.	CMU-AU 510	local market
	Fusarium sp.	CMU-AU 202	local market
	Fusarium sp.	CMU-AU 208	local market
	Fusarium sp.	CMU-AU 912	local market
	Trichocladium pyriforme	CMU-AU 210	local market
	Tulasnella sp.	CMU-AU 212	local market
	Xylaria sp.	CMU-AU 009	local market

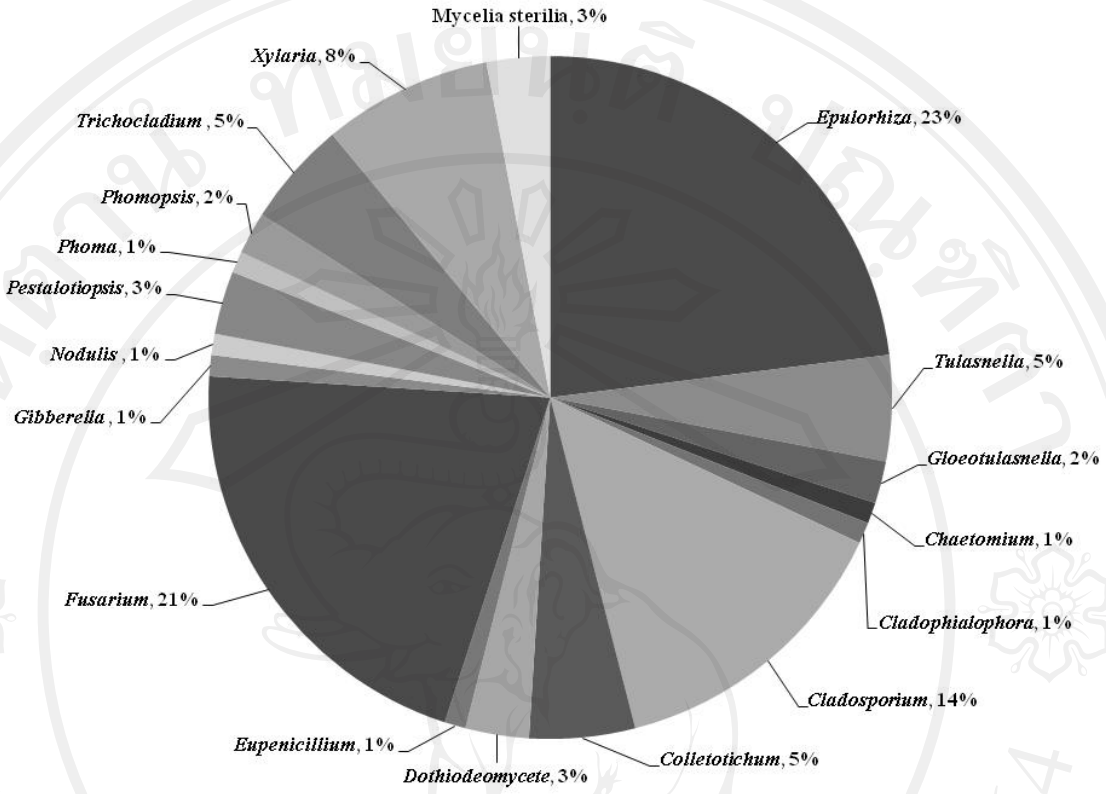

Figure 3.10 Percentages of the occurrence of endophytic fungal taxa isolated from roots of six terrestrial orchids (*Doritis pulcherrima*, *Eulophia spectabilis*, *Paphiopedilum bellatullum*, *Pecteilis susannae*, *Phaius tankervilleae*, and *Spathoglottis affinis*)

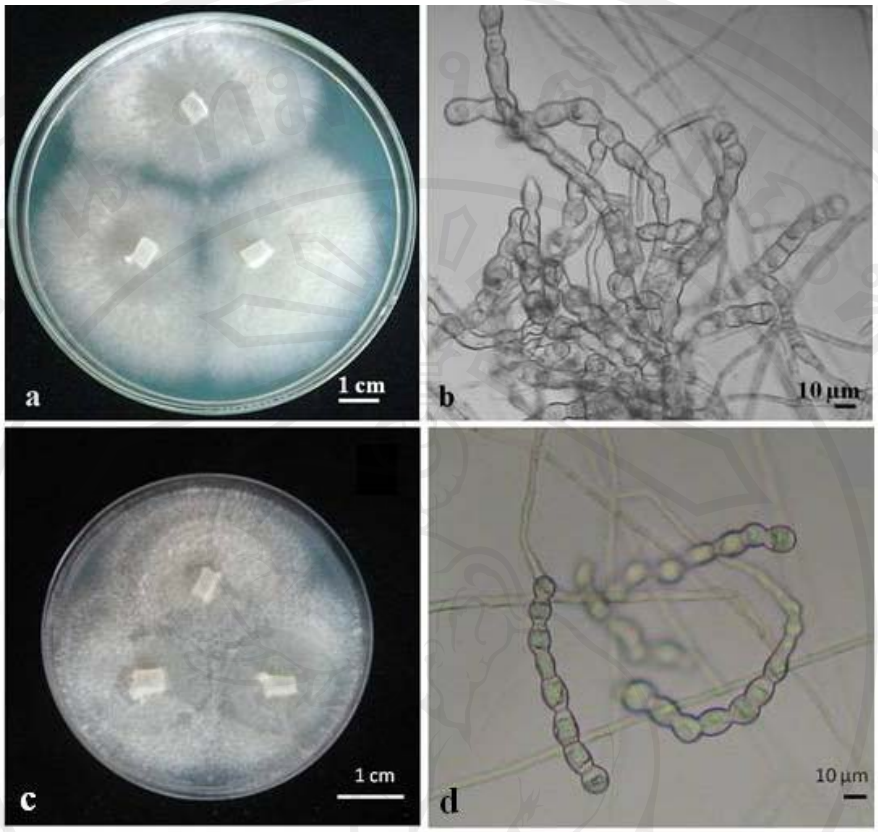

Figure 3.11 *Epulorhiza* isolates; (a) and (c) fungal colonies cultured on Potato dextrose agar, (b) and (d) chains of monilioid cells (ellipsoid to subglobose), short swollen compartments of fungal isolates CMU-AUG 013 and CMU-STE 004, respectively

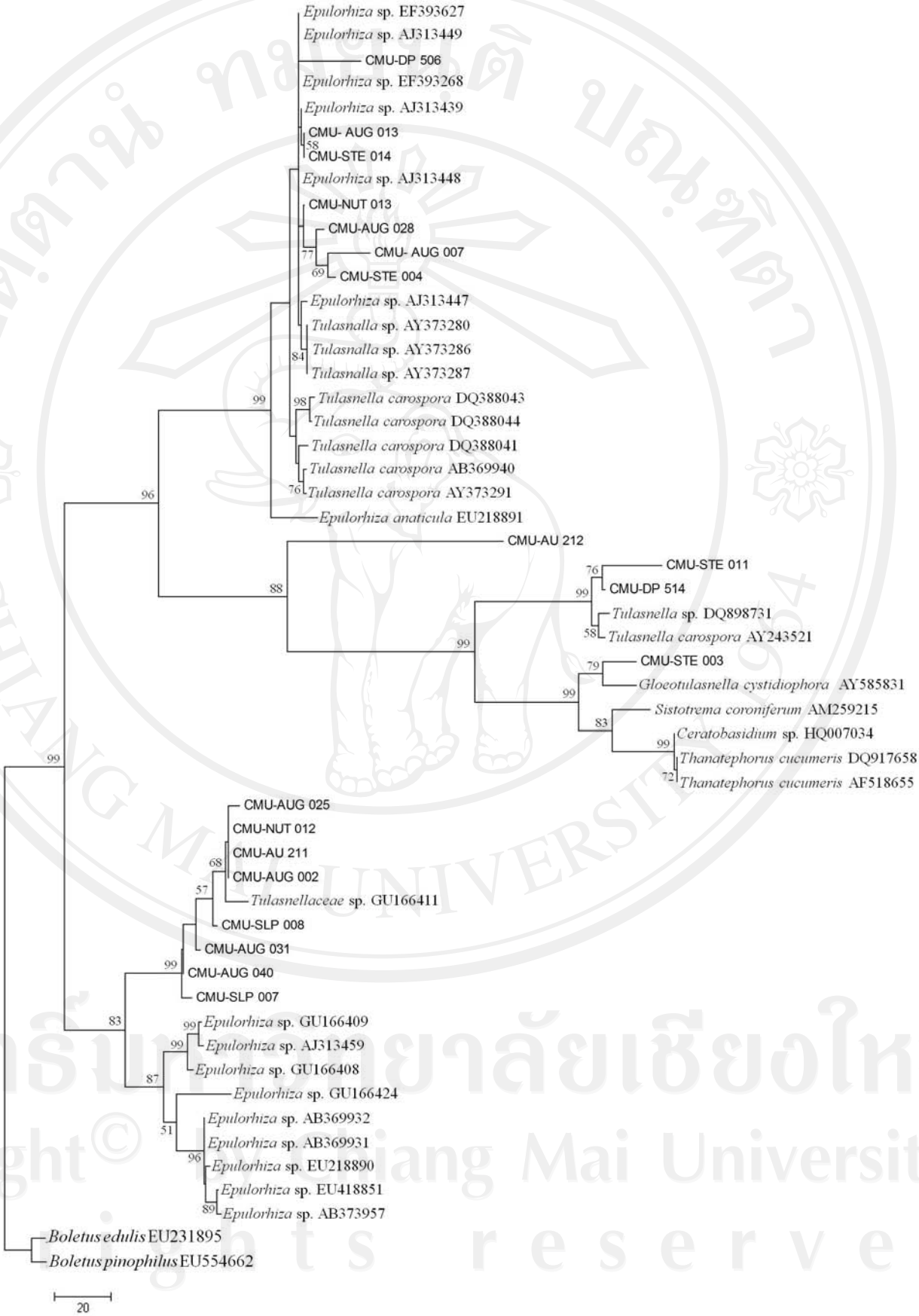

Figure 3.12

Figure 3.12 Maximum-parsimony trees based on an alignment of internal transcribed spacer1, 5.8S ribosomal RNA gene and internal transcribed spacer 2 of 19 fungal endophytes isolated from roots of six terrestrial orchids (*Pecteilis susannae*, *Paphiopedilum bellatulum*, *Phaius tankervilleae*, *Eulophia spectabilis*, *Doritis pulcherrima* and *Spathoglottis affinis*). The tree was rooted with *Boletus edulis* and *Boletus pinophilus*. Bootstrap values (calculated from 1000 re-samplings) higher or equal to 50% are shown at each branch. The bar represents 20 substitutions per nucleotide position

3.4 Discussion

The determination of mycorrhizal colonization in roots of 6 terrestrial orchid samples (*D. pulcherrima*, *E. spectabilis*, *P. bellatullum*, *P. susannae*, *P. tankervilleae*, and *S. affinis*) indicated that the roots of all orchid samples were heavily colonized with peloton structures of mycorrhizal fungi. Under the microscope, it was possible to select mycelium that had grown out of pelotons onto agar and these were subcultured. This colonization confirms that Thai terrestrial orchids depend upon mycorrhizal associations in nature as described by other worker elsewhere (McKendrick *et al.* 2000, 2002; Smith and Read, 2008; Swart and Dixon 2009; Wright *et al.* 2009).

Based on identification of endophytic fungi isolated from roots of all terrestrial orchid samples, the recovered orchid mycorrhizal fungi belonging to the genus *Epulorhiza* was dominant, being present in all terrestrial orchid samples. This finding supports the view that the genus *Epulorhiza* is one of the most common and distinctive form-genera of *Basidiomycetes* forming mycorrhizal

associations with orchids (Currah *et al.* 1997; Zettler and Hofer 1998; Ma *et al.* 2003; Stewart and Kane 2006; Taylor and McCormick 2007; Zhu *et al.* 2008; Shimura *et al.* 2009). Moreover, the genus *Tulasnella* and *Gloeotulasnella* also have been isolated from roots of terrestrial orchids and reported as orchid mycorhizal fungi (Kristiansen *et al.* 2001; Lee 2002; Shefferson *et al.* 2005; Porras-Alfaro and Bayman, 2007). In addition to the two fungal taxa mentioned above, Sangthong (2002) reported the same 4 endophytic fungal taxa (*Xylaria, Fusarium, Phomopsis*, and *Colletotrichum*) were isolated from roots of epiphytic orchids collected from northern Thailand. For roots of *Lepanthes* (*Orhidaceae*) in which 29% of the isolated fungi belonged to the genus *Xylaria* (Bayman *et al.* 1997). Moreover, *Fusarium* also has been reported as endophytic fungi isolated from roots of orchids (Ovando *et al.* 2005).

The classical method for fungal identification, using morphological characteristics, is difficult for orchid mychorrizal fungi as most species being anamorphic or mycelia sterillia on culture agar. The orchid fungi which form sexual stages in culture belong to the *Basidiomycetes* and rarely produce sexual fruiting structures (Robert 1999; Sivasithamparam *et al.* 2002). Therefore, the molecular approach for identification has become the favorite method and was used to confirm preliminary classification of the fungal isolates. In this chapter, the fungal ITS sequencing and phylogenetic analysis showed differences in the sequences among the fungal isolates. The 19 endophytic fungi associated with the 6 Thai terrestrial orchid species were clustered with orchid mycorrhizal fungi and were closely related with mycorrhizal associations known to be present in some other orchid species (Ma *et al.* 2003; McCormick *et al.* 2004; Suarez *et al.* 2006;

Shefferson *et al.* 2005; Porras-Alfaro and Bayman, 2007; Taylor and McCormick, 2007; Shimura *et al.* 2009; Nontachaiyapoom *et al.* 2010).

In conclusion, it was demonstrated that all 6 terrestrial orchid species sampled were heavily colonized by mycorrhizal fungi. The dominant fungal genus obtained from all terrestrial orchids was *Epulorhiza*. In spite of this research, the diversity and distribution of orchid mycorrhizal fungi remain poorly studied as there are only a small number of studies has been undertaken so far in Thailand (Sangthong, 2002; Athipunyakom *et al.* 2004a; 2004b; Nontachaiyapoom *et al.* 2010). Thus, further study on fungal diversity and distribution of orchid mycorrhiza are required for future application in the conservation of Thai terrestrial orchids, especially the threatened species.