

Endophytic fungi from *Pecteilis susannae* (L.) Rafin (Orchidaceae), a threatened terrestrial orchid in Thailand

Ruangwut Chutima · Bernard Dell ·
Suyanee Vessabutr · Boonsom Bussaban ·
Saisamorn Lumyong

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Abstract Eight endophytic fungi were isolated from roots of the threatened terrestrial orchid, *Pecteilis susannae* (L.) Rafin. Phylogenetic analysis based on an alignment of internal transcribed spacer regions of nuclear rDNA indicated that seven isolates belonged to the genus *Epulorhiza* and one to *Fusarium*. All fungal isolates were cultured with orchid seeds collected from three field sites near Doi Suthep-Pui National Park, Chiang Mai, Thailand. Seed germination and protocorm development were evaluated up to 70 days after sowing. Percent symbiotic seed germination was highest (86.2%) when seeds were cultured with *Epulorhiza* (CMU-Aug 013). The protocorm development was the most advanced up to stage 2, continued embryo enlargement, or rupture of the testa, and the highest percentage was 17.8% when seeds were cultured with *Epulorhiza* (CMU-Aug 007). Without fungi, seed germination and protocorm development were 62.1% and 11.1%, respectively. The dependency of *P. susannae* on fungal symbionts for early seedling development is yet to be determined. Optimizing seed germination and seedling

fitness will assist the conservation of this threatened orchid in Thailand.

Keywords Endophytic fungi · *Epulorhiza* · Orchid mycorrhiza · *Pecteilis susannae* · Seed germination · Terrestrial orchid

Introduction

Orchids are one of the largest families of flowering plants worldwide, with over 35,000 species in some 750 genera. The highest number of orchid species occurs in the tropical zone, and diversity decreases with increasing distance from the equator (Brundrett et al. 2001). In Thailand, there are about 170 genera and 1,230 species, of which 150 species are considered to be endemic to the country (Nanakorn and Indharamusika 1998; Santisuk et al. 2006). Of these, 80% are epiphytic, and the remainder are mostly terrestrial orchids. The number of the native orchids in northern Thailand is estimated to be up to 600 species (Nanakorn and Indharamusika 1998). This region has a rich diversity of plant communities and is the center of plant distribution of Southeast Asia. However, most of the forests in the region are secondary or degraded, and there is a high level of human impact due to slash and burn agriculture in the uplands which is having a negative impact on many orchids.

To conserve orchids, it is necessary to protect the plant species and their ecological niche and assist translocation from hostile environments (Swarts and Dixon 2009). Therefore, the orchid propagation is necessary and important to increase the population of plant species before transferral to natural habitats. Asymbiotic seed germination

R. Chutima · B. Bussaban · S. Lumyong (✉)
Department of Biology, Faculty of Science,
Chiang Mai University,
Chiang Mai 50200, Thailand
e-mail: scboi009@chiangmai.ac.th

B. Dell
Sustainable Ecosystems Research Institute,
Faculty of Sustainability, Environmental and Life Sciences,
Murdoch University,
Perth 6150, Australia

S. Vessabutr
Queen Sirikit Botanic Garden,
Mae-Rim, Chiang Mai 50180, Thailand

using a synthetic medium for culture of orchid seeds and seedlings has become a favored method especially for tropical orchids. However, this approach has had limited success with some terrestrial orchids (Arditti et al. 1981, 1990; Smith and Read 1997). Batty et al. (2006b) reported that the survival rate of Australian terrestrial orchid seedlings (*Caladenia arenicola*, *Diuris magnifica*, and *Thelymitra crinita*) after transferral to soil was low due to the orchids not producing tubers that are essential for survival through the summer dormancy period. Nonetheless, the survival rate of orchid seedlings from symbiotic sowing was higher than from asymbiotic sowing. In nature, most orchid seeds cannot germinate or can imbibe but will not develop unless they are colonized with the compatible mycorrhizal fungi which supply young plants with carbon and inorganic nutrients (Brundrett et al. 1996, 2003; McKendrick et al. 2000, 2002; Swarts and Dixon 2009; Wright et al. 2009). During symbiotic seed germination, orchid seeds are cultured and infected with compatible fungi, usually on a medium consisting of powdered oats with yeast extract (Brundrett et al. 2001, 2003). An efficient symbiotic seed germination protocol to germinate seeds of the rare subtropical terrestrial orchid, *Habenaria macroceratitis*, was described, and the seed germination percent was highest when seeds were sown with a fungal mycobiont, *Epulorhiza* sp. (Stewart and Kane 2006). Batty et al. (2006a) found that the seed of several Australian temperate terrestrial orchid taxa, *C. arenicola* and *Pterostylis sanguinea*, germinated best when seeds were sown with mycorrhizal fungi, and the seedling survival improved when actively growing symbiotic seedlings were transferred to natural habitats during the growing season.

In Thailand, there are only a few reports on orchid mycorrhizal fungi and their efficacy in facilitating seed germination and plant growth. Athipunyakom et al. (2004a) reported on seven genera and 14 species of orchid mycorrhizal fungi isolated from 11 terrestrial orchid species. In another study, the seed of one terrestrial orchid, *Spathoglottis plicata*, inoculated with *Epulorhiza repens* and *Rhizoctonia globularis*, presented the initiation of leaves 60 days after seed sowing (Athipunyakom et al. 2004b).

Pecteilis susannae (L.) Rafin is a widely distributed terrestrial orchid in dry dipterocarp forests and rainforests throughout Thailand (Nanakorn and Indharamusika 1998; Santisuk et al. 2006). However, the species has become threatened with extinction due to the loss of its natural habitat as well as over-harvesting for sale in illegal trade. The orchid capsules appear to have few viable seeds. Micropropagation using tissue culture techniques is not yet available (Santisuk et al. 2006; Suyanee Vessabutr, personal communication). Therefore, this study aimed to investigate the orchid mycorrhizal fungi associated with *P. susannae* and determine their benefit for seed germination. The data

collected from this study will be used for further propagation and conservation of this orchid.

Materials and methods

Orchid source

Rhizosphere soil and whole plants of *P. susannae* (Fig. 1) were collected from three field sites in natural habitat near Doi Suthep-Pui National Park, Chiang Mai province, Thailand (site 1: Mae-Sa, site 2: Sa-Luang, and site 3: Queen Sirikit Botanic Garden) in July, August, and September 2005–2007 (Table 2). The orchid samples were kept in plastic bags and were used within 48 h.

Colonization

Orchid roots were washed with tap water and cleared using 10% (w/v) KOH solution, stained in 0.05% (w/v) trypan blue in lactoglycerol (1:1:1 lactic acid, glycerol, and water), and observed under a compound microscope. Peloton structure and colonization by mycorrhizal fungi were assessed (Brundrett et al. 1996).

Fungal isolation

Endophytic fungi were isolated using a modification procedure of Yamato et al. (2005). Orchid root segments were detached, rinsed with tap water to remove debris, and cut into 1-cm segments. The segments were sterilized in 70% ethanol for 3 min then a solution containing 95% ethanol–5.25% NaOCl–sterile distilled water (1:1:1 v/v/v) for 1 min and rinsed with sterile distilled water three times. The segments were crushed in a Petri dish using a sterile glass rod. Approximately 20 ml of cooled (45°C) autoclaved modified Czapek Dox agar medium containing sucrose 0.5 g, NaNO₃ 0.33 g, KH₂PO₄·7H₂O 0.2 g, MgSO₄·7H₂O 0.1 g, KCl 0.1 g, yeast extract 0.1 g, agar 15 g, and distilled water 1,000 ml was poured into each Petri dish and mixed before solidification. The plates were incubated at 25°C in the dark, and microscopic observations were made daily for 21 days. Fungal colonies emerging from root tissues and cells containing peloton structure (Fig. 1f) were transferred to potato dextrose agar (PDA) and assigned 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.

Fungal identification and DNA extraction

Endophytic fungi were identified based on morphological characteristics and molecular approach. Morphology was

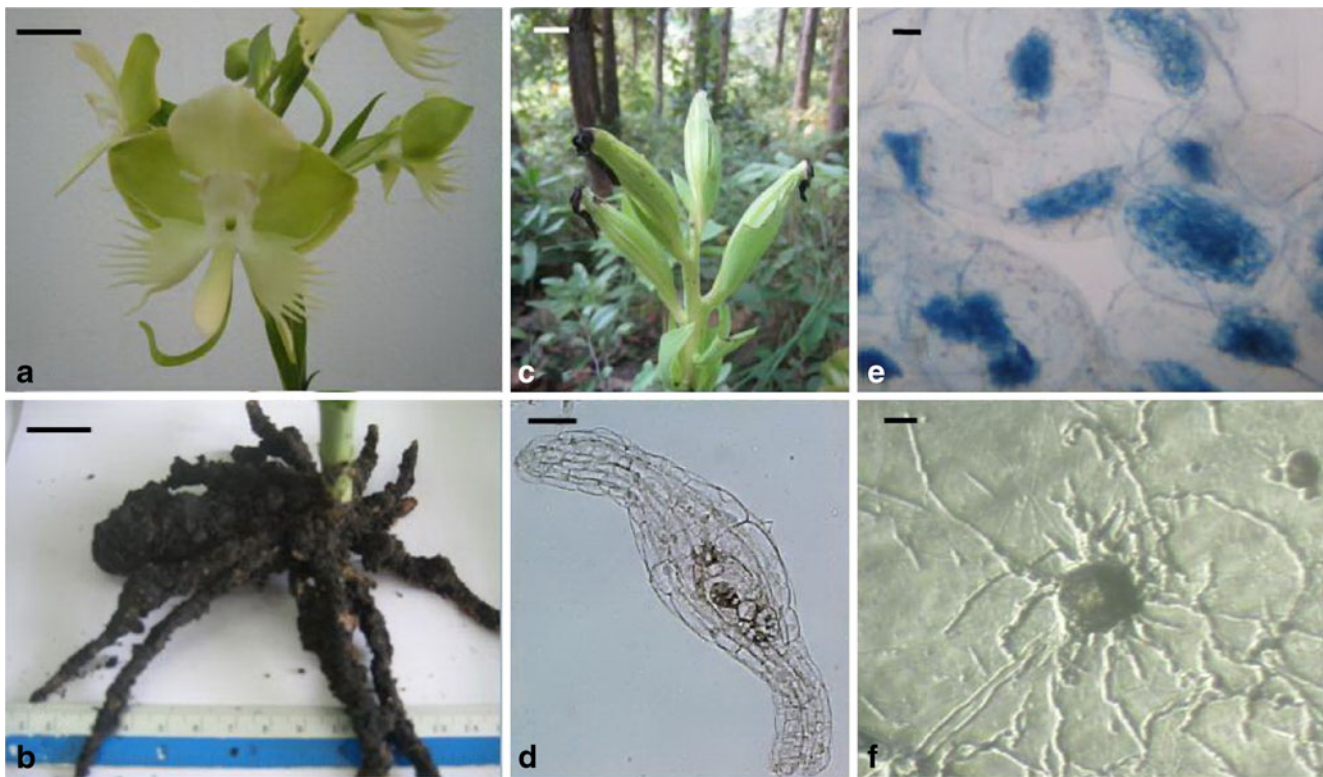


Fig. 1 *P. susannae*: **a** flower, **b** root system, **c** capsules of orchid in natural habitat, **d** orchid seed, **e** peloton structures, coiled hyphae, in cortical cells of roots stained with 0.05% (*w/v*) trypanblue in

lactoglycerol, and **f** fungal colony emerging from root cell containing peloton structure, single peloton. Bar: **a** 2 cm, **b** 2 cm, **c** 3 cm, **d** 0.10 mm, **e** 20 μ m, and **f** 40 μ m

examined using keys and methods outlined by Barnett and Hunter (1987), Currah and Zelmer (1992), Currah et al. (1997), Robert (1999), and Athipunyakom et al. (2004a). The molecular approach was modified from the procedure of Promputtha et al. (2005) and Yamato et al. (2005). Mycelium was scraped directly off culture plates, transferred into 1.5-ml centrifuge tubes, and freeze-dried using a lyophilizer (Dura-Dry™, USA). The freeze-dried mycelium was mixed with sterile white quartz sand and 600 μ l of preheated (60°C) 2 \times 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 μ l 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. Phenol–chloroform (1:1) extraction was repeated two 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 and 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 μ l of TE buffer

(10 mM Tris–HCl, 1 mM EDTA, pH 8) containing 20 μ g/ml RNase and incubated at 37°C for 60 min. DNA samples were checked for purity by electrophoresis in 1% (*w/v*) agarose gel stained with ethidium bromide under UV light.

Fungal internal transcribed spacer 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 light. PCR products were purified using PCR cleanup Gel extraction NucleoSpin® Extract II purification Kit (Macherey-Nagel, Dueren, Germany) following the manufacturer's protocol. The purified PCR products were directly sequenced. Sequencing reactions were performed, and the sequences were automatically determined in a genetic analyzer (1st Base, Selangor, 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

Table 1 Seed germination and protocorm developmental stages use for determination of growth and development of *Pecteilis susannae* in vitro

Stage	Description
0	No germination, viable embryo
1	Swollen embryo, production of rhizoids
2	Continued embryo enlargement, rupture of testa, further production of rhizoids
3	Appearance of protomeristem
4	Emergence of first leaf
5	Elongation of first leaf

Adapted from Stewart and Kane (2006)

tree was constructed using the PAUP beta 10 software version 4.0 (Swofford 2002). Branch and bound searches were performed with the criterion of maximum parsimony with tree-bisection–reconnection branch swapping algorithm. Starting trees were obtained via stepwise addition with 100 random sequence input orders. The parsimony tree scores, including tree length and consistency, retention, rescaled consistency, and homoplasy indices (TL, CI, RI, RC, and HI), also were calculated. All molecular characters were unordered and given equal weight during analysis. Relative branch support was estimated with 1,000 bootstrap replications for the analyses (Felsenstein 1985).

Orchid seed collection

Seeds (Fig. 1d) were obtained from mature capsules (Fig. 1c) in natural habitat (Site 2; Sa-Luang, Chiang Mai, Thailand) in October–November 2008. Immediately following collection, capsules were dried over silica gel for 2 weeks at 25°C, followed by storage at 4°C in the dark until used, within 4 months (Stewart and Kane 2006). Viability of seeds was determined using the tetrazorium test (Brundrett et al. 2001).

Symbiotic seed germination

The symbiotic effects of fungal isolates on seed germination of *P. susannae* were evaluated using a modified procedure of Stewart and Kane (2006). Seeds were sown from capsules after surface sterilization for 1 min in 95%

ethanol–5.25% NaOCl–sterile distilled water (1:1:1 v/v/v). Seeds (50–100) were placed over the surface of a 1×4 cm filter strip (Whatman No.4) within a 9-cm-diameter Petri dish containing 20 ml of sterile oat meal agar (pH 6.0). Each plate was inoculated with a 1×1-cm plug of each fungal inoculum taken from the hyphal edge 5 days after culturing on PDA. Uninoculated plates served as a control. Each treatment was replicated on four plates. Plates were sealed with parafilm and wrapped in aluminum foil to exclude light and maintained in the dark at 25°C for 70 days. The plates were examined weekly using a stereomicroscope and returned to the dark conditions. Seed germination and protocorm development were scored on a scale of 0–5 (Table 1). Seed germination and protocorm development percentages were calculated by dividing the number of seeds in each germination and development stage by the total number of viable seeds in the sample. The data were statistically analyzed using SPSS V16.0 for one-way analysis of variance (ANOVA), and means were compared by Duncan's Multiple Range test ($P \leq 0.05$).

Results

Peloton structure

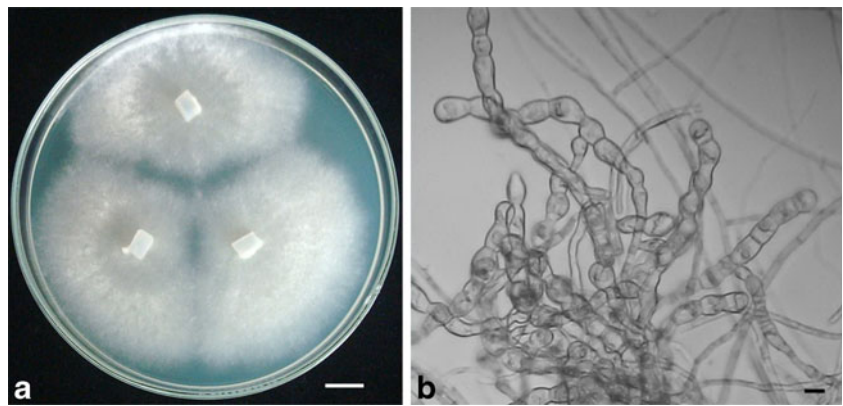
Peloton structures, coils of hyphae, of orchid mycorrhizal fungi (Fig. 1e) were found in cortical cells of all orchid roots after staining with 0.05% trypan blue in lactoglycerol. All root samples taken were heavily colonized (100%).

Table 2 Endophytic fungi isolated from roots of *Pecteilis susannae* and used in symbiotic seed germination

Fungal isolate	Taxa/GenBank accession number	Orchid collection time	Location
CMU-Aug 002	<i>Epulorhiza</i> sp. GQ856216	July 2005	Site 1
CMU-Aug 007	<i>Epulorhiza</i> sp. GQ856215	August 2007	Site 2
CMU-Aug 013	<i>Epulorhiza</i> sp. GQ856214	August 2007	Site 2
CMU-Aug 021	<i>Fusarium</i> sp. GQ862347	August 2007	Site 2
CMU-Aug 025	<i>Epulorhiza</i> sp. FJ882028	September 2007	Site 3
CMU-Aug 028	<i>Epulorhiza</i> sp. GQ862346	July 2005	Site 1
CMU-Aug 031	<i>Epulorhiza</i> sp. FJ940903	July 2007	Site 3
CMU-Aug 040	<i>Epulorhiza</i> sp. FJ873174	August 2007	Site 2

Site 1: Mae-Sa, Site 2: Sa-Luang, Site 3: Queen Sirikit Botanic Garden, all three sites located in Mae-Rim, Chiang Mai, Thailand

Fig. 2 *Epulorhiza* sp., CMU-Aug 013, **a** fungal colony cultured on potato dextrose agar for 14 days, *bar*=1 cm and **b** chain of monilioid cells (ellipsoid to subglobose), short swollen compartments, *bar*=10 μ m



Fungal isolation and identification

Eight endophytic fungal isolates were recovered from the roots of *P. susannae*; seven isolates were identified as members of the genus *Epulorhiza*; and one isolate was from the genus *Fusarium* based on morphological characteristics and molecular data (Table 2, Figs. 2, 3, and 4). The features of *Rhizoctonia*-like fungi, monilioid hyphae or chains of short swollen compartments (Fig. 2b), were presented by

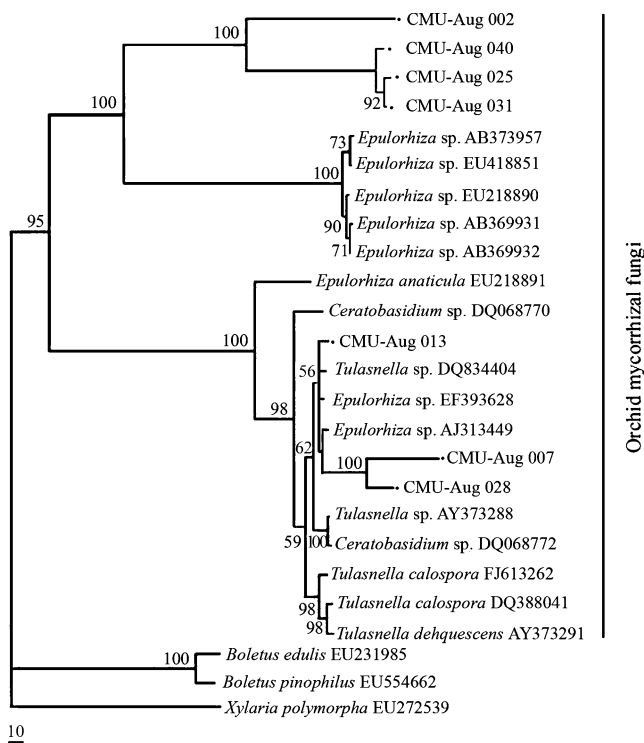


Fig. 3 Maximum-parsimony tree based on an alignment of internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2. The tree was rooted with *Boletus edulis*, *Boletus pinophilus*, and *Xylaria polymorpha*. Bootstrap values (calculated from 1,000 resamplings) higher or equal to 50% are shown at each branch (TL=1,201, CI=0.7594, HI=0.2406, RI=0.8909, RC=0.6765). The *bar* represents ten substitutions per nucleotide position

Epulorhiza isolates after being cultured on PDA for 21 days.

Parsimony and distance phylogenetic analyses showed that all seven fungal endophytes identified as members of the genus *Epulorhiza* belonged to the same clade of orchid mycorrhizal fungi, containing *Epulorhiza*, *Tulasnella*, and *Ceratobasidium*, with high bootstrap support of 95% (Fig. 3). Four fungal endophyte isolates (CMU-Aug 002, CMU-Aug 025, CMU-Aug 031, and CMU-Aug 040) formed a branch that was closely related to the genus *Epulorhiza* (anamorph of *Tulasnella*), and three fungal endophyte isolates (CMU-Aug 007, CMU-Aug 013, and CMU-Aug 028) were clustered with *Epulorhiza*, *Tulasnella*, and *Ceratobasidium*. The remaining endophytic fungus isolate CMU-Aug 021 was closely related to *Fusarium oxysporum*, in clade I, with 100% bootstrap support (Fig. 4).

Symbiotic seed germination

The tetrazorium test revealed *P. susannae* (L.) Rafin seeds to be 51.25% viable. All symbiotic and asymbiotic seed (control) treatments began to swell and germinate within 14 days after sowing. An effect of endophytic fungi on seed germination of *P. susannae* was found and evaluated after sowing for 70 days as shown in Fig. 5. Seed germination was significantly higher than the uninoculated control (62.1%) when seeds were inoculated with fungal isolates CMU-Aug 013 (86.2%), CMU-Aug 007 (79.9%), and CMU-Aug 028 (77.3%). These three fungal isolates also promoted protocorm developmental stage 1 (>60%). However, only two fungal isolates, CMU-Aug 013 (17.6%) and CMU-Aug 007 (17.7%), significantly promoted protocorm developmental stage 2 when compared to the control (11.1%; Fig. 6).

Discussion

The phylogenetic tree constructed by the sequences of ITS 1, ITS 2, and 5.8 ribosomal RNA gene of all fungal

Fig. 4 Maximum-parsimony tree based on an alignment of internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2. The tree was rooted with *Claviceps fusiformis* and *Cladosporium cladosporioides*. Bootstrap values (calculated from 1,000 resamplings) higher or equal to 50% are shown at each branches (TL=320, CI=0.8781, HI=0.1219, RI=0.8636, RC=0.7584). The bar represents ten substitutions per nucleotide position

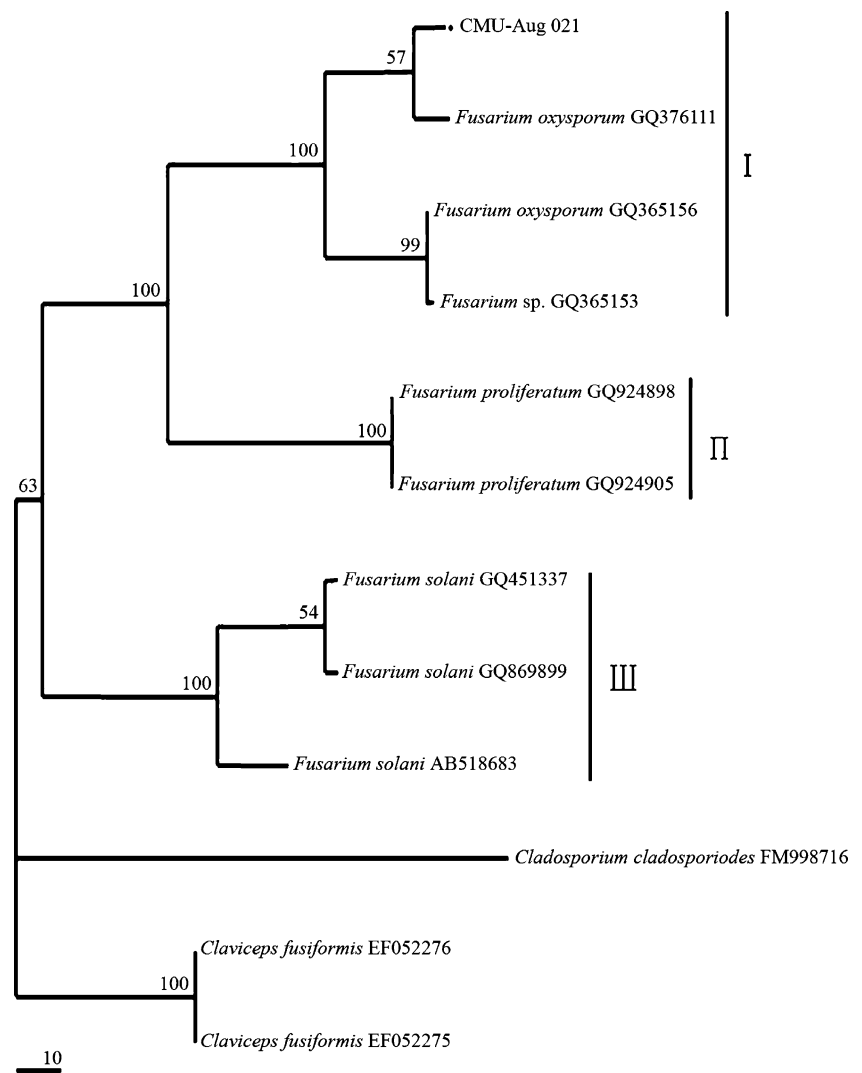


Fig. 5 Symbiotic seed germination and protocorm developmental stages of *P. susannae* using fungal endophyte *Epulorhiza* sp., CMU-Aug 013, cultured on oat meal agar 70 days after sowing; **a** stage 0; **b**, **c** stage 1; and **d** stage 2 of protocorm developments

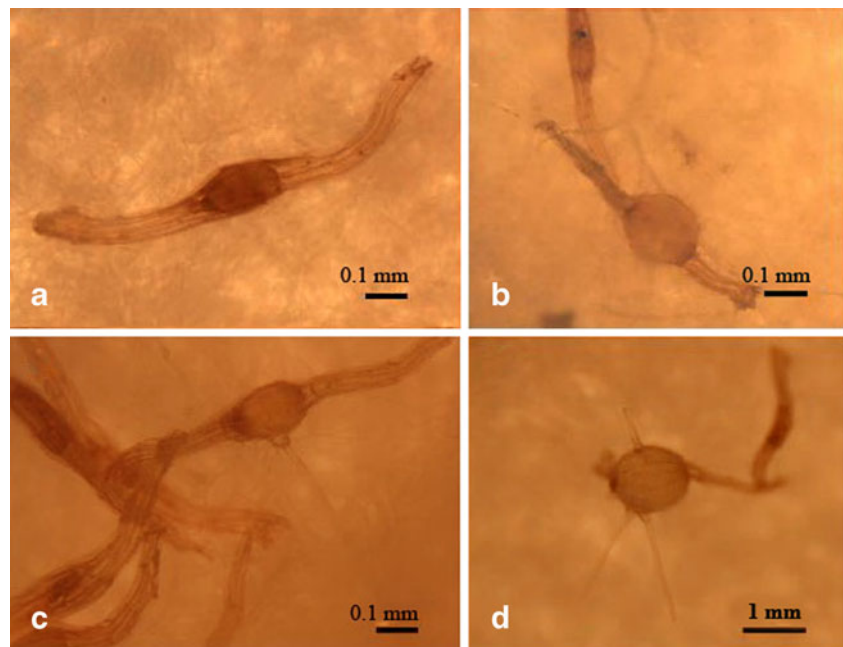
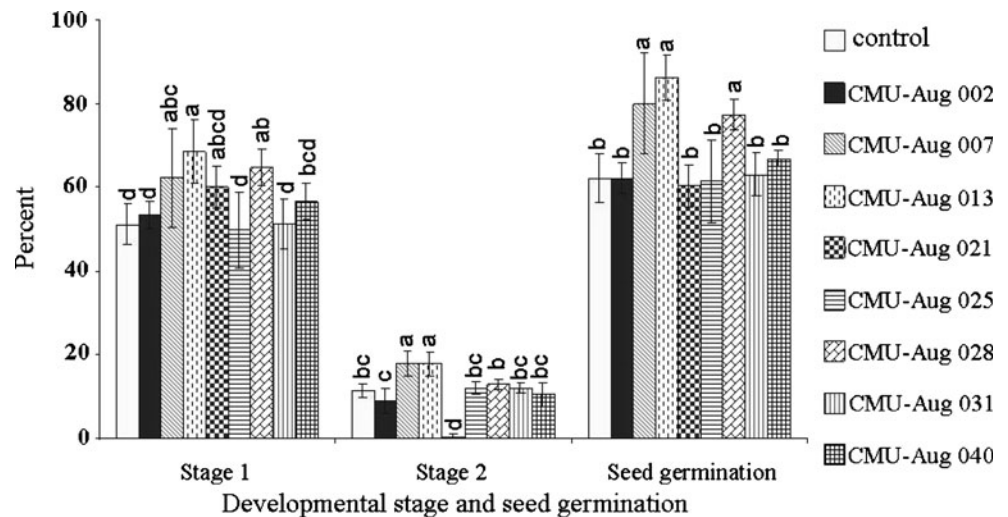


Fig. 6 Effects of eight endophytic fungi on protocorm development and seed germination of *P. susannae* 70 days after sowing. The results are the mean of four replicates with bars indicating the standard deviation. Mean values with the same letter are not significantly different (ANOVA and means were compared by Duncan's Multiple Range test; $P < 0.05$)



endophytes and known sequences in the GenBank database indicated that the fungi associated with *P. susannae*, seven *Epulorhiza* isolates (CMU-Aug 002, CMU-Aug 007, CMU-Aug 013, CMU-Aug 025, CMU-Aug 028, CMU-Aug 031, and CMU-Aug 040), were clustered in the clade of orchid mycorrhizal fungi and closely related with mycorrhizal associates known to be present in some other orchid species (Ma et al. 2003; McCormick et al. 2004; Suarez et al. 2006; Porras-Alfaro and Bayman 2007; Taylor and McCormick 2007; Shimura et al. 2009). This study supports the view that the anamorphic genus *Epulorhiza* is one of the most common and distinctive form-genera of *Basidiomycota* 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). However, some studies reported successful isolation and *in vitro* seed germination of terrestrial orchids by other fungi, e.g., *Ceratorhiza*, *Moniliopsis*, and *Fusarium* (Zelmer et al. 1996; Stewart and Zettler 2002; Kristiansen et al. 2004; Ovando et al. 2005; Johnson et al. 2007).

In addition, the fungal endophyte CMU-Aug 021 was identified as a *Fusarium* sp., and the phylogenetic analyses based on ITS 1, ITS 2, and 5.8 ribosomal RNA gene showed that this fungal isolate was closely related with *F. oxysporum* (GenBank accession number GQ376111), an endophytic species mainly associated with *Acortus calamus*, sweet flag. Although this plant species is not closely related with orchids, previous studies have reported that *Fusarium* obtained from orchids can promote the growth of orchid seedlings and form endomycorrhiza (Ovando et al. 2005; Johnson et al. 2007). Further, molecular analysis using genomic DNA extracted from orchid roots will be used to identify the endophytic fungi and compare with the isolated fungi.

In vitro symbiotic seed germination has become a favored and useful methodology for orchid seed propagation and use in plant reintroduction. However, there are few reports that address *in vitro* symbiotic seed germination of Thai native terrestrial orchid species (Athipunyakom et al. 2004b). This is the first study that describes *in vitro* symbiotic seed germination of *P. susannae*. In this study, the percentages of symbiotic seed germination was very high (86.2%, 79.9%, and 77.3%), and the differences in percentages of seed germination between symbiotic and asymbiotic (control, 62.1%) treatments were 24.1%, 17.8%, and 15.2% by fungal isolates CMU-Aug 013, CMU-Aug 007, and CMU-Aug 028, respectively. The most advanced protocorm development (stage 2) was promoted by *Epulorhiza* isolates 70 days after seed sowing. This is similar to the findings of Stewart and Kane (2006). In their study, seeds of *H. macroceratitis*, a terrestrial orchid in Florida, were cultured with six fungi originating from the roots of *H. macroceratitis*. All six fungal isolates were identified as belonging to the genus *Epulorhiza*, resulting in a maximum of 65.7% germination and maximum protocorm development of stage 2 after seed sowing for 58 days. The difference in percentage of seed germination between fungal and control treatments was 14.6%.

However, in other terrestrial orchid species, the difference between control and inoculated seed germination treatments can be quite large, indicating a greater dependency on mycorrhizal associations in recruitment. For example, Stewart and Zettler (2002) reported that the highest seed germination percentages for *Habenaria quinqueseta*, *Habenaria repens*, and *H. macroceratitis* (18.1%, 55.1%, and 50.8%, respectively) were found after incubation with a *Ceratorhiza* isolate, while uninoculated control had less than 1% seed germination. Athipunyakom et al. (2004b) isolated *E. repens* and *R. globularis* from roots of *S. plicata*, a Thai native terrestrial orchid. These two fungal

isolates promoted seed germination of *S. plicata* by 42.8% (*E. repens*) and 12.6% (*R. globularis*) after seeds were sown for 127 days. No germination occurred in the control treatment in their study. By contrast, some terrestrial orchid species are able to germinate without fungal partners. The lady's slipper orchid (*Cypripedium calceolus*) in Britain was successfully propagated by asymbiotic seed germination (Ramsay and Stewart 2008). However, mycorrhizal associations are probably beneficial in the life cycle of most orchids because of increased plant fitness (Brundrett et al. 2001; Dearnaley 2007).

Based on our results, the *Epulorhiza* isolates are the most effective fungal endophytes to promote seed germination of the tropical orchid, *P. susannae*. Nevertheless, the difference between percentages of symbiotic and asymbiotic seed germination was not high, and the final protocorm developmental stage is not sufficient for propagation and conservation of this terrestrial orchid species at the present time. There is a complicated relationship between orchid and endophytic fungi, and it is not clear whether the mycorrhizal fungi found in adult plants are the same fungi necessary for seed germination (Porrás-Alfaro and Bayman 2007). Shimura et al. (2009) reported different phylogenetic groups of mycorrhizal fungi isolated from adult flowering and juvenile plants of *Cypripedium macranthos*, a temperate terrestrial orchid in Japan. Moreover, some reports suggest there are specific endophytic fungi for different stages of growth in an orchid's life cycle (Kristiansen et al. 2004; Stewart and Kane 2006; Dearnaley 2007; Tao et al. 2008). For orchid conservation, not only in *in vitro* seed germination but also in *in situ* seed germination, establishment of orchid seedlings is essential (Batty et al. 2006a, b). Further studies are, therefore, needed in order to understand the effect of endophytic fungi on each step of the life cycle of *P. susannae*. An *in situ* seed-sowing study is proposed for this threatened species to trap possible candidate fungi.

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