Research paper

Axenic Synthesis of Ericoid Mycorrhiza in *Rhododendron formosanum* with *Phialocephala* Species

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【Summary】

Formosan rhododendron (*Rhododendron formosanum* Hemsl.) is an endemic species of Ericales in Taiwan. The Rf28 fungal strain was isolated from the roots of Formosan rhododendron and identified as an endophytic species of *Phialocephala* based on its morphological characteristics. This identification was further supported by a DNA sequence analysis. This species is a new addition to the fungal flora of Taiwan. In ericoid-mycorrhizal synthesis, Rf28-inoculated Formosan rhododendron seedlings exhibited vigorous growth, and an examination of the root ultrastructure revealed the occurrence of hyphal complexes and hyphal coils in cortical cells. These results demonstrated that Formosan rhododendron can form ericoid mycorrhiza in vitro with *Phialocephala* spp.

Key words: Ericoid mycorrhiza, Ericoid mycorrhizal fungus, *Phialocephala* spp., *Rhododendron formosanum*.

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研究報告

台灣杜鵑根部與內生菌*Phialocephala* spp.純合成為杜鵑類菌根

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摘 要

台灣杜鵑為台灣原生種杜鵑科植物。本研究自台灣杜鵑根系分離純化杜鵑類內生菌Rf28菌株，並以其ITS1-F/TW13序列與基因庫已知的杜鵑類內生菌資料比對，鑑定為*Phialocephala*菌株，此種真菌為台灣絲孢菌類新紀錄種。以Rf28菌株接種台灣杜鵑無菌苗再合成菌根試驗發現，菌根合成苗生長良好，且自其根部皮層細胞的微細構造中發現具有菌絲複合體和菌絲圈，此結果證實*Phialocephala* spp.內生菌能與台灣杜鵑形成典型的杜鵑類菌根。

關鍵詞：杜鵑類菌根、杜鵑類菌根菌、*Phialocephala* spp.、台灣杜鵑。

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INTRODUCTION

Ericoid mycorrhizas (ERM) belong to endomycorrhizas that have intracellular, septate hyphae and hyphal coils, but without arbuscular hyphae, a mantle, a Hartig net, or vesicles (Harley 1969). Currently, 2 types of ericoid mycorrhizal fungi (ERMF), ascomycetes and hyphomycetes, have been identified. The well-known species *Pezoloma ericae* (Read) Baral belongs to the ascomycetes type (Baral and Krieglsteiner 2006); whereas *Oidiodendron maius* Barron (Couture et al. 1983, Dalpé 1986, Douglas et al. 1989), *Phialocephala fortinni* Wang and Wilcox (Stoyke and Currah 1991, Hambleton and Currah 1997), *Cryptosporiopsis ericae* Sigler, and *C. brunnea* Sigler (Sigler et al. 2005) are members of the hyphomycetes.

Although numerous sterile fungi have been isolated from roots of the Ericaceae and Epacridaceae, they have not been identified to the species level due to their inability to form telemorphs and conidia. (Burgeff 1961, Peretto et al. 1990, Stoyke and Currah 1991, Hutton et al. 1994, Xiao and Berch 1996, Hambleton and Currah 1997). However, in recent years, internal transcribed spacers (ITSs) of ribosomal DNA have successfully been used to clarify the phylogenetic relationships and exhibit the genetic diversity of ERMF (Liu et al. 1998, Chambers et al. 1999, McLean et al. 1999, Monreal et al. 1999, Sharples et al. 2000, Usuki et al. 2003). This ITS method can speed up research on ERMF.

The main objectives of this study were to determine the specific taxonomic disposition of an isolated fungus (Rf28) from endemic *R. formosanum* roots using morphological characteristics and sequence analyses of ITSs of rDNA and to characterize the nature of its associations with host roots by a mycorrhizal resynthesis technique.

MATERIALS AND METHODS

Fungal strain

A specimen of Rf28 was deposited at the Forest Mycobiont Laboratory of National Chaiyi University, Chaiyi, Taiwan. This endophyte was isolated from roots of *R. formosanum* (120°47'31.35"E, 23°38'9.85"N) located in Sanlinchi Recreational Park in Chushang Township, Nantou County, Taiwan (Lin et al. 2010).

Morphology and growth of the colony

The colony of Rf28 was transferred to 2% malt extract agar (MEA: 20 g L^{-1} agar, 15 g L^{-1}, BD Bioscience PharMingen, San Jose, CA), potato dextrose agar (PDA: 39 g L^{-1}, BD) and oat meal agar (OAT: 30 g L^{-1}, Himedia, Mumbai, India) media and incubated in 2 separate growth chambers at 15 and 22℃, respectively. After 12 d, the growth rates of the colony were recorded, while the morphology of the colony was observed every day (Stoyke and Currah 1991, Hambleton and Currah 1997).

Morphology of hyphae

Corn meal agar (CMA: 50 g L^{-1} corn meal and 15 g L^{-1} agar, Sigma, St. Louis, MO, USA) medium was used to culture the endophyte. After 20 d, plugs from different media were transferred to slides for further culturing under a non ultraviolet (nUV) growth light to induce sporulation of the endophyte. After 40 d of culture on slides, 1% of aniline blue was applied to stain the hyphae, and observations on the morphology were made using a light microscope, and photos were taken for records (Hutton et al. 1994).

DNA extraction, sequencing, and phylogenetic analysis

Methods described by Sigler et al. (2005) were followed. Mycelium for DNA extraction was scraped from the surface of PDA agar cultures. Genomic DNA was extracted using Puregene Proteinase K (Qiagen, St. Louis, MO, USA). Total fungal DNA was used as a template for amplification with primers ITS1-F and TW13 (Sigler et al. 2005). Polymerase chain reaction (PCR) products were sequenced by Genomics BioSci and Tech Company (Taipei, Taiwan). Sequences were assembled, and related sequences were searched using BLAST searches.

Phylogenetic relationships were analyzed by MEGA (Molecular Evolutionary Genetics Analysis) (Tamura et al. 2007). Bootstrapping was performed using a Neighbor-joining method (Saitou and Nei 1987).

Resynthesis

Pure resynthesis was done following the method of Dalpé (1986). After surface cleaning, seeds of *R. formosanum* were sterilized with a 10% sodium hypochlorite solution for 15 min and rinsed 3 times with sterilized distilled water, then transferred to test tubes containing 1% agar for germination. Germinated seedlings were transplanted to modified Mitchell and Read medium (MMR medium: 32 mg L^{-1} NH_4Cl, 43.5 mg L^{-1} $CaCl_2 \cdot 7H_2O$, 10 mg L^{-1} $MgSO_4 \cdot 7H_2O$, 5.5 mg L^{-1} KCl, 3.75 mg L^{-1} $FeCl_3$, 2 g L^{-1} sucrose, 210 mg L^{-1} KH_2PO_4, 100 μg L^{-1} pyridoxine, 100 μg L^{-1} thiamine, 10 g L^{-1} agar). Seven days later, the aseptic seedlings were inoculated with the Rf28 fungal strain and grown in a growth chamber at 22℃ and 16 h of light at 5000 lux.

Morphology

After 2 mo of culture, the roots of inoculated seedling were sampled and cleaned with water in a supersonic oscillator (Upson et al. 2007). The morphology of mycorrhiza was observed with a stereomicroscope (Usuki and

Narisawa 2005).

For the ultrastructural study, root samples were fixed with 2.5% glutaraldehyde and 4% paraformaldehyde fixative in a phosphate-buffered solution (0.1 M, pH 7.0) for 4 h at room temperature, then rinsed with the phosphate-buffered solution 3 times each time for 15 min followed by serial dehydration in 30, 50, 70, 85, 95, and 100% ethanol and 100% acetone, and finally dried in a critical-point dryer using liquid carbon dioxide. Dried materials were mounted on an aluminum stub with twin adhesives, coated, and observed with a scanning electron microscope (Nakamura et al. 2002).

Statistical analyses

Differences in growth rates among treatments were analyzed using a one-way analysis of variance (ANOVA). Tukey's multiple-range test was applied for further analysis.

RESULTS AND DISCUSSION

Morphology of the strain

The Rf28 endophyte was incubated on different media (MEA, OAT, and PDA) and at 2 temperatures (15 and 22℃) for the morphological study. Colonies of Rf28 cultured on OAT at 15 and 22℃ showed a light olive-green color with white edges in the at early stage. After 27 d (Fig. 1C, D), the surfaces of the colonies were covered with white and grayish-white hyphae, while the bottoms of the colonies were still olive-green. The average growth rate of the colony at 22℃ (2.07 ±0.02 mm d^{-1}) was significantly higher than that at 15℃ (1.56±0.04 mm d^{-1}) (Fig. 1, Table 1).

Colonies of Rf28, grown on MEA at 22℃ showed thin air hyphae on the surfaces with a dark olive-green color with white edges (Fig. 1A, B). The average growth rate of the colony at 22℃ (2.76±0.02 mm d^{-1}) was significantly higher than that at 15℃ (1.56± 0.06 mm d^{-1}) (Fig. 1, Table 1).

Colonies of Rf28, grown on PDA at 22℃ showed a light olive-green color with white edges in the early stage (Fig. 1E, F). After 12 d, the surfaces of the colonies were covered with thick white and grayish-white hyphae. The average growth rate of colonies at 22℃ (1.74±0.02 mm d^{-1}) was significantly higher than that at 15℃ (1.57±0.02 mm d^{-1}) (Fig. 1, Table 1).

Taken together, these results indicated that the averaged growth rates of Rf28 on different media were always significantly higher at 22℃ than that at 15℃, and MEA medium allowed the fungus to grow better at 22℃ (Table 1).

Morphology of hyphae

After treating the slide cultures of CMA with nUV for 40 d, only dark-brown, thick-walled, septate and sparingly branched hyphae (2.7~3.4 μm in width) were found (Fig. 2).

Molecular analyses

BLAST searches showed that the 5.8S rDNA/ITS sequences of Rf28 determined in this study were more than 89% identical to previously published sequences from *Phialocephala fortinii* (AY394915). *Cryptosporiopsis ericae* Sigler was used as an outgroup in this study. Thus, Rf28 was classified into the same group of *P. fortinii* (AY394915) by the NJ method (Fig. 3, Table 2). Also, the Rf28 endophyte might be a new species belonging to *Phialocephala*.

An earlier study by Tseng and Lu (2003) indicated that there are 15 native *Rhododendron* species in Taiwan. Among them, 11 species are considered endemic, accounting for 73% of these native species. Very little

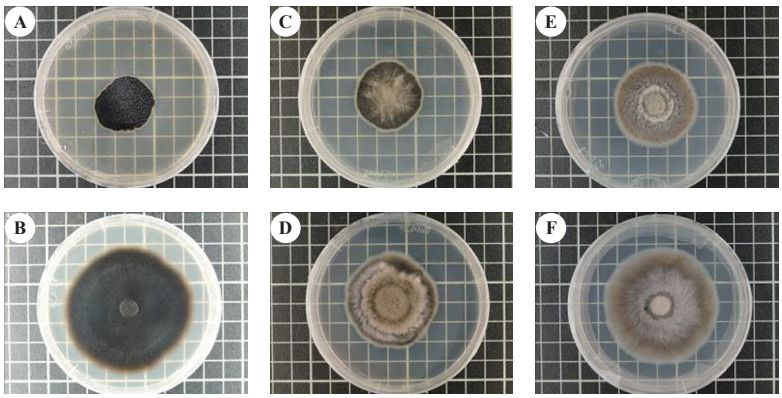

Fig. 1. Growth of the Rf28 strain cultured on different media and at 2 temperatures after 27 d. A and B, on MEA medium at 15 and 22℃, respectively; C and D, on OAT medium at 15 and 22℃, respectively; E and F, on PDA medium at 15 and 22℃, respectively.

Table 1. Growth rates of the Rf28 strain on different media and at 2 temperatures after 12 d of culture

Strain	Medium	Temperature	Mean ± SD (mm d^{-1})
Rf28	OAT	15℃	1.56 ± 0.04^{d}
Rf28	OAT	22℃	2.07 ± 0.02^{b}
Rf28	MEA	15℃	1.56 ± 0.06^{d}
Rf28	MEA	22℃	2.76 ± 0.02^{a}
Rf28	PDA	15℃	1.57 ± 0.02^{d}
Rf28	PDA	22℃	1.74 ± 0.02^{c}

All values are reported as the mean ± standard deviation of 4 replicates of culture. Values with different superscript letters significantly differ at $p < 0.05$.

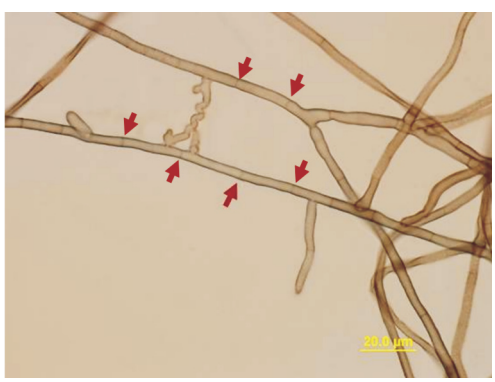

Fig. 2. Morphology of Rf28 hyphae on a CMA slide culture showing branched, brown, and thick-walled hyphae. Arrows indicate septa.

research had been done on ERM due to the fact that isolated endophytes from *Rhododendron* sp. cannot form telemorphs and conidia. However, ITS technology made the identification of these endophytes from *Rhododendron* sp. possible.

Pure resynthesis

After 2 mo of cultivation, seedlings of Formosan rhododendron inoculated with the Rf28 strain grew well (Fig. 4A). The root associations did not exhibit significant features under a stereomicroscope (Fig. 4B). However, features of a hyphal complex of ERM were

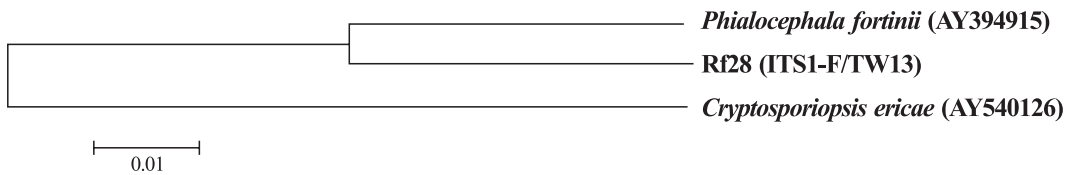

Fig. 3. Neighbor-joining phylogenetic tree based on rDNA ITS sequence data from the Rf28 strain isolated from *Rhododendron formosanum* along with known ericoid mycorrhizal fungi and selected fungal species with high similarity from GenBank. Data for the percent similarity are presented in Table 2.

Table 2. Similarity matrix derived from a molecular analysis of the Rf28 strain using ITS rDNA sequences

Strain	Rf28	*P. fortinii*	*C. ericae*
Rf28	100	89.8	82.8
Phialocephala fortinii (AY394915)	89.8	100	84.5
Cryptosporiopsis ericae (AY540126)	82.8	84.5	100

Data are presented as percentages.

Fig. 4. Root association of a *Rhododendron formosanum* seedling inoculated with the Rf28 strain. A, Seedling; B, root-fungus association (arrows); C, ultrastructure of the hyphal complex (arrow).

discovered in cortical cells of root associations in resynthesized seedlings (Fig. 4C).

CONCLUSIONS

In this study, 2 primers (ITS1-F/TW13) were used to amplify the sequence of 5.8S rDNA/ITS from the Rf28 endophyte by PCR. The sequence of the PCR product was compared with the database on the NCBI website by BLAST. The comparison showed that the Rf28 sequence exhibited a 89.8% similarity to that of *Phialocephala fortinii* Wang and Wilcox (AY394915). Based on the phylogenetic tree analysis (Fig. 3, Table 2), the endophyte (Rf28) isolated from Formosan rhododendron and *P. fortinii* belonged to the same group. Thus, it could be inferred that Rf28, a new addition to the fungal flora of Taiwan, belongs to the genus *Phialocephala*.

In the mycorrhizal resynthesis study (Fig. 4), hyphal complexes and the structure of ERM were discovered in cortical cells of root associations of seedlings inoculated with Rf28. This result supports the notion that Formosan rhododendron can form a symbiosis with endophytic fungal *Phialocephala* spp. to form ericoid mycorrhiza, which could be very

important for its adaptation to poor-nutrient environments.

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