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Research Article
Characterization of *Pisolithus orientalis* from Taiwan and its Compatibility with *Cyclobalanopsis glauca*

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Abstract

Background and Objective: The mycorrhizae of most Fagaceae species is known to be ectomycorrhizae. The objective of this study was to investigate the colony morphology, growth rate on different media and on the modified Melin-Norkrans medium at different pH levels and to analyze ITS rDNA of the Cms01 isolate. Finally, the basidiospore inoculum is used to induce mycorrhizal synthesis. **Materials and Methods:** In this study, these sporocarps were collected from a Fagaceae spp., stand located in Jhongpu, Chiayi county. Then, basidiospores were isolated from the sporocarps and examined under light and scanning electron microscopy. **Results:** Basidiospores were small, globose and possessed secondary spines. Morphological and molecular analyses indicated that isolate Cms01 belongs to *Pisolithus orientalis* and that it grows optimally on modified Melin-Norkrans medium at a pH of 6.5. The mycorrhizal association experiment showed that roots of colonized seedlings produced the mantle and hartig net characteristic of ectomycorrhizae. **Conclusions:** These results showed that this strain (Cms01) was a newly recorded species in the fungal flora of Taiwan and it could form a typical ectomycorrhiza with *Cyclobalanopsis glauca* seedling and it also belonged to an ectomycorrhizal fungus.

Key words: *Cyclobalanopsis glauca*, ectomycorrhizae, ectomycorrhizal fungus, mycorrhizal synthesis, *Pisolithus orientalis*

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Pisolithus spp., are well-known ectomycorrhizal fungi, belonging to the family Pisolithaceae[1,2]. The genus is found in temperate to tropical regions of the world and has mycorrhizal associations with members of the Casuarinaceae, Dipterocarpaceae, Pinaceae, Fagaceae and Myrtaceae[1,3,4]. *Pisolithus* has viewed as monotypic[5-7] and is well-studied, mainly based on ectomycorrhizal studies, as *P. tinctorius* (P. Micheli ex Pers.)[1,8,9]. Rauschert[10] suggested that this species was conspecific with *Polysaccum arhizum* Scop., which he redefined as *Pisolithus arhizus* (Scop.) Rauschert. However, molecular studies later indicated that *P. tinctorius* is a species complex[11-13]. At least 11 phylogenetic species were proposed by Martin *et al*.[14]. Recently, Phosri *et al*.[15] described a new species, *P. orientalis*, based on morphological and ITS nrDNA sequence analyses.

Mycorrhiza is a type of symbiosis in which plant roots associate intimately with fungi. Ninety eight percent of all plant species have symbiotic fungal partners associated with their root systems[16]. The various forms of mycorrhizal associations are ubiquitous in nature and play important roles in plant nutrition and nutrient cycling. They also influence the structure and dynamics of the plant communities within which they exist[17]. Functions of mycorrhizae include being mutualistic[18] and tolerant[19-21]. A previous study revealed that the Cms01 isolate of *P. orientalis* was a new species in the fungal flora of Taiwan[22]. However, the morphology of the basidiospores, the Internal Transcribed Spacer (ITS) rDNA sequence and other characteristics of the isolate remain unknown. The objectives of this study were to examine the compatibility of *P. orientalis* with *Cyclobalanopsis glauca* var. *glauca* and further characterize the Cms01 isolate.

MATERIALS AND METHODS

Strains: The Cms01 isolate was obtained previously from a sporocarp within a Fagaceae plantation (120°34 '41" E, 23°26' 4" N; altitude, 246 m), located in Jhongpu Township, Chiayi County, Taiwan[22]. Specimens of Cms01 were deposited at the Tree Mycorrhiza Laboratory of National Chiayi University and its ITS genomic sequences were deposited in GenBank (KU221036).

Seeds: Seeds of *Cyclobalanopsis glauca* were collected from the campus of National Chiayi University in November, 2014.

Colony morphology and growth rate: Mycelia from isolate Cms01 were transferred to dishes containing modified Melin-Norkrans medium (MMN)[23], Potato Dextrose Agar (PDA), Oat Flake Medium (OFM) or yeast phosphate soluble starch (YpSs)[24] and then placed in a growth chamber at 23°C. Colony radial growth rates were recorded after incubation for 14 days.

Basidiospore characteristics: Observations of basidiospore morphology were made with a light microscope and photomicrographs were taken for further examination[25]. The ultrastructures of the basidiospores were examined with a Scanning Electron Microscope (SEM) and photographed according to the method described by Yanaga *et al*.[26] with modifications.

DNA extraction, sequencing and phylogenetic analysis: The methods described by Phosri *et al*.[27] were followed for ITS rDNA analysis. Mycelia for DNA extraction, amplification and sequencing were scraped from the surfaces of PDA cultures. Genomic DNA was extracted using puregene proteinase K. Total fungal DNA was used as the template for amplification with primers ITS1-F and ITS4[28]. The PCR products were sequenced by Genomics BioSci and Tech Company. Sequences were assembled and related sequences were analyzed using BLAST searches.

The phylogenetic relationships were analyzed by Molecular Evolutionary Genetics Analysis software[29]. Bootstrapping was performed using neighbor-joining[30].

Growth rates at different pH: The method for evaluating optimal culture conditions for the fungus was modified from that of Garcia-Rodriguez *et al*.[31]. Optimal culture conditions were determined by placing a 0.5 mm plug of mycelium onto each MMN dish, the pH levels of sample media ranged from 4.5-7.5. Dishes were sealed with Parafilm and incubated at 23°C in the dark for 30 days. After 30 days, the colonies were measured and growth rates were calculated. Colony morphology was observed daily.

Inoculation with endophytes: Basidiospore inoculum was prepared according to the procedures described by Marx *et al*.[32]. To induce mycorrhizal synthesis in tree seedlings in grow tubes (tubelings), test tubes (4 cm diameter and 18 cm height) containing a mixture of peat and vermiculite (1:1 v/v, previously sterilized at 121°C for 60 min) were used. The tubeling technique was modified from the methods of Molina[33] and Hu *et al*.[34]. When the seedlings were transplanted into the tubes, the inoculum (10 mL) was placed near the root. The Cms01-inoculated seedlings were grown, watered and fertilized in a greenhouse. Standard fertility inputs were added to each tube along with a total of 100 mL sterile MMN solution[23] for incubation.

Observation of colonization in the seedling root systems: After 3 months of culture, the roots of inoculated seedling were sampled and cleaned with water in a supersonic oscillator. Mycorrhiza morphology was observed with a stereo microscope and morphotypes were distinguished[35].

For SEM examination, root samples were fixed with 2.5% glutaraldehyde and 4% paraformaldehyde fixative in phosphate buffer solution (0.1 M, pH 7.0) for 4 h at room temperature, then rinsed with phosphate buffer solution three times (15 min each rinse), followed by serial dehydration in 10, 20, 30, 50, 70, 80, 90, 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 adhesive, sputter-coated with gold and observed under SEM[36,37].

Statistical analysis: SPSS version 12.0 (Chicago, IL, USA) for Windows was used to perform the statistical analyses. Means of three separate experiments±standard deviation (n = 3) were calculated from collected data. Tukey's multiple range test at $p \leq 0.05$ was used to determine the differences in colony growth in media and pH values.

RESULTS AND DISCUSSION

Basidiospore morphology: After washing, basidiospores were observed by light microscope to be relatively small, globose and possess spines (Fig. 1a). SEM observations revealed that the isolate possessed smaller, globose basidiospores with secondary spines (Fig. 1b).

Phosri *et al.*[15] proposed *Pisolithus orientalis* as a new species based on morphological and ITS nrDNA sequence analyses. Differs from *P. tinctorius* by smaller globose basidiospores ornamented by isolated groups of narrow cones that adhere together to form secondary spines. According to our results, the basidiospores in this study belong to *P. orientalis*.

Morphology and growth rate: Isolate Cms01 was grown on MMN, OFM, PDA and YpSs dishes at 23°C. After 14 days, colonies of Cms01 cultured on MMN and PDA showed a brown-yellow color (Fig. 2a, b), while those cultured on OFM (Fig. 2c) and YpSs (Fig. 2d) displayed gray-brown and white colors, respectively. The average growth rate of the colonies on MMN (0.87±0.02 mm day^{-1}) was significantly higher than those grown on OFM, PDA and YpSs (0.76±0.06, 0.40±0.05 and 0.37±0.02 mm day^{-1}, respectively) (Table 1).

Isolate Cms01 was grown for 30 days on MMN medium with pH ranging from 4.5-7.5 (Fig. 3). The greatest average growth rate was on pH 6.5 (0.91±0.03 mm day^{-1}), although, growth on pH 7.5 (0.88±0.03 mm day^{-1}) and pH 4.5 (0.86±0.06 mm day^{-1}) was not significantly different from that observed at pH 6.5 (Table 2). Growth at pH 5.5 (0.79±0.04 mm day^{-1}) ($p<0.05$) was significantly less than

Fig. 1(a-b): Morphology of basidiospores by (a) Light and (b) Scanning electron microscopy

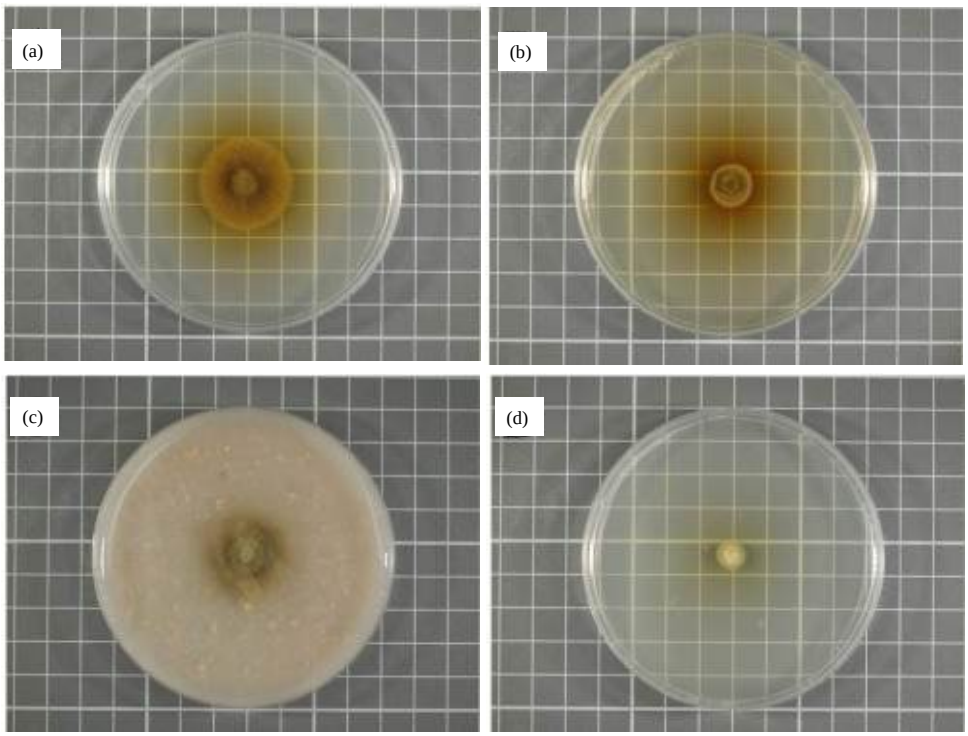

Fig. 2(a-d): Growth of isolate Cms01 on different media after 14 days. Media are modified (a) Melin-Norkrans medium, (b) Potato dextrose agar, (c) Oat flake medium and (d) Yeast phosphate soluble starch

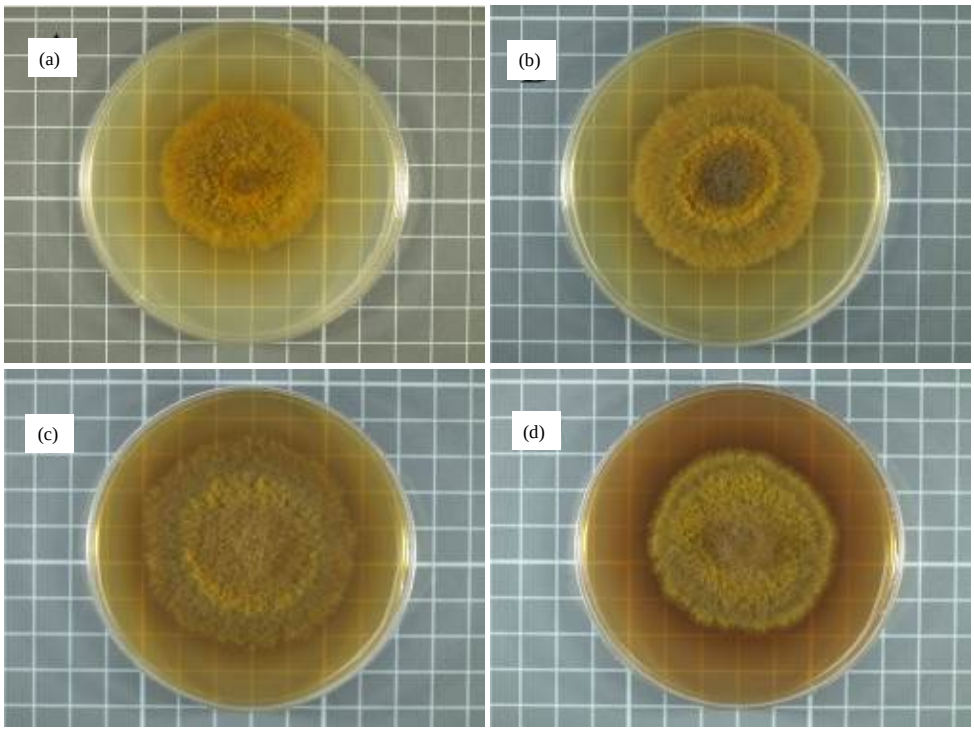

Fig. 3(a-d): Growth of isolate Cms01 on modified Melin-Norkrans medium at different pH levels after 30 days. pH levels are (a) 4.5, (b) 5.5, (c) 6.5 and (d) 7.5

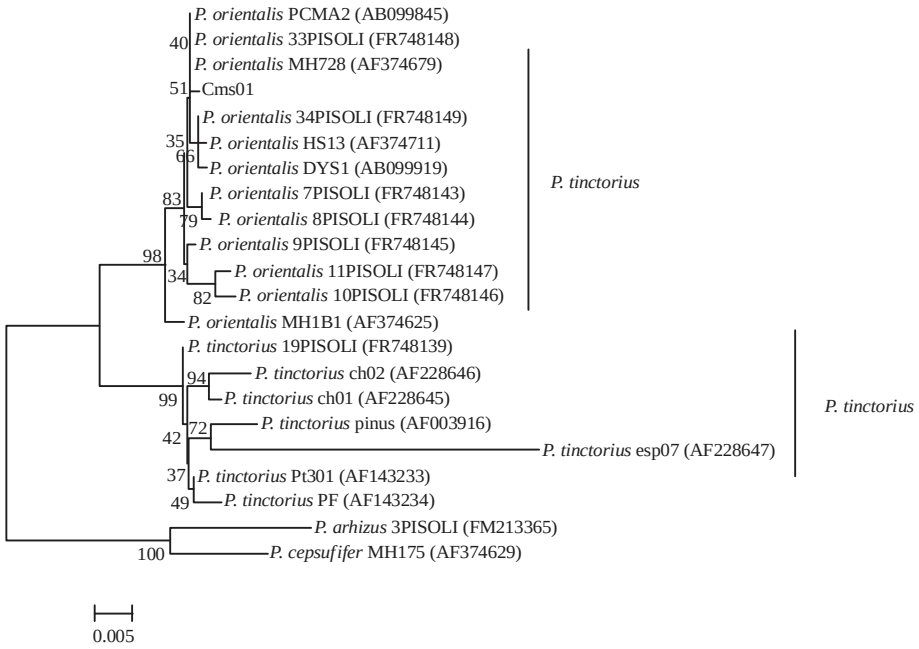

Fig. 4: Neighbor-joining phylogenetic tree based on rDNA ITS sequence data from the isolate Cms01 with *Pisolithus tinctorius* and *P. orientalis*[15] sequences obtained from GenBank. Numerical values above the branches indicate bootstrap percentiles from 1000 replicates. Bootstrap numbers over 50% are indicated. Horizontal branch lengths are proportional to the scale of base pair substitutions

Table 1: Growth rates of isolate Cms01 on different media after 14 days of culture

Media	Mean±SD (mm day^{-1})
MMN	0.87±0.02^{c}
OFM	0.76±0.06^{b}
PDA	0.40±0.05^{a}
YpSs	0.37±0.02^{a}

All values are Means±Standard Deviation of three replicate cultures ($p<0.05$), values in the same column with different letters are significantly different ($p<0.05$), MMN: Melin-Norkrans medium, OFM: Oat flake medium, PDA: Potato dextrose agar and YpSs: Yeast phosphate soluble starch

Table 2: Growth rates of isolate Cms01 on modified Melin-Norkrans medium at different pH levels after 30 days

pH value	Mean±SD (mm day^{-1})
4.5	0.86±0.06^{ab}
5.5	0.79±0.04^{a}
6.5	0.91±0.03^{b}
7.5	0.88±0.03^{ab}

All values are Means±Standard Deviation of three replicate cultures ($p<0.05$), values in the same column with different letters are different significantly different ($p<0.05$)

that at pH 6.5 but not different from that observed at pH 7.5 and pH 4.5 (Table 2). It has been suggested that the optimum growth conditions for *P. tinctorius*[38] are between pH 6 and 7 on either MMN or PDA[31]. However, the optimum growth conditions for *P. orientalis* remain unclear. In our study, better growth was found for isolate Cms01 on MMN than on PDA medium. Based on the above results, isolate Cms01 showed little difference from *P. tinctorius* in terms of its preferred growth conditions.

Molecular phylogenetic analysis: Based on DNA sequences serve to reevaluate earlier classifications and provide more accurate species delimitation[14,39]. Studies of phylogeny and its implications for understanding the biogeography of ectomycorrhizal fungi showing cryptic speciation have been progressing significantly in recent years as a result of DNA sequencing[40-42].

Taxonomic affinities are assigned to Cms01 based on BLAST sequence similarity analysis (http://blast.ncbi.nlm.nih.gov/Blast.cgi) including several most closely matched sequences. The ITS sequences of Cms01 can be matched to *P. orientalis* (NR119745); therefore, they are grouped in the neighbor-joining analysis (98% bootstrap) (Fig. 4) and all sequences of the nearest species from Phosri *et al.*[15]. The neighbor-joining analysis (Fig. 4) further suggests that isolate Cms01 is not closely related to *P. tinctorius*. Thus, isolate Cms01 belongs to *P. orientalis*.

Morphology and colonization in inoculated seedlings: All treated seedlings survived after 3 months of incubation. Features of root associations of inoculated-seedling were observed by stereomicroscope and SEM.

The Cms01-inoculated seedlings grew well (Fig. 5). The features of root associations were pyramidal (Fig. 6a) and had gold-yellow external hyphae (Fig. 6b) when

Fig. 5: Morphology of *Cyclobalanopsis glauca* seedling inoculated with isolate Cms01 shown after incubation for 3 months

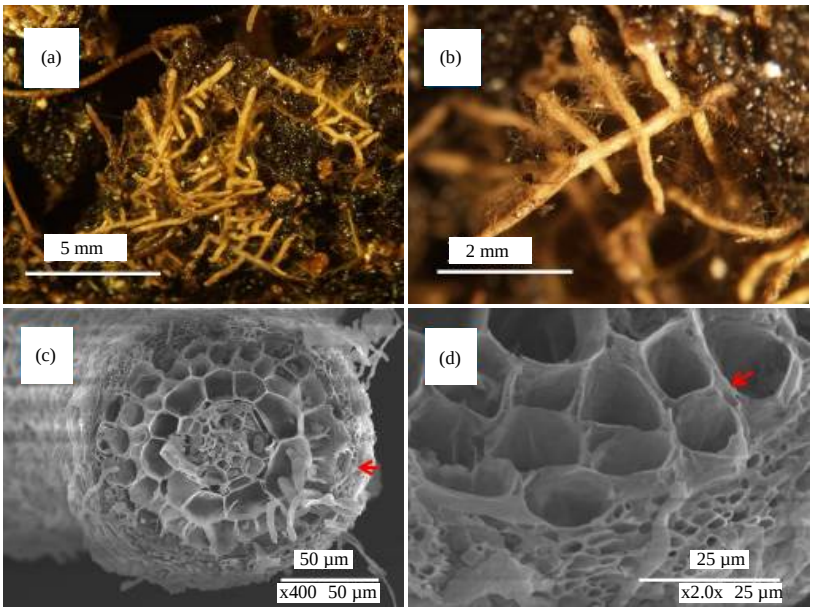

Fig. 6(a-d): Root structure of *Cyclobalanopsis glauca* inoculated seedlings after incubation for 3 months, (a) Stereomicroscope images showing pyramidal, (b) Gold-yellow external hyphae, (c) Scanning electron microscopy images showing the mantle (arrow) and (d) Hartig net (arrow)

viewed under a stereomicroscope. The characteristic features of ectomycorrhizae, the mantle (Fig. 6c) and Hartig net (Fig. 6d)[43] were observed in inoculated roots examined under SEM.

CONCLUSION

The results of this study confirmed the characteristics of isolate Cms01. Traditional and molecular analyses suggest that

this isolate should be classified into the *P. orientalis* group, this study represents a new record of this species from Taiwan. Furthermore, the isolate Cms01 is ectomycorrhizal with *Cyclobalanopsis glauca*.

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