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Pezicula neosporulosa, a fungal endophyte from *Chamaecyparis formosensis* (Cupressaceae) newly recorded in Taiwan

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ABSTRACT

Endophytic fungi are fungi inhabiting in plants without causing any symptoms. This study examined endophytes isolated from a *Chamaecyparis formosensis* tree in Taiwan. From the 38 isolates, twenty-nine isolates belong to Xylariaceae (Sordariomycetes), eight isolates belong to Dermateaceae (Leotiomycetes), and one isolate belongs to Microthyriaceae (Dothideomycetes). The multilocus phylogenetic analyses revealed a newly recorded species of Dermateaceae in Taiwan, *Pezicula neosporulosa*.

Key words: Endophytic fungi, Leotiomycetes, Dermateaceae, *Pezicula*

Introduction

Chamaecyparis formosensis Mats. is an endemic gymnosperm distributed at 1000–2800 m in Taiwan, usually mixing with *C. obtuse* var. *formosana* (Hayata) Rehder. or forming pure forest. Fungal endophytes in this plant have not been studied. Fungal endophytes are fungi inhabiting in plants without causing apparent symptoms; they are likely symbiotic fungi with beneficial roles to plants. Previous studies showed some endophytes can improve plant resistance to pathogens, herbivores, and stress (Arnold et al. 2003; Busby et al. 2015; Clay et al. 1985; Rodriguez et al. 2008). Endophytes are globally distributed and highly diverse, but relatively few species have been identified due to lack of sexual structures in cultures. The identification of the targeted species is required to discover the microbial resources. Using internal transcribed spacer (ITS) sequences can only identified endophytes to the higher taxonomic level. The public sequence database, such as GenBank, contains a lot of endophyte sequences named to class level. Several endophytes have been described as new fungal species in the recent decades (e.g., Peterson et al. 2005; Suwannarach et al. 2010; Chaverri et al. 2011; Sharma et al. 2014; Aime et al. 2018). Endophyte communities within plants might include unknown or cryptic species of fungi. This study examined fungal endophytes

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isolated from *C. formosensis* and identified a newly recorded species, *Pezicula neosporulosa* Y. Zhilin & G.J.M. Verkley.

Materials and Methods

Endophyte isolation

Three randomly selected branches of healthy leaves in ca. 30-cm length of a *C. formosensis* tree were collected at Chinese Hemlock National Trail in Taipingshan (Ilan County, Taiwan). To randomize the isolation, three short branches in ca. 10-cm of each collected branch were cut into ca. 2 mm segments after briefly washed by tap water. The leaf segments from all branches were then pooled together and soaked in 95% ethanol for 10 s, 0.5% NaOCl for 2 min, and 70% ethanol for 2 min. After surface sterilization, 50 leaf segments were randomly selected and placed on a 2% malt extract agar (MEA) slant in individual 1.5 mL microtubes. The 50 MEA slants with leaf segments were then stored in a Ziploc bag and incubated at room temperature for 2 months. Each week, the slants were examined for fungal growth, and a small piece of mycelia was collected for DNA extraction and cultured onto 2% MEA for preservation.

DNA extraction, PCR and sequencing

Genomic DNA was extracted from fungal mycelia using the REDExtract-N-Amp™ Plant Tissue PCR Kit (Sigma-Aldrich, MI, USA) followed the manufacturer's instruction. The genomic DNA was stored at -20°C until PCR amplification. ITS1F/LR3 primers (Gardes and Bruns 1993; Hopple and Vilgalys 1994) were used to amplify the nuclear ribosomal DNA fragment containing the partial small subunit (SSU), ITS1, 5.8S, ITS2, and D1-D2 region of the large subunit (LSU). PCR conditions followed U'Ren et al. (2010). Additional loci of protein coding genes were amplified for multilocus phylogenetic analyses. 983F/2218R (Rehner and Buckley 2005), Bt-Bt-LEV-Up4/Bt-LEV-Lo1 (Jong et al. 2001), and fRPB2-5F/fRPB2-7cR (Liu et al. 1999) primers were used for amplification of elongation factor 1-alpha (*ef1-α*), β-tubulin (*tub*) and the second largest subunit of RNA polymerase II (*rpb2*), respectively. Each 20 μL PCR reaction contained 0.8 μL of each primer 10 μM, 4 μL genomic DNA, 10 μL of REDExtract-N-Amp™ PCR ReadyMix, and 4.4 μL of sterile distilled water. For Bt-LEV-Up4/Bt-LEV-Lo1 primers, PCR protocol was started from 95°C for 2 min and followed by 35 cycles of 95°C for 1 min, 50 to 52°C for 1.5 min, 72°C for 2 min, and then 72°C for 10 min before ended and stored at 4°C. The PCR was run twice, and the PCR product of the first PCR was diluted 4X as the template for the second PCR. Touchdown PCR was necessary for PCR using fRPB2-5F/fRPB2-7cR and 983F/2218R primers. The PCR program started from 94°C for 2 min, followed by 8 cycles of 94°C for 40 sec, 58°C for 40 sec (decrease 1°C per cycle) and 72°C for 2 min, and then 30 cycles of 72°C for 2 min, 51°C for 1.5 min, 72°C for 2 min (increase 1 sec per cycle), and a final step of 72°C for 10 min before ended and stored at 4°C. All PCR were conducted using SimpliAmp™ thermal cycler (Applied Biosystems; MA, USA). To confirm PCR success, PCR products were loaded in a 1 % agarose gel in 1X TAE buffer with 5 % HealthView nucleic acid stain (Genomics, Taipei, Taiwan) and run for 25 minutes at 100V. Gels were visualized using a G:Box Mini with GeneSys software (SYNGENE, MD, USA).

Table 1. *Pezicula* isolates used for phylogenetic analyses in this study. Sequences generated in this study are indicated by asterisk.

Taxon name	*ITS*	*ef1-α*	*rpb2*	*tub*
Cryptosporiopsis actinidiae M79	HM595534	KF376276	KF376216	KF376336
Pezicula acericola CBS239.97	KF376154	KF376223	KF376214	KF376283
Pezicula acericola CBS245.97	KF376153	KF376222	KF376213	KF376282
Pezicula aurantiaca CBS201.46	KF376150	KF376225	KF376210	KF376335
Pezicula carpinea CBS921.96	KF376155	KF376220	KF376159	KF376280
Pezicula carpinea CBS324.97	KF376156	KF376218	KF376160	KF376278
Pezicula carpinea CBS923.96	NR_144927	KF376219	KF376158	KF376279
Pezicula cinnamomea CBS239.96	KF376102	KF376264	KF376165	KF376323
Pezicula cinnamomea CBS240.96	KF376105	KF376265	KF376163	KF376325
Pezicula cinnamomea CBS625.96	KF376104	KF376266	KF376164	KF376322
Pezicula cinnamomea CBS626.96	KF376103	KF376268	KF376162	KF376324
Pezicula corylina CBS249.97	KF376106	KF376221	KF376161	KF376281
Pezicula eucrita CBS259.97	KF376145	KF376271	KF376205	KF376333
Pezicula eucrita CBS262.97	KF376147	KF376272	KF376206	KF376331
Pezicula eucrita CBS325.96	KF376146	KF376270	KF376207	KF376334
Pezicula eucrita CBS656.96	KF376144	KF376269	KF376208	KF376332
Pezicula frangulae CBS100244	KF376152	KF376226	KF376211	KF376285
Pezicula frangulae CBS778.96	KF376151	KF376227	KF376212	KF376286
Pezicula neosporulosa CBS101.96	KF376130	KF376260	KF376193	KF376305
Pezicula neosporulosa CBS102.96	KF376132	KF376237	KF376181	KF376318
Pezicula neosporulosa P15	KF376118	KF376243	KF376195	KF376297
Pezicula neosporulosa P32	KF376113	KF376258	KF376197	KF376287
Pezicula neosporulosa P41	KF376131	KF376251	KF376166	KF376315
Pezicula ocellata CBS949.97	KF376149	KF376224	KF376215	KF376284
Pezicula rubi CBS253.97	KF376100	KF376274	KF376204	KF376329
Pezicula rubi CBS593.96	KF376101	KF376273	KF376203	KF376330
Pezicula sporulosa CBS224.96	KF376108	KF376229	KF376201	KF376326
Pezicula sporulosa CBS225.96	KF376107	KF376228	KF376202	KF376327
18TP088	MT183931	*OP995806	*OP995800	*OP995812
18TP089	MT183932	*OP995807	*OP995801	*OP995813
18TP090	MT183933	*OP995808	*OP995802	*OP995814
18TP099	MT183942	*OP995809	*OP995803	*OP995815
18TP103	MT183946	*OP995810	*OP995804	*OP995817
18TP116	MT183959	*OP995811	*OP995805	*OP995816

For each sample, the PCR product and 1 μL of 10 μM primer were sent to the Mission Biotech Ltd. (Taipei, Taiwan) for Sanger sequencing by ABI 3730 xl DNA Analyzer (Thermo Fisher Scientific Inc; MA, USA). Each isolate was sequenced bidirectionally, and both sequences were assembled with Phred and Phrap in Mesquite version 3.7 (Maddison and Maddison 2021a). Assembled sequences were edited manually using Chromaseq Package version 1.53 (Maddison and Maddison 2021b).

Phylogenetic analyses

The isolates 18TP088, 18TP089, 18TP090, 18TP093, 18TP099 and 18TP116 showed

99.79% sequence identity to both *Pezicula sporulosa* and *P. neosporulosa*. The later species was an endophyte isolated from *Abies* in China (Yuan and Verkley 2015). Five of the isolates (18TP093 was excluded because the ITS sequence is identical to 18TP090) and the isolate 18TP103 (99.36% identical to *P. carpinea*.) were selected for further multilocus phylogenetic analyses.

To confirm the identity of the *Pezicula* isolates, selected isolates with ITS, *ef1-α*, *rpb2* and *tub* sequences from Yuan and Verkley (2015) were

Table 2. BLAST results of endophytes isolated from *Chamaecyparis formosensis*. *Pezicula* isolates are shown in boldface.

Isolate No.	Top-named hit	Query Cover	E value	Per. Ident
18TP087	*Nemania diffusa* isolate PH18	100%	0	100.00%
18TP088	***Pezicula sporulosa* strain CBS 634.96**	**100%**	**0**	**99.79%**
18TP089	***Pezicula sporulosa* strain CBS 634.96**	**100%**	**0**	**99.79%**
18TP090	***Pezicula sporulosa* strain CBS 634.96**	**100%**	**0**	**99.79%**
18TP091	*Xylaria cubensis* isolate NC1046	100%	0	97.98%
18TP092	*Xylaria cubensis* isolate NC1217	100%	0	98.18%
18TP093	***Pezicula sporulosa* strain CBS 634.96**	**100%**	**0**	**99.79%**
18TP094	*Xylaria cubensis* isolate NC1217	100%	0	98.18%
18TP095	*Neofabraea inaequalis* CBS 326.75	100%	0	99.36%
18TP096	*Xylaria cubensis* isolate NC1217	100%	0	98.18%
18TP097	*Xylaria cubensis* isolate NC1217	100%	0	98.18%
18TP098	*Xylaria cubensis* isolate NC1217	100%	0	98.18%
18TP099	***Pezicula sporulosa* strain CBS 634.96**	**100%**	**0**	**99.79%**
18TP100	*Paramicrothyrium chinensis* isolate IA20	95%	0	95.12%
18TP101	*Xylaria cubensis* isolate NC1217	100%	0	98.18%
18TP102	*Xylaria cubensis* isolate NC1217	100%	0	98.18%
18TP103	***Pezicula carpinea* GB4547**	**100%**	**0**	**99.36%**
18TP104	*Xylaria cubensis* isolate NC1217	100%	0	98.18%
18TP105	*Xylaria cubensis* isolate NC1046	100%	0	97.98%
18TP106	*Nemania* sp. voucher KoLRI_EL005386	100%	0	99.79%
18TP107	*Xylaria curta* strain SGLMf32	100%	0	100.00%
18TP108	*Nemania yunnanensis* KUMCC 20-0267	100%	0	99.01%
18TP109	*Xylaria cubensis* isolate NC1217	100%	0	98.18%
18TP110	*Xylaria anisopleura* strain FS11	100%	0	96.98%
18TP111	*Nemania diffusa* isolate KoRLI047373	100%	0	99.80%
18TP112	*Xylaria cubensis* isolate NC1217	100%	0	98.18%
18TP113	*Xylaria curta* strain SGLMf32	100%	0	100.00%
18TP114	*Xylaria cubensis* isolate NC1217	100%	0	98.18%
18TP115	*Xylaria cubensis* isolate NC1217	100%	0	98.18%
18TP116	***Pezicula sporulosa* strain CBS 634.96**	**100%**	**0**	**99.79%**
18TP117	*Xylaria cubensis* isolate NC1046	100%	0	97.98%
18TP118	*Xylaria cubensis* isolate NC1110	100%	0	99.60%
18TP119	*Xylaria cubensis* isolate NC1046	100%	0	97.98%
18TP120	*Xylaria cubensis* isolate NC1046	100%	0	97.98%
18TP121	*Xylaria cubensis* isolate PH79	100%	0	100.00%
18TP122	*Xylaria cubensis* isolate NC1046	100%	0	97.78%
18TP123	*Xylaria cubensis* isolate NC1046	100%	0	97.98%
18TP124	*Xylaria cubensis* isolate NC1046	100%	0	97.77%

used in the phylogenetic analyses (Table 1). All sequences of each locus were individually aligned using MUSCLE (Edgar 2004) on EMBL-EBI (Madeira et al. 2022). The phylogenetic tree of each locus was inferred by maximum likelihood method using RAxML (Stamatakis 2014) on CIPRES Science Gateway (Miller et al. 2010). After removing the conflict taxa, the concatenated alignment was analyzed by PartitionFinder2 (Lanfear et al. 2017) to determine the best-fit partitioning scheme for phylogenetic analyses. A final multilocus phylogenetic tree was inferred with GTR+I+G model by RAxML with 1000 bootstrap replicates.

Morphology

Colony morphology was examined and compared on 2% MEA, potato dextrose agar (PDA) and oatmeal agar (OA). The fungi were cultured on 2% MEA for 7 days, and a 4-mm plug of the mycelia was transferred to a 9-cm Petri dish containing the selected medium. Cultures were incubated in dark at 25 °C. The colony morphology was examined and recorded by photograph on day 7, 14, 21 and 28 after transferring to the selected medium. Hypha and conidium morphology were examined by light microscopy.

Results

Thirty-eight fungal endophytes were isolated from the 50 MEA slants. The isolation frequency was 76%. The ITS1-5.8S-ITS2 sequence of each isolate was detected by ITSx 1.1 (Bengtsson-Palme et al. 2013), and the extracted sequences were searched in the GenBank database by Basic Local Alignment Search Tool (BLAST) with blastn suite. The results of BLAST were listed in Table 2. Twenty-nine isolates belonged to Xylar-

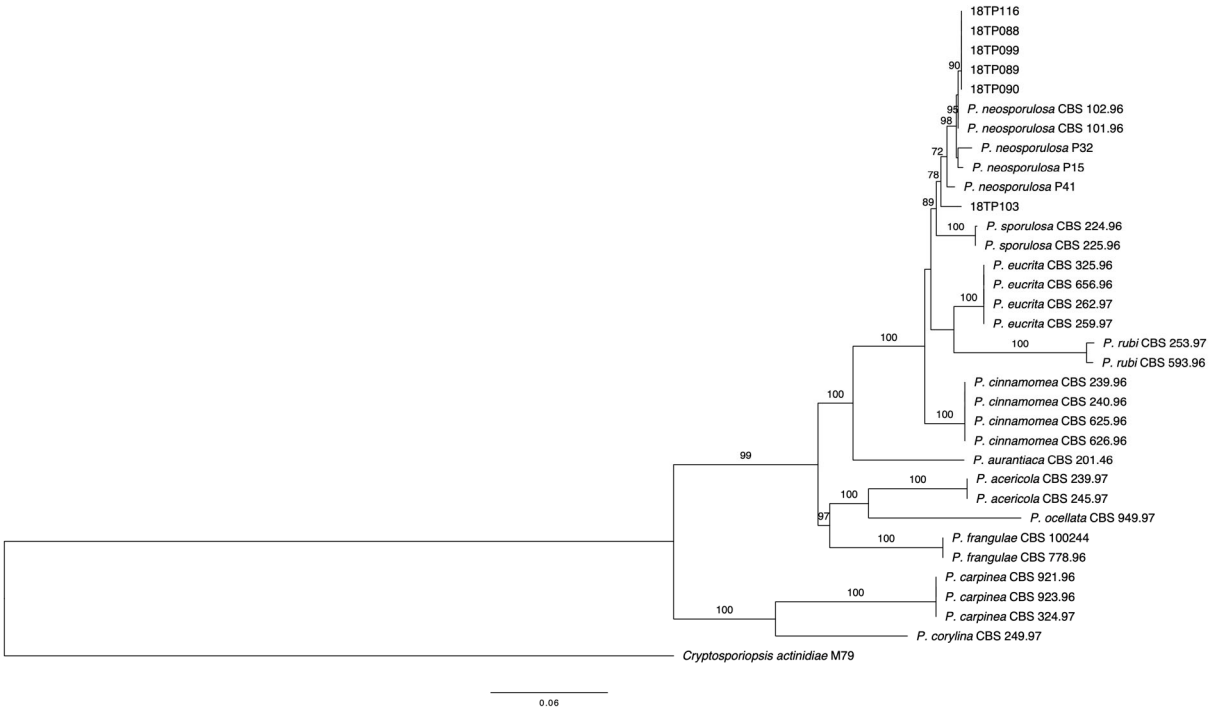

Fig. 1. Maximum likelihood phylogenetic tree of *Pezicula* isolates. The tree is concatenated of *ITS*, *ef1-α*, *rpb2* and *tub* genes. The best tree is inferred by GTR+I+G model using RAxML with 1000 bootstrap replicates. Branches are proportional to the branch length. Bootstrap support values greater than 70 are shown on the branches.

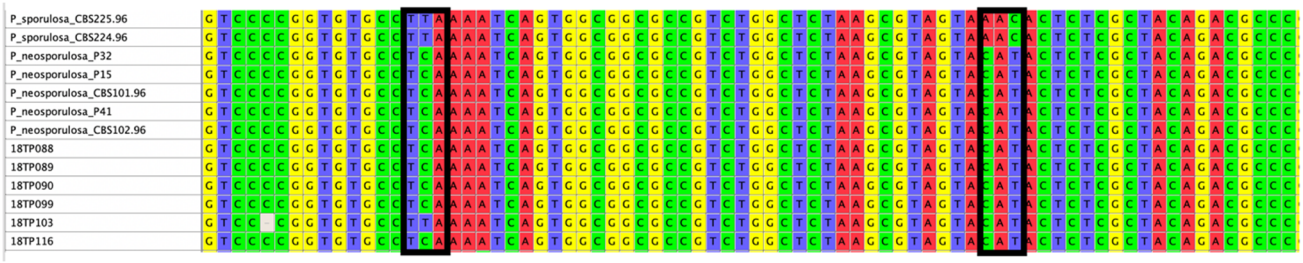

Fig. 2. Sequence alignments of ITS2 at position no.40–42 (left square) and no. 80–82 (right square) are different between *Pezicula sporulosa* and *P. neosporulosa*. The *Pezicula* endophytes isolated from this study have identical nucleotides at both positions to *P. neosporulosa* except the isolate 18TP103.

iaceae (Sordariomycetes), including *Xylaria cubensis*, *X. curta*, *Nemania diffusa*, etc.; eight isolates belonged to Dermateaceae (Leotiomycetes); and one isolate belonged to Microthyriaceae (Dothideomycetes).

The four-gene concatenated maximum likelihood phylogenetic tree of *Pezicula* isolates was shown in Fig. 1. The six endophytes isolated from this study were in the *P. neosporulosa* clade which was sister to *P. sporulosa*. The isolate 18TP103 formed an individual clade sister to the other *P. neosporulosa*, and the other 5 isolates were clustered into a single clade with other *P. neosporulosa*. *P. sporulosa* and *P. neosporulosa* are distinguishable by the no. 40–42 (TTA/TCA) and no. 80–82 (AAC/CAT) nucleotides of ITS2 locus (Yuan and Verkley 2015). The ITS2 sequences of the isolates 18TP088, 18TP089, 18TP088, 18TP090, and 18TP116 are identical to *P. neosporulosa* at both positions (Fig. 2). The nucleotides of isolate 18TP103 were identical to *P. sporulosa* at the position no. 40–42 but were identical to *P. neosporulosa* at the position no. 80–82. The results revealed the isolates 18TP088, 18TP089, 18TP088, 18TP090, and 18TP116 are *P. neosporulosa*.

The colony morphology of the 6 isolates clustered with *P. neosporulosa* on MEA, PDA and OA were shown in Fig. 3. Although the morphology was variable among the 6 isolates, the growth rate of isolate 18TP103 on PDA was apparently faster than the other isolates, which was in consistent with the phylogenetic analyses. Additionally, the five *P. neosporulosa* isolates did not sporulate in cultures, but the isolate 18TP103 produced conidia on PDA and OA.

Taxonomy

Pezicula neosporulosa Y. Zhilin & G.J.M. Verkley, Mycoscience 56: 206. 2015 **Fig. 3**

Description. No sexual morph developed in culture. See Yuan and Verkley (2015) for the description of sexual morph. Colony morphology varied by isolates. On MEA, colonies off white and flat, and some isolates becoming pale yellow in the center after 14 days. Margins initially entire and turning into undulate to filiform later in some isolates. Mycelium slightly woolly in center after 14 days. The colony diameter reaching 45–54 mm on day 21. On PDA, colonies flat to raised, and colors varied by isolates from white, pale yellow, to light salmon pink. Rings with

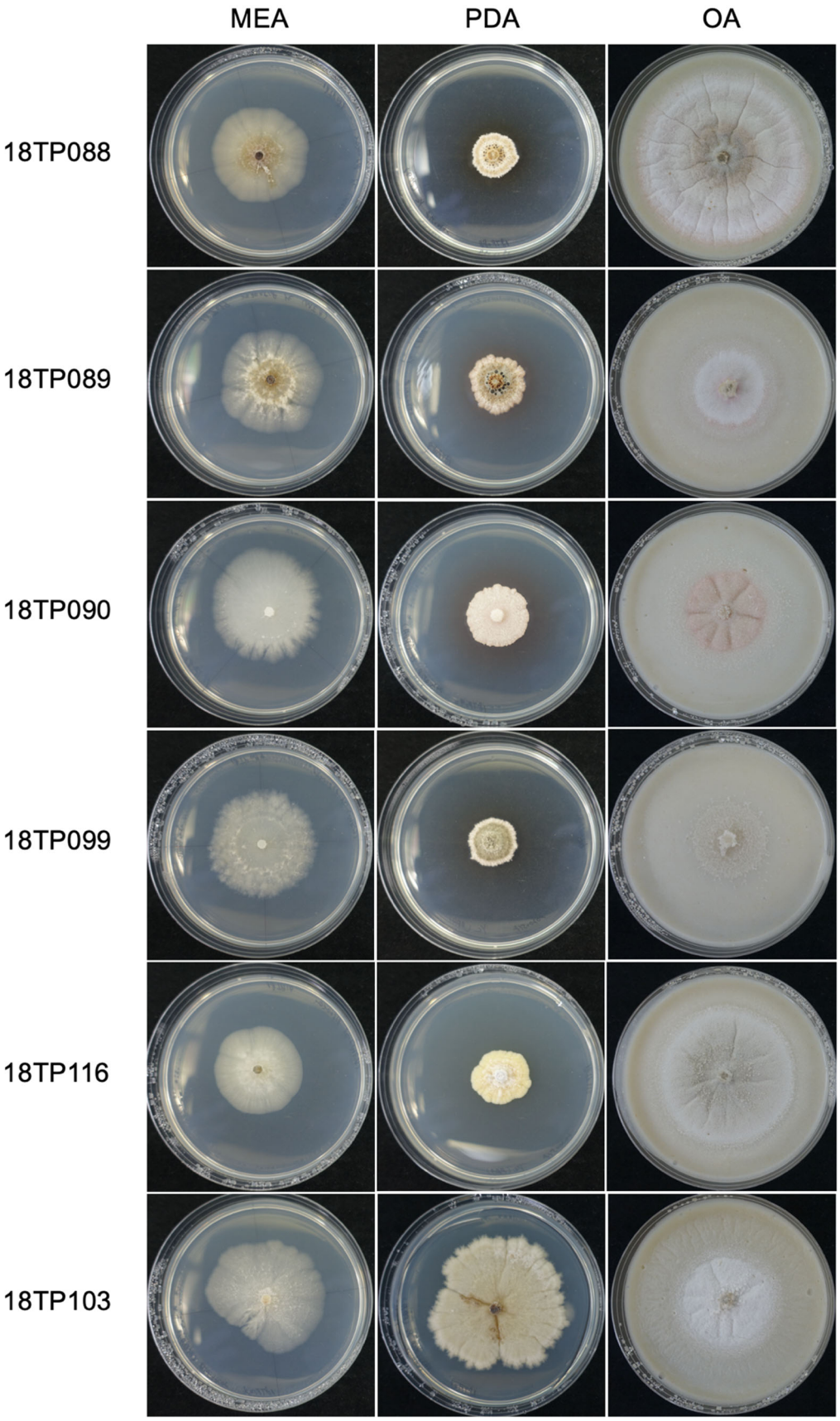

Fig. 3. Culture morphology of *Pezicula* isolates on MEA, PDA and OA. All colonies are incubated at 25°C for 21 days.

dark colors appearing on colonies after 7 days. Margins undulate, mycelium floccose. Brown water droplets produced at center after 14 days in some isolates. The colony diameter reaching 24–30 mm on day 21. On OA, colonies initially flat to granular, and cottony to woolly at the center. Some isolates becoming rugose after day 14. Colony colors white, and varied at the center, including white, pale yellow, gray, and pink in different isolates. Colored rings or bands formed after day 14. The colony diameter reaching 66–80 mm on day 21. Endophytic isolates did not sporulate in cultures.

Ecology. Endophytes of *Chamaecyparis formosensis* (this study), *Abies beshanzuensis* (Yuan and Verkley 2015) and *Pseudowintera colorata* (Purushotham et al. 2021). Apothecia on bark of *Abies alba* (Yuan and Verkley 2015). In soil (Das et al. 2020).

Distribution. Taiwan, Korea, China, New Zealand, and Netherlands.

Materials examined. TAIWAN. Taipinshan, Ilan County, isolated from leaves of *Chamaecyparis formosensis*, April 25, 2018, Y.-L. Huang. Living cultures (18TP088, 18TP089, 18TP090, 18TP099, 18TP116) deposited at the National Museum of Natural Science.

Discussion

Fungal endophytes of *Chamaecyparis formosensis* include Xylariaceae, Dermateaceae and Microthyriaceae species. Species of Xylariaceae are frequently isolated as endophytes (Petrini and Petrini 1985, Rajulu et al. 2013). The most dominant species, *Xylaria cubensis*, is a widely distributed fungus of the world and is usually found producing stromata on decaying wood. It has been isolated as endophytes from many plants including *Chamaecyparis thyoides* in North America (Bills and Polishook 1992). Other xylariaceous endophytes isolated from this study such as *X. curta* and *Nemania diffusa* are also common endophytes found in tropical Asia (Rajulu et al 2013, Okane et al. 2012).

The genus *Pezicula* is currently associated with 143 species epithets in the MycoBank database and 165 in Index Fungorum. Two species have been reported in Taiwan: *P. subcarnes* was found on bark of *Dendropanax pelucidopuncata* (Liou and Chen 1977), and *P. ericae* was isolated from *Pinus taiwanensis* as a mycorrhizal fungus (Song et al. 2020). *Pezicula neosporulosa* isolated from this study is a newly recorded species in Taiwan.

The culture morphology on PDA of *P. neosporulosa* is highly divergent (Yuan and Verkley 2015), and the current study also shows the variations of cultural characteristics among isolates (Fig. 3). Previous studies on *P. neosporulosa* isolated from soil produce conidiomata on OA (Das et al. 2020), and the in vitro cultures from the ascospores also produce conidia on OA (Yuan and Verkley 2015), but neither the sexual structures nor the conidiomata are developed in culture in this study. The identification based on the morphology of the endophytic isolates is difficult. In addition, the BLAST search shows that the ITS sequences of the isolates are 99.79% identical to both *P. neosporulosa* and *P. sporulosa*, so the identity of the isolates cannot be determined by the BLAST search. However, the multilocus phylogenetic analysis reveals the five isolates are *P. neosporulosa* with a strong support.

The culture morphology of the isolate 18TP103 is apparently different from the other isolates. The isolate 18TP103 produces conidia on PDA and OA, and the colony growth on PDA is much faster than the other isolates. The BLAST result indicates that the ITS sequence of 18TP103 is 99.36% identical to *P. carpinea*, but our phylogenetic analyses show a distant relationship between 18TP103 and *P. carpinea.* The phylogenetic tree shows that 18TP103 is sister to the *P. neosporulosa* clade but is not sister to the *P. sporulosa* clade. Moreover, the ITS2 sequence of 18TP103 does not have identical nucleotides at the positions which was used to distinguish the two species. Therefore, the isolate 18TP103 remains for further studies to confirm the identity.

Acknowledgements

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無柄盤菌屬植物內生真菌 *Pezicula neosporulosa*：分離自紅檜之臺灣新紀錄種

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摘　　要

植物內生真菌係生長於植物體中卻不會對植物體造成病害的真菌，本研究檢視紅檜之植物內生真菌，在分離出的 38 株菌株中，有 29 株屬於炭角菌科，8 株屬於皮盤菌科，1 株屬於小盾殼科。透過多基因座分子系統樹分析發現一臺灣新紀錄種，為無柄盤屬之 *Pezicula neosporulosa*。

關鍵詞：植物內生真菌、錘舌菌綱、皮盤菌科、無柄盤屬