

New Records of Endophytic Fungi Associated with the Araucariaceae in Taiwan

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Abstract. *Araucaria cunninghamii* and *A. heterophylla* are popular ornamental trees in Taiwan, and *Wollemia nobilis* was introduced to Taiwan in 2005. Branches of these araucariaceous plants which had died back were sampled for botryosphaeriaceous pathogens, and isolates were identified using morphological and molecular taxonomic techniques. We found that fungi associated with these araucariaceous hosts included *Botryosphaeria dothidea*, *Lasiodiplodia theobromae*, *Neofusicoccum mangiferae*, and *N. parvum*. They were distributed in wider phylogenetic clades than in previous reports, and did not reveal host specificity. We suggest that this phenomenon might be associated with a regional pathogen pool which caused this unique host pattern in Taiwan.

Keywords: Araucariaceae, Botryosphaeriaceae, latent pathogen, new record.

INTRODUCTION

The Araucariaceae with 41 extant species is divided into three genera: *Agathis*, *Araucaria*, and *Wollemia*. They are limited to the southern hemisphere with unique biogeography patterns (Kershaw and Wagstaff, 2001). Several are very popular ornamental trees at gardens in subtropical regions, including *Araucaria cunninghamii* and *A. heterophylla*. *Wollemia nobilis* is the single species in the genus *Wollemia*. It was first discovered in 1994 in a remote area of the Wollemi National Park, 150 km northwest of Sydney, Australia (Jones *et al.*, 1995). This species is highly endangered in its natural environment with only 100 adult trees in three sites. Since the introduction of common pathogens into a population with low genetic diversity could be destructive if the trees are susceptible to them, examination of those pathogens is significant for their conservation (Bullock *et al.*, 2000).

The Botryosphaeriaceae is a family of common endophytes of a wide variety of

woody plants worldwide. Some members of the Botryosphaeriaceae are latent pathogens. Following physical damage or stress to a host, they often become aggressive and kill large parts of the host (Slippers and Wingfield, 2007). Based on the molecular phylogeny, they are distinguished into seven genera: *Bortyosphaeria*, *Diplodia*, *Dothiorella*, *Lasiodiplodia*, *Neofusicoccum*, *Neoscytalidium*, and *Pseudofusicoccum* (De Wet *et al.*, 2008). Host-generalist and -specialist species were observed in all clades of the Botryosphaeriaceae, and there is little evidence of host-associated coevolution. Ancestors of the Botryosphaeriaceae most likely evolved on angiosperms, then independently shifted to gymnosperms in different clades (De Wet *et al.*, 2008). Four of seven lineages occur on both angiosperms and gymnosperms. *Diplodia* and *Lasiodiplodia* are sister clades. *Diplodia* species are predominantly associated with gymnosperms, with a few species on both gymnosperms and angiosperms. In contrast, *Lasiodiplodia*, *Neofusicoccum*, and *Bortyosphaeria* species occur mainly on angiosperms, and only occasionally on gymnosperms (De Wet *et al.*, 2008). Characteristics of a wide host range, a latent

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phase which can be neglected by quarantine, and their ability to rapidly cause disease when their hosts are under stress make these fungi a significant threat to agricultural and native forest ecosystems (Slippers and Wingfield, 2007).

In this study, materials were sampled from araucariaceous plants which were in a declining state. Fungi were isolated and identified using morphological and molecular taxonomic techniques. We found that isolates were distributed in wider phylogenetic clades than in previous reports, and host specificity was not revealed. We suggest that this might be associated with the regional pathogen pool which caused this unique host pattern in Taiwan.

MATERIALS AND METHODS

Fungal isolation and morphological characterization

Samples were collected from declining and cankered trees from various regions and hosts (Table 1). Fungal isolations were made on plates of malt extract agar (MEA; Difco, Kansas City, MO, USA) and potato dextrose agar (PDA; Difco) from the discolored pith of branches (Fig. 1). The plates were left on a bench at room temperature. The isolates were induced to sporulate on water agar amended with pine needles as substratum and observed under a dissecting microscope. Measurements were made with material mounted in distilled water. Morphological characteristics (Crous *et al.*, 2006) were examined and photographed by stereomicroscopy (Leica Wild M10, Wetzlar, Germany) and light microscopy (Zeiss Axioskop, Jena, Germany). Specimens and cultures were deposited in the herbarium of the National Museum of Natural Science (TNM), Taichung, Taiwan.

Molecular identification and phylogenetic analysis

DNA was extracted using a Qiagen DNA mini kit (Valencia City, CA, USA) following instructions in the manual. Regions 1 and 2 of the internally transcribed spacer (ITS) and the 5.8S ribosomal subunit were amplified by universal primers (ITS1/ITS4) (White *et al.*, 1990). Polymerase chain reaction (PCR) products were visualized on a 1% agarose gel containing Cyber Green using UV illumination. The PCR

products were sequenced using an ABI PRISM 3730 DNA sequencer (Applied Biosystems, Foster City, CA, USA). Other sequence data for various Botryosphaeriaceae species not generated in this study were obtained from GenBank. The GenBank accession nos. in this study were JN688952~N688956 (Table 1).

A maximum-likelihood (ML) tree was created in PAUP (Phylogenetic Analysis Using Parsimony) vers. 4.0b10 (Swofford, 2003) using the parameters determined with jModelTest (Posada, 2008). Bootstrap support was determined after 1000 replications, and only groups with frequencies of > 60% were retained.

Pathogenicity tests

Because seedlings of *W. nobilis* were not available, 6-month-old seedlings of *Araucaria heterophylla* and *A. cunninghamii* were used to determine the relative pathogenicity of the fungi isolated from *W. nobilis*. Five seedlings were wounded on the stem with a 3-mm cork borer to a depth of 2 mm. Inoculation consisted of inserting 3-mm mycelium plugs into the wounds and wrapping them with parafilm. Sterile MEA plugs applied to an equal number of seedlings with the same method served as the controls (Dakin *et al.*, 2010).

RESULTS

Molecular identification and phylogenetic analysis

A collection of Botryosphaeriaceae isolates from diverse hosts were included in this dataset to determine their identity, and to derive information regarding their specificity. In this study, the five isolates were distributed in three clades, and the phylogenetic pattern did not reveal host specificity. The isolate found in *W. nobilis* was in the *Neofusicoccum ribis/N. parvum* complex of the *Neofusicoccum* clade, and the ITS sequence was identical to that of *N. parvum* (Fig. 2, Table 1). Isolates found in *A. heterophylla* were in the *Lasiodiplodia* and *Neofusicoccum* clades, and the ITS sequences were identical to that of *L. theobromae* and highly similar to that of *N. mangiferae*, respectively (Fig. 2, Table 1). Isolates found in *A. cunninghamii* were in the *Lasiodiplodia* and *Botryosphaeria* clades, and the ITS sequence were identical to those of *L. theobromae* and *B. dothidea*, respectively (Fig.

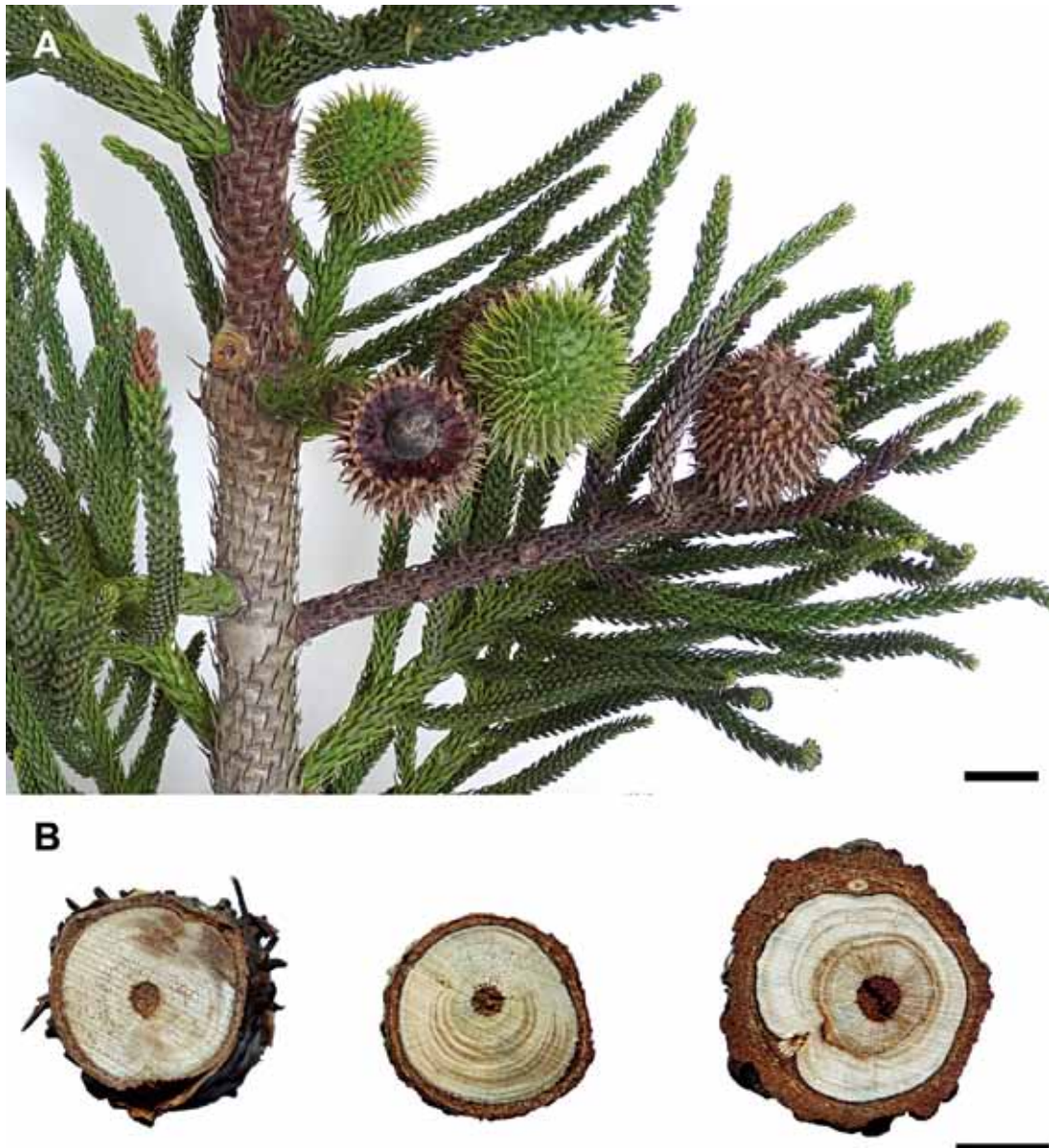


Fig. 1. (A) Dieback associated with *Lasiodiplodia theobromae* on *Araucaria cunninghamii*. (B) Stem cross-section showing internal wood decay and black discoloration of the pith material of *A. cunninghamii* (left), *A. heterophylla* (middle), and *Wollemia nobilis* (right). Bars = 2 cm in A and 1 cm in B.

2, Table 1). In previous studies, isolates occurred on Araucariaceae plants only located in the *Neofusicoccum* clade (Fig. 2). Isolates collected in this study further extended to the *Lasiodiplodia* and *Botryosphaeria* clades. *Neofusicoccum mangiferae* was also the first record of occurrence on a gymnosperm. *Botryosphaeria dothidea*, *L. theobromae*, *N. mangiferae*, and *N. parvum* are all generalists that could occur on a wide range of hosts (Table 1) (De Wet *et al.*, 2008).

Fungal isolation and morphological characterization

Four botryosphaeriaceous fungi were identified in this study. No teleomorph was induced on the substratum. The morphological characteristics in culture of these species are described here.

Botryosphaeria dothidea (Moug.: Fr.) Ces. & De Not., *Comm. Soc. Crit. It.* 1: 215. 1863.
Anamorph: *Fusicoccum aesculi* Corda, in Stürm, *Deutschlands Flora*, 2: 111. 1829.

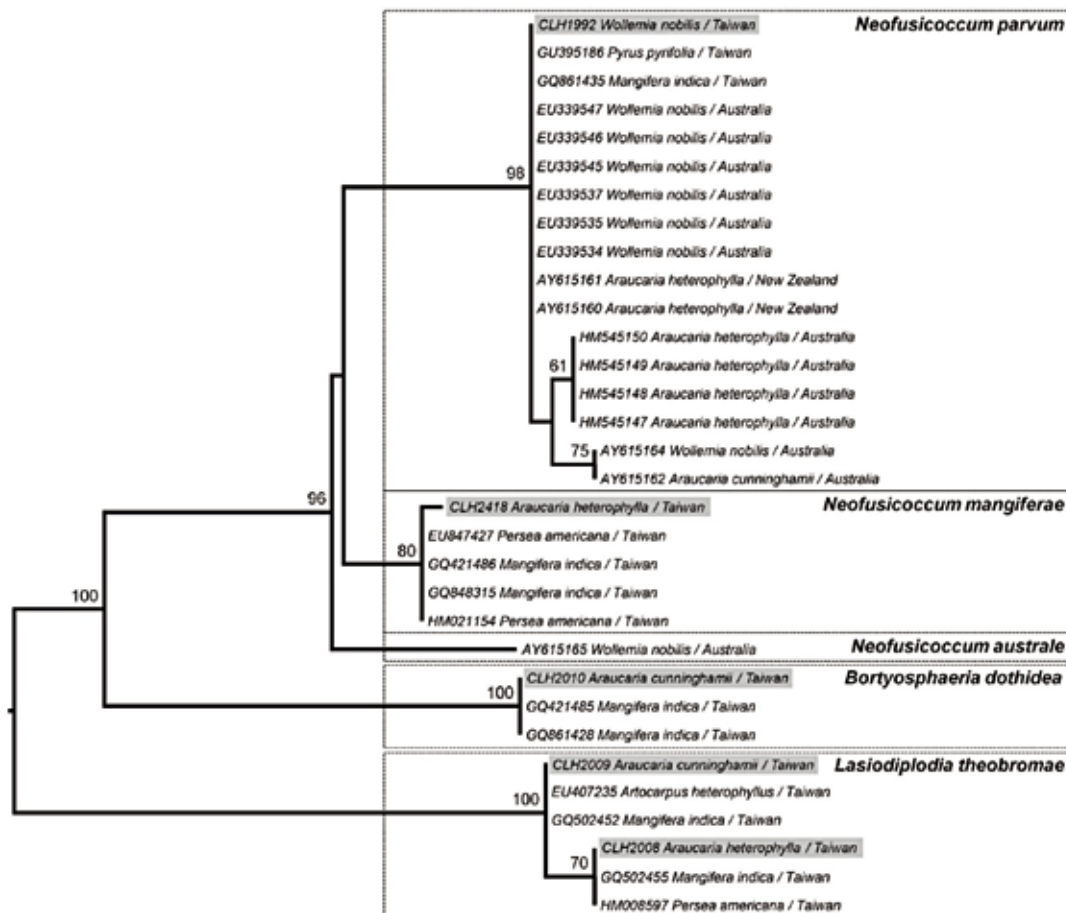


Fig. 2. Phylogram constructed for the internally transcribed spacer and 5.8S rDNA based on a maximum-likelihood analysis with bootstrap values. Only groups with bootstrap values of > 60% were retained. Isolates found on the Araucariaceae and other isolates found in Taiwan with the most similar sequences counterparts in this study are included. The isolates, hosts, and locations in this study are marked in gray.

Colonies on MEA rapidly spread, reaching 7-7.5 cm in diam. in 7 days at room temperature. Mycelium white at first, turning grayish-white then blackish-brown, floccose, forming scattered, rope-like hyphal strands. Pycnidia superficial, blackish-brown, globose to pyriform, ostiolate, 200-500 μm wide, covered with yellow-olivaceous hyphae. Conidia hyaline, fusiform with a flat base, smooth, 24-32 x 5-7 μm (L/B = 4.6), not becoming septate with age.

Specimen examined. Taichung, National Museum of Natural Science, on *A. cunninghamii*, coll. C. L. Huang (CLH2010), Apr. 18, 2011 (TNM F24923).

The most distinctive feature of this taxon is its conidia, which are large, unicellular, hyaline, and narrowly fusiform. In Taiwan, this species was reported as a causal agent of various fruit

diseases (Ni *et al.*, 2010; Ko *et al.*, 2011); this is the first report of *B. dothidea* associated with *A. cunninghamii*.

Lasiodiplodia theobromae (Pat.) Griff. & Maubl., Bull. trimest. Soc. Mycol. Fr. 25: 57. 1909. Teleomorph: *Botryosphaeria rhodina* (Berk. & M.A. Curtis) Arx, Gen. Fungi Sporulating in Culture (Lehr.): 143. 1970.

Colonies on MEA rapidly spread, covering entire Petri dish in 1 week at room temperature. Mycelium grayish at first, turning black and fluffy. Pycnidia superficial, pyriform, setose, papillate, 0.2-0.5 mm wide, blackish-brown. Conidia unicellular, hyaline, granulose, ellipsoid, 22-27 x 12-15 μm (L/B = 1.8), mature conidia 1-septate, pale-brown, longitudinally striate.

Specimens examined. Tainan, Beiho, on *A. heterophylla* coll. C. L. Huang (CLH2008), Apr. 12, 2011 (TNM F24921). Tainan, Beiho, on *A. cunninghamii*. coll. C. L. Huang (CLH2009), Apr. 12, 2011 (TNM F24922).

This species was reported to cause stem-end rot of mango, papaya, and banana in Taiwan (Ni *et al.*, 2008). *Araucaria cunninghamii* and *A. heterophylla* are new hosts of *L. theobromae*.

Neofusicoccum mangiferae (Syd. & P. Syd.) Crous, Slippers & A. J. L. Phillips, Stud. Mycol. 55: 248. 2006.

Colonies on MEA rapidly spread, covering entire Petri dish in 7 days at room temperature. Mycelium grayish-white at first, turning olivaceous-brown, floccose, forming scattered, grayish rope-like strands. Pycnidia superficial, globose to pyriform, ostiolate, 120-200 µm wide, sometimes aggregated up to 1 mm wide, blackish-brown. Conidia hyaline, broadly ellipsoid with a flat base, smooth, 12-15 x 7-8 µm (L/B = 1.75).

Specimen examined. Taipei City, on *A. heterophylla*, coll. C. L. Huang (CLH2418), June 6, 2011 (TNM F24924).

This species was reported to cause fruit rot of avocado and mango in Taiwan (Ni *et al.*, 2009, 2010). This isolate differs from that described by Slippers *et al.* (2005) by the broader conidia (12-15 x 7-8 µm vs. 12-14 x 4-6 µm) and lacking vegetative toruloid cells.

Neofusicoccum parvum (Pennycook & Samuels) Crous, Slippers & A. J. L. Phillips, Stud. Mycol. 55: 248. 2006.

Teleomorph: *Botryosphaeria parva* Pennycook & Samuels Mycotaxon 24: 455. 1985.

Colonies on MEA rapidly spread, covering entire Petri dish in 5 days at room temperature. Mycelium white at first, turning grayish-brown, floccose, forming scattered, grayish rope-like strands, reaching lid of Petri dish. Pycnidia superficial, globose to pyriform, ostiolate, 75-150 µm wide, sometimes aggregated up to 1 mm wide, blackish-brown. Spermatia abundant, ellipsoid to short-cylindrical, smooth, hyaline, 4-10 x 2-2.5 µm, occasionally 1-septate. Conidia hyaline to light-brown; ellipsoid with a flat base, smooth, 10-17.5 x 5-6 µm (L/B = 2.5), becoming 1-septate with age.

Specimens examined. Taichung, Lishan, on twig of *Pyrus bretschneideri*, coll. Y. M. Shen, (SYM 00020), Sept. 13, 2010 (TNM F24062). Taichung, National Museum of Natural Science, on *W. nobilis*, coll. C. L. Huang (CLH1992), Nov. 11, 2010 (TNM F24920).

This species was first reported from New Zealand as *Fusicoccum parvum* (Pennycook and Samuels, 1985) and was later assigned to *Neofusicoccum* by Crous *et al.* (2006). It was recently reported to cause stem canker and dieback of Asian pear trees in Taiwan (Shen *et al.*, 2010).

Pathogenicity tests

After 2 months of incubation, one of the tested *A. cunninghamii* seedlings exhibited cankers and dieback, and discolored pith was found from a section of its stem. The other inoculated seedlings exhibited dark lesions around the wounded area, while the controls remained symptomless. The *N. parvum* pathogen was reisolated from the discolored pith of inoculated stems, thus fulfilling Koch's postulate.

DISCUSSION

The *Neofusicoccum* clade includes two major species complexes: *N. ribis/N. parvum* and *N. luteum/N. australe* that occur on a wide variety of angiosperm and gymnosperm hosts (De Wet *et al.*, 2008). Isolates associated with araucariaceous plants were only reported from these two species complexes (Fig. 2). The pathogenicity tests of *N. parvum* showed characteristics of a latent pathogen. It only became aggressive when a host became weak. The *N. mangiferae* isolate was first reported from *A. heterophylla* in this study. It has a single-nucleotide polymorphism (SNP) and broader conidia that significantly differ from the most similar isolates (Figs. 2, 3). These genetic and morphological variations might be unique to gymnosperm hosts. More samples are needed to confirm this result.

The *Botryosphaeria* clade included two species. One of which was *B. dothidea* that occurs on a wide variety of angiosperms and occasionally on gymnosperms (De Wet *et al.*, 2008). In the *Lasiodiplodia* clade, isolates of *L. theobromae* were all grouped together, and they originated from a wide variety of hosts including both angiosperms and gymnosperms (De Wet *et*

al., 2008). All four species found in this study are host generalists, and the same species with identical or almost-identical sequences have been found on various fruit trees in Taiwan since 2008 (Table 1). Infection of the Araucariaceae most likely recently occurred in different groups via a host shift, as there appears to be little evidence for host-associated coevolution among species of the Botryosphaeriaceae. Understanding these patterns of host shift is important, because they can often lead to disease or epidemic outbreaks (Slippers and Wingfield, 2007).

After a host is infected by Botryosphaeriaceae pathogens, stress or non-optimal growth conditions of trees can further induce serious disease expression (Stanosz *et al.*, 2001). Stress conditions include drought stress (most commonly), extensive physical damage by hail or pathogens, frost or heavy snow, or planting on unsuitable sites (elevation, soil type, temperature, etc.). In Taiwan, *W. nobilis* was first introduced

to the Botanical Garden, National Museum of Natural Science (Taichung, Taiwan) in 2005. In addition to the purposes of exhibit and education, the garden is an *ex situ* conservation site for this species in a subtropical area. Once a species is introduced to a new place, it will be challenged by new environmental conditions and pathogens. One of the Wollemi pines died after being transplanted at our museum in 2010, and *N. parvum* was isolated from a stem that had died back. Lethal disease expression might have further arisen by the stress of the transplantation process. Being a host generalist as the Botryosphaeriaceae means that its members can cross-infect native and introduced hosts, moving in both directions causing infections and disease (Parker and Gilbert, 2004; Slippers and Wingfield, 2007). The monitoring and control of these pathogens should be a new task for agricultural, plantation, and botanical garden management in the future.

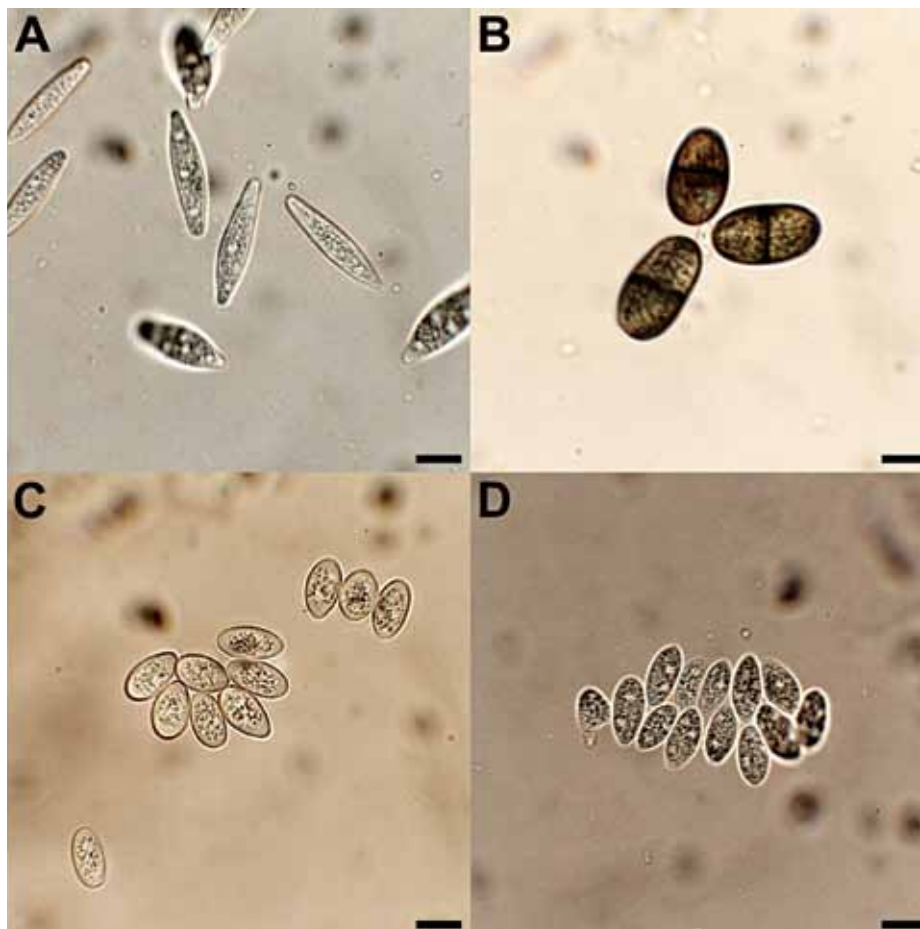


Fig. 3. Conidia of (A) *Botryosphaeria dothidea*, (B) *Lasiodiplodia theobromae*, (C) *Neofusicoccum mangiferae*, and (D) *N. parvum*. Bars = 10 µm.

Table 1. List of Botryosphaeriaceae isolates included in this study and some of most similar internally transcribed spacer counterparts in GenBank. Samples in this study are indicated by a gray background.

Species	Isolate	Host	Location	GenBank
<i>Botryosphaeria dothidea</i>	CLH2010	<i>Araucaria cunninghamii</i>	Taiwan	JN688955
		<i>Mangifera indica</i> (mango)	Taiwan	GQ421485
		<i>Mangifera indica</i> (mango)	Taiwan	GQ861428
		<i>Sophora japonica</i>	China	AB369469
		<i>Prunus persica</i> (peach)	China	HQ660456
		<i>Olea europaea</i> (olive)	Greece	AY640254
		<i>Olea europaea</i> (olive)	Italy	EF638749
		<i>Quercus phellos</i>	USA: North Carolina	GQ996070
<i>Lasiodiplodia theobromae</i>	CLH2008	<i>Araucaria heterophylla</i>	Taiwan	JN688953
		<i>Mangifera indica</i> (mango)	Taiwan	GQ502455
		<i>Persea americana</i> (avocado)	Taiwan	HM008597
		<i>Arachis hypogaea</i> (peanut)	China	FJ594747
		<i>Eucalyptus</i> sp.	China	HQ332191
		<i>Gmelina arborea</i>	Costa Rica	EF622077
		<i>Costus villosissimus</i>	Ecuador	HM855219
		<i>Grevillea robusta</i>	Kenya	FJ904834
<i>L. theobromae</i>	CLH2009	<i>Araucaria cunninghamii</i>	Taiwan	JN688954
		<i>Mangifera indica</i> (mango)	Taiwan	GQ502452
		<i>Artocarpus heterophyllus</i> (jackfruit)	Taiwan	EU407235
		<i>Eucalyptus</i> sp.	China	HQ332193
		<i>Gossypium</i> sp. (cotton)	India	DQ212767
		<i>Anacardium excelsum</i>	Panama	EF423533
		<i>Mangifera indica</i> (mango)	Brazil	EU915208
		<i>Leucospermum</i> sp.	USA: Hawaii	FJ150695
		<i>Cecropia insignis</i>	Panama	FJ612603
		<i>Pterocarpus angolensis</i>	South Africa	FJ888468
		<i>Eucalyptus</i> sp.	Kenya	FJ904840
<i>Neofusicoccum mangiferae</i>	CLH2418	<i>Araucaria heterophylla</i>	Taiwan	JN688956
		<i>Mangifera indica</i> (mango)	Taiwan	GQ421486
		<i>Mangifera indica</i> (mango)	Taiwan	GQ848315
		<i>Persea americana</i> (avocado)	Taiwan	EU847427
		<i>Persea americana</i> (avocado)	Taiwan	HM021154
		<i>Eucalyptus</i> sp.	China	EU675679
		<i>Mangifera indica</i> (mango)	Australia	AY615185
		<i>Syzygium cordatum</i>	South Africa	DQ316081
<i>N. parvum</i>	CLH1992	<i>Wollemia nobilis</i>	Taiwan	JN688952
		<i>Mangifera indica</i> (mango)	Taiwan	GQ861435
		<i>Pyrus pyrifolia</i> (pear)	Taiwan	GU395186
		<i>Punica granatum</i> (pomegranate)	China	GU997685
		<i>Vernicia fordii</i>	China	HQ844226
		<i>Actinidia deliciosa</i> (kiwifruit)	China	JF439214
		<i>Bruguiera sexangula</i> (mangrove)	China	FJ441608
		<i>Wollemia nobilis</i>	Australia	EU339534
		<i>Eucalyptus grandis</i>	Australia	EU339551
		<i>Araucaria heterophylla</i>	New Zealand	AY615160
		<i>Coffea arabica</i>	Colombia	EF672311

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內共生真菌寄生南洋杉科植物的台灣新記錄

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南洋杉科（裸子植物）的瓦勒邁杉於2005年首度引進台灣，肯氏南洋杉及小葉南洋杉亦為主要的庭園景觀樹種。由這些南洋杉科植物枯枝，我們分離出數種潛伏性病原的葡萄座腔菌科真菌，以DNA序列及形態特徵的比對，確認這些菌株為*Botryosphaeria dothidea*，*Lasiodiplodia theobromae*，*Neofusicoccum mangiferae*及*N. parvum*。除了由瓦勒邁杉分離出的*N. parvum*，其它三種菌株為第一次發現於南洋杉科植物的寄生菌，且並未呈現對寄主的專一性。進一步由DNA序列比對資料，發現台灣近年來已有報導完全相同或近乎相同的DNA序列菌株引起許多的果樹（被子植物）病害。我們推測台灣已存在葡萄座腔菌科病原菌庫的散佈，因而造成此由被子植物轉移至裸子植物寄主的現象。

關鍵詞：葡萄座腔菌科，南洋杉科，潛伏性病原菌，新記錄。