

Research paper

## Investigation of *Camellia* Tree Diseases in Taiwan and Establishment of a Rapid Detection Method for *Colletotrichum* spp.

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### [ Summary ]

*Camellia* is commonly used for afforestation and as an important oil-producing crop with high economic value. Diseases that occur on aboveground parts of *Camellia* are considered to be major reasons causing declines in the quality and quantity of tea oil, but little is known about them. In this study, we investigated disease occurrence of *Camellia* in Taiwan and discovered that blister blight, leaf spot, and soft rot were major symptoms on *Camellia* farmlands in the north, while leaf spot and anthracnose were found on *Camellia* farmlands in central Taiwan. Through tissue isolation, fungal pathogen candidates of *Camellia* diseases were identified; 15 fungal isolates were obtained, subjected to nucleic acid extraction, and sequenced. Sequence blast results showed that these isolates included *Colletotrichum* spp. (known as an anthracnose disease pathogen), *Alternaria* sp., *Botryosphaeria dothidea*, *Botryotinia fuckeliana* (*Botrytis cinerea*), *Lasiodiplodia pseudotheobromae*, *Neofusicoccum parvum*, *Pestalotiopsis* spp., *Phomopsis* sp., and *Sclerotinia sclerotiorum*. All isolates were reported to be plant pathogens, but *Camellia*-related research is still lacking. A phylogenetic tree constructed with internal transcribed spacers (ITSs) of the 15 isolates in this study showed that pathogens of *Camellia* anthracnose were clustered into 2 species complexes of *C. gloeosporioides* and *C. acutatum*. *Colletotrichum* spp. isolates from different regions were all classified into the same group and were evolutionally closer than other potential *Camellia* leaf pathogens. Anthracnose is considered to be the most important *Camellia* disease with 2 species complexes, and a rapid detection method was first established in this study. Three primer sets were designed for the rapid detection by a polymerase chain reaction (PCR) based on our ITSs to identify anthracnose disease on *Camellia*. The primer sets designed in this study showed high specificity and sensitivity and could successfully detect *Colletotrichum* spp. and distinguish between the *C. gloeosporioides* and *C. acutatum* species complexes. This will contribute to achieving prevention strategies and early disease prevention of *Camellia* anthracnose. In this study, *Colletotrichum* spp. and 8 other potential fungal pathogens were isolated from *Camellia* leaves, and results will be useful in understanding potential pathogens. With the establishment of a rapid detection method for serious anthracnose disease of *Camellia*, our study can be used as an important reference for early diagnosis, which will be valuable for disease control and management.

**Key words:** *Camellia*, *Colletotrichum* spp., molecular rapid detection, polymerase chain reaction.

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## 研究報告

## 臺灣油茶病害調查及油茶炭疽病菌 快速檢測方法之建立

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### 摘要

油茶為臺灣早期的造林樹種，也是近年重要油料作物。油茶地上部病害為造成油茶產量與品質下降的主要原因之一，但卻缺少相關之研究。本研究經由臺灣北部及中部油茶園的病害調查，發現在北部油茶園葉部病害以餅病、葉斑病和軟腐病為主，中部則以葉斑病和炭疽病最為常見。進一步將感病油茶葉片進行組織分離，獲得真菌分離菌株，並依菌落型態進行分群後，從中選取15株真菌分離株，萃取核酸並定序與序列比對，發現油茶葉片及果實的植物病原真菌，以炭疽病菌屬(*Colletotrichum* spp.)真菌所占比例最高。此外，自油茶罹病葉上分離而得的菌株還包含*Alternaria* sp.、*Botryosphaeria dothidea*、*Botryotinia fuckeliana* (*Botrytis cinerea*)、*Lasiodiplodia pseudotheobromae*、*Neofusicoccum parvum*、*Pestalotiopsis* sp.、*Phomopsis* sp.、*Sclerotinia sclerotiorum*。這些真菌分離株皆有被報導為植物病原菌，但在油茶上的研究仍相當缺乏。進一步將真菌分離株的ITS序列進行親緣關係分析，可以發現感染油茶的炭疽病菌，主要來自於*C. gloeosporioides*和*C. acutatum*這兩個種群，並與自其他作物上分離到的常見炭疽病菌歸為同一系群，與其他葉部潛在病原真菌在類緣關係上有所區別。有鑑於炭疽病菌的兩個種群為油茶主要病原菌，本研究首次針對油茶上此兩個重要炭疽病菌種群發展快速檢測方式。透過真菌核酸ITS區域，設計3組油茶炭疽病菌之專一性引子對，利用分子檢測技術(聚合酶鏈鎖反應，PCR)來進行快速檢測。結果顯示本研究設計之引子對油茶炭疽病菌具良好專一性及靈敏度，並可成功區分在油茶上主要造成炭疽病菌的不同種群。透過快速檢測方式的建立，未來將有助於病原菌在潛伏感染時期的檢測。本研究自臺灣油茶罹病葉片上分離出炭疽病菌屬及其他8種真菌，有助於了解油茶葉部之潛在病原菌，同時針對危害嚴重之油茶炭疽病發展快速檢測技術，可作為未來油茶病害防治與健康管理之用，提供病害早期發現早期防治的管理模式。

關鍵詞：油茶、炭疽病菌、分子快速檢測、聚合酶鏈鎖反應。

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## INTRODUCTION

The *Camellia* tree is described as a small-sized evergreen tree of the genus *Camellia* which is one of the four largest woody oil crops in the world. After the fruits are harvested and exposed to the sun, the seeds are hulled out and then squeezed to produce a slightly bitter oil. It is commonly known as the “bitter tea oil” and also referred to “Oriental olive oil” based on its beneficial effects on human health and its fatty acid composition which is comparable to that of olive oil (Chen et al. 1996, Chen and Sun 2011).

The planting of *Camellia* in Taiwan has mainly been for afforestation instead of seed harvesting and oil extraction. Extensive field cultivation and management are commonly adopted. In recent years as food safety issues have become of greater concern coupled with the low ratio of domestic self-produced oil materials, *Camellia* trees have the potential to be developed into an important local economic oil crop. Nowadays, the main species of domestic *Camellia* trees in Taiwan are *Camellia oleifera*, *C. tenuifolia*, and *C. brevistyla*. The total planted area is about 1000 ha, and groves are mainly distributed in central, southern, and eastern counties, among which Chiayi County has the largest planted area, with the rest mainly distributed in Nantou, Hualien, Miaoli, and Taoyuan (Xie and Huang 2013, Zhang 2014).

*Camellia* trees prefer a warm and humid growing environment, mildly acidic soil with abundant organic matter and good drainage, and sufficient light. The applicable planting period of *Camellia* trees is from November to March. As planted *Camellia* seedlings grow slowly, good weed management, fertilizer management, pruning, and field pest management are required to promote the growth and improve yields and the quality of tea fruits (Chen and Sun 2011).

Generally, the first harvest of *C. oleifera* occurs in about the fourth year after planting. In the early stage after planting, the severity of pests and diseases can affect seedling survival, which requires disease and pest control. Mature *Camellia* trees show stronger resistance to pests and diseases. If we better understood the disease occurrence on *Camellia* farms, we could identify major diseases and develop preventive strategies through good field management. According to the monitoring and investigation of the occurrence of diseases on domestic *Camellia* farms, the main occurrence seasons and months of various diseases and pests can be determined which can be used as references for preventing diseases and pests. Common diseases on *Camellia* farms include anthracnose (also known as brown blight), net blister blight, leaf blight, and sooty mold. Among them, *Camellia* anthracnose is considered to be dominant (Wu et al. 2013, Lu et al. 2017).

Anthracnose is a widespread plant disease. Anthracnose pathogens are classified to the genus *Colletotrichum* which contains many different species and can infect the leaves and fruits of many crops. In the early phase of leaf infection, brown round or irregular spots can be observed, and sometimes concentric round patterns can also emerge. Late-phase leaf lesions are often grayish-white with black dots in the central area; infections can produce necrotic spots or cracking on the fruit. In severe cases, it can cause premature fruit drop (Cannon et al. 2012, da Silva et al. 2020). *Camellia* infected by *Colletotrichum* spp. shows many symptoms, such as blackening of the branches, a tendency to break, tree trunk cancer, fruit cracking, and large-scale dropping of leaves and fruits. It also affects the growth vigor of trees and fruit yields; the entire plant may wilt and die (Wu et al. 2013).

The disease mainly occurs in warm and

humid environments and can be observed throughout the year in Taiwan. The peak period of disease occurrence is June to October. The pathogen can be spread by wind, and in severe cases, it can reduce production by more than 50% in planted areas resulting in serious economic losses (Liu et al. 2019).

In previous studies, the main pathogen causing anthracnose was reported to be *C. gloeosporioides* according to morphological characters. With the rapid progress of sequencing technology, the alignment of multiple gene sequences and bioinformatics analytical techniques are widely used to effectively classify and identify *Colletotrichum* spp., and many new species of anthracnose pathogens have been reported. The concept of “species” has been further developed into different clades based on results of phylogenetic analyses of DNA sequences. In recent years, *Colletotrichum* spp. were identified into at least two different groups as *C. gloeosporioides* and *C. acutatum* “species complexes”, which contain many similar species (Damm et al. 2012, Weir et al. 2012).

Although *Camellia* trees are important domestic afforestation tree species and oil-bearing crops, related disease research is still limited. In this study, we observed the disease occurrence and investigated the pathogen community on *Camellia* farms to understand dominant and potential pathogenic threats. While anthracnose caused by *Colletotrichum* spp. was considered the most important aboveground disease of *Camellia* in previous studies, we also investigated isolates of *Colletotrichum* spp. Although there have been several PCR primer pairs designed for detecting anthracnose on different crops with acceptable sensitivities, more-practical and -useful primer pairs were developed for *Colletotrichum* spp. detection in this study. With latent infection and destructive characters

of *Colletotrichum* spp., specific primer pairs were designed to develop a sensitive and specific method for the rapid detection of anthracnose. These primer pairs can also be used to distinguish between the *C. gloeosporioides* and *C. acutatum* species complexes that cause anthracnose on *Camellia* trees. In addition, rapid detection would be valuable for monitoring *Camellia* anthracnose diseases, and potentially benefit disease control in the future.

## MATERIALS AND METHODS

### Investigation of the disease occurrence of *Camellia*

To evaluate the disease occurrence and identify the most important diseases of *Camellia* in Taiwan, 3 *Camellia* farming sites were selected. The first sample plot was located in Longtan, Taoyuan (in northwestern Taiwan), and the 2 other sample sites were in Lienhuachih, Nantou and Dongshi, Taichung (both in the central area of Taiwan). All samples were collected in 2017 to 2018, and leaf symptoms observed in the field were recorded to identify the most common diseases on *Camellia* farms. Several diseased leaves were collected for further pathogen isolation to identify fungal pathogen groups infecting *Camellia*.

### Isolation and incubation of fungal pathogens

Fresh *Camellia* leaves with disease symptoms were collected in the field. Potential fungal pathogens were isolated from infected plant tissues of samples by the following process. About 0.5~1 cm<sup>2</sup> of plant tissues containing healthy-diseased tissue junctions were cut out. The excised samples were immersed in 1% NaClO for 90 s for surface sterilization. Then, leaf segments were transferred to sterilized distilled water 3 times for 30 s each to remove

any residual NaClO. These leaf segments were then placed on sterilized filter paper to remove any residual distilled water and finally placed on 2% water agar. The water agar plates were incubated at room temperature for 2~4 d. A single slice of fungal hyphae was cut from the edge of a leaf segment and transferred to potato dextrose agar (PDA) for further pure-culture incubation. After 2~4 d of incubation, hyphae from the edge of each mycelium were cut and transferred to new PDA plates for incubation to produce pure fungal isolates. All fungal isolates were stored in sterilized distilled water for further use.

#### **Fungal genomic DNA extraction**

The DNA extraction method was modified from that of Hung et al. (1999). Fungal mycelium (0.16 g; about 0.4×0.4-cm agar plate covered with growing fungal hyphae of isolates) was scraped and placed into a 1.5-ml plastic tube. Hyphae were then homogenized with a plastic pestle. DNA extraction buffer (0.9 ml at pH 8.0; 100 mM Tris-HCl, 100 mM EDTA, and 250 mM NaCl) and 0.1 ml 20% Sarkosyl buffer were added. The suspension was vortexed and incubated at 55°C for 1 h. The tube was centrifuged at 6000 rpm for 5 min. The supernatant (0.8 ml) was saved, and 100 µl of 5 M NaCl and 100 µl of cetyltrimethylammonium bromide (CTAB)/NaCl (10% CTAB in 0.7 M NaCl) were added and incubated at 65°C for 10 min. An equal volume of chloroform/isoamyl alcohol (24 : 1) was added, mixed thoroughly, and centrifuged at 11,000 rpm for 5 min. The aqueous suspension was saved. Equal volumes of phenol/chloroform/isoamyl alcohol (25 : 24 : 1) were added, mixed thoroughly, and centrifuged at 11,000 rpm for 5 min. The aqueous suspension was saved. Isopropanol (0.6 volume) was added to precipitate nucleic acids, and the mixture was centrifuged at 12,000 rpm for 30 min. The pel-

let was washed with 70% ethanol to remove any residual CTAB. Briefly, the pellet was dried and resuspended in 100 µl of TE buffer (pH 8.0 : 10 mM Tris and 1 mM EDTA) and stored at -20°C. Genomic DNA (gDNA) of all 15 fungal isolates was extracted, and the internal transcribed spacer (ITS) sequences were amplified by a PCR using the universal ITS4 and ITS5 primers to identify each isolate.

#### **Fungal ITS sequence amplification**

Fungal ITS sequences were amplified by a PCR using the primer sets ITS5 (5'-GGAAGTAAAAGTCGTAACAAGG-3') and ITS4 (5'-TCCTCCGCT-TATTGATATGC-3'). A PCR was conducted in 25-µl reaction mixtures (10 mM Tris-HCl at pH 8.7, 50 mM KCl, 2 mM MgCl<sub>2</sub>, 0.25 mM of each dNTP, 1.25 units of Taq DNA polymerase, 0.2 µM each primer, and 2 µl of extracted DNA). All PCR reagents were procured from Promega (Madison, WI, USA). The PCR was carried out with the following setup: initial incubation at 94°C for 2 min, followed by 30 cycles of a denaturing step at 94°C for 30 s, an annealing step at 56°C for 30 s, and extension at 72°C for 1.5 min, and a final extension of 5 min at 72°C. The amplified DNA was sequenced for further identification.

#### **Phylogenetic analysis**

ITS regions of fungal isolates derived from diseased *Camellia* leaves were amplified to examine their molecular characteristics. Newly obtained sequences of each fungal isolate in this study were used to blast on the National Center for Biotechnology Information (NCBI) database. Sequences of model species with 99~100% similarity to our sequences were derived from the database for further phylogenetic tree construction. Molecular Evolutionary Genetic Analysis (MEGA)

software vers. 6.0 (Tamura et al. 2013) was used for the sequence analysis. ClustalW implemented in MEGA vers. 6 was used for multiple sequence alignments of each ITS sequence which were manually adjusted to allow for maximum sequence similarity (Tamura et al. 2013). The phylogenetic analysis was inferred using the maximum-likelihood method based on the Kimura 2-parameter model (Kimura 1980). Each clade was assessed using a bootstrap analysis with 1000 replicates. All positions containing gaps were eliminated (Tamura et al. 2013).

### ***Colletotrichum* sp. primer pair design and PCR amplification**

Representative *Colletotrichum* ITS sequences derived from the NCBI database (GeneBank) were used to evaluate specific regions for primer design (Appendix Fig. 1). ITS sequences were aligned with ClustalW software, and regions for primer design were artificially selected. Conserved sequences between the ITS5 and ITS4 regions were used for designing 3 primer sets, including 1 genus-specific primer pair and 2 species complex-specific primer pairs. Sequences were as follows: genus-specific primer pair of CgG-F1: 5'-CGAAATGCGATAAGTAATGT-3' and CgG-R1: 5'-TACTACGCAAAGGAGGCT-3'; 1st species complex-specific primer pair of CgS-F1: 5'-CTCTTGGTCTGGCATCG-3' and CgS-R1: 5'-TACCTTTGAGGGCCTACAT-3'; and 2nd species complex-specific primer pair of CgS-F2: 5'-GCAGCGAAATGCGATAAG-3' and CgS-R2: 5'-CCCAGTGCGAGACGTAA A-3'. Two genus-specific primer pairs from previous studies were used for comparison: Col1: 5'-AACCTTTGTGAACRTACCTA-3' and Col2: 5'-T T A C T A C G - C A A A G G A G G C T - 3' (Martinez-Culebras et al. 2003) and Col-F: 5'-AACCTTTGT-

GAACATACCT-3' and Col-R: 5'-CCACT-CAGAAGAAACGTCGTT-3' (Cano et al. 2004). All primers used in this study are listed in Table 1. PCR conditions were the same as those described above.

### **PCR specificity and sensitivity tests of *Colletotrichum* sp. primer pairs**

Fifteen fungal isolates obtained from *Camellia* leaves were subjected to primer pair specificity tests. The 5 primer pairs examined included CgG-F1/CgG-R1, CgS-F1/CgS-R2, CgS-F2/CgS-R2, Col1/Col2, and Col-F/Col-R. Nucleic acids of pure cultured *Colletotrichum* sp. isolates were serially diluted 10, 10<sup>2</sup>, 10<sup>3</sup>, 10<sup>4</sup>, 10<sup>5</sup>, 10<sup>6</sup>, and 10<sup>7</sup> times, and sensitivities of individual primer pairs were tested. PCR conditions were the same as those described above.

### **Practical application tests of *Colletotrichum* disease on *Camellia***

*Camellia* leaves suspected of being infected were collected from the field. Lesioned leaf tissues were cut out and used for total nucleic acid extraction following the instruction manual of the Plant Genomic DNA Mini Kit (Geneaid Biotech, New Taipei City, Taiwan). Three primer pairs designed in this study were tested for practical application. Nucleic acids of pure-cultured *Colletotrichum* sp. were used as a positive control, whereas nucleic acids of healthy leaves and sterile water were used as negative controls. PCR conditions were the same as those described above.

## **RESULTS**

### **Disease occurrence on *Camellia***

In this study, 3 sampling sites were selected to investigate the disease occurrence on *Camellia* in northern and central areas in Taiwan. Symptoms of leaves were observed

and recorded to represent *Camellia* disease occurrences. All different disease symptoms of *Camellia* diseases observed in the 2-yr investigation were classified and are shown in Fig. 1. Blister blight, algal spots, and soft rot were major symptoms observed at the sampling site in Longtan, Taoyuan. Anthracnose and leaf spots were major symptoms at Lienhuachih, Nantou, while anthracnose was the major disease in Dongshi, Taichung. The findings reflected that anthracnose and leaf spots were commonly observed in the central area, while blister blight and soft rot were only observed in northern Taiwan with its cooler climate.

### Isolation, incubation, and identification of fungal pathogens

Fungal isolates derived from *Camellia* leaves exhibiting different symptoms included anthracnose, leaf spot, and soft rot. All fungal isolates were first classified based on the colony morphology. Then we grouped all of the fungal isolates into 15 different groups. Representative morphologies of pure fungal isolates are shown in Fig. 2. Based on ITS sequences, 5 fungal isolates were identified as *Colletotrichum* spp., 3 isolates were *Pestalotiopsis* spp., and 7 other isolates were *Neofusicoccum parvum*, *Lasiodiplodia pseu-*

*dotheobromae*, *Alternaria* sp., *Phomopsis* sp., *Botryotinia fuckeliana*, *Botryosphaeria dothidea*, and *Sclerotinia sclerotiorum* (Table 2). Overall, *Colletotrichum* spp. causing anthracnose in the field occupied the largest proportion of total fungal isolates. Together with results of field disease occurrence investigations, anthracnose was generally considered one of the dominant diseases on *Camellia* farms we investigated in Taiwan. While *Pestalotiopsis* spp. were considered to be potential fungal pathogens causing leaf spots, their pathogenicity still needs to be evaluated. However, several potential fungal pathogens were also considered according to harmful records on other plants. It was mentioned that *Sclerotinia sclerotiorum* was the only fungus isolated from *Camellia* leaves with soft rot symptoms. These symptoms were observed on the northern *Camellia* farm in Taoyuan and could potentially be regarded as a new *Camellia* disease in Taiwan. Although numerous symptoms and potential pathogenetic fungal isolates were observed in our study, the following experiments focused on anthracnose because of its high occurrence rate and characteristics of latent infections.

A phylogenetic tree of all the aforementioned 15 fungal isolates was constructed with other representative fungal ITS se-

**Table 1. Primers used in this study**

Genus-specific primer pair		Reference
CgG-F1	5'-CGA AAT GCG ATA AGT AAT GT -3'	This study
CgG-R1	5'-TAC TAC GCA AAG GAG GCT- 3'	
Col1	5'-AAC CCT TTG TGA ACR TAC CTA-3'	Martinez-Culebras et al. 2003
Col2	5'-TTA CTA CGC AAA GGA GGC T-3'	
Col-F	5'-AAC CCT TTG TGA ACA TAC CT-3'	Cano et al. 2004
Col-R	5'-CCA CTC AGA AGA AAC GTC GTT-3'	
Species complex-specific primers		This study
CgS-F1	5'-CTC TTG GTT CTG GCA TCG-3'	This study
CgS-F2	5'-GCA GCG AAA TGC GAT AAG-3'	
CgS-R2	5'-CCC AGT GCG AGA CGT AAA-3'	

quences derived from the NCBI, with a special focus on *Colletotrichum* spp. According to the maximum-likelihood tree constructed with MEGA vers. 6, the fungal isolates were classified into different clades with different taxonomic statuses. Among all fungal isolates from *Camellia*, the *Colletotrichum* spp. clade was closest to the *Phomopsis* spp. clade, then to the *Pestalotiopsis* spp. clade (Fig. 3). *Alternaria* sp., *Botryotinia fuckeliana*, and *Sclerotinia sclerotiorum* were separated into a quite-distinct group compared to other isolates. *Alternaria* sp. showed the highest difference with other fungi and was considered an outer group for phylogenetic tree construction. We identified that there were at least 2 *Colletotrichum* groups found: one was grouped as the *C. gloeosporioides* species complex and the other was the *C.*

*acutatum* species complex. We considered that besides *C. gloeosporioides*, *C. acutatum* could also be a potential anthracnose pathogen to *Camellia* in Taiwan.

#### Specific PCR established for the rapid detection of *Camellia* anthracnose

Three primer sets of CgG-F1/CgG-R1, CgS-F1/CgS-R1, and CgS-F2/CgS-R2, were first designed. After basic PCR amplification tests, the CgS-R1 primer showed less specificity and was eliminated from further studies. Since CgS-R2 showed good specificity with CgS-F1 and CgS-F2, the 3 primer sets of CgG-F1/CgG-R1, CgS-F1/CgS-R2, and CgS-F2/CgS-R2 were selected as the genus- or species-specific primers. While CgG-F1/CgG-R1 was designed as the *Colletotrichum* genus-specific primer set,

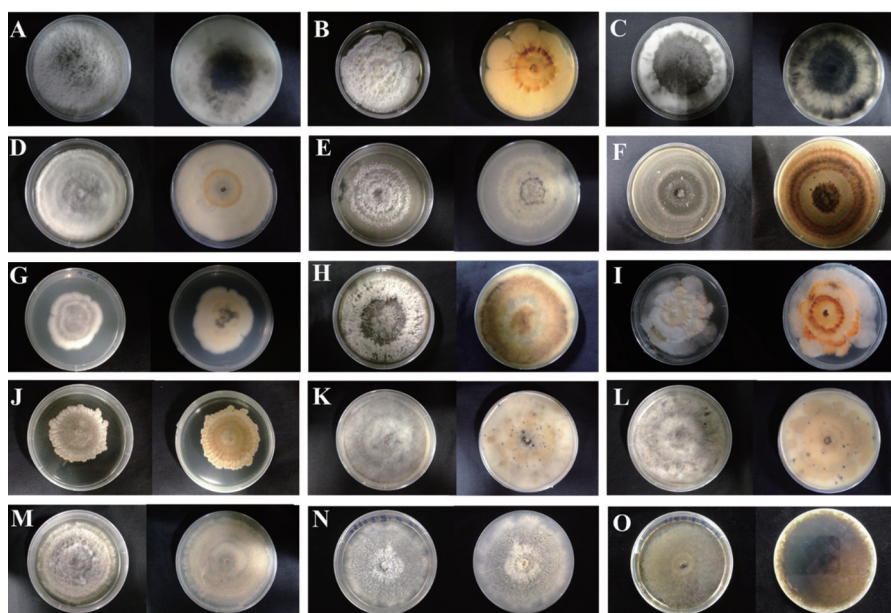


**Fig. 1.** Investigation of the disease occurrence of *Camellia* in Taiwan. Symptoms of blister blight, algal spots, and soft rot were observed in Longtan, Taoyuan (A to C); anthracnose (D to F) and leaf spot were observed in Lienhuachih, Nantou (G and H); and anthracnose was observed in Dongshi, Taichung (I).



CgS-F1/CgS-R2 and CgS-F2/CgS-R2 were designed as species-specific primers which could only amplify the DNA region of the *C. gloeosporioides* species complex. To demonstrate the specificity of each primer set, 2 *Colletotrichum* spp.-specific primer sets, Col1/Col2 and Col-F/Col-R designed in a previous study, were used for comparison with the newly developed primer sets in this study, and ITS4/ITS5 were used as positive controls. All 15 representative fungal DNAs were used as targets to evaluate the specificity of each primer set. DNA fragments amplified by Col1/Col2, Col-F/Col-R, CgG-F1/CgG-R1, CgS-F1/CgS-R2, and CgS-F2/CgS-R2 were 416, 138, 203, 254, and 227 bp, respectively (Fig. 4B-F). Specificities of

the genus-specific primer sets of Col1/Col2 and CgG-F1/CgG-R1 were demonstrated when only *Colletotrichum* isolates showed the 416- and 203-bp DNA bands, while the remaining 10 isolates of other species had no PCR products with the 2 primer sets (Fig. 4B, D). Otherwise, CgS-F1/CgS-R2 and CgS-F2/CgS-R2 respectively amplified 254- and 227-bp DNA fragments of all *C. gloeosporioides* isolates (Fig. 4E, F). Also no other signal of PCR products of *C. acutatum* or other fungal isolates was detected by the 2 primer sets. However, the Col-F and Col-R primers considered to be a *Colletotrichum* genus-specific primer set in a previous study (Cano et al. 2004) showed no identification ability with the *C. acutatum* isolate with



**Fig. 2.** Morphologies of fungal isolates from diseased *Camellia* leaves. Fungal isolates of *Neofusicoccum parvum*, *Pestalotiopsis* sp., *Lasiodiplodia pseudotheobromae*, *Colletotrichum gloeosporioides*, *Botryosphaeria dothidea*, *Alternaria* sp., *Colletotrichum acutatum*, *Phomopsis* sp., *Pestalotiopsis* sp., and *Botryotinia fuckeliana* from Lienhuachih, Nantou (A to J); isolates of *Colletotrichum gloeosporioides* from Dongshi, Taichung (K to M); isolates of *Plestalotiopsis mangiferae* and *Sclerotinia sclerotiorum* from Longtan, Taoyuan are shown (N and O). *Sclerotinia sclerotiorum* was the only fungal isolate derived from soft rot leaves and was considered to be its pathogen.

**Table 2. List of investigated sites and fungal isolates in this study**

No.	Fungal isolate	Host	Collection site	Symptoms	<i>Colletotrichum</i> detection*
1	<i>Neofusicoccum parvum</i>	<i>Camellia oleifera</i>	Lienhuachih, Nantou	Anthracnose/Leaf spots	-
2	<i>Pestalotiopsis</i> sp.	<i>C. oleifera</i>	Lienhuachih, Nantou	Anthracnose/Leaf spots	-
3	<i>Lasiodiplodia pseudohebroneae</i>	<i>C. oleifera</i>	Lienhuachih, Nantou	Anthracnose/Leaf spots	-
4	<i>Colletotrichum gloeosporioides</i>	<i>C. oleifera</i>	Lienhuachih, Nantou	Anthracnose	+
5	<i>Bortyosphaeria dothidea</i>	<i>C. brevispyla</i>	Lienhuachih, Nantou	Leaf spots	-
6	<i>Alternaria</i> sp.	<i>C. brevispyla</i>	Lienhuachih, Nantou	Anthracnose/Leaf spots	-
7	<i>Colletotrichum acutatum</i>	<i>C. brevispyla</i>	Lienhuachih, Nantou	Anthracnose	+
8	<i>Phomopsis</i> sp.	<i>C. brevispyla</i>	Lienhuachih, Nantou	Anthracnose/Leaf spots	-
9	<i>Pestalotiopsis</i> sp.	<i>C. brevispyla</i>	Lienhuachih, Nantou	Anthracnose/Leaf spots	-
10	<i>Bortyotinia fuackiana</i>	<i>C. brevispyla</i>	Lienhuachih, Nantou	Anthracnose/Leaf spots	-
11	<i>Colletotrichum gloeosporioides</i>	<i>Camellia</i> sp.	Dongshi, Taichung	Anthracnose	+
12	<i>Colletotrichum gloeosporioides</i>	<i>Camellia</i> sp.	Dongshi, Taichung	Anthracnose	+
13	<i>Colletotrichum gloeosporioides</i>	<i>Camellia</i> sp.	Dongshi, Taichung	Anthracnose	+
14	<i>Pestalotiopsis mangiferae</i>	<i>Camellia</i> sp.	Longtan, Taoyuan	Anthracnose/Leaf spots	-
15	<i>Sclerotinia sclerotiorum</i>	<i>Camellia</i> sp.	Longtan, Taoyuan	Soft rot/Water-soaked spots	-

\* Detection of *Colletotrichum* spp. was conducted by a PCR with CgG-F1/CgG-R1. +/- refers to positive or negative signals after PCR amplification.

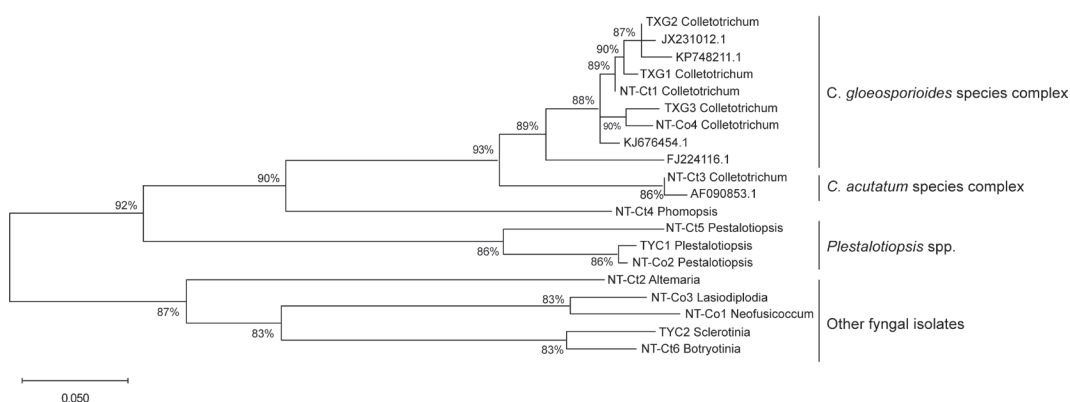
no amplified PCR products observed but showed a non-specific band with *Alternaria* sp. isolates (Fig. 4C).

Results showed that the CgG-F1/CgG-R1, CgS-F1/CgS-R2, and CgS-F2/CgS-R2 primer sets developed in this study had good specificities according to their original *Colletotrichum* genus-specific or *C. gloeosporioides* species-specific purpose. By combining the CgG-F1/CgG-R1 *Colletotrichum* genus-specific primer set and one of the *C. gloeosporioides* species-specific sets of CgS-F1/CgS-R2 or CgS-F2/CgS-R2, we could also generally identify fungal isolates belonging to the *C. gloeosporioides* or *C. acutatum* species complex (Fig. 4, Table 2). While anthracnose is regarded as the most serious *Camellia* disease and usually occurs with a period of latent infection, we concluded that the specific PCR established in this study with 3 newly developed primer sets will be valuable for *Camellia* anthracnose detection based on their high specificity. The

sensitivity and practical application of the PCR-based rapid detection method were further evaluated in this study.

### Sensitivities of the PCR primer sets

gDNA extracted from 1 pure *C. gloeosporioides* isolate was used to verify the sensitivity of each aforementioned primer set (including 2 previous and 3 newly designed primer sets). The original DNA concentration was approximately 11.3 ng/ $\mu$ l and was 10-fold serially diluted from  $10\times$  to  $10^7\times$  with sterile double-distilled H<sub>2</sub>O. Each concentration of *C. gloeosporioides* DNA was amplified by the 5 primer sets. Sensitivities of the Col1/Col2, Col-F/Col-R, and CgG-F1/CgG-R1 primer sets were quite similar and could amplify *C. gloeosporioides* gDNA with the  $10^{-4}$  dilution, i.e., about 1.13 pg/ $\mu$ l gDNA in a 20- $\mu$ l PCR. Comparing the above 3 primer sets, Col-F/Col-R and CgG-F1/CgG-R1 showed stronger PCR-amplified signals under lower concentrations and also



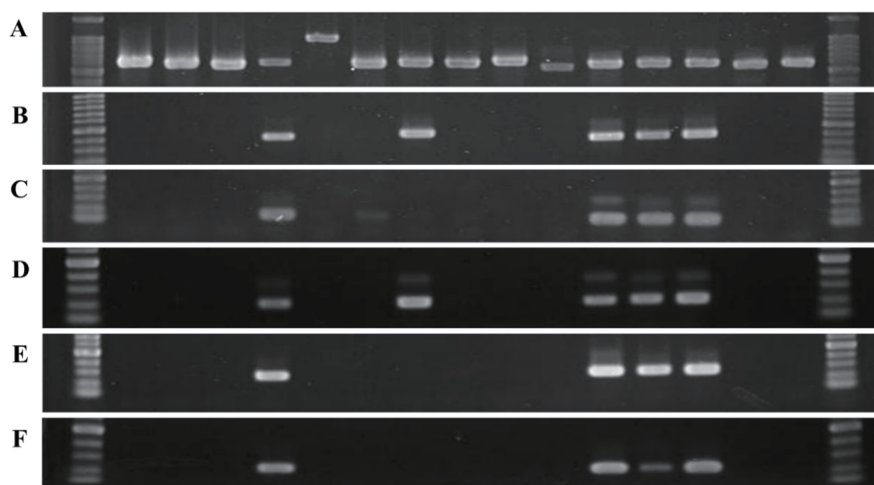
**Fig. 3.** Phylogenetic tree of fungal isolates from *Camellia* leaves. Internal transcribed spacer (ITS) sequences derived from the NCBI database are shown with their accession numbers, and other ITS sequences with isolate numbers were derived in this study. KP748211.1, JX231012.1, KJ676454.1, and FJ224116.1 were representative sequences of the *Colletotrichum gloeosporioides* species complex while AF090853.1 was considered to be the *C. acutatum* species complex. The phylogenetic analysis was conducted using the maximum-likelihood method with MEGA 6. Associated taxa clustered together in the bootstrap test (with 1000 replicates) are shown as percentages.

demonstrated better sensitivities than Col1/Col2 (Fig. 5A-C).

The 2 *C. gloeosporioides* species-specific primer sets designed in this study were demonstrated to have higher sensitivities than all 3 above *Colletotrichum* genus-specific primer sets. The sensitivity limitations of the 2 primer sets were certified to be able to detect about 0.1 pg/μl gDNA of *C. gloeosporioides* (Fig. 5D, E). It was exciting to discover that the primer sets designed in our study had good specificities and sensitivities that could be further used in *Camellia* anthracnose disease detection in the field. In addition, they can be used for early disease diagnosis of harvested fruit.

### Practical applied validation

Anthracnose-diseased *Camellia* leaves collected from *Camellia* farms were used for validation of the practical rapid detection method. Total DNA was extracted from both healthy and diseased leaves and was amplified by the 3 primer sets designed in this study. gDNA extracted from pure *C. gloeosporioides* mycelium was used as a positive control (Fig. 6). PCR results demonstrated that all 3 primer sets successfully amplified target fragments from diseased samples without false-positive signals from healthy samples. Considering the specificity and sensitivity of the practical tests, the 3 primer sets were judged to

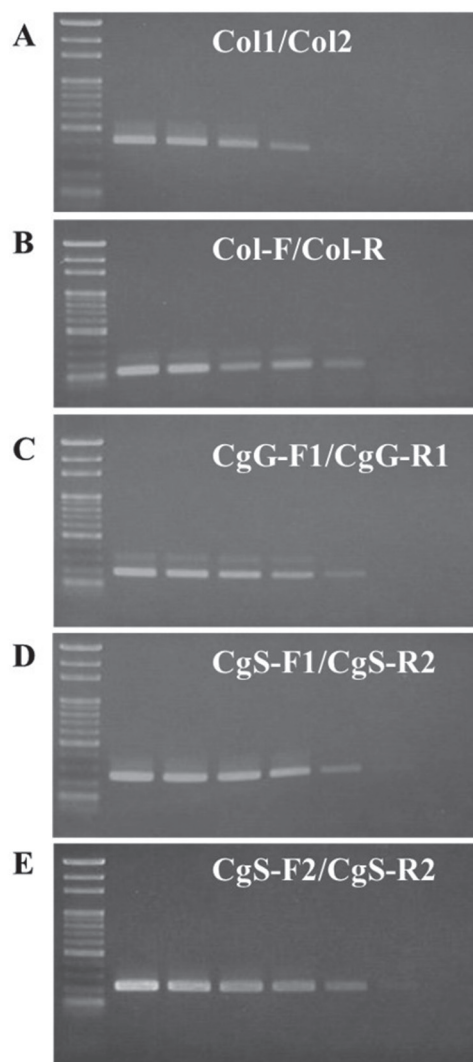


**Fig. 4.** Rapid detection and specificity test of primers designed for *Camellia* anthracnose disease detection. All 15 fungal isolates from *Camellia* leaves were used to evaluate the specificity of each primer set. The fungal universal primer set ITS4/5 was used as a positive control (A). Amplification results of 2 *Colletotrichum* genus-specific primer sets, Col1/Col2 (with a 416-bp amplicon) and Col-F/Col-R (with a 138-bp amplicon), from previous studies are shown (B, C). One *Colletotrichum* genus-specific primer set (CgG-F1/CgG-R1) and 2 *C. gloeosporioides* species complex-specific primer sets (CgS-F1/CgS-R2 and CgS-F2/CgS-R2) designed in this study were estimated by PCR to have 203-, 254-, and 227-bp amplicons, respectively (D-F). Samples of each lane from left to right are molecular marker, *Neofusicoccum parvum*, *Pestalotiopsis* sp., *Lasiodiplodia pseudotheobromae*, *C. gloeosporioides*, *Botryosphaeria dothidea*, *Alternaria* sp., *C. acutatum*, *Phomopsis* sp., *Pestalotiopsis* sp., *Botryotinia fuckeliana*, *C. gloeosporioides*, *C. gloeosporioides*, *C. gloeosporioides*, *Pestalotiopsis mangiferae*, *Sclerotinia sclerotiorum*, and molecular marker.

be reasonably reliable for the rapid detection of *Colletotrichum* and for distinguishing between the *C. gloeosporioides* and *C. acutatum* species complexes. This may also contribute to anthracnose disease control in the future.

## DISCUSSION

Disease occurrences of *Camellia* in the field in Taiwan during 2017 to 2018 were investigated in this study. Based on our investigation, we demonstrated that the aboveground diseases of *Camellia* were similar to diseases on other tea plants, including blister blight, leaf spot, soft rot, and anthracnose (TPS 2019). Among the diseases caused by fungal pathogens on *Camellia* leaves, soft rot caused by *S. sclerotiorum* could potentially be considered a newly recorded disease on *Camellia*, and the disease usually occurred in cool weather. Anthracnose is considered the most serious disease on *Camellia*, causing a decline in the quality of camellia oil, and it was also reported to be one of the most destructive disease on other crops, fruits, and afforestation trees, e.g., pepper, strawberry, tea trees, mango trees, stout camphor trees, etc. (Carlos et al. 2008, Kamle et al. 2013, Chung et al. 2020, Lin et al. 2020). According to a field investigation of leaf symptoms, anthracnose was a more-dominant disease than leaf spot; soft rot was only observed in several regions during specific seasons, i.e., late winter or early spring (Lu et al. 2019). Clearly, our findings showed that anthracnose and leaf spot were major *Camellia* foliage symptoms observed on *Camellia* farmlands in the northern and central areas of Taiwan. Both of these diseases seemed to be closely connected to temperature, with higher occurrences observed with higher temperatures in central Taiwan.

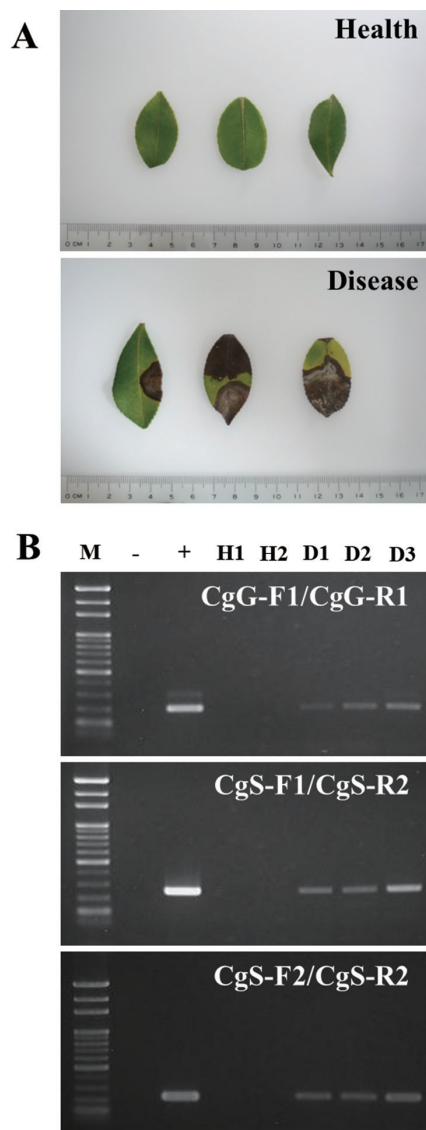


**Fig. 5.** Sensitivity test of specific primers designed for *Camellia* anthracnose disease detection. The DNA of *Colletotrichum gloeosporioides* was used with 10-fold serial dilution to evaluate the sensitivity of each primer set. All 5 primer sets of Col1/Col2 (A), Col-F/Col-R (B), CgG-F1/CgG-R1 (C), CgS-F1/CgS-R1 (D), and CgS-F2/CgS-R2 (E) used in this study were tested at 7 different concentrations. Lanes 1 to 8 represent a molecular marker and PCR products of different concentrations with a series of 10-fold dilutions (at  $1 \times$ ,  $10^{-1} \times$ ,  $10^{-2} \times$ ,  $10^{-3} \times$ ,  $10^{-4} \times$ ,  $10^{-5} \times$ , and  $10^{-6} \times$ ).

Since some anthracnose and leaf spot symptoms were atypical, further isolation was conducted to identify the compositions of the fungal pathogens on diseased leaves.

These findings generally corresponded to the pathogenic fungi isolated from diseased *Camellia* leaves. In the identification of the fungal compositions of potential pathogens, at least 15 different fungal isolates were selected according to their colony types and further specified into 9 different species according to their micro-morphological structure and ITS sequences (Table 2). Fungal isolates included *Colletotrichum* spp., *Pestalotiopsis* spp., *N. parvum*, *L. pseudotheobromae*, *Alternaria* sp., *Phomopsis* sp., *Botryotinia fuckeliana*, *Botryosphaeria dothidea*, and *S. sclerotiorum*. Some of these fungi were previously verified to be pathogenic to *Camellia*, i.e., *Colletotrichum* spp., *Pestalotiopsis* spp. and *S. sclerotiorum* (Vogelgsang et al. 1998, Thomma 2003, Lisa et al. 2006). Other fungi were also considered to be potential pathogens or secondary pathogens according to their pathogenicity to at least 1 other host plant (Vogelgsang et al. 1998, Thomma 2003, Lisa et al. 2006). Since the study involved only disease occurrence and fungal isolation, further inoculation experiments are still needed to clarify the pathogenicity of each fungal isolate. Despite the uncertified pathogenicity tests of potential fungal pathogens, our findings provide the first systemic study of the fungal composition of the field disease occurrence in *Camellia* in Taiwan. These results could help us infer potential and dominant pathogens on *Camellia* farms. These results can serve as a valuable reference for *Camellia* disease management in Taiwan.

On the other hand, isolation of *Colletotrichum* from *Camellia* anthracnose-infected leaves revealed that both *C. gloeosporioides*



**Fig. 6.** Practical application test of *Camellia* anthracnose disease-specific primers. Healthy and diseased samples used for the practical application test (A). The 3 primer sets designed in this study were used for the practical application test (B). Lane 1, molecular marker; Lane 2, negative control ( $H_2O$  as a template); Lane 3, positive control (DNA of *Colletotrichum gloeosporioides* as a template); Lanes 4 and 5, DNA of healthy leaves; Lanes 6 to 8, DNA of diseased leaves.

and *C. acutatum* were anthracnose-causing pathogens. While fungi of the genus *Colletotrichum* are distributed into several species complexes, *C. gloeosporioides* and *C. acutatum* are the 2 major species complexes (Damm et al. 2012, Weir et al. 2012). Our results expanded our previous knowledge and revealed that there could be additional *Colletotrichum* species causing anthracnose on *Camellia* (Chen et al. 2017). However, the pathogenicities of different *Colletotrichum* isolates especially belonging to different *Colletotrichum* species complexes have still not been determined. It would be interesting to clarify the different pathogenicities or virulences of isolates among different *Colletotrichum* species complexes derived from *Camellia*, which would also be useful for evaluating plant resistance. We intend to continue pursuing this investigative lead in further experimental studies.

Since early symptoms and various spots caused by *Colletotrichum* species are sometimes hard to diagnose by traditional methods, one of the major objectives of our study was to develop a rapid detection method with higher sensitivity and specificity for detecting *Colletotrichum* spp. Among the 3 primer sets we designed in this study, one was used for *Colletotrichum* genus-specific detection and the other two were used for *C. gloeosporioides* species complex-specific detection. All 3 primer sets showed good specificity and sensitivity and were quite useful for detecting diseased plant samples. It is also worth noting that the 2 species complex-specific primer sets of *C. gloeosporioides*, CgS-F1/CgS-R2 and CgS-F2/CgS-R2, demonstrated higher sensitivities. The 2 primer sets showed 10-fold sensitivity compared to all other primer sets tested in this study and could be used to detect pathogens under as low a DNA concentration as 0.1

pg. Although application of the primer sets in this study still needs further evaluation, preliminary results indicated their possibilities for long-term monitoring of changes in pathogen populations and disease management in the early stage.

The primer sets developed in our study can be applied to identify *Colletotrichum* spp. (Torres-Calzada et al. 2011). Using different combinations of primer sets, the genus-specific primer set (CgG-F1/CgG-R1) together with 1 species complex-specific primer set (CgS-F1/CgS-R2 or CgS-F2/CgS-R2), we could detect *Colletotrichum* spp. and distinguish between the *C. gloeosporioides* and *C. acutatum* species complexes. When fungal DNA could be amplified by both genus-specific and species complex-specific primer sets, the fungus belonged to the *C. gloeosporioides* species complex. In contrast, if the fungal DNA was only amplified by the *Colletotrichum* genus-specific primer, the fungus should belong to other *Colletotrichum* spp. such as those in the *C. acutatum* species complex.

While anthracnose widely occurs in different plants and *Colletotrichum* is composed of many species complexes (Armitage et al. 2020), the genus-specific CgG-F1/CgG-R1 primer pair was supposed to be capable of widely amplifying all *Colletotrichum* spp. even from different plants. Similar applications could also be extended to the CgS-F1/CgS-R2 and CgS-F2/CgS-R2 primer sets for detecting *Colletotrichum* species. Although amplification was only tested on fungal isolates from *Camellia* and many more *Colletotrichum* isolates should be acquired for further assessment, our study still provides a crucial step toward anthracnose rapid detection and *Colletotrichum* species identification.

## CONCLUSIONS

This research first revealed *Camellia* disease occurrences in northern and central Taiwan. According to leaf symptoms, we classified the symptoms into blister blight, algal spot, leaf spot, soft rot, and anthracnose. These symptoms were quite similar to those of diseases on tea trees. To understand the fungal composition of potential pathogens, fungal isolates from diseased *Camellia* leaves were identified as *Colletotrichum* spp., *Pestalotiopsis* spp., *Neofusicoccum parvum*, *Lasiodiplodia pseudotheobromae*, *Alternaria* sp., *Phomopsis* sp., *Botryotinia fuckeliana*, and *Sclerotinia sclerotiorum*. Among all the aboveground diseases, anthracnose was considered the most important one with its high observation and isolation rates that were consistent with previous considerations. It is worth noting that *C. gloeosporioides* was not the only species of *Colletotrichum* causing anthracnose on *Camellia*, while *C. acutatum* was also isolated from diseased *Camellia* leaves. Traditionally, anthracnose disease control has been quite difficult based on time-consuming identification and latent infection by *Colletotrichum* spp. In this study, a rapid detection PCR method was established with newly designed genus-specific and species complex-specific primer sets. All the 3 primer sets showed good specificity and sensitivity and could be applied to detect pathogens on plant tissues. They can be further used for field disease management even with a large number of samples. On the other hand, combinations of our primer sets were also useful in distinguishing between the *C. gloeosporioides* and *C. acutatum* species complexes. To our knowledge, there has been no comprehensive study of *Camellia* diseases. In this study, we investigated potential fungal pathogens of *Camellia* and established a rapid detection method for *Colletotrichum* identifica-

tion which can be helpful for *Camellia* disease management in Taiwan.

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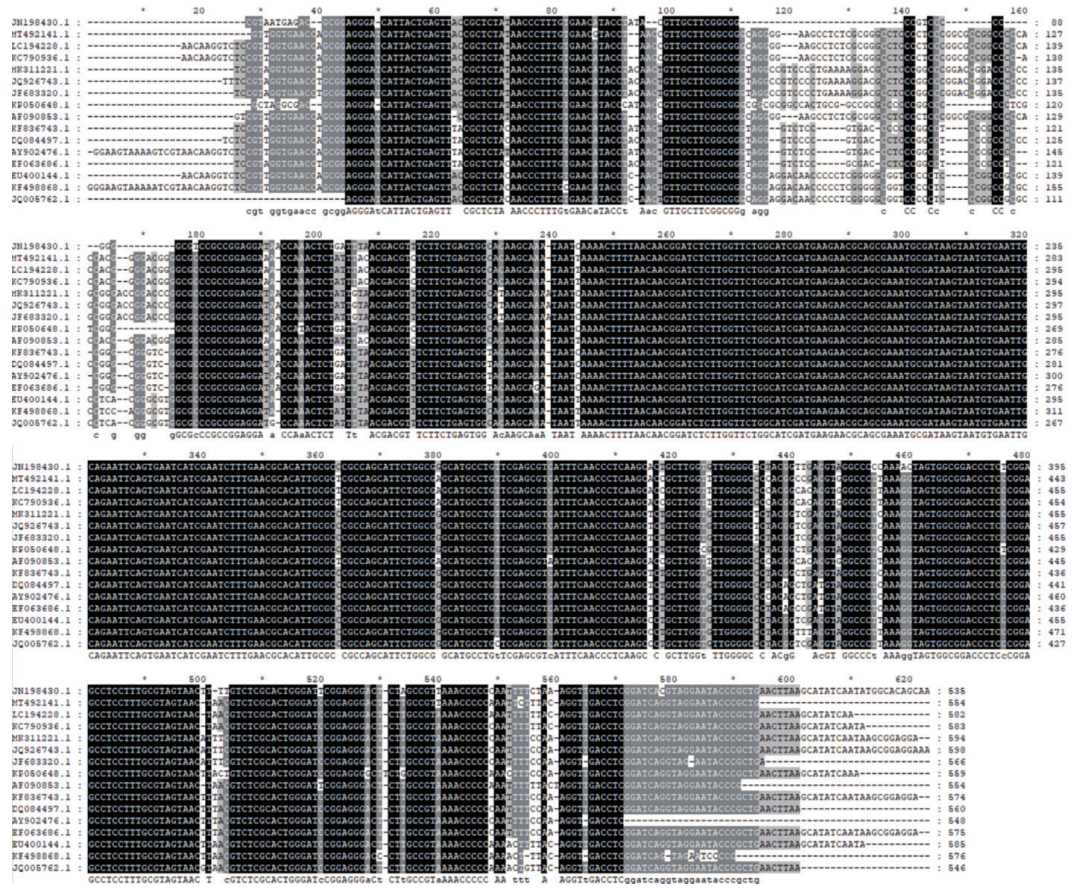
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Appendix Fig. 1. Sequences used for *Colletotrichum*-specific primer design. Representative internal transcribed spacer (ITS) sequences of *Colletotrichum* spp. were derived from the NCBI. Sequences belonging to the *C. gloeosporioides* species complex are KF836743.1, DQ084497.1, EF063686.1, and AY902476.1. Sequences belonging to the *C. acutatum* species complex are AF090853.1, KC790936.1, and LC194228.1. Other sequences of *Colletotrichum* spp. included *C. fiorinae* (MT492141.1), *C. boninense* (MK311221.1, JQ926743.1, and JF683320.1), *C. crassipes* (KP050648.1 and JN198430.1), and *C. fuscum* (KF498868.1, JQ005762.1, and EU400144.1).

