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臺灣草莓炭疽病之探討

The study of strawberry anthracnose in Taiwan

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臺灣草莓炭疽病之探討

The study of strawberry anthracnose in Taiwan

本論文係鐘珮哲君（D05633001）在國立臺灣大學植物病理與微生物學系完成之博士學位論文，於民國 111 年 08 月 24 日承下列考試委員審查通過及口試及格，特此證明。

The undersigned, appointed by the Department of Plant Pathology and Microbiology on 24 August 2022 have examined a PhD dissertation entitled above presented by Pei-Che Chung (D05633001) candidate and hereby certify that it is worthy of acceptance.

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研究所畢業後經由高考分發至行政院農業委員會苗栗區農業改良場服務，由於在第一線輔導農民植物保護相關問題，很幸運地可以充分發揮在校所學。然而當欲進一步深入探討田間問題時，越是發現自己的不足，因而毅然決然回母系攻讀博士班。時光荏苒，很開心也很有成就感的是，總算順利以在職進修身分畢業了！

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中文摘要

草莓 (*Fragaria* × *ananassa* Duch.) 為具高經濟價值且深受消費者喜愛之作物，其栽培面積於臺灣約為 500 公頃。在臺灣炭疽病為草莓生產最大的限制因子之一，尤以感病品種'桃園 1 號'受害最為嚴重。調查國內草莓主要產區，本病害除可感染草莓全株各部位外，高達 50%以上之病株呈現典型冠腐病徵。本研究於 2010 至 2018 年由新竹縣、苗栗縣、南投縣及嘉義縣等地區之草莓罹病植株分離 52 株菌，透過病原菌型態及 internal transcribed spacer (ITS)、glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*)、chitin synthase (*CHS1*)、actin (*ACT*)、β-tubulin (*TUB2*)、calmodulin (*CAL*) 及 intergenic region between *Apn2* and *MAT1-2-1* (ApMAT) 等序列進行多基因親緣分析，確認造成臺灣草莓炭疽病之病原菌包含 *Colletotrichum siamense*、*C. fructicola* (*C. gloeosporioides* species complex)、*C. karstii*、*C. boninense* (*C. boninense* species complex) 及一新種 *C. miaoliense* (*C. acutatum* species complex)。*C. siamense* 及 *C. fructicola* 為主要致病菌，在 25°C 或 30°C 下不論葉片有無傷口皆產生較大之病斑，而其他三種病原菌僅在有傷口條件下產生微小病斑。由於炭疽病菌能潛伏感染寄主，種植健康不帶菌的草莓苗，將可大幅降低本田期病害的發生，同時減少化學藥劑的使用。而為生產健康草莓苗，準確、靈敏、快速又合乎成本效益的檢測技術便是其中最重要的關鍵。本研究分析已發表之 29 種炭疽病菌菌株之全基因體序列，找到位於 L-arabinitol 4-dehydrogenase (*ladA*) 及 NAD(P)H-dependent D-xylose reductase (*xyl1*) 兩基因間之非保守序列進行引子設計，開發出巢式聚合酶鏈鎖反應技術。本技術可以偵測最主要的炭疽病菌 *C. siamense* 與 *C. fructicola*，但不會偵測到其他草莓病原菌或土壤中常見的腐生菌，可偵測到低至 1 pg 之 *C. siamense* DNA (約 15 個細胞)，代表具有高度專一性及靈敏度。本研究釐清臺灣草莓炭疽病菌種類及特性，有助於防治策略之擬定與執行，及未來草莓抗病育種之篩

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選。本研究所開發之檢測技術已直接應用於草莓種苗病害驗證作業，期望加速推廣草莓健康種苗之使用。

關鍵詞：炭疽病、多基因親緣分析、潛伏感染、健康種苗、比較基因體學、巢式聚合酶鏈鎖反應

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ABSTRACT

In Taiwan, strawberry (*Fragaria* × *ananassa* Duch.) is a high-value and popular crop with an average annual cultivated area of ~500 ha. Anthracnose disease is one of the key limiting factors of strawberry production in Taiwan, especially for the susceptible cultivar 'Taoyuan no.1'. We surveyed anthracnose in major strawberry cultivation areas, and observed that it could infect all parts of the strawberry plant and more than 50% of diseased plants showed typical anthracnose crown rot symptoms. A total of 52 isolates were collected from Hsinchu County, Miaoli County, Nantou County and Chiayi County during 2010 to 2018. Based on morphological characterization and multi-gene phylogenetic analysis using the sequences of transcribed spacer (ITS), glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), chitin synthase (*CHS1*), actin (*ACT*), β-tubulin (*TUB2*), calmodulin (*CAL*) and intergenic region between *Apn2* and *MAT1-2-1* (ApMAT), we revealed that *Colletotrichum siamense*, *C. fructicola* (*C. gloeosporioides* species complex), *C. karstii*, *C. boninense* (*C. boninense* species complex) and *C. miaoliense* sp. nov. (*C. acutatum* species complex) were associated with strawberry anthracnose in Taiwan. The predominant species *C. siamense* and *C. fructicola* caused larger lesions at 25°C or 30°C on leaves with or without wounds, whereas the other three species caused tiny lesions only on wounded leaves. Because *Colletotrichum* spp. could cause latent infections, use of healthy and pathogen-free strawberry runner plants will greatly reduce the occurrence of anthracnose rot in the field and the usage of fungicides. To produce healthy runner plants, it is important to diagnose anthracnose at the stage of latent infection. We conducted comparative genomics analysis of 29 known *Colletotrichum* spp. genomes to search for non-conserved regions suitable for the design of specific primers which are located between L-arabinitol 4-dehydrogenase (*ladA*) and NAD(P)H-

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dependent D-xylose reductase (*xyl1*). We developed a nested PCR assay which can specifically detect the predominant anthracnose pathogens *C. siamense* and *C. fructicola*, but not other pathogens and saprophytes associated with strawberry plants. It could detect as low as 1 pg genomic DNA of *C. siamense*, which corresponds to 15 cells of *C. siamense*, suggesting the high sensitivity of this new detection technique. This study clarified the species identity and characteristics of strawberry anthracnose pathogens in Taiwan, which can help develop and apply effective control strategies as well as disease resistance breeding in the future. The detection method developed in this study has been applied to the voluntary pathogen-free certification system for strawberry propagation, which is expected to accelerate the promotion of healthy strawberry runner plants.

Keywords: anthracnose, multilocus phylogenetic analysis, latent infection, healthy nursery, comparative genomics, nested PCR

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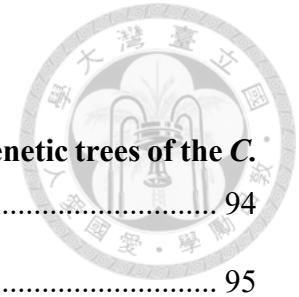

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Chapter 1

Introduction

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1.1 Research motivations

Strawberries (*Fragaria* x *ananassa*) have good taste, pleasant appearance, rich nutritional value, and are deeply loved by consumers. In Taiwan, the strawberry cultivated area is about 500 hectares and over 90% of the strawberry cultivation is in Miaoli County. Combined with tourism and leisure agriculture, it has become a very representative sixth-level industry (Chung et al. 2019). Strawberry is a very distinctive and important crop in the jurisdiction area of Miaoli District Agricultural Research and Extension Station (MDAIS), Council of Agriculture, Executive Yuan. The relevant researchers of MDAIS are responsible for the development of the strawberry industry. The breeding period for strawberry is usually April to September, and the annual demand for strawberry runner plants is 27.5 million plants (吳及鐘 2021). However, the biggest bottleneck in the nursery breeding period is disease control, especially during the high temperature and rainy months from July to August that anthracnose is prone to occur. Since 2010, due to the occurrence of severe anthracnose, farmers have faced the problem of lacking runner plants in the production fields (鐘及彭 2013). Most of the runner plants are still propagated by farmers from the mother plants which reproduced from field plants. However, whether the asymptomatic mother plants are pathogen free or not is unknown. During the summer, leaf spots or wilted symptoms of anthracnose usually appear after frequent thunderstorms, and farmers are used to spraying fungicides after the rain. Excessive use of fungicides could not only lead to the occurrence of phytotoxicity, but also triggered the pathogen to develop fungicide resistance under the high selection pressure. Because *Colletotrichum* spp. have the characteristics of latent infection, even if the strawberry plants are healthy in appearance, a high percentage of plants eventually show disease symptoms and even die after transplanting.

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Anthracnose is a major threat to the strawberry industry. However, the *Colletotrichum* spp. that caused the serious death of strawberries has not been clarified, and whether the strawberry plants are infected cannot be identified by appearance. In order to improve the strawberry industry, this study started from identifying the species of *Colletotrichum* that caused strawberry anthracnose. Because of the disease outbreak in 2010, we started systematic investigation for the *Colletotrichum* species of strawberry anthracnose. In this study, diseased plants were collected from several strawberry cultivated areas in Taiwan, and the *Colletotrichum* spp. were identified through multilocus molecular phylogenetic analyses, and the virulence of the isolates was subsequently assayed by inoculation experiments. After identifying the predominant species of *Colletotrichum*, it will help to screen disease-resistant varieties through inoculation tests at the breeding stage. Since application of healthy runner plants is an important strategy to prevent anthracnose, nested PCR detection method is developed to screen the anthracnose-free healthy runner plants and reduce the incidence of anthracnose in the field. By clarifying the species of *Colletotrichum* of strawberry in Taiwan, this study lays important foundation on disease management. The detection method developed by this study can be applied to the current voluntary pathogen-free certification system for strawberry propagation, which will contribute to the propagation of healthy strawberry runner plants and further enhance the development of the strawberry industry. We hope this study can help the strawberry industry to produce high-quality strawberries by integrated pest management (IPM) based on understanding the pathogenic ecology.

1.2 Strawberry cultivation in Taiwan

According to the statistics of the Food and Agriculture Organization (FAO) in 2020 (FAOSTAT 2022), China, USA, Egypt, Mexico, Turkey, Spain, Brazil, Russia, Poland,

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Morocco, Japan, Korea, Germany, United Kingdom, and Italy are the top 15 strawberry producing countries. Notably, the planting area of China (126,644 ha) is more than 7 times of the USA (17,400 ha), but the total yield between the two countries is only 3 times different.

Although Taiwan has a subtropical climate, there are still about 500 hectares of strawberry cultivation area (Chung et al. 2019). Strawberry cultivation in Taiwan could be traced back to 1934. At that time, a small number of strawberry plants were introduced from Japan and planted in Yangmingshan. Initial attempts at commercial cultivation and promotion failed due to the high susceptibility of strawberry to diseases. In 1958, people in Dahu Township introduced strawberry from Luzhou Township for a small scale of cultivation. This was the beginning of strawberry cultivation in Dahu. In the early years after introduction, the cultivation of strawberry was based on the rotation with rice. After harvesting of rice in the second crop season, the soil was prepared and strawberry was planted, and the surface of the soil was covered with straw to prevent weeds. The harvest period of strawberry was from February to May, and rice seedlings were transplanted into the field after the harvesting of strawberry. The strawberries produced at that time were contracted with food factories and mainly used as raw materials for processing. In 1973, the price per kilogram was only NT$13. Therefore, farmers were not willing to plant them, and the cultivation area was only about tens of hectares. Since 1980, sightseeing and fruit picking had become popular, farmers would rather break the contract than provide their strawberries to food processors. Since then, strawberries had changed from processed raw materials to fresh fruits, and the price had doubled. As a result, farmers in Dahu switched to plant strawberries, making Dahu the hometown of strawberries (張等 2009). In 1985, a variety of strawberry ‘Toyonoka’ was introduced from Japan, and ‘Taoyuan No. 1’,

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which was selected from 'Toyonoka', was officially named in 1990 and became the major variety.

Currently in Taiwan, over 90% of strawberry cultivation is in Miaoli County. The rest of the cultivation distributes sporadically in Taipei City, Taoyuan City, Hsinchu County, Taichung City, Nantou County, Tainan City, and Kaohsiung City from north to south (the counties/cities listed here are the ones with > 3 hectares of strawberry cultivation) (https://agr.afa.gov.tw/afa/afa_frame.jsp). However, the strawberry industry has been threatened by diseases and pests, especially anthracnose for more than ten years.

1.3 The evolution of the taxonomy of *Colletotrichum* spp.

The genus *Colletotrichum* includes pathogens, endophytes, and saprophytes. In particular, *Colletotrichum* is a scientific and economic important pathogen which was considered the 8th most influential plant pathogenic fungus by many experts and scholars (Dean et al. 2012). Almost all crops from tropical to temperate regions are susceptible to one or more species of *Colletotrichum*. Due to the latent infection characteristics, *Colletotrichum* spp. cause serious post-harvest diseases in many fruits (Prusky 1996).

The classification of *Colletotrichum* spp. was quite confusing in early 2000s. For example, a new taxon was erected when *Colletotrichum* sp. Infected a new host genus which no disease had previously been reported. The method was based on host specificity, even though the new species of *Colletotrichum* has no morphological difference (von Arx 1957). However, many members of this genus can cause diseases on different crops. Therefore, a better classification system is particularly important for developing control strategies. Around 1957, von Arx first formally classified about 750 names of *Colletotrichum* into 11 taxa based on morphology (von Arx 1957), among that *C. gloeosporioides* covers about 600 synonyms (Cannon et al. 2012). Sutton accepted 22

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and 39 anthracnose species according to morphological and cultural characteristics in 1980 and 1992, respectively. However, only some distinctive characteristics were used for classification, resulting in frequently misidentification of *Colletotrichum* spp. (Cannon et al. 2012; Hyde et al. 2009a; Jayawardena et al. 2016b). Taylor et al. (2000) proposed the application of Genealogical Concordance Phylogenetic Species Recognition (GCPSR) to identify phylogenetic species, and GCPSR was proven to be a powerful tool to resolve *Colletotrichum* spp. by Cai et al (2009). Taxonomy of *Colletotrichum* spp. should be based on multi-gene phylogeny, compared with type specimens, and a good phylogenetic lineage should be combined with identifiable characters such as morphology, physiology, pathogenicity, culture characteristics, and secondary metabolites (Cai et al. 2009).

Cai et al (2009) proposed a protocol for describing new species and epitypes of *Colletotrichum* spp.: (1) A detailed morphological description of the fungus must be provided, including colony characters, shape and size of conidia, appressoria, setae, ascus and ascospore, and growth rate. (2) Except for identifiable morphological or other phenotypic characters, the proposed new species should show sufficient evolutionary divergence from other closely related taxa based on multilocus molecular phylogenetic analyses, and any comparison must be made with type specimens. (3) It is recommended to use ITS, *TUB2*, *GAPDH*, *ACT* and others for multilocus molecular phylogenetic analyses. The sequences should be deposited in a recognized international database. (4) The medium used for description and comparison can vary according to different needs (e.g. mating test on Czapek-dox agar medium), where a possible description should also be provided from a collection on host tissues. (5) The ex-type cultures of new species should be deposited in more than two internationally recognized culture collections, and all information must be registered in MycoBank. (6) Detailed growth rates in standard

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medium, standard temperature, and growth conditions should be provided. (7) When considering a specific specimen and derived culture as epitype and ex-epitype, the strain must come from the original host, original geographic locality, have well-matched morphology and other phenotypic characters as the type which should be examined if practical and preferentially described. (8) Pathogenicity testing may be useful and should be performed whenever possible (Cai et al. 2009).

Hyde et al. (2009b) also provided a comprehensive overview of 66 commonly used and 19 doubtable names of *Colletotrichum* spp., further emphasizing the need for molecular approaches to redefine the taxonomy of this genus. Since then, the classification of *Colletotrichum* spp. has entered a new era based on molecular methods.

Weir et al. (2012) performed the phylogeny analysis of *C. gloeosporioides* species complex with the multi-gene sequence of 8 genes, including internal transcribed spacer (ITS), glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), calmodulin (*CAL*), β-tubulin (*TUB2*), actin (*ACT*), chitin synthase (*CHS-1*), glutamine synthetase (*GS*), manganese-superoxide dismutase (*SOD2*). Among these, *CAL*, *CHS-1*, *GS*, or *SOD2* can be used to distinguish *C. fructicola* from other species by single gene sequence; *CAL* or *TUB2* can be used to distinguish *C. siamense* from other species. Phylogenetic analysis of combined sequences of intergenic sequence between *Apn2* DNA lyase and *Mat1-2* (ApMat) and *GS* was found very useful for clearly delineating the species in the *C. gloeosporioides* species complex (Liu et al. 2015). On the other hand, analysis of ITS, *ACT*, *TUB2*, *CHS-1*, *GAPDH*, and histone3 (*HIS3*) was suggested for resolving *C. acutatum* species complex, and analysis of ITS, *ACT*, *TUB2*, *CHS-1*, *GAPDH*, *HIS3*, and *CAL* was suggested for resolving *C. boninense* species complex (Damm et al. 2012a; Damm et al. 2012b).

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In recent years, the classification of *Colletotrichum* spp. has been continuously updated; from 119 species (Cannon et al. 2012), 190 species and 11 species complexes (Jayawardena et al. 2016b), 247 species and 14 species complexes (Jayawardena et al. 2020), 248 species and 15 species complexes (Bhunjun et al. 2021), to 280 species and 16 species complexes in 2022 (Liu et al. 2022) were updated. The continual discovery of new species indicates that the genus is highly diverse.

The classification of strawberry anthracnose pathogen has also been changed with time. In 1931, *C. fragariae* was the first reported *Colletotrichum* spp. associated with a disease of strawberry in Florida (Brooks 1931). At that time, Brooks (1931) considered anthracnose is primarily a disease of the stolons but occasionally infected the petioles of the nursery plants. Thereafter, *C. fragariae* caused crown rot and fruit rot in the fruit production field were reported (Horn and Carver 1963; Howard 1972). In 1965, *C. acutatum* sp. Nov. was reported causing ripe fruit rot of strawberry in Queensland (Simmonds 1965). In Florida, *C. gloeosporioides* (= *C. fragariae*) caused black leaf spot of strawberry in 1983 (von Arx 1957; Howard 1983). In 2012, through multilocus molecular phylogenetic analyses, *C. fragariae* was determined in synonymy with *C. theobromicola* (Weir et al. 2012), and *C. acutatum* (Damm et al. 2012a) and *C. gloeosporioides* (Weir et al. 2012) remained in the original classification. So far, *Colletotrichum* spp. associated with strawberry anthracnose were classified into 5 species complexes, including *C. acutatum*, *C. boninense*, *C. dematium*, *C. gloeosporioides*, and *C. truncatum* species complexes. Until 2020, the species known in each species complex are shown in Fig. 1.1. The species of strawberry anthracnose pathogens in Taiwan included *C. siamense* and *C. fructicola* belonging to *C. gloeosporioides* species complex, *C. boninense* and *C. karstii* belonging to *C. boninense* species complex, and *C. miaoliense*

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sp. Nov. belonging to *C. acutatum* species complex (details described in Chapter 2) (Chung et al. 2020).

1.4 Nutrient acquisition strategies and lifestyles of *Colletotrichum*

According to nutrient acquisition strategies, *Colletotrichum* spp. were classified into plant pathogens, endophytes, saprotrophs, and rarely entomopathogens and opportunistic human pathogens. It is worth noting that the same species will have different nutrition modes depending on environmental conditions (Jayawardena et al. 2016a).

1.4.1 *Colletotrichum* spp. as plant pathogens

As a complex genus, *Colletotrichum* has different lifestyles, and many members of it can switch lifestyles (O'Connell et al. 2012; Promputtha et al. 2007). The lifestyles of plant pathogens can be classified into biotrophic, necrotrophic, and hemi-biotrophic (Chethana et al. 2021b; Chethana et al. 2021a; van Kan et al. 2014). In the biotrophic lifestyle, the pathogens remain in living host plant cells and absorbs nutrients, but without killing plant cells (Mendgen and Hahn 2002). Different strategies are needed to escape from the defense response of the host plant. For instance, deacetylation of the surface-exposed chitin of the cell wall can mask the invasive hyphae. To manipulate the plant host's physiological and biochemical environment which could suppress the plant defense responses, a variety of effector proteins are secreted by pathogens (De Silva et al. 2017; Dou and Zhou 2012; Münch et al. 2008; O'Connell et al. 2012). During the necrotrophic stage, the pathogen kills host cells by secretion toxic substances and cell wall degrading enzymes, which subsequently break down plant tissue and consume it for their own growth (O'Connell et al. 2012; van Kan 2006). Secondary hyphae develops and symptom

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appears during this stage. Pathogen survives on dead or dying cells and complete its life cycle (Gan et al. 2013; Laluk and Mengiste 2010).

Most phytopathogenic *Colletotrichum* spp. are considered hemibiotropic pathogens. They have an initial biotrophic stage and then necrotrophic stage in their life cycles. Many *Colletotrichum* spp. can change their relationships with host plants at different stages of their life cycles. Such changes may be associated with the physiological maturity and resistance of host plants, environmental factors and virulence genes of the fungal pathogen. Transformation in cell morphology and infection modes was observed during the lifestyle transition (O'Connell et al. 2012; Stergiopoulos and Gordon 2014). For *C. lindemuthianum* in bean, the penetration hyphae forms swollen vesicle and primary hyphae in the epidermal cell (Mendgen and Hahn 2002). These structures are surrounded by the invaginated plant plasma membrane. At this stage, the host is still symptomless defined as biotrophic stage. After 1-2 days, plant plasma membrane disintegration starts, causing plant cell death. The ensuing secondary hyphae secretes large amounts of cell-wall-degrading enzymes to disintegrate the cell wall and enter the necrotrophic phase. For *C. higginsianum* in *Arabidopsis thaliana* (Jayawardena et al. 2021; O'Connell et al. 2012), the conidia germinates in 2 hours, forms appressoria within 22 hours, and penetrates the host within 26 hours. *C. higginsianum* is at biotrophic phase within 48 hours, switches to necrotrophy at 60 hours (symptoms gradually appeared), and is at necrotrophic phase after 72 hours. Conidiophores are formed 6 days later and conidia are spread out by rain-splash.

For pathogenic *Colletotrichum* spp. to establish interactions with the host plants, the first step is the landing and attachment of the spores on the host surface (Mendgen et al. 1996; Tucker and Talbot 2001). The spores produce germ tubes, from which appressoria are formed. Appressorium is defined as a specialized cell or an adherent structure from

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which a penetration peg develops to pierce host tissue (Emmett and Parbery 1975). Appressoria can be classified into two types (Chethana et al. 2021a). The first type is unicellular appressoria formed terminally of the germ tube or laterally on the hyphae. The dark or pigmented appressoria produced by *Colletotrichum* spp. belong to this category (Ryder and Talbot 2015). The second type refers to compound or multi-cellular appressoria, including the infection cushions of *Botrytis cinerea* (Demoor et al. 2019), infection plaques of *Pseudocercosporella herpotrichoides* (Daniels et al. 1991), and expressoria of *Epichloë festucae* (Green et al. 2019).

Colletotrichum spp. with latent, quiescent or dormant parasitic relationship refers to the pathogens in the life of the host plant exist in a quiescent manner, until under specific circumstances, the pathogen become active (Verhoeff 1974). The conidia, appressoria and penetration pegs of these pathogens have evolved physiologically inactive mechanisms that delay activation, this mechanism helps evade host defense responses (Latunde-Dada 2001). In the case of *C. gloeosporioides*, when the conidia lands on unripe fruit of avocado, germinate within several hours and form appressoria within 19 hours. The penetration peg branches in the fruit cuticle to form a dendritic structure that penetrates cells in the fruit epidermis to produce a swollen hyphal structure. These structures remain quiescent until the fruit harvested and ripen then differentiate into thin and long necrotrophic hyphae causing postharvest disease (Prusky et al. 2013). It keeps a dynamic balance among the host, pathogen and environment during quiescent period, and the host plant with symptomless. Physiological and physical changes in the host plant, the environment, or both trigger changes in the original equilibrium to prompt the pathogen to restart aggression (Jarvis 1994; Prusky 1996).

For strawberry anthracnose, the primary inoculum in the field mostly come from strawberry nurseries. In particular, the pathogen can be introduced by infected but

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asymptomatic runner plants (Mcinnes et al. 1992). At the early stage of the colonization of *C. gloeosporioides* on strawberry leaf, it presents a quiescent infection form and continues to multiply (Rahman and Louws 2017). When the primary conidia of *C. acutatum* lands on strawberry surface, appressoria forms and secondary conidia will also be produced. The secondary conidia can spread to other plants with splashing water (Leandro et al. 2001). During this period, strawberry tissue is still symptomless. Appressoria and secondary conidia are considered to be important inoculum sources of severe anthracnose in fruit-producing fields. The length of the latent period is affected by environmental factors such as temperature, humidity and the amount of fertilizer (especially nitrogen). The duration is 2-3 days at 25°C and 6-17 days at 5°C. The latent period of *C. acutatum* was slightly shorter than that of *C. gloeosporioides* at 5-10°C, but similar at higher temperatures (King et al. 1997; Leandro et al. 2003; Smith 2008).

1.4.2 *Colletotrichum* spp. as endophytes

Endophytes can be classified into clavicipitalean (grass-inhabiting) and non-clavicipitalean (non-grass-inhabiting) (Carroll 1988; Hyde and Soytong 2008). The grass-inhabiting endophytes produce alkaloid compounds which make the host plant toxic or unattractive to herbivores (Clay 1996). Some non-grass-inhabiting endophytes were found to have the roles of increasing the growth and the drought and disease resistance of their host plants (Hyde and Soytong 2008). Several endophytes are latent pathogens, and these endophytic “pathogens” are not highly pathogenic because they have co-evolved with their host plants (Brown et al. 1998; Sieber 2007). These “pathogens” sporulate under certain conditions, such as when the leaf becomes senescent, when the plants are stressed, or when the fruits are ripened (Hyde and Soytong 2008).

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Many *Colletotrichum* spp. isolated from healthy plant tissues are considered to act as both endophytes and pathogens. However, this does not necessarily mean that all endophytes can switch to a necrotrophic lifestyle (Cannon et al. 2012), and differentiating between the two life strategies is difficult. On the other hand, so far 33 endophytic *Colletotrichum* species were reported as beneficial organisms in the host plant (Liu et al. 2022). Some *Colletotrichum* spp. can be pathogenic to one plant species and endophytic to another plant species. For example, the strawberry pathogens *C. dematium* is an endophyte of grapevine (*Vitis vinifera*) (Hyde et al. 2009b), *C. fructicola* is an endophyte of *Dendrobium* spp. (Ma et al. 2018), and *C. karstii* is an endophyte of aquatic plant (*Potamogeton wrightii*) (Zheng et al. 2022).

1.4.3 *Colletotrichum* spp. as saprotrophs

The sources of nutrients for saprotrophs are derived from nonliving organic materials or dead plant tissues. *Colletotrichum* spp. that cause severe plant diseases are also frequently isolated from dead plant materials and are classified as saprotrophs. *C. gloeosporioides* was isolated from decaying leaves of *Dracaena lourieri*, and *C. artocarpicola* was isolated from *Artocarpus heterophyllus* in Thailand (Bhunjun et al. 2019; Thongkantha et al. 2008). The conidia of *C. acutatum* and *C. gloeosporioides* isolated from strawberry survived for a year in autoclaved soil, but rapidly reduced their viability within days in the untreated soil at 22% moisture (Freeman et al. 2002). In Finland, *C. acutatum* can survive in strawberry residues on the soil surface (or covered with soil) for a winter. Greenhouse experiments showed that *C. acutatum*-colonized weed debris can be the inoculum for infection of young strawberry plants (Parikka et al. 2006).

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1.4.4 *Colletotrichum* spp. as entomopathogens

Some *Colletotrichum* species have been reported to have the ability to infect insects. It was first found that *C. gloeosporioides* could cause an epidemic of the important pest *Orthezia praelonga* in Brazil (Damm et al. 2012a). Marcelino et al. (2008) reported the epidemics occurred in *Fiorinia externa* in New York, Connecticut, Pennsylvania and New Jersey, and the pathogen was identified as *C. acutatum* var. *fioriniae* (Marcelino et al. 2008). Interestingly, *C. acutatum* var. *fioriniae* and *C. gloesporioides* f. sp. *Ortheziidae* were found at different stages of host adaptation. While *C. acutatum* var. *fioriniae* still maintained the ability to invade plants, *C. gloesporioides* f. sp. *Ortheziidae* appears to have lost this ability (Marcelino et al. 2009).

1.4.5 *Colletotrichum* spp. as human pathogens

A few *Colletotrcichum* species are opportunistic human pathogens. Cano et al. (2004) reported that *C. coccodes*, *C. crassipes*, *C. dematium*, *C. gloeosporioides*, and *C. graminicola* are clinically important pathogens in Spain (Cano et al. 2004). *C. fructicola*, *C. fusiforme*, *C. tropicale*, and *C. truncatum* can cause keratitis, and some of the patients were found exposing to soil or dirty water during farming operations (Hung et al. 2020; Llamos et al. 2016). In Australia, *C. gloeosporioides* has been reported to infect through a stab wound from a lemon tree and cause deep tissue mycosis in human (Figtree et al. 2013).

1.5 Detection of strawberry anthracnose

Due to the latent infection characteristics of anthracnose, whether the mother plant is latently infected has a great influence on the propagation of strawberry runner plants. Several culture- and PCR-based detection methods have been developed for detecting

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latent infection of strawberry anthracnose (Table 1.1 and 1.2). The culture-based methods include the treatments of paraquat (Cerkauskas 1988), freezing (Mertely and Legard 2004), and ethanol (SDEI, Simple Diagnosis using Ethanol Immersion) (Ishikawa 2003). Culture-based methods are low cost and easier for farmers or nursery operators to apply, but require 7 to 14 days to observe conidial mass and professional knowledge to identify the morphology of the conidia. The advantage of PCR-based detection methods is high specificity and sensitivity, but the cost of testing is relatively high, and needs a laboratory to conduct relevant experiments.

1.5.1 Culture-based detection methods

Paraquat

Since the 1980s, the herbicide paraquat (1,1'-dimethyl-4,4'-bipyridinium dichloride) has been used to detect *Colletotrichum* in symptomless plant materials (Biggs 1995; Cerkauskas 1988; Mertely and Legard 2004). The plant tissue was completely washed, surface sterilized, treated with 0.03 to 0.6% paraquat, and incubated in a moist chamber. The chlorophyll-containing tissue is killed rapidly, and the process accelerates fungal growth and sporulation. The potential pathogen can subsequently be identified based on the conidia. In 1993, researches at the Central Science Laboratory in Great Britain began to use paraquat to detect *C. acutatum* on strawberry petioles of imported strawberry plants (Cook 1993).

Freezing

Since paraquat is toxic when ingested, inhaled, or absorbed through the skin or eyes, freezing was investigated as an another option in Florida (Mertely and Legard 2004). After freezing symptomless tissues at -15°C then incubated them in a moist chamber for 5 to 7 days, the conidia mass of *Colletotrichum* spp. is formed. Using this method, *C.*

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acutatum and *C. gloeosporioides* were detected on asymptomatic petioles of field-grown plants, and *C. acutatum* and *C. dermatium* were detected on asymptomatic transplants from northern (Canadian) nurseries (Mertely and Legard 2004).

Simple Diagnosis using Ethanol Immersion (SDEI)

A simple diagnostic method using ethanol immersion treatment (SDEI) was developed to detect latent infection by *Glomerella cingulate* on strawberry leaves (Ishikawa 2003). Leaves changed to dark brown and salmon-pink conidial masses were produced within 5 to 10 days after treated with SDEI. Different levels of tolerance of strawberry varieties to anthracnose did not influence the rate of conidial mass formation after SDEI.

1.5.2 PCR-based detection methods

Conventional PCR

C. acutatum was a quarantine organism on strawberries in the European Union (EU) during the 1990s (Parikka and Lemmetty 2004). The latent infection of *C. acutatum* on many other plant species might serve as a potential inoculum source for strawberry infection (Freeman et al. 2001). Strawberry plants imported into United Kingdom (UK) and Finland were tested for black spots caused by *C. acutatum*. It took about 7 days to complete the isolation of the pathogen, and the plants had to be kept in cold storage during the time, which may destroy the fresh runners. In order to develop a more efficiently method, primers of conventional PCR were designed based on ITS region to detect *C. acutatum* on asymptomatic strawberry plants (Freeman et al. 2001; Parikka and Lemmetty 2004; Sreenivasaprasad et al. 1996).

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PCR-microtube hybridization (MTH) method

PCR-MTH is a PCR method complimented with microtube hybridization. The lower part of old petioles were cut into ~1-cm segments, surface-sterilized, then incubated on potato sucrose agar (PSA) for 3 days to promote the growth of the hyphae. DNA extracted from the hyphae were used for PCR. The primers targeted a region in MAT1-2 were designed for specific identification of *C. acutatum* and *G. cingulata*. Conventional PCR was followed by MTH, which uses the PCR product as the probe to hybridize pathogen-specific sequence spotted on a strip of nylon membrane. Since the laborious electrophoresis step was omitted, the PCR-MTH method was more suitable for high-throughput screening (Furuta et al. 2017).

Nested PCR

For detection of *C. acutatum* on symptomless strawberry leaves, a nested PCR assay was developed in 2008 (Perez-Hernandez et al. 2008). Two sets of primers were designed based on the ITS region, and the detection limit was 1 fg of fungal DNA. The nested PCR assay combined with FISA (detached leaves frozen for 3 h, incubated for 2 days at 27°C, immersed in Tween 20, then sonicated for 30 min and agitated for 1 min) could reliably detect *C. acutatum* in artificially inoculated leaves and symptomless leaves from the field. To reduce the disease incidence in mother plants, a nested PCR assay for detecting *C. siamense* and *C. fructicola* on asymptomatic strawberry plants were developed in this thesis (Chung et al. 2022). Through comparative genomics analysis, a non-conserved region located between the L-arabinitol 4-dehydrogenase (*ladA*) and NAD(P)H-dependent D-xylose reductase (*xyl1*) genes were selected for primer design. The assay could detect as little as 1 pg of *C. siamense* genomic DNA and had applied to facilitate the production of healthy strawberry runner plants in Taiwan.

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Quantitative real-time PCR (qPCR)

Debode et al. (2009) developed qPCR assays for *C. acutatum* using primers targeting the ITS region and *β-tubulin* gene (Debode et al. 2009). The TaqMan assay based on ITS could reliably detected as little as 50 fg genomic DNA, which corresponds to 100 copies of target DNA. However, the assay based on *β*-tubulin was about 66 times less sensitive, thus less suitable for the detection purpose. *C. acutatum* on naturally infected symptomless strawberry leaves collected from production fields could be detected by this assay. Extensive latent spread of the pathogen was detected in the field, with a within-row dispersal distance up to at least 1.75 m per week (Debode et al. 2015). To distinguish four prevalent species causing strawberry anthracnose in China, including *C. fructicola*, *C. gloeosporioides*, *C. aenigma*, and *C. siamense,* a sensitive qPCR assay targeting cutinase gene was developed (Yang et al. 2022). The assay, with detection limit of 53 pg, could be used for rapid and high-throughput diagnosis of these pathogens in field plant samples.

High resolution melt (HRM) analysis

Rahman et al. (2019) developed an HRM analysis that could simultaneously detect *C. acutatum* and *C. gloeosporioides* latently infected in strawberry leaves (Rahman et al. 2019). The primer pair targeted the ITS region, and the assay allowed stable detection of the two *Colletotrichum* spp. in the middle-aged leaves. With the leaf samples at appropriate leaf stage, both SYBR® Green-based and TaqMan® assays could clearly identify *C. gloeosporioides* (84.3°C) and *C. acutatum* (85.4°C) by melt curve analysis. Another multiplex HRM assay was developed in Florida to rapidly and accurately detect the primary microorganisms causing strawberry crown rot including *Colletotrichum* spp., *Phytophthora* spp., and *Macrophomina phaseolina* (Wang et al. 2021). The assay could

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detect and differentiate these pathogens directly from symptomatic samples without electrophoresis. Although a special advanced equipment was essential to carry out the assay, an estimated reagent cost of US $0.20 per reaction made HRM economically feasible to apportion the initial costs in the long run (Verma et al. 2018).

Loop-mediated isothermal amplification (LAMP) assay

Two sets of primer pairs targeting the ITS region and *β-tubulin* gene were designed for LAMP assay (Zhang et al. 2016). While the LAMP assay using Ltub2 was specific for *C. acutatum*, the assay using LITSG1 not only detected *C. acutatum* but also *C. gloeosporioides* and *C. fragariae*. The detection limit of LITSG1 (20 pg) was 10-fold lower than Ltub2 (200 pg). For the detection on symptomless leaves, the freeze-incubate-sonicate-agitate (FISA) method was applied. The samples were first frozen, followed by incubated at 26°C in darkness for 2 days to promote sporulation. The conidia dislodged by sonication and agitation steps were used for DNA extraction and LAMP assay. Field trials in Florida and Iowa proved that both primer sets could successfully detect *C. acutatum* on asymptomatic leaves. In China, another LAMP assay targeting *β-tubulin* and *cytochrome b* (*cyt* b) genes were developed to detect *C. gloeosporioides* and G143A mutations associated with high resistance to pyraclostrobin (Wu et al. 2019). The detection limit was 10 pg, which was at least 10-fold more sensitive than conventional PCR. The process of diagnosis, including crude DNA extraction using a lateral-flow devise (LFDs) within 2 min and LAMP assay, could be accomplished in an hour. The LAMP assay could detect 1 pg of DNA on the ethidium bromide-stained agarose gel and 10 pg of DNA under natural light for hydroxynaphthol blue (HNB) color changed by naked eye. The detection of G143A mutation and determination of minimum inhibitory concentrations (MICs) of field isolates can provide useful information for chemical

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control of strawberry anthracnose. In 2021, Liu et al. combined LAMP and PCR to detect and identify the latently infected *Colletotrichum* spp. on strawberry (Liu et al. 2021). After 10 minutes of plant lysis, direct and rapid diagnosis of *Colletotrichum* spp. (at the species complex level) on strawberry could be done within an hour. The primers of LAMP assay were designed based on the conserved regions in *β-tubulin* gene from 13 *Colletotrichum* species of *C. gloeosporioides* and *C. acutatum* species complexes. The sensitivity was 100 pg of genomic DNA. PCR primers specific to ApMat, Marker 1 (the gene CGGC5_05943 encoding a hypothetical protein), and Marker 2 (an intergenic region) could subsequently be used to further determine the latent infection at the species level (Gan et al. 2017).

1.6 Current certification programs for strawberry nurseries in different countries and regions

To ensure the health of strawberry runner plants, disease inspection standards and certification programs were established in Europe, the United States, Japan, and Taiwan (Table 1.3 and Fig. 1.2).

1.6.1 The certification scheme for strawberry in European Union

The European and Mediterranean Plant Protection Organization (EPPO) certification scheme for strawberry was approved in 1994 and revised in 2008 (Anonymous 2008). The scheme describes the production process of strawberry material tested for pathogens. The whole process includes “Candidate plant”, “Nuclear stock”, “Propagation stock I”, “Propagation stock II” and “Certified material”. By combining hot air treatment and tissue culture, it removes viruses and virus-like organisms, and also fungi and bacteria. Plants produced through the above process, and then confirmed to be

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free of specific pathogens, are called “Candidate plants”. Different inspection standards were set up for different certified stages. For the nuclear stock, all plants must not be detected with specific pests and diseases. Propagation stock I without any symptoms is randomly tested, if any plant is detected with *Phytophthora fragariae* var. *fragariae*, *Colletotrichum acutatum*, or *Phytophthora cactorum*, the certification of the whole batch of plants will be refused. Infestation by various pests and diseases should not exceed the limits in propagation stock II stage: the tolerance of *Chaetosiphon fragaefolii* in visual inspection is only 1% of the plants; the tolerance of viruses, *Verticillium dahlia*, *V. albo-atrum* is 2%, Phytoplasmas, *Phytophthora cactorum*, *Rhizoctonia fragariae*, *Chaetosiphon fragaefolii* is 1% and plant presence of live mites (*Phytonemus pallidus fragariae*) is 0.1% in the certified material stage. Notably, the occurrence and detection of *C. acutatum* is not allowed at all stages of certification. In addition, the origin of each plant in the certified process should be recorded so that any health or trueness-to-type can be traced. The certified material must be marked with an official label, indicating the certification authority, the nursery supplier, and the certification status of the plants.

1.6.2 Strawberry Clean Plant Program in California, USA

In California, strawberry cultivars are developed by the Plant Sciences Department at the Davis campus of the University of California (UC Davis), patented and licensed by UC Davis Technology Transfer Services, and preserved and distributed by Foundation Plant Services (FPS 2008). FPS combines heat treatment and tissue culture to remove viruses and other pathogens to produce healthy strawberry planting stock. Relevant studies showed that even if the plants have no virus detected, heat treatment and tissue culture procedures helped the plants grow more vigorously and produce more daughter plants (at least 50% to 400%) than conventional propagation methods. Every year, FPS

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conducts disease testing of strawberry cultivars from UC Davis to ensure that the propagation stocks are free of key pathogens. The targeted pathogens and detection methods include viruses by PCR, graft index or herbaceous host index; phytoplasmas by PCR; *Phytophthora fragariae* var. *fragariae* by visual inspection; *Xanthomonas fragariae* by PCR and *Aphelenchoides besseyi* by visual inspection.

1.6.3 The UF/IFAS Strawberry Clean Plant Program in Florida, USA

In Florida, strawberries are cultivated in approximately 10,000 acres with annual production value up to US $300 millions (USDA 2018). Most growers use cultivars developed by the University of Florida/ The Institute of Food and Agricultural Sciences (UF/IFAS) strawberry breeding program at the UF/IFAS Gulf Coast Research and Education Center. The UF/IFAS Strawberry Clean Plant Program produces pathogen-tested planting stock according to the requirements of nurseries and fruit growers (Moyer 2019). The new cultivars are grown in tissue culture to ensure specific-pathogen free. Pathogen-tested planting stock are distributed to nurseries with transfer agreements and/or licensing contracts. Since the mother plant is derived from tissue culture, it has the juvenile characteristics of slow vegetative growth and late flowering and fruiting. Therefore, the whole propagation process takes at least about three years. Depending on the practices and conditions of the nursery, a single meristem plant can produce about one million plants in a three-year cycle, or about 20 million plants in a four-year cycle. Since strawberry nurseries use pathogen-tested meristem plants as the initial source of propagation, the spread of pathogens through mother plants should be less likely to occur. However, to ensure the production of healthy runner plants, further pathogen detection during the propagation in nurseries is necessary.

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1.6.4 Strawberry healthy nursery propagation system in Tochigi, Japan

Tochigi Prefecture is the largest strawberry producing area in Japan. It has more than 600 hectares of strawberry cultivation, and the strawberry healthy nursery propagation system has been implemented for decades. At present, 100% of farmers used the runners propagated following the healthy nursery supply system (鐘 2017). Most of the new cultivars released from Tochigi Prefecture are virus-free through tissue culture to obtain the nuclear stock. Four major viral diseases of strawberry in Japan, including strawberry vein banding virus (SVBV), strawberry crinkle virus (SCV), strawberry mottle virus (SmoV), and strawberry mild yellow edge virus (SMYEV), are detected by PCR. The plants of the nuclear stock in the provenance greenhouse are virus-free, and then the original stock is propagated from the nuclear stock. Plants propagated from the original stock are moved to the original nursery for cultivation, and these are the mother plants subsequently provided to farmers. Runners propagated from the mother plants in the nurseries are used for transplanting into the field (石川 2012). Because virus infection can reduce strawberry production by 30-50%, virus-free nuclear stocks are the basic requirement of Japan's strawberry nursery supply system. In addition, fungicides are frequently used during the nursery period to prevent the infection and spread of anthracnose rot. With the latent infection characteristics, it's difficult to identify whether the asymptomatic plants are infected by naked eyes. Therefore, anthracnose is also listed as the detected pathogen in the healthy nursery supply system.

1.6.5 A voluntary pathogen-free certification system for strawberry propagation in Taiwan

In Taiwan, most strawberry runner plants have been propagated by farmers themselves. However, since about 2010, anthracnose rot has become more and more

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serious, often causing an average of 20% of the runner plants infected during the nursery propagation period. The rate of replanting in a single field was even more than 70% in the early stage of planting. High temperature and humidity with frequent rain showers are very likely to cause an outbreak of anthracnose rot in the nursery stage during summer. To break through this dilemma, Bureau of Animal and Plant Health Inspection and Quarantine (BAPHIQ), Seed Improvement and Propagation Station (TSS), Taiwan Agricultural Research Institute (TARI), Miaoli District Agricultural Research and Extension Station (MDAIS), and Research Center for Plant Medicine of National Taiwan University jointed to draw up the Instructions for Verification Diseases of Strawberry Nurseries, which was announced in 2018. The target diseases for inspection included fungal diseases caused by *Fusarium oxysporum* and *Colletotrichum* spp., viral disease caused by strawberry mild yellow edge virus (SMYEV), and nematode disease caused by the root-rot nematode (*Pratylenchus* sp.). The propagation process consists of four stages: nuclear stock (G0) refers to the materials that have been inspected with specific pathogens and then tissue cultured; Generation I (G1) refers to the plants derived from tissue culture and are being acclimated. The plants of Generation II (G2) serve as healthy mother plants, from which Generation III (G3) was propagated in the nursery and used for transplanting into the production field.

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1.8 Tables

Table 1.1. Culture-based methods for detection latent infection of *Colletotrichum* spp. in strawberry.

Detection method[a]	*Colletotrichum* spp.	Tissues for detection	Processing time	References
Paraquat	*C. acutatum*	Petiole	3 days	Cook (1993)
SDEI	*Glomerella cingulata*	Leaf	10 days	Ishikawa (2003)
Freezing	*C. acutatum*, *C. gloeosporioides*, *C. dematium*	Petiole	5-7 days	Mertely and Legard (2004)

[a]SDEI: simple diagnosis using ethanol immersion.

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Table 1.2. PCR-based methods for detection latent infection of *Colletotrichum* spp. in strawberry.

Detection method[a]	*Colletotrichum* spp.	Primer target region[b]	Sensitivity[c]	References
Conventional PCR	*C. acutatum*	ITS	100 fg	Sreenivasaprasad et al. (1996)
			-	Freeman et al. (2001)
			-	Parikka and Lemmetty (2004)
PCR-MTH	*C. acutatum*, *Glomerella cingulata*	*MAT1-2*	-	Furuta et al. (2017)
Nested PCR	*C. acutatum*	ITS	1 fg	Perez-Hernandez et al. (2008)
	C. siamense, *C. fructicola*	intergenic region between *ladA*/*xyl1*	1 pg	Chung et al. (2022) This thesis
Real-time PCR	*C. acutatum*	ITS	50 fg	Debode et al. (2009)
	C. acutatum, *C. gloeosporioides*	ITS	-	Rahman et al. (2019)
	C. fructicola, *C. aenigma*, *C. siamense* and *C. gloeosporioides*	*cutinase*	53 pg	Yang et al. (2022)

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LAMP	*C. acutatum*, *C. fragariae*, *C. gloeosporioides*	ITS	20 pg	Zhang et al. (2016)
	C. acutatum	*TUB2*	200 pg	
	C. gloeosporioides		10 pg	Wu et al. (2019)
	Colletotrichum spp.		100 pg	Liu et al. (2021)
HRM	*C. acutatum*, *C. gloeosporioides*	ITS	1 pg	Wang et al. (2021)

[a]PCR: polymerase chain reaction; MTH: microtube hybridization; LAMP: Loop-mediated isothermal amplification; HRM: High resolution melt.

[b]ITS: internal transcribed spacer; *MAT1-2*: mating type gene; *ladA*: L-arabinitol 4-dehydrogenase; *xyl1*: NAD(P)H-dependent D-xylose reductase; *TUB2*: beta-tubulin.

[c]-: not available/applicable.

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Table 1.3. Current certification programs for strawberry nurseries in different countries and regions.

Country/region	Tissue Culture	Pathogens tested[a]	Detection methods[b]	Certification standards[c] 1	2	3	4
European Union	Yes	Viruses (SCV, SMYEV, SMoV, SVBV etc.)	Graft or PCR	0%	0%	0%	2%
		Phytoplasmas	PCR	0%	0%	0%	1%
		Xanthomonas fragariae	PCR	0%	0%	0%	0%
		Phytophthora cactorum	Microscopic inspection (sporangia)	0%	0%	0%	1%
		Phytophthora fragariae var. *fragariae*	Bait test	0%	0%	0%	0%
		Colletotrichum acutatum	Paraquat test	0%	0%	0%	0%
		Stem nematode (*Ditylenchus dipsaci*) and leaf and bud nematodes (*Aphelenchoides besseyi*, *A. blastophthorus*, *A. fragariae* and *A. ritzemabosi*)	Baermann funnel technique	0%	0%	0%	0%
California in USA	Yes	Viruses (SCV, SMYEV, SMoV, SVBV etc.)	PCR, Graft index	Annual testing for mother plants			
		Phytoplasmas	PCR				
		Xanthomonas fragariae	PCR				
		Phytophthora fragariae var. *fragariae*	Visual inspection				
		Aphelenchoides besseyi	Visual inspection				
		Viruses (SCV, SMYEV, SMoV, SVBV etc.)	PCR or ELISA	0%			

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Florida in USA	Yes	Phytoplasmas	PCR	0%	No description		
		Xanthomonas fragariae	PCR	0%			
		Phytophthora spp.	ELISA	0%			
		Colletotrichum spp.	Selective medium	0%			
Tochigi in Japan	Yes	Viruses (SCV, SMYEV, SMoV, SVBV)	PCR	0%	No description		
		Colletotrichum spp.	SDEI	0%			
		Fusarium oxysporum f. sp. *fragariae*	SDEI	0%			
Taiwan	Yes	*Fusarium oxysporum*	Selective medium	0%	0%	0%	0%
		Colletotrichum spp.	Nested PCR and SDEI	0%	0%	0%	0%
		Virus (SMYEV)	PCR	0%	0%	0%	0%
		Pratylenchus sp.	Visual inspection or PCR	0%	0%	0%	0%

[a]SCV: strawberry crinkle virus; SMYEV: strawberry mild yellow edge virus; SMoV: strawberry mottle virus; SVBV: strawberry vein banding virus. Only the common detection virus types are listed in the table, other detection targets in each region are as follows:

- European Union: arabis mosaic virus (ArMV), raspberry ringspot virus (RpRSV), strawberry latent ringspot virus (SLRV) and tomato black ring virus (TBRV).
- California in USA: arabis mosaic virus (ArMV), raspberry ringspot virus (RpRSV), strawberry latent “C” virus (SLCV), strawberry latent ringspot virus (SLRV), tobacco necrosis virus (TNV), tobacco ringspot virus (TRSV), strawberry necrotic shock virus (SNSV), tomato black ring virus (TBRV), tomato bushy stunt virus (TBSV), and tomato ringspot virus (ToRSV).

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- Florida in USA: arabis mosaic virus (ArMV), beet pseudo yellows clostero virus (BPYV), raspberry ringspot virus (RpRSV), strawberry latent ringspot virus (SLRV), strawberry necrotic shock virus (SNSV), strawberry pallidosis virus (SPV), tobacco ringspot virus (TRSV), tobacco streak virus (TSV), tomato black ring virus (TBRV), and tomato ringspot virus (ToRSV).

[b]PCR: polymerase chain reaction; ELISA: enzyme-linked immunosorbent assay; SDEI: simple diagnosis using ethanol immersion.

[c]Certification scheme of different country or organization:

- EPPO: 1-Nuclear stock, 2-Propagation stock I, 3-Propagation stock II, 4-Certified material.
- Florida, Tochigi, and Taiwan: 1-Nuclear stock, 2-Generation I, 3- Generation II, 4- Generation III.

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1.9 Figures

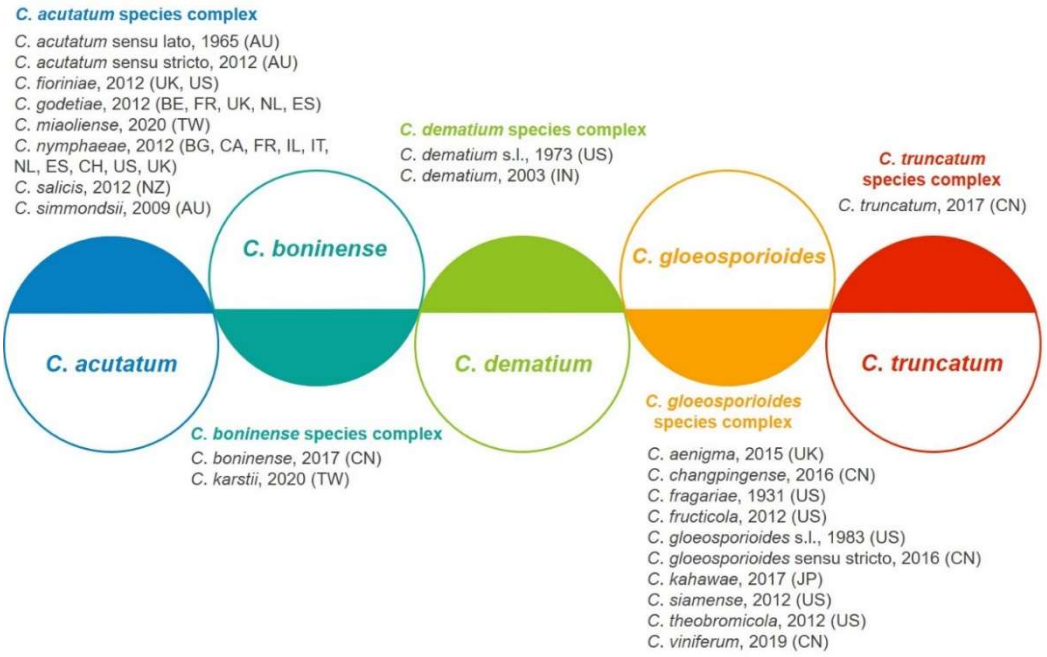

Fig. 1.1. *Colletotrichum* spp. associated with strawberry.

AU: Australia. UK: United Kingdom. US: United States of America. BE: Belgium. FR: France. NL: Netherlands. ES: Spain. TW: Taiwan. BG: Bulgaria. CA: Canada. IL: Israel. IT: Italy. CH: Switzerland. NZ: New Zealand. CN: China. IN: India. JP: Japan.

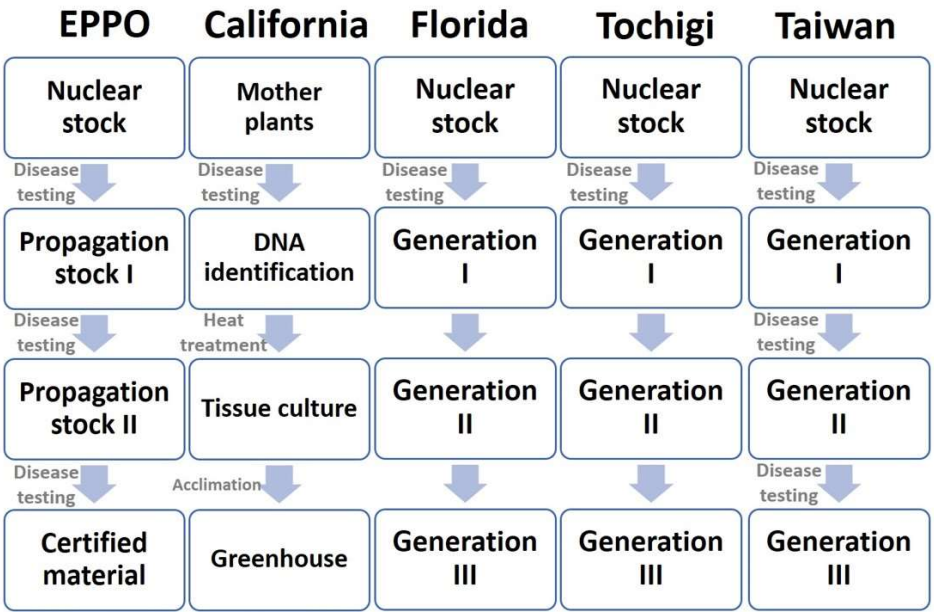

Fig. 1.2. Certification procedures in different countries and regions.

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Chapter 2

Diversity and pathogenicity of *Colletotrichum* species causing strawberry anthracnose in Taiwan and description of a new species, *Colletotrichum miaoliense* sp. nov.

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2.1 Abstract

Strawberry is a small fruit crop with high economic value. Anthracnose caused by *Colletotrichum* spp. poses a serious threat to strawberry production, particularly in warm and humid climates, but knowledge of pathogen populations in tropical and subtropical regions is limited. To investigate the diversity of infectious agents causing strawberry anthracnose in Taiwan, a disease survey was conducted from 2010 to 2018, and *Colletotrichum* spp. were identified through morphological characterization and multilocus phylogenetic analysis with internal transcribed spacer, glyceraldehyde 3-phosphate dehydrogenase, chitin synthase, actin, beta-tubulin, calmodulin, and the intergenic region between *Apn2* and *MAT1-2-1* (ApMAT). Among 52 isolates collected from 24 farms/nurseries in Taiwan, a new species, *Colletotrichum miaoliense* sp. nov. (6% of all isolates), a species not previously known to be associated with strawberry, *Colletotrichum karstii* (6%), and three known species, *Colletotrichum siamense* (75%), *Colletotrichum fructicola* (11%), and *Colletotrichum boninense* (2%), were identified. The predominant species *C. siamense* and *C. fructicola* exhibited higher mycelial growth rates on potato dextrose agar and caused larger lesions on wounded and non-wounded detached strawberry leaves. *Colletotrichum boninense*, *C. karstii*, and *C. miaoliense* only caused lesions on wounded leaves. Understanding the composition and biology of the pathogen population will help in disease management and resistance breeding.

2.2 Introduction

Strawberry (*Fragaria* × *ananassa* Duch.) is a popular small fruit crop with high economic and nutritive value. Strawberry is in high demand globally. From 2008 to 2018, the annual worldwide cultivation of strawberries increased from approximately 400 to 483 thousand hectares (FAOSTAT 2020). Although strawberries are native to temperate

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regions, they can also be grown in tropical and subtropical regions (sometimes under high-altitude conditions). The land areas devoted to strawberry cultivation in Colombia, Peru, Guatemala, Bolivia, and Taiwan in 2018 were 1482 ha, 1453 ha, 690 ha, 522 ha, and 506 ha, respectively (FAOSTAT 2020).

Anthracnose caused by *Colletotrichum* spp. is a serious threat to strawberry production, especially in warm and humid climates (Howard et al. 1992). Rain-splashed conidia of *Colletotrichum* spp. serve as the major inoculum causing epidemics of strawberry anthracnose disease (Penet et al. 2014). After landing on the plant surface, the conidia germinate, form appressoria, then penetrate the epidermal cells (Perfect et al. 1999). *Colletotrichum* spp. can infect various strawberry tissues, causing black spots or irregular spots on leaves, sunken black spots or necrosis lesions on petioles, stolons, and fruits, and wilting of the whole plant due to crown rot (Howard et al. 1992). Under high humidity, concentric rings of acervuli with orange conidial masses can be observed on necrotic tissues. In the US state of Florida, anthracnose causes the death of up to 80% of seedlings in the nursery and yield losses of over 50% in the field (Howard et al. 1992). In Taiwan, strawberry seedlings are propagated from March to September, and the high temperature, high humidity and heavy rainfall during this period provide a suitable environment for epidemics. From 2010 to 2016, anthracnose crown rot caused the loss of 30%-40% of seedlings and ~20% of plants after transplanting (Chung et al. 2019).

Colletotrichum spp. have traditionally been classified based on the shape of the conidia and appressorium, the presence of a seta or perithecium, and culture characteristics (Gunnell and Gubler 1992; Cannon et al. 2012). Using these criteria, early studies reported *C. acutatum*, *C. gloeosporioides*, and *C. fragariae* as strawberry anthracnose pathogens (Freeman and Katan 1997; Howard et al. 1992). However, *Colletotrichum* spp. share similar features, and morphological characteristics can be

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influenced by environmental factors including culture media, light, and temperature (Cai et al. 2009; Hyde et al. 2009; Vieira et al. 2017). Therefore, a polyphasic approach based on morphology and genetic characteristics was proposed for identification of *Colletotrichum* species (Cai et al. 2009). A combination of multiple gene sequences, including internal transcribed spacer (ITS), glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*), chitin synthase (*CHS-1*), actin (*ACT*), beta-tubulin (*TUB2*), calmodulin (*CAL*), and the intergenic region between *Apn2* and *MAT1-2-1* (ApMAT), can provide more molecular features to resolve different species in a *Colletotrichum* species complex (Damm et al. 2012a; Weir et al. 2012). Through multilocus sequence analysis coupled with morphological characterization, recent studies have identified many additional *Colletotrichum* species associated with strawberry, namely *C. acutatum*, *C. fioriniae*, *C. godetiae*, *C. nymphaeae*, *C. salicis* and *C. simmondsii* (*C. acutatum* species complex), *C. aenigma*, *C. changpingense*, *C. fructicola*, *C. gloeosporioides*, *C. siamense* and *C. theobromicola* (syn. *C. fragariae*) (*C. gloeosporioides* species complex) and *C. boninense* (*C. boninense* species complex) (Baroncelli et al. 2015; Bi et al. 2017; Chen et al. 2020; Damm et al. 2012a; Gunnell and Gubler 1992; Han et al. 2016; Jayawardena et al. 2016; Weir et al. 2012).

Although strawberry is of great economic importance in Taiwan and anthracnose has become more destructive in the past decade, the pathogen population in Taiwan has not been systematically investigated. The causal agents of strawberry anthracnose were previously reported to be *C. gloeosporioides* (Tzean et al. 2019), *C. dematium*, *C. fragariae*, and *C. acutatum* (Plant Protection Information System; https://otserv2.tactri.gov.tw/ppm/), but information about the isolation, pathogenicity, morphology, and sequences of these species is not sufficient for species identification. Recently, based on analysis of multiple gene sequences, we identified *C. siamense* as the

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pathogen causing anthracnose crown rot (Chung et al. 2019). To provide accurate information for disease control and resistance breeding, in this study we aimed to reveal the population composition of the infectious agents associated with strawberry anthracnose in Taiwan. Samples collected from the major strawberry-producing areas of Taiwan from 2010 to 2018 were subjected to morphological and multi-gene phylogenetic analyses. To further understand the *in vitro* and *in planta* aggressiveness of different *Colletotrichum* spp. at different temperatures, multiple representative isolates of each species were tested for mycelial growth rates in an artificial medium as well as the ability to cause lesions on wounded or non-wounded strawberry leaves. Since population analysis of *Colletotrichum* spp. causing strawberry anthracnose has only been reported for species from the UK (Baroncelli et al. 2015) and China [Anhui, Hainan (only one isolate), Hebei, Hubei, Liaoning, Shandong, and Zhejiang Provinces and Beijing and Shanghai cities] (Chen et al. 2020; Han et al. 2016; Jayawardena et al. 2016), which, with the exception of Hainan, are geographical regions located at higher latitudes (30-53° N) relative to Taiwan (24.5° N), this study will provide insights into the biology of strawberry anthracnose disease in subtropical regions.

2.3 Materials and methods

2.3.1 Sample collection and pathogen isolation

From 2010 to 2018, different strawberry tissues (including the leaf, stolon, fruit, root and crown) showing anthracnose disease symptoms were collected from 24 farms and nurseries located in Miaoli, Hsinchu, Nantou, and Chiayi Counties in Taiwan. From 2009 to 2018, the strawberry-cultivated areas in Miaoli, Hsinchu, Nantou, and Chiayi accounted for approximately 89.6%, 2.8%, 2.6%, and 0.2% of the total strawberry-cultivated area in Taiwan, respectively. Pure cultures of all fungal isolates were obtained

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by the single hyphal tip isolation method. Approximately 2 × 2 mm fragments bordering healthy and necrotic zones in diseased tissues were surface-sterilized with 0.5%-1% sodium hypochlorite, rinsed with sterile deionized water three times, then placed onto 1.5% water agar. After 2-3 days of incubation at 25°C, single hyphal tips were transferred to potato dextrose agar (PDA, BD Difco) and cultured for further use. A total of 52 *Colletotrichum* spp. isolates were used in this study (Table 2.1): 26 (50%) isolated from crowns, 11 (21.2%) from leaves, 5 (9.6%) from fruits, 5 (9.6%) from roots, and 5 (9.6%) from stolons (Table 2.1). Type specimens in this study were deposited in the herbarium of the Department of Plant Pathology and Microbiology, National Taiwan University (NTUH). Ex-type living cultures were deposited in the Culture Collection of the Department of Plant Pathology and Microbiology, National Taiwan University (NTUCC), Bioresource and Collection Research Center (BCRC), and Miaoli District Agricultural Research and Extension Station (MDAIS). Nomenclature and taxonomic information were deposited in MycoBank (Crous et al. 2004) (www.mycobank.org). *Colletotrichum* spp. were preserved as mycelium discs in ddH_2O at 4°C for short-term storage and in 10% glycerol with 5% lactose at -80°C for long term storage. Before conducting experiments, each isolate was revived by culturing on PDA for 5-7 days at 25°C under a 12-h/12-h photoperiod.

2.3.2 DNA extraction, PCR amplification, and sequencing

For each *Colletotrichum* spp. isolate, the mycelium was taken from a 7-day-old culture grown on PDA medium. The mycelium was frozen in liquid nitrogen and ground into a fine powder using a sterile mortar and pestle. Genomic DNA was extracted using the Plant Genomic DNA Extraction Miniprep System Kit (VIOGENE) according to the manufacturer's instructions. Seven genetic fragments, namely ITS, *GAPDH*, *CHS-1*, *ACT*,

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TUB2, *CAL*, and ApMAT, were amplified with the primers listed in Supplementary Table 5 using the Biometra Thermal Cycler (Biometra TRIO). Each PCR reaction contained 1 µl of genomic DNA (20-50 ng), 5 µl of 10X reaction buffer [with Tris-HCl (pH 9.0), PCR enhancers, $(NH_4)_2SO_4$, 20 mM $MgCl_2$], 2 µl of dNTPs (2.5 mM each), 1 µl of 10 µM forward primer, 1 µl of 10 µM reverse primer, 0.5 µl (2.5 U) of Prime Taq DNA Polymerase (GenetBio Inc.), and 39.5 µl of ddH_2O. The thermal cycling parameters were 1 cycle of 95°C for 4 min and 30-35 cycles of 95°C for 30 s, 52-62°C for 30 s, and 72°C for 60 s followed by a final extension step of 72°C for 7 min. The optimal annealing temperatures for different genetic regions were: ITS: 58°C, *GAPDH*: 52°C, *CHS-1*: 58°C, *ACT*: 58°C, *TUB2*: 58°C, *CAL*: 59°C, and ApMAT: 62°C. Amplicons were bidirectionally sequenced using the dideoxy termination method on the ABI 3730 DNA Analyzer (Tri-I Biotech, Taiwan). Raw sequence chromatograms were manually examined, and the sequences of each fragment were assembled in ApE v2.0.55 (A Plasmid Editor, M. Wayne Davis at the University of Utah, Salt Lake City, UT).

2.3.3 Multilocus phylogenetic analysis and species recognition

Newly generated sequences from each isolate were blasted against the GenBank nr database, and searches were restricted to type materials for initial determination of the closest matching species and species complex. Related gene sequences (ITS, *GAPDH*, *CHS-1*, *ACT*, *TUB2*, *CAL*, and ApMAT) of *Colletotrichum* spp. from recent publications were downloaded from GenBank (Damm et al. 2012a; Damm et al. 2012b; Weir et al. 2012). For each gene, sequences from the isolates belonging to the same species complex were aligned using the MAFFT v7 online server (https://mafft.cbrc.jp/alignment/server/) (Katoh and Standley 2013). The aligned sequences were manually edited using MEGA

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v10 (Kumar et al. 2018) to improve the alignment. The post-alignment sequences of multiple genes/loci were concatenated in a text editor.

BI, ML, and MP approaches for each individual locus and the concatenated sequences were used to identify closely related taxa. Best-fit models of nucleotide substitution were selected using the Akaike information criterion implemented in MrModeltest v.2.4 (Nylander 2004) and run in PAUP v.4.0 (L. Swofford 2002) (Supplementary Table 6). BI analyses were performed using MrBayes v.3.2.6 (Ronquist and Huelsenbeck 2003). Two independent analyses of four Markov Chain Monte Carlo (MCMC) chains (3 heated, 1 cold) were run from a random tree for 2 x 10^6 (for the *C. acutatum* species complex), 4 x 10^6 (for the *C. boninense* species complex) and 6 x 10^6 (for the *C. gloeosporioides* species complex) generations or until the average standard deviation of split frequencies was below 0.01. The analysis was sampled every 1000 generations, and the first 25% of the generations were discarded as burn-in. The effective sample size and convergence were monitored with Tracer v1.7.1 (Rambaut et al. 2018). MP analyses were performed in PAUP v.4.0 (L. Swofford 2002) using the heuristic search option with Tree Bisection Reconnection branch swapping and 100 random sequence addition. Maxtrees were set to 5000 and bootstrap analysis was performed with 1000 replicates. ML analyses were performed in RAxML v8.2.10 (Stamatakis 2014) using the GTR-gamma substitution model with 1000 bootstrap replicates. Phylogenetic trees were visualized in FigTree v1.4.3. The concatenated alignments and phylogenetic trees were deposited in TreeBASE (www.treebase.org) with the study ID 26665.

We applied the Genealogical Concordance Phylogenetic Species Recognition (GCPSR) method (Dettman et al. 2003; Taylor et al. 2000) for species delimitation of *Colletotrichum* taxa. A novel species was considered novel the clade was strongly supported as monophyletic by BI (posterior probability ≥ 0.95), ML (bootstrap ≥ 70%),

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and MP (bootstrap ≥ 70%) analyses in the multilocus phylogenetic tree and in the majority of individual gene trees.

2.3.4 Morphological characterization

Morphological characterization of one selected isolate from each *Colletotrichum* species was conducted following the procedures of Weir et al. (2012) and Damm et al. (2012) (Damm et al. 2012a; Damm et al. 2012b; Weir et al. 2012). Cultures were grown on PDA and 1/4-strength PDA (1/4 PDA) (Su et al. 2012) for 2-3 weeks at 25°C and 30°C under a 12-h/12-h photoperiod. (Our preliminary tests showed that compared with PDA, the low nutrient medium 1/4 PDA could stimulate sporulation without affecting the size and shape of conidia.) The experiment was performed in two independent trials, each consisting of two to three plates per isolate. Conidiomata were investigated using a dissecting microscope (Leica M125). Conidia, conidiophores, setae, asci, ascomata and appressoria were examined using a light microscope (Leica DM2500). To induce the formation of appressoria, 20-30 µl of conidial suspension (prepared using sterile dH_2O) was dropped onto a microscope slide, covered with a cover slip, then incubated in a moist chamber at 25°C for 2-3 days (Yang et al. 2009). The lengths and widths of 55 conidiogenous cells, 100 conidia and 30 appressoria were measured using ImageJ software (Rueden et al. 2017).

2.3.5 Effect of temperature on mycelial growth

Mycelium-agar discs (6 mm in diameter) were cut with a sterilized cork borer from the advancing edge of 5- to 7-day-old *Colletotrichum* spp. colonies then placed (with the mycelium-side down) onto the center of a 90 mm petri dish containing 25 ml PDA. Colony diameters were measured after 7 days of incubation at different temperatures under a 12-h/12-h photoperiod in a growth chamber (Firstek, GC-560H). The mycelial

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growth rate (mm/day) was calculated as "(the diameter of the colony – the diameter of the mycelium-agar disc) / 7". For one selected isolate from each *Colletotrichum* species, the mycelial growth rates at 18°C, 22°C, 25°C, 28°C, 30°C, and 32°C were determined in two to three independent trials, each consisting of three PDA plates per isolate per temperature. The optimum temperature for the mycelial growth rate was estimated based on the Gaussian process (4 parameter) for nonlinear regression in SigmaPlot 14 (Systat Software, San Jose, CA). Growth of an additional two representative isolates of each *Colletotrichum* species, selected from distinct subclades within the species based on the multilocus phylogenetic analysis, was measured at 25°C and 30°C in two independent trials, each consisting of four PDA plates per isolate per temperature.

2.3.6 Pathogenicity assay

The susceptible cultivar 'Taoyuan No. 1' was used for all inoculation tests in this study. The pathogenicity of *Colletotrichum* spp. (one isolate from each *Colletotrichum* species) was examined via Koch's postulates. Strawberry seedlings at the four- to five-leaf stage were inoculated by spraying a spore suspension on the leaves until runoff (10^6 spores/ml), and also applying 1 ml of spore suspension (10^6 spores/ml) on the crown region after removal of one to two old leaves. After inoculation, the seedlings were covered with plastic bags (> 90% relative humidity) for 3 days at 30°C and then incubated in a growth chamber for 11 days at 30°C and 70% relative humidity under a 12-h/12-h photoperiod. The fungi were re-isolated from lesions of diseased tissues (as mentioned above using the single hypha tip isolation method), then identified based on morphological characteristics and ITS sequences as described above.

The virulence levels of different *Colletotrichum* spp. isolates were determined by inoculation of wounded and non-wounded detached strawberry leaves. Fully expanded

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healthy leaves were collected from strawberry seedlings at the four- to five-leaf stage. For inoculation of wounded leaves, each leaflet was punctured with a sterile syringe needle on the left and right sides of the midrib, and 10 μl of a spore suspension (1 x 10^6 spores/ml) was deposited on the left wound site, and sterile dH_2O was deposited on the right wound site as a control. Similarly, inoculations of non-wounded leaves were performed in the same way as mentioned above. After inoculation, the leaves were kept in a moist chamber (a plastic box with dH_2O at the bottom; the cut end of the petiole was submerged in the water) at 25°C or 30°C under a 12-h/12-h photoperiod. Lesion size was measured at 7 and 14 days post inoculation (dpi). Lesions smaller than 0.1 cm in diameter were considered unsuccessful infections. The same isolates for the "effect of temperature on mycelial growth" test were used for the pathogenicity assay. For one selected isolate from each *Colletotrichum* species, inoculations of wounded and non-wounded leaves were performed at 25°C and 30°C in three independent trials, each consisting of 12 leaflets (4 leaves from 4 seedlings) per isolate per treatment. An additional two representative isolates of each *Colletotrichum* species, selected from distinct subclades within the species based on the multilocus phylogenetic analysis, were used for wound inoculation at 30°C. The experiment was performed in two independent trials, each consisting of 12 leaflets (4 leaves from 4 seedlings) per isolate per treatment.

2.3.7 Statistical Analysis

Data were analyzed by analysis of variance (ANOVA) using the software SPSS v18. Tukey's range test or Student's *t*-test was used to test for significant differences among or between different treatments at a significance threshold of $P < 0.05$.

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2.4 Results

2.4.1 Molecular identification and phylogenetic analysis

Colletotrichum spp. isolates were first identified at the species complex level. Among 52 *Colletotrichum* spp. isolates sampled from the major strawberry-producing areas of Taiwan, 45 (86.5%) isolates belonged to the *C. gloeosporioides* species complex, 4 (7.7%) belonged to the *C. boninense* species complex, and 3 (5.8%) belonged to the *C. acutatum* species complex (Table 2.1).

To further analyze the *C. acutatum* species complex, 3 isolates together with 40 reference isolates, including the outgroup *C. orchidophilum* (CBS 632.80), were used to construct phylogenetic trees with five gene sequences (ITS, *GAPDH*, *CHS-1*, *ACT*, and *TUB2*) (Table 2.1 and Supplementary Table 1) following Damm et. al. (2012) (Damm et al. 2012a) and Fu et. al. (2019) (Fu et al. 2019). The final data matrix contained a total of 1821 characters with gaps (ITS: 1–540, *GAPDH*: 541–799, *CHS-1*: 800–1081, *ACT*: 1082–1329, *TUB2*: 1330–1821), of which 237 characters were parsimony informative, 174 parsimony uninformative, and 1410 constant. After 2,000,000 generations of topological convergence via Bayesian inference (BI) analysis, 2,378 trees were obtained. The first 25% of the trees were discarded, representing the burn-in phase of the analyses, and the remaining trees were used to calculate the Bayesian posterior probabilities in the majority rule consensus tree (Fig. 2.1). The maximum likelihood (ML) analysis resulted in a best scoring RAxML tree with a final optimized likelihood value of -6726.174303. The most parsimonious tree resulted from the maximum parsimony (MP) analysis received tree length = 692, consistency index (CI) = 0.714, and retention index (RI) = 0.843. All three isolates (ML1040, ML1042, ML1794) were grouped in a distinct clade with significant statistical support in the multilocus phylogenetic analysis (1/100/100, BI/ML/MP) (Fig. 2.1) and the single gene trees of *GAPDH*, *CHS-1*, and *TUB2*

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(Supplementary Fig. S1). This clade was distinct from all other known species, and is herein described as a new species, *C. miaoliense* sp. nov.

To analyze the phylogeny of the *C. boninense* species complex, six gene sequences (ITS, *GAPDH*, *CHS-1*, *ACT*, *TUB2* and *CAL*) from 4 isolates together with 31 reference isolates, including the outgroup sequence of *C. gloeosporioides* (IMI 356878), were used to construct phylogenetic trees (Table 2.1 and Supplementary Table 1). The final data matrix contained a total of 2363 characters with gaps (ITS: 1–558, *GAPDH*: 559–852, *CHS-1*: 853–1132, *ACT*: 1133–1411, *TUB2*: 1412–1914, *CAL*: 1915–2363), of which 365 characters were parsimony informative, 407 parsimony uninformative, and 1591 constant. After 1,187,000 generations of topological convergence via BI analysis, 492 trees were obtained. The first 25% of the trees were discarded, representing the burn-in phase of the analyses, and the remaining trees were used to calculate the Bayesian posterior probabilities in the majority rule consensus tree (Fig. 2.2). The ML analysis resulted in a best scoring RAxML tree with a final optimized likelihood value of -10025.941645. The most parsimonious tree resulted from the MP analysis received tree length = 1281, CI = 0.774, and RI = 0.839. In single gene trees of *GAPDH*, *CHS-1*, and *TUB2* and the multilocus phylogenetic tree, three isolates (ML351, 442, 1792) clustered with strong statistical support in the clade containing the type strain CGMCC 3.14194 and other related isolates of *C. karstii* (Fig. 2.2; single gene trees not shown). In single gene trees of *GAPDH* and *CAL* and the multilocus phylogenetic tree, the isolate ML521 clustered with strong statistical support in the clade containing the type strain CBS 123755 and other related isolates of *C. boninense* (Fig. 2.2; single gene trees not shown).

To identify species in the *C. gloeosporioides* species complex, a combination of seven gene sequences (ITS, *GAPDH*, *CHS-1*, *ACT*, *TUB2*, *CAL* and ApMAT) from 45 isolates together with 47 reference isolates, including the outgroup sequence of *C.*

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boninense (CBS 123755), were used to construct phylogenetic trees (Table 2.1 and Supplementary Table 1). The final data matrix contained a total of 3571 characters with gaps (ITS: 1–553, *GAPDH*: 554–808, *CHS-1*: 809–1051, *ACT*: 1052–1325, *TUB2*: 1326–2028, *CAL*: 2029–2728, ApMAT: 2729–3571), of which 655 characters were parsimony informative, 735 parsimony uninformative, and 2181 constant. After 6,574,000 generations of topological convergence via BI analysis, 9,864 trees were obtained. The first 25% of the trees were discarded, representing the burn-in phase of the analyses, and the remaining trees were used to calculate the Bayesian posterior probabilities in the majority rule consensus tree (Fig. 2.3). The ML analysis resulted in a best scoring RAxML tree with a final optimized likelihood value of -18514.217014. The most parsimonious tree resulted from the MP analysis received tree length = 2400, CI = 0.722, and RI = 0.876. In single gene trees of *TUB2*, *CAL*, and ApMAT and the multilocus phylogenetic tree, 39 isolates clustered with strong statistical support in the clade containing the type strain CBS 130417 and other related isolates of *C. siamense* (Fig. 2.3; single gene trees not shown). The 39 isolates formed a subclade with a high support value (1/100/98, BI/ML/MP) (Fig. 2.3). In single gene trees of *GAPDH*, *ACT*, *TUB2*, *CAL*, and ApMAT and the multilocus phylogenetic tree, six isolates clustered with strong statistical support in the clade containing the type strain CBS 130416 and other related isolates of *C. fructicola* (Fig. 2.3; single gene trees not shown).

2.4.2 Taxonomy

Based on morphological traits and multilocus phylogenetic analysis, the 52 isolates were assigned to five *Colletotrichum* spp. including one new taxon (*C. miaoliense* sp. nov.) (Fig. 2.4; described in detail below), one newly recorded taxon in strawberry (*C. karstii*), and three species known to be associated with strawberry anthracnose (*C.*

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boninense, *C. fructicola* and *C. siamense*) (Supplementary Fig. S2-S5). The colony features that developed at 25°C on PDA and 1/4 PDA were all white to grey, with orange conidia ooze. *C. siamense* ML133 and *C. karstii* ML351 produced abundant conidia when cultured on 1/4 PDA at 25°C; *C. boninense* ML521 produced more conidia on PDA at 25°C; *C. fructicola* ML348 and *C. miaoliense* ML1040 sporulated more abundantly on 1/4 PDA at 30°C. The conidium and appressorium measurements of the five *Colletotrichum* spp. (isolates from this study and the type strains) are listed in Supplementary Table 2. The conidia produced by *C. miaoliense* ML1040 were longer [length to width (L/W) ratio = 3.4] (Fig. 2.4) than the conidia of the other four species in this study (L/W ratio = 2.3-3) (Fig. S2-S5; Supplementary Table 2).

***Colletotrichum miaoliense* sp. nov. P. C. Chung & H. Y. Wu. Fig. 2.4.**

MycoBank number MB835424.

Etymology: The epithet *miaoliense* specifically refers to Miaoli County, Taiwan, where the new taxon was discovered.

Sexual morph not observed. *Asexual morph* observed on 1/4 PDA [BCRC FU31304 (= NTUCC 20-001-1, ML1040]. Vegetative hyphae 3–6 µm in diameter, hyaline, smooth-walled, septate, branched. Chlamydospores not observed. Sporodochia developed, conidiophores formed directly on hyphae. Conidiophores hyaline, smooth-walled, simple or branched. Conidiogenous cells hyaline, smooth-walled, cylindrical to ampulliform, often integrated, occasionally polyphialidic; phialides discrete, 5.9–26.4 µm ($\overline{x}$ = 13.3 ± 4.8, n = 55) in length, apical opening 1.1–2.6 µm in diameter (1.7 ± 0.3, n = 55). Conidia hyaline, smooth-walled, aseptate, straight, fusiform to cylindrical, acute ends, 11.2–17 × 3.3–5 µm ($\overline{x}$ = 14.2 ± 1.1 × 4.1 ± 0.3 µm, n = 100), L/W ratio = 3.4. Conidia from aerial hyphae varied in size (6.6–20 × 2.9-4.9 µm, $\overline{x}$ = 11.2 ± 2.5 × 3.8 ± 0.4 µm, n=100), L/W ratio = 3.0. Seta absent. Appressoria single or in loose clusters, pale brown, smooth-

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walled, elliptical to clavate, entire edge, 5.9–9.1 × 4–6.0 μm ($\overline{x}$ = 7.5 ± 1.1 × 5 ± 0.6 μm, n = 27), L/W ratio = 1.5.

Culture features: Colonies on PDA flat to somewhat raised, margin entire; mycelium partly floccose, white to pale olivaceous grey; sporodochia orange, scattered in rings, reverse bright orange to orange; average 4.2 cm in diameter in 7 days at 25 °C. Conidia ooze was visible as an orange mass.

Material examined: Taiwan, Miaoli County, Shitan Township, from crown rot of *Fragaria* × *ananassa*, 28 Oct. 2016, P.-C. Chung; holotype NTUH 20-001-1, ex-holotype living culture BCRC FU31304 (= NTUCC 20-001-1, ML1040).

Additional materials examined: Taiwan, Nantou County, Renai Township, from leaf spot of *Fragaria* × *ananassa*, 24 Nov. 2016, P.-C. Chung, NTUH 20-001-2; living culture NTUCC 20-001-2 (= ML1042). Taiwan, Nantou County, Renai Township, from leaf spot of *Fragaria* × *ananassa*, 4 Jul. 2018, P.-C. Chung; NTUH 20-001-3, living culture NTUCC 20-001-3 (= ML1794). Known distribution: Miaoli and Nantou Counties, Taiwan.

Notes: Three isolates of *C. miaoliense* were collected from Miaoli County and Nantou County, Taiwan. Multilocus analysis indicated that *C. miaoliense* forms a robust clade clearly distinct from all the other known species in the *C. acutatum* species complex. Of the six *Colletotrichum* species in this complex (*C. acutatum*, *C. fioriniae*, *C. godetiae*, *C. nymphaeae*, *C. salicis*, and *C. simmondsii*) that have been reported as anthracnose pathogens of strawberry, *C. miaoliense* is phylogenetically most closely related to *C. nymphaeae* and *C. simmondsii*. Morphologically, *C. miaoliense* differs from *C. nymphaeae* (CBS 515.78) in the size of conidia (16.1 ± 2.3 × 4.9 ± 0.7 μm versus 14.2 ± 1.1 × 4.1 ± 0.34 μm), the shape of conidia (one end round and one end rounded to acute in contrast to the new species, in which both ends are acute), the size of appressoria (8.7

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± 2.5 × 5.5 ± 1.0 versus 7.5 ± 1. 1 × 5 ± 0.6 μm) (Supplementary Table 2), and the shape of appressoria (*C. miaoliense* and *C. simmondsii* appressoria are elliptical to clavate, whereas the appressoria of *C. nymphaeae* are clavate or irregular in outline, entire, and have an undulate to lobate margin (Damm et al. 2012a)). Compared with *C. simmondsii*, the conidia of *C. miaoliense* are longer (mean length 14.2 μm versus 8.1 μm). In addition, the conidia of *C. simmondsii* are cylindrical with one end round and one end acute or both ends acute. Although the appressoria of *C. miaoliense* and *C. simmondsii* are similar in shape and L/W ratio, the appressoria of *C. simmondsii* are larger (Supplementary Table 2).

2.4.3 Effect of temperature on mycelial growth

A representative isolate selected from each of five *Colletotrichum* species was grown on PDA at 18°C to 32°C. The maximum growth rate of *C. siamense* ML133 was estimated at 27.9°C, whereas the maximum growth rates of *C. fructicola* ML348, *C. karstii* ML351, *C. boninense* ML521 and *C. miaoliense* ML1040 were at 26.0°C, 26.9°C, 24.0°C, and 26.5°C, respectively (Fig. 2.5 and Supplementary Table 3). The growth rate of *C. boninense* ML521 drastically decreased at 32°C (Fig. 2.5). *C. miaoliense* ML1040 exhibited the slowest growth rate at all tested temperature regimes except 32°C (Fig. 2.5). The ranking of species by growth rates at higher temperatures (28°C, 30°C and 32°C) is as follows: *C. siamense* ML133 > *C. fructicola* ML348 > *C. karstii* ML351 and *C. boninense* ML521 (28°C and 30°C) > *C. miaoliense* ML1040 > *C. boninense* ML521 (32°C) (Fig. 2.5 and Supplementary Table 3).

The growth rates of *C. siamense* ML133 were also compared with those of another two representative isolates selected from *C. siamense*, *C. fructicola*, *C. karstii*, and *C. miaoliense* (Supplementary Table 4). The ranking of isolates by mycelial growth rates at

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both 25°C and 30°C on PDA was as follows: *C. siamense* ML133 and ML540 > *C. siamense* ML612 and *C. fructicola* ML368 > *C. fructicola* ML356 and *C. karstii* ML1792 > *C. karstii* ML442 > *C. miaoliense* ML1042 and ML1794.

2.4.4 Pathogenicity assay

Pathogenicity was tested using Koch's postulates for *C. siamense* ML133, *C. fructicola* ML348, *C. karstii* ML351, *C. boninense* ML521, and *C. miaoliense* ML1040. These isolates all caused leaf and/or crown necrosis in strawberry seedlings (Fig. S6). *C. siamense* ML133 caused the most severe symptoms with 100% disease incidence. The disease incidences for *C. fructicola* ML348, *C. karstii* ML351, *C. boninense* ML521, and *C. miaoliense* ML1040 were only 30%, 30%, 30% and 50%, respectively. Notably, after spray inoculation of the seedlings with *C. karstii* ML351, leaf spots scarcely occurred, and no leaf lesions were observed for *C. boninense* ML521. Even though there were few visible symptoms, *C. boninense* ML521 could be re-isolated from surface-sterilized inoculated leaves.

Virulence of the five selected isolates was subsequently assayed using wounded and non-wounded detached leaves at 25°C and 30°C (Fig. 2.6). For all five isolates, inoculation of wounded leaves resulted in typical anthracnose lesions, which were first observed at 2-4 days post inoculation (dpi). *C. siamense* ML133 caused the largest brown necrotic lesions, sometimes with chlorotic or reddish margins (Fig. 2.6a). The necrotic lesions caused by *C. fructicola* ML348, *C. karstii* ML351, *C. boninense* ML521, and *C. miaoliense* ML1040 were significantly smaller (Fig. 2.6b; *C. fructicola* ML348 was slightly more virulent than *C. karstii* ML351, *C. boninense* ML521, and *C. miaoliense* ML1040). At 7 dpi, *C. siamense* ML133 caused significantly larger lesions at 30°C (1.26 cm in diameter) than 25°C (0.65 cm in diameter), whereas the sizes of lesions caused by

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other *Colletotrichum* species were similar (0.07-0.35 cm in diameter) at different temperatures (Fig. 2.6b).

In regard to inoculations of unwounded leaves, necrotic lesions caused by *C. siamense* ML133 and *C. fructicola* ML348 first appeared at 4-7 dpi, but no lesions occurred in the plants inoculated with the other three *Colletotrichum* spp. isolates (Fig. 2.6a). Inoculation of unwounded leaves with *C. siamense* ML133 resulted in larger lesions than inoculation with the other *Colletotrichum* spp., and the lesion sizes at 14 dpi were significantly larger at 30°C (1.35 cm in diameter) than at 25°C (0.35 cm in diameter) (Fig. 2.6c).

Inoculations of wounded leaves were conducted at 30°C for *C. siamense* ML133, *C. boninense* ML521, and two additional representative isolates of *C. siamense*, *C. fructicola*, *C. karstii*, and *C. miaoliense* (Fig. S7). The results showed that *C. siamense* (ML133, ML540, ML612) and *C. fructicola* ML356 caused significantly larger lesions (1.21-1.74 cm in diameter at 7 dpi; 3.06-3.47 cm in diameter at 14 dpi) than *C. fructicola* ML368, *C. karstii* (ML442, ML1792), and *C. miaoliense* (ML1042, ML1794) (0.42-0.76 cm in diameter at 7 dpi; 1.06-2.15 cm in diameter at 14 dpi).

2.5 Discussion

Over the past decade, our knowledge of fungi and their relationships with plant hosts has seen an exponential growth due to the progress in bioinformatics and molecular phylogenetics. Cryptic taxa identification is progressing rapidly and groups of fungi, including important plant pathogens, are now mainly classified using molecular data-based phylogenetic inference. For instance, the *C. acutatum*, *C. boninense*, and *C. gloeosporioides* species complexes now each contain over 20 species (Damm et al. 2012a; Damm et al. 2012b; Weir et al. 2012). Several *Colletotrichum* spp. with the capacity to

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cause strawberry anthracnose in temperate regions have been reported (Adhikari et al. 2019; Baroncelli et al. 2015; Bi et al. 2017; Bobev et al. 2002; Braganca et al. 2016; Damm et al. 2012a; Gan et al. 2017; Jayawardena et al. 2016; Latinovic et al. 2012; Nam et al. 2008; Novotny et al. 2007; Polizzi et al. 2011; Porta-Puglia and Mifsud 2006; Weir et al. 2012); however, knowledge of the composition of the pathogen populations in tropical and subtropical regions is limited. Through morphological characterization, phylogenetic analyses involving five to seven loci (ITS, *GAPDH*, *CHS-1*, *ACT*, *TUB2*, *CAL* and ApMAT), and inoculation tests on strawberry seedlings and detached leaves, the present study revealed that five *Colletotrichum* species cause strawberry anthracnose in Taiwan. In addition to the known strawberry anthracnose pathogens *C. boninense* (Bi et al. 2017), *C. fructicola* (Gan et al. 2017; Hirayama et al. 2018), and *C. siamense* (Chen et al. 2020; Han et al. 2016; Weir et al. 2012), one new species, *C. miaoliense*, and one species not previously known to infect strawberry, *C. karstii*, were identified. *C. karstii* was previously isolated from a wide range of plants such as anthurium, apple, citrus, and chili pepper (Damm et al. 2012b; Diao et al. 2017; Guarnaccia et al. 2017), but not from strawberry. In this study, *C. siamense*, *C. fructicola*, and *C miaoliense* were isolated from different tissues, and all five *Colletotrichum* species were proved to be pathogenic to strawberry leaves and crowns (Fig. S6). The lack of tissue specificity is in agreement with previous observations of *C. acutatum* in strawberry (Howard et al. 1992; Peres et al. 2005).

The predominance of *C. siamense* (75%) and *C. fructicola* (11%) in the strawberry anthracnose pathogen population in Taiwan can be attributed to their higher levels of pathogenicity and aggressiveness. While all five *Colletotrichum* spp. were pathogenic to wounded leaves, only *C. siamense* and *C. fructicola* were able to cause lesions on non-wounded leaves. In addition, *C. siamense* (ML133, ML540, ML612) and *C. fructicola* (ML356) caused significantly larger lesions at 25°C and 30°C (Fig. 2.6 and Fig. S7). A

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similar phenomenon was observed for *Colletotrichum* spp. causing strawberry anthracnose in Zhejiang, China (latitude ~30°N) (Chen et al. 2020): *C. fructicola* (53% of the isolates) and *C. siamense* (23%) dominated the population, and *C. fructicola* exhibited the highest level of pathogenicity (only 25°C was tested) (Chen et al. 2020). Although *C. boninense*, *C. karstii*, and *C. miaoliense* were much less virulent, wounds caused by natural agencies (wind, rain, insects and animals) as well as human activities (e.g., trimming old leaves, which is a common agricultural practice employed by most strawberry farmers in Taiwan) would provide potential infection sites allowing these pathogens to bypass the first line of defense (e.g., the cuticle) (Ranathunge et al. 2012; Sm Auyong 2015; Wang et al. 2017) in strawberry.

Among the five *Colletotrichum* species identified in this study, *C. siamense* exhibited greater mycelial growth rates on PDA, especially at higher temperatures. The fitness advantage of *C. siamense* in warm temperature weather may explain its current geographical distribution. In the literature, *C. siamense* was most reported in tropical and subtropical regions (Han et al. 2016; Silva et al. 2012; Weir et al. 2012), whereas *C. boninense*, *C. fructicola*, and *C. karstii* were reported in regions across a wide range of latitudes (Damm et al. 2012b; Fu et al. 2019; Guarnaccia et al. 2017; Hu et al. 2015; Li et al. 2019; Weir et al. 2012; Yang et al. 2009). Temperature is among the key environmental factors affecting a pathogen's survival (Velásquez et al. 2018). A recent study based on published observations of 612 crop pests and pathogens from 1960 onwards revealed significant positive latitudinal shifts of many important pests and pathogens under climate change (Bebber et al. 2013). More research on the genetic and biological characteristics of different *Colletotrichum* species from diverse geographical areas will be needed to understand the emergence and spread of anthracnose diseases. With rising global

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temperatures, it will be particularly important to monitor the expansion of the heat-adapted *C. siamense* toward high latitudes.

C. boninense, *C. fructicola*, *C. siamense*, and *C. karstii* have been isolated from diverse plants other than strawberry in different countries/regions (Damm et al. 2012b; Weir et al. 2012). In Taiwan, *C. fructicola* has been reported as an anthracnose pathogen in mango, wax apple, and chili (de Silva et al. 2019; Duan et al. 2018), *C. siamense* in lychee, star fruit, and mango (Ni et al. 2017; Wu et al. 2020), *C. karstii* in passion fruit (Lin 2015), and *C. boninense* in pitaya (Lin et al. 2017). Previous studies have demonstrated that *Colletotrichum* spp. from strawberry are pathogenic to other crops and even weeds. For example, *C. acutatum* could not only infect pepper, eggplant, tomato, and bean but also latently colonize weeds such as *Vicia* spp. and *Conyza* spp. (Freeman et al. 2001) In one study, *C. fructicola* was frequently isolated from leaves of *Amaranthus blitum*, and artificial inoculation of *C. fructicola* caused brown leaf spots on *A. blitum* (Hirayama et al. 2018). To determine whether weed control is necessary to minimize the primary infection in the field, it is worth investigating whether the five *Colletotrichum* spp. we identified could colonize the weeds commonly present in and nearby strawberry fields in Taiwan. More sampling and artificial inoculation assays will be required to understand the host range of the new species *C. miaoliense*.

Anthracnose is a key limiting factor for strawberry production in Taiwan and many other areas. Outbreaks of anthracnose in strawberry nurseries and fields have caused yield losses of up to 50%-80% (Chen et al. 2020; Forcelini and Peres 2018; Howard et al. 1992; Xie et al. 2010). This study demonstrated the diversity of pathogenic *Colletotrichum* species associated with strawberry in Taiwan. The findings offer precise information about pathogen identity, which is valuable for screening of resistant varieties and development of effective disease management strategies. Regardless of whether it was

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inoculated on wounded or non-wounded leaves, the predominant pathogen *C. siamense* caused larger lesions at 30°C than 25°C, which is meaningful in subtropical Taiwan and areas with a similar phenology. Because no significant difference was observed between the mycelial growth rates of *C. siamense* at 25°C, 28°C, and 30°C, higher disease severity at 30°C could be due to reduced resistance of strawberry against anthracnose at higher temperatures (Mori 1998; Smith and Black 1987). In Taiwan, the susceptible cultivar 'Taoyuan No. 1' has been widely cultivated for over 30 years. Development of temperature-independent resistant cultivars will be particularly important for strawberry breeding programs in Taiwan and other tropical and subtropical regions. Future work will focus on monitoring pathogen population changes, investigating the fungicide sensitivity levels of different *Colletotrichum* species, and developing molecular detection methods to aid the production of strawberry seedlings without latent infection of major *Colletotrichum* species.

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2.7 Tables

Table 2.1. List of *Colletotrichum* spp. associated with strawberry anthracnose in Taiwan.

Species	Isolate[a]	Strawberry tissue	Collection date	Sampling site	GenBank accession number[b]						
					ITS	*GAPDH*	*CHS1*	*ACT*	*TUB2*	*CAL*	ApMAT
C. acutatum species complex											
C. miaoliense sp. nov.	**ML1040**	crown	2016/10/28	Shitan Township, Miaoli County	MK908419	MK908470	MK908522	MK908573	MK908624	-[c]	-
	ML1042	leaf	2016/11/24	Renai Township, Nantou County	MK908420	MK908471	MK908523	MK908574	MK908625	-	-
	ML1794	leaf	2018/07/04	Renai Township, Nantou County	MK908421	MK908472	MK908524	MK908575	MK908626	-	-
C. boninense species complex											
C. boninense	**ML521**	leaf	2013/01/21	Taian Township, Miaoli County	MK908424	MK908475	MK908527	MK908578	MK908629	MK908677	-
C. karstii	**ML351**	leaf	2012/07/06	Shitan Township, Miaoli County	MK908422	MK908473	MK908525	MK908576	MK908627	MK908675	-
	ML442	leaf	2012/07/04	Nanchuang Township, Miaoli County	MK908423	MK908474	MK908526	MK908577	MK908628	MK908676	-
	ML1792	leaf	2018/05/16	Renai Township, Nantou County	MK908425	MK908476	MK908528	MK908579	MK908630	MK908678	-
C. gloeosporioides species complex											
C. fructicola	**ML348**	leaf	2012/07/06	Shitan Township, Miaoli County	MK908461	MK908513	MK908564	MK908615	MK908666	MK908714	MK908758
	ML353	root	2012/07/10	Shitan Township, Miaoli County	MK908462	MK908514	MK908565	MK908616	MK908667	MK908715	MK908759
	ML356	crown	2012/07/10	Shitan Township, Miaoli County	MK908463	MK908515	MK908566	MK908617	MK908668	MK908716	MK908760
	ML368	stolon	2012/07/24	Dahu Township, Miaoli County	MK908464	MK908516	MK908567	MK908618	MK908669	MK908717	MK908761
	ML818	crown	2016/07/06	Renai Township, Nantou County	MK908468	MK908520	MK908571	MK908622	MK908673	MK908721	MK908765
	ML1012	leaf	2016/11/09	Renai Township, Nantou County	MK908469	MK908521	MK908572	MK908623	MK908674	MK908722	MK908766
C. siamense	ML040	fruit	2010/03/22	Gongguan Township, Miaoli County	MK908426	MK908477	MK908529	MK908580	MK908631	MK908679	MK908723
	ML041	fruit	2010/03/24	Dahu Township, Miaoli County	MK908427	MK908478	MK908530	MK908581	MK908632	MK908680	MK908724
	ML048	fruit	2010/05/12	Dahu Township, Miaoli County	MK908428	MK908479	MK908531	MK908582	MK908633	MK908681	MK908725
	ML076	stolon	2010/08/27	Dahu Township, Miaoli County	MK908429	MK908480	MK908532	MK908583	MK908634	MK908682	MK908726

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Species	Isolate[a]	Strawberry tissue	Collection date	Sampling site	GenBank accession number[b]						
					ITS	*GAPDH*	*CHS1*	*ACT*	*TUB2*	*CAL*	ApMAT
	ML133	crown	2011/10/28	Dahu Township, Miaoli County	MK174223[a]	MK908481	MK174224[a]	MK174225[a]	MK174226[a]	MK174227[a]	MK174228[a]
	ML177	fruit	2012/03/09	Dahu Township, Miaoli County	MK908430	MK908482	MK908533	MK908584	MK908635	MK908683	MK908727
	ML275	crown	2012/06/04	Dahu Township, Miaoli County	MK908431	MK908483	MK908534	MK908585	MK908636	MK908684	MK908728
	ML284	crown	2012/06/04	Shitan Township, Miaoli County	MK908432	MK908484	MK908535	MK908586	MK908637	MK908685	MK908729
	ML293	root	2012/4/25	Guanxi Township, Hsinchu County	MK908433	MK908485	MK908536	MK908587	MK908638	MK908686	MK908730
	ML294	crown	2012/06/13	Dahu Township, Miaoli County	MK908434	MK908486	MK908537	MK908588	MK908639	MK908687	MK908731
	ML296	crown	2012/06/13	Dahu Township, Miaoli County	MK908435	MK908487	MK908538	MK908589	MK908640	MK908688	MK908732
	ML320	crown	2012/06/27	Taian Township, Miaoli County	MK908436	MK908488	MK908539	MK908590	MK908641	MK908689	MK908733
	ML328	crown	2012/07/02	Shitan Township, Miaoli County	MK908437	MK908489	MK908540	MK908591	MK908642	MK908690	MK908734
	ML372	crown	2012/07/26	Shitan Township, Miaoli County	MK908438	MK908490	MK908541	MK908592	MK908643	MK908691	MK908735
	ML393	stolon	2012/07/26	Shitan Township, Miaoli County	MK908439	MK908491	MK908542	MK908593	MK908644	MK908692	MK908736
	ML416	crown	2012/08/15	Dahu Township, Miaoli County	MK908440	MK908492	MK908543	MK908594	MK908645	MK908693	MK908737
	ML418	crown	2012/08/15	Dahu Township, Miaoli County	MK908441	MK908493	MK908544	MK908595	MK908646	MK908694	MK908738
	ML419	root	2012/08/06	Shitan Township, Miaoli County	MK908442	MK908494	MK908545	MK908596	MK908647	MK908695	MK908739
	ML443	crown	2012/07/26	Shitan Township, Miaoli County	MK908443	MK908495	MK908546	MK908597	MK908648	MK908696	MK908740
	ML458	crown	2012/08/15	Dahu Township, Miaoli County	MK908444	MK908496	MK908547	MK908598	MK908649	MK908697	MK908741
	ML461	leaf	2012/09/12	Gongguan Township, Miaoli County	MK908445	MK908497	MK908548	MK908599	MK908650	MK908698	MK908742
	ML462	crown	2012/09/12	Gongguan Township, Miaoli County	MK908446	MK908498	MK908549	MK908600	MK908651	MK908699	MK908743
	ML463	root	2012/09/12	Gongguan Township, Miaoli County	MK908447	MK908499	MK908550	MK908601	MK908652	MK908700	MK908744
	ML464	crown	2012/09/13	Shitan Township, Miaoli County	MK908448	MK908500	MK908551	MK908602	MK908653	MK908701	MK908745
	ML469	crown	2012/09/18	Dahu Township, Miaoli County	MK908449	MK908501	MK908552	MK908603	MK908654	MK908702	MK908746
	ML471	crown	2012/09/18	Dahu Township, Miaoli County	MK908450	MK908502	MK908553	MK908604	MK908655	MK908703	MK908747

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Species	Isolate[a]	Strawberry tissue	Collection date	Sampling site	GenBank accession number[b]						
					ITS	*GAPDH*	*CHS1*	*ACT*	*TUB2*	*CAL*	ApMAT
	ML476	root	2012/09/18	Dahu Township, Miaoli County	MK908451	MK908503	MK908554	MK908605	MK908656	MK908704	MK908748
	ML477	crown	2012/09/18	Dahu Township, Miaoli County	MK908452	MK908504	MK908555	MK908606	MK908657	MK908705	MK908749
	ML485	crown	2012/10/04	Guoxing Township, Nantou County	MK908453	MK908505	MK908556	MK908607	MK908658	MK908706	MK908750
	ML490	crown	2012/11/13	Gongguan Township, Miaoli County	MK908454	MK908506	MK908557	MK908608	MK908659	MK908707	MK908751
	ML491	crown	2012/11/13	Gongguan Township, Miaoli County	MK908455	MK908507	MK908558	MK908609	MK908660	MK908708	MK908752
	ML494	crown	2012/12/10	Dahu Township, Miaoli County	MK908456	MK908508	MK908559	MK908610	MK908661	MK908709	MK908753
	ML513	crown	2013/01/11	Shitan Township, Miaoli County	MK908457	MK908509	MK908560	MK908611	MK908662	MK908710	MK908754
	ML540	stolon	2013/04/22	Gongguan Township, Miaoli County	MK908458	MK908510	MK908561	MK908612	MK908663	MK908711	MK908755
	ML608	stolon	2013/07/10	Shitan Township, Miaoli County	MK908459	MK908511	MK908562	MK908613	MK908664	MK908712	MK908756
	ML612	leaf	2013/07/10	Shitan Township, Miaoli County	MK908460	MK908512	MK908563	MK908614	MK908665	MK908713	MK908757
	ML617	crown	2013/07/18	Dahu Township, Miaoli County	MK908465	MK908517	MK908568	MK908619	MK908670	MK908718	MK908762
	ML754	leaf	2016/03/17	Chiayi County	MK908466	MK908518	MK908569	MK908620	MK908671	MK908719	MK908763
	ML762	fruit	2016/03/28	Dahu Township, Miaoli County	MK908467	MK908519	MK908570	MK908621	MK908672	MK908720	MK908764

[a]Isolates in bold are representatives of each *Colletotrichum* spp. selected for mycelial growth and pathogenicity assays.

[b]ITS: internal transcribed spacer; *GAPDH*: glyceraldehyde 3-phosphate dehydrogenase; *CHS-1*: chitin synthase; *ACT*: actin; *TUB2*: beta-tubulin; *CAL*: calmodulin; ApMAT: intergenic sequence between *Apn2* DNA lyase and *MAT1-2-1*.

[c]-: not available.

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2.8 Figures

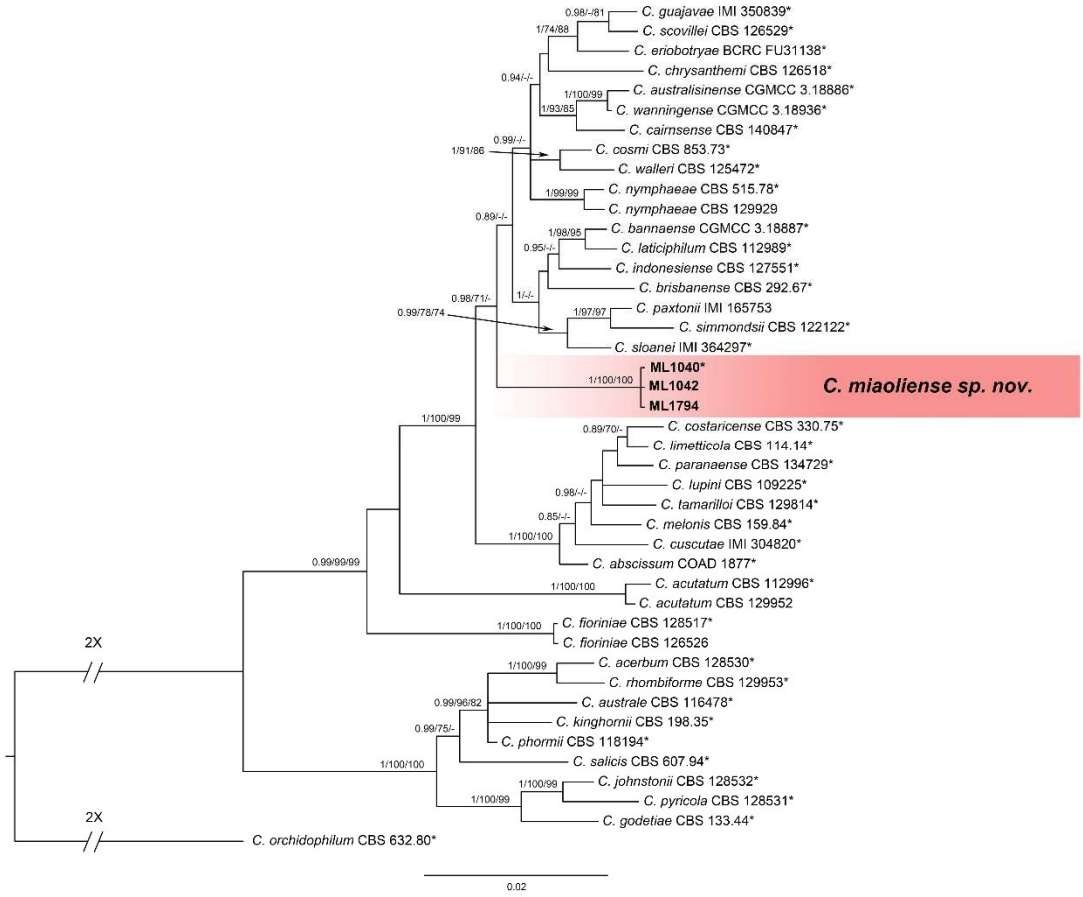

Fig. 2.1. A Bayesian inference phylogenetic tree of the *C. acutatum* species complex. The phylogenetic tree was built using concatenated sequences of the ITS and the *GAPDH*, *ACT*, *CHS-1* and *TUB2* genes. Bayesian inference (BI) posterior values above 0.9 and bootstrap support values from maximum likelihood (ML) and maximum parsimony (MP) above 70% are shown at each node (BI/ML/MP). *C. orchidophilum* CBS 632.80 was used as the outgroup. * indicates the ex-type strains. Strains isolated in this study are shown in bold.

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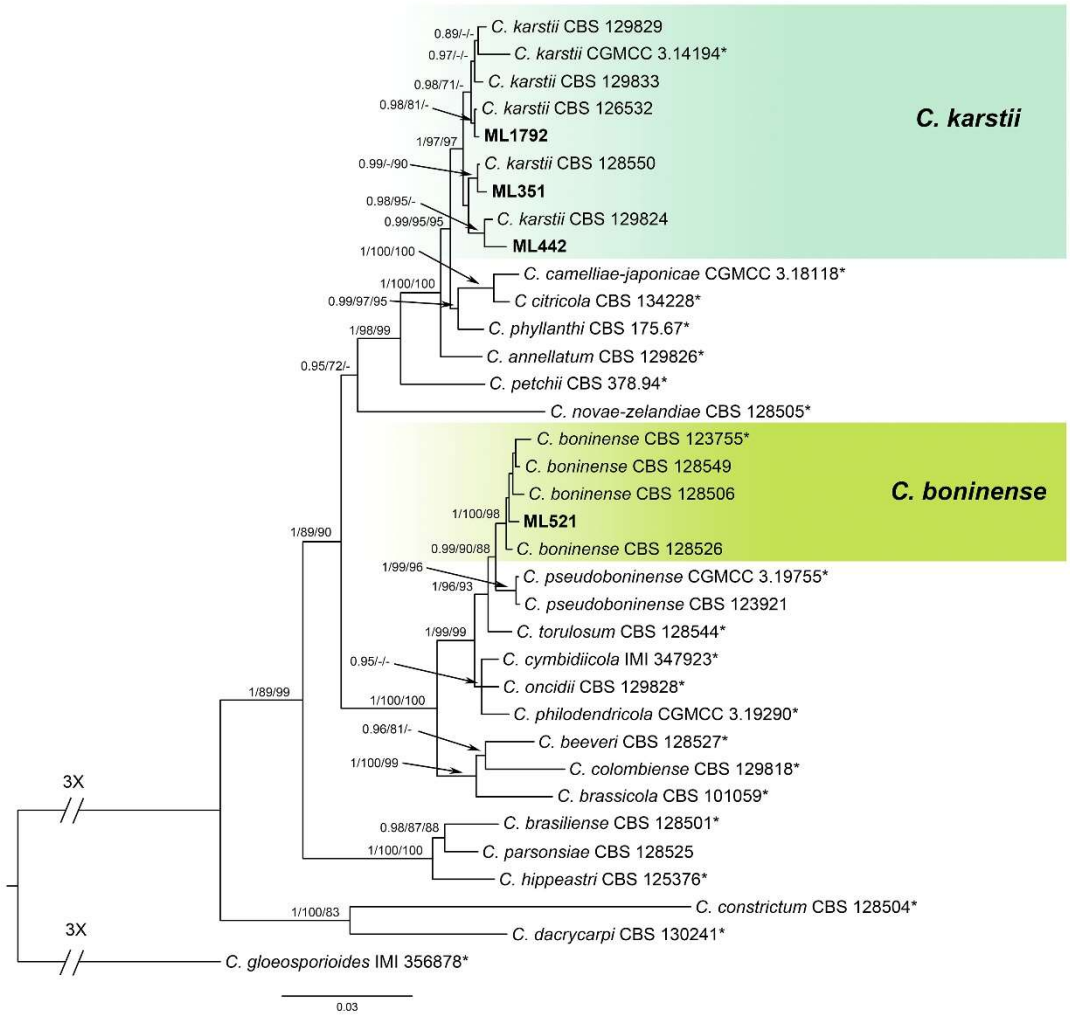

Fig. 2.2. A Bayesian inference phylogenetic tree of the *C. boninense* species complex. A phylogram was built using concatenated sequences of the ITS and the *GAPDH*, *ACT*, *CHS-1*, *TUB2* and *CAL* genes. Bayesian inference (BI) posterior values above 0.9 and bootstrap support values from maximum likelihood (ML) and maximum parsimony (MP) above 70% are shown at each node (BI/ML/MP). *C. gloeosporioides* IMI 356878 was used as the outgroup. * indicates the ex-type strains. Strains isolated in this study are shown in bold.

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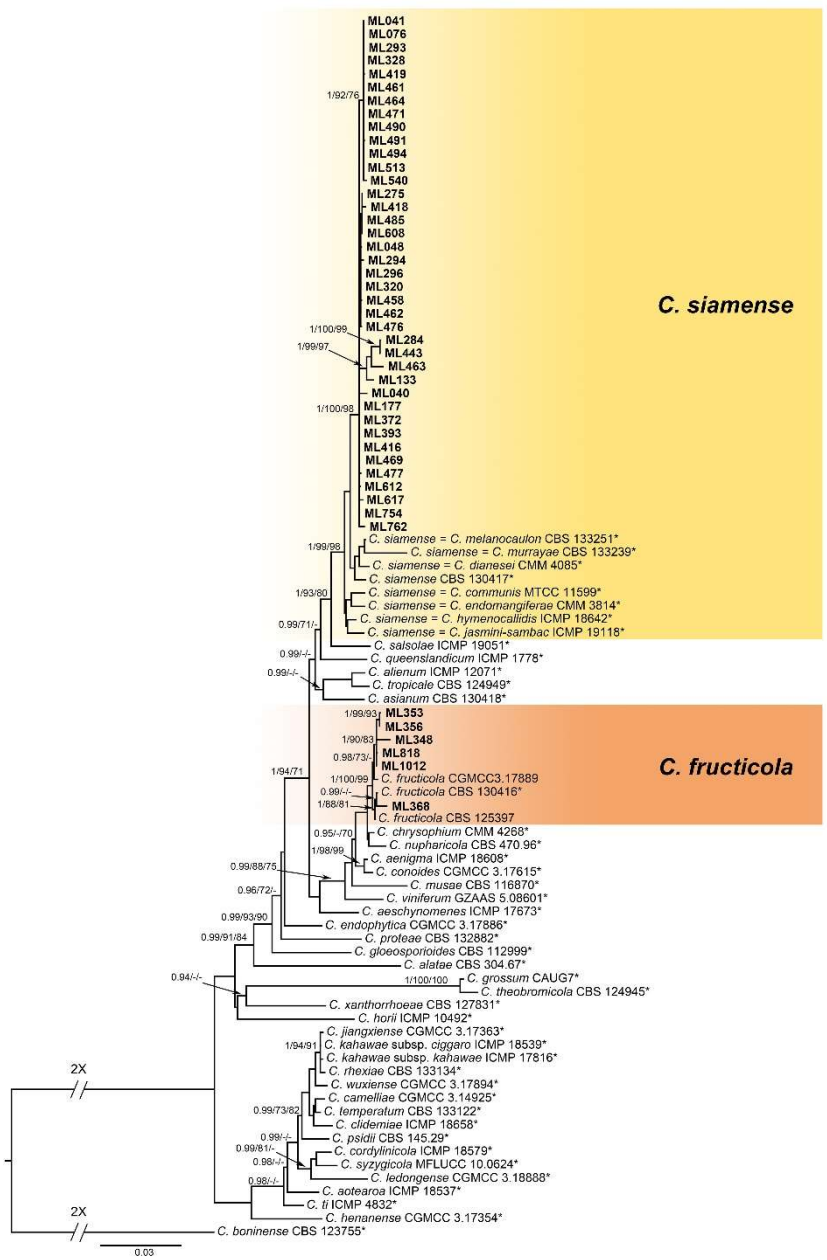

Fig. 2.3. A Bayesian inference phylogenetic tree of the *C. gloeosporioides* species complex.

The phylogenetic tree was built using concatenated sequences of the ITS, ApMAT, and the *GAPDH*, *ACT*, *CHS-1*, *TUB2*, *CAL* genes. Bayesian inference (BI) posterior values above 0.9 and bootstrap support values from maximum likelihood (ML) and maximum parsimony (MP) above 70% are shown at each node (BI/ML/MP). *C. boninense* CBS 123755 was used as the outgroup. * indicates the ex-type strains. Strains isolated in this study are shown in bold.

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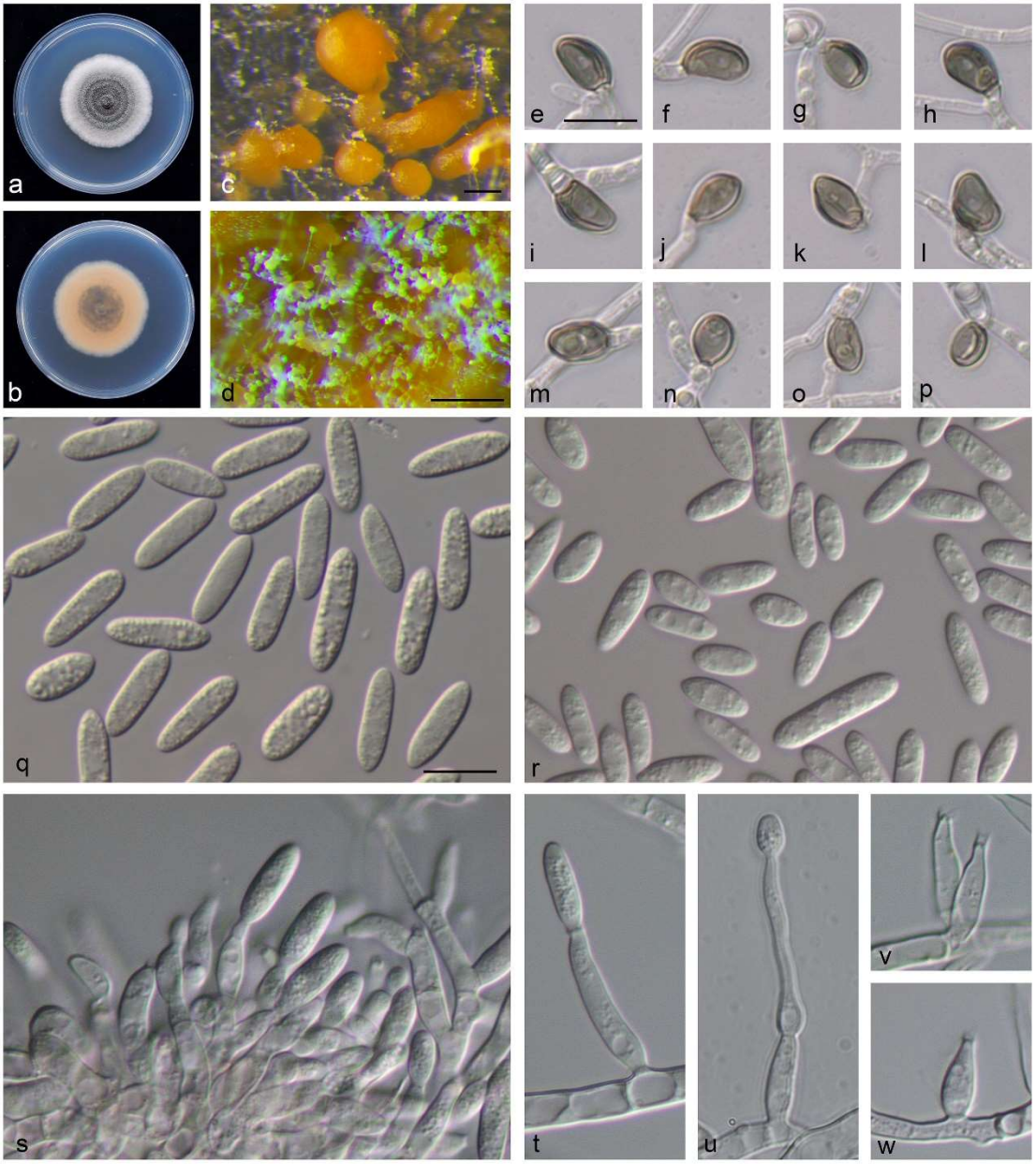

Fig. 2.4. *Colletotrichum miaoliense* sp. nov. ML1040.

(a) Upper side of colony; (b) reverse side of colony; (c-d) conidiomata; (e-p) appressorium (induced in dH_2O on a microscope slide); (q) conidia from conidiomata; (r) conidia from aerial mycelium; (s-w) conidiophores. a-b on potato dextrose agar (PDA); c-d and q-w on 1/4-strength PDA. Scale bars: c, d = 0.2 mm, e = 10 μm, applies to f-p, q = 10 μm, applies to r-w.

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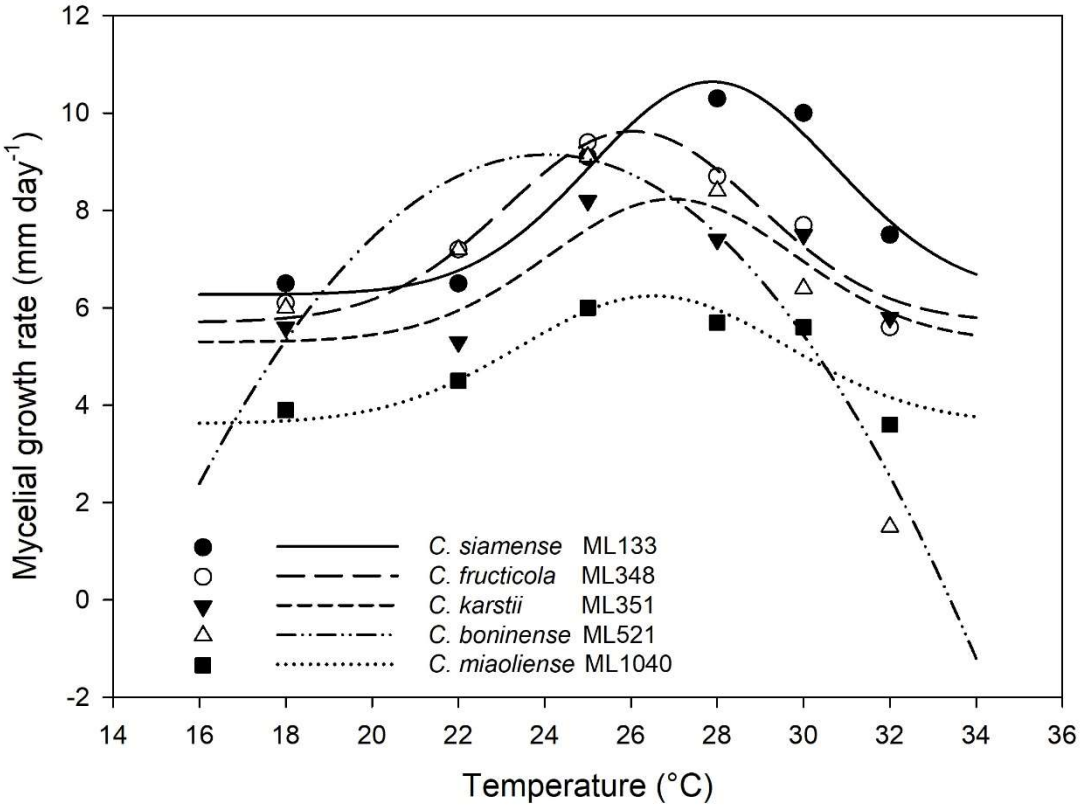

Fig. 2.5. Mycelial growth rates of *Colletotrichum* spp. at different temperatures.
Different symbols represent the mean growth rates of different species at the tested temperatures. Gaussian process regression was used to estimate the optimum temperature for mycelial growth.

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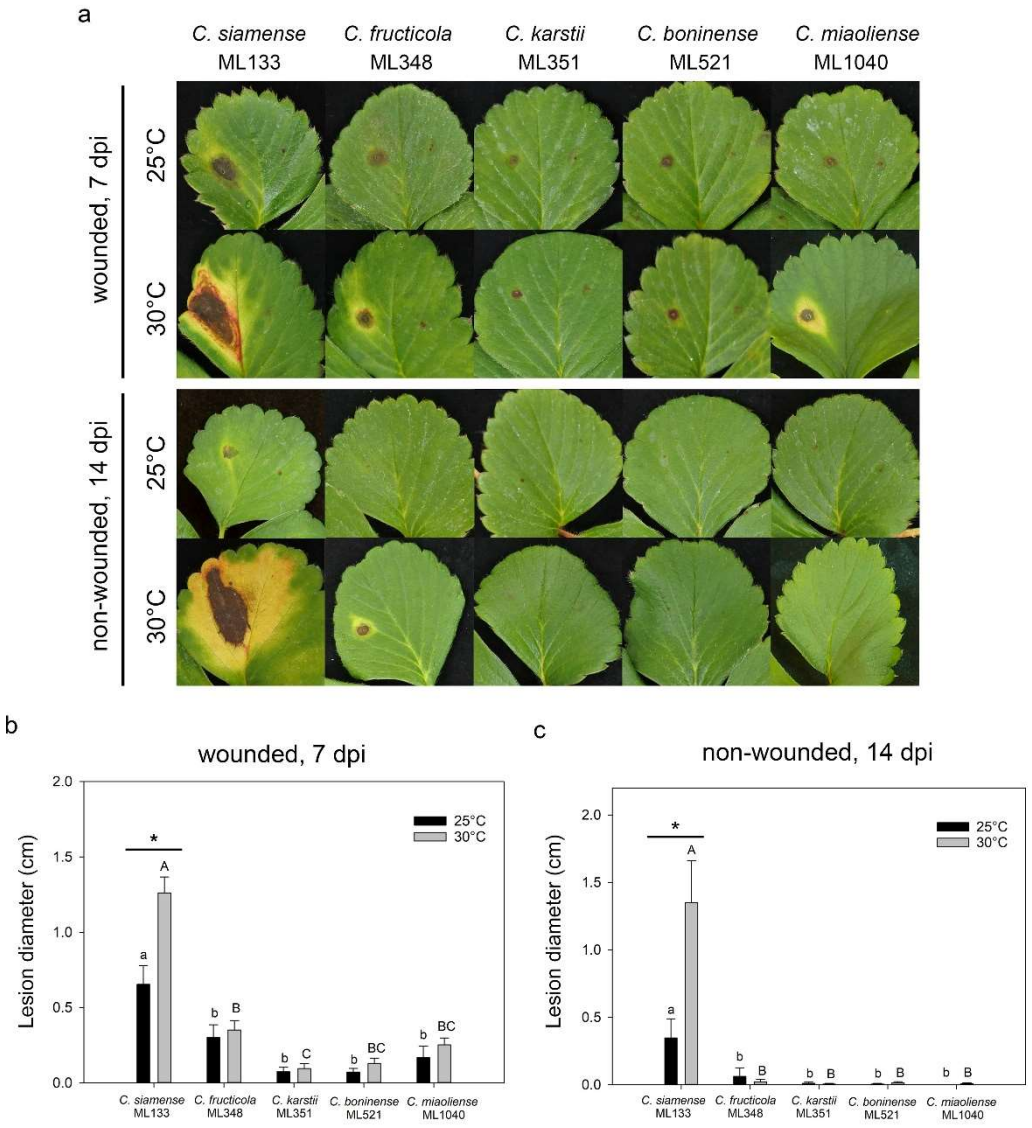

Fig. 2.6. Inoculation of *Colletotrichum* spp. on detached wounded and non-wounded strawberry leaves at different temperatures.

(a) Lesions at 7 days post inoculation (dpi) on wounded leaves or at 14 dpi on non-wounded leaves incubated at 25°C or 30°C. The left side of each leaflet was inoculated with 10 µl spore suspension (10^6 spores/ml) and the right side with water (control). (b-c) Lesion sizes resulting from inoculation of wounded and non-wounded leaves. The results from the same temperature were analyzed together. Data (mean ± standard error) with different letters are significantly different based on Tukey's range test at $P < 0.05$ (n = 12). For each species, the difference between 25°C and 30°C was analyzed by Student's *t*-test (* denotes $P < 0.05$).

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2.9 Supplemental Materials

Supplemental Table 2.1. List of *Colletotrichum* spp. strains used for phylogenetic analysis.

Species	Culture[a]	Host/Tissue	Country/Region	GenBank accession number[b]							Ref.
				ITS	*GAPDH*	*CHS1*	*ACT*	*TUB2*	*CAL*	ApMAT	
C. acutatum species complex											
C. abscissum	COAD 1877*	*Citrus sinensis* cv. Pera	Brazil	KP843126	KP843129	KP843132	KP843141	KP843135	-[c]	-	Fu et al. (2019)[1]
C. acerbum	CBS 128530*	*Malus domestica*	New Zealand	JQ948459	JQ948790	JQ949120	JQ949780	JQ950110	-	-	Fu et al. (2019)[1]
C. acutatum	CBS 112996*	*Carica papaya*	Australia	JQ005776	JQ948677	JQ005797	JQ005839	JQ005860	-	-	Fu et al. (2019)[1]
C. acutatum	CBS 129952	*Olea europaea*	Portugal	JQ948364	JQ948695	JQ949025	JQ949685	JQ950015	-	-	Guarnaccia et al. (2017)[2]
C. australe	CBS 116478*	*Trachycarpus fortunei*	South Africa	JQ948455	JQ948786	JQ949116	JQ949776	JQ950106	-	-	Fu et al. (2019)[1]
C. australisinense	CGMCC 3.18886*	*Hevea brasiliensis*	China, Guangxi	MG209623	MG241962	MG241981	MG241947	MG209645	-	-	Liu et al. (2018)[3]
C. bannaense	CGMCC 3.18887*	*Hevea brasiliensis*	China, Yunnan	MG209638	MG242006	MG241996	MG242002	MG209660	-	-	Liu et al. (2018)[3]
C. brisbanense	CBS 292.67*	*Capsicum annuum*	Australia	JQ948291	JQ948621	JQ948952	JQ949612	JQ949942	-	-	Fu et al. (2019)[1]
C. cairnsense	CBS 140847*	*Capsicum annuum*	Australia	KU923672	KU923704	KU923710	KU923716	KU923688	-	-	Fu et al. (2019)[1]
C. chrysanthemi	CBS 126518*	*Carthamus* sp.	Netherlands	JQ948271	JQ948601	JQ948932	JQ949592	JQ949922	-	-	Diao et al. (2017)[4]
C. cosmi	CBS 853.73*	*Cosmos* sp, seed	Netherlands	JQ948274	JQ948604	JQ948935	JQ949595	JQ949925	-	-	Fu et al. (2019)[1]
C. costaricense	CBS 330.75*	Coffea arabica	Costa Rica	JQ948180	JQ948510	JQ948841	JQ949501	JQ949831	-	-	Fu et al. (2019)[1]
C. cuscutae	IMI 304802*	*Cuscuta* sp.	Dominica	JQ948195	JQ948525	JQ948856	JQ949516	JQ949846	-	-	Fu et al. (2019)[1]
C. eriobotryae	BCRC FU31138*	*Eriobotrya japonica*	Taiwan	MF772487	MF795423	MN191653	MN191648	MF795428	-	-	Damm et al. (2020)[5]
C. fioriniae	CBS 128517*	*Fiorinia externa*	USA	JQ948292	JQ948622	JQ948953	JQ949613	JQ949943	-	-	Diao et al. (2017)[4]
C. fioriniae	CBS 126526	*Primula* sp., leaf	Netherlands	JQ948323	JQ948653	JQ948984	JQ949644	JQ949974	-	-	Fu et al. (2019)[1]
C. godetiae	CBS 133.44*	*Clarkia hybrida* cv. kelvon	Denmark	JQ948402	JQ948733	JQ949063	JQ949723	JQ950053	-	-	Fu et al. (2019)[1]
C. guajavae	IMI 350839*	*Psidium guajava*	India	JQ948270	JQ948600	JQ948931	JQ949591	JQ949921	-	-	Diao et al. (2017)[4]
C. indonesiense	CBS 127551*	*Eucalyptus* sp.	Indonesia	JQ948288	JQ948618	JQ948949	JQ949609	JQ949939	-	-	Damm et al. (2012)[6]
C. johnstonii	CBS 128532*	*Solanum lycopersicum*, fruit	New Zealand	JQ948444	JQ948775	JQ949105	JQ949765	JQ950095	-	-	Fu et al. (2019)[1]
C. kinghornii	CBS 198.35*	*Phormium* sp.	UK	JQ948454	JQ948785	JQ949115	JQ949775	JQ950105	-	-	Fu et al. (2019)[1]
C. laticiphilum	CBS 112989*	*Hevea brasiliensis*	India	JQ948289	JQ948619	JQ948950	JQ949610	JQ949940	-	-	Fu et al. (2019)[1]
C. limetticola	CBS 114.14*	*Citrus aurantifolia*	USA, Florida	JQ948193	JQ948523	JQ948854	JQ949514	JQ949844	-	-	Damm et al. (2012)[6]
C. lupini	CBS 109225*	*Lupinus albus*	Ukraine	JQ948155	JQ948485	JQ948816	JQ949476	JQ949806	-	-	Fu et al. (2019)[1]

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Species	Culture[a]	Host/Tissue	Country/Region	GenBank accession number[b]							Ref.
				ITS	*GAPDH*	*CHS1*	*ACT*	*TUB2*	*CAL*	ApMAT	
C. melonis	CBS 159.84*	*Cucumis melo*	Brazil	JQ948194	JQ948524	JQ948855	JQ949515	JQ949845	-	-	Damm et al. (2012)[6]
C. nymphaeae	CBS 515.78*	*Nymphaea* sp.	Netherlands	JQ948197	JQ948527	JQ948858	JQ949518	JQ949848	-	-	Diao et al. (2017)[4]
C. nymphaeae	CBS 129929	*Fragaria* × *ananassa*	USA	JQ948229	JQ948559	JQ948890	JQ949550	JQ949880	-	-	Damm et al. (2012)[6]
C. orchidophilum	CBS 632.80*	*Dendrobium* sp.	USA	JQ948151	JQ948481	JQ948812	JQ949472	JQ949802	-	-	Fu et al. (2019)[1]
C. paranaense	CBS 134729*	*Malus domestica*	Brazil, Parana	KC204992	KC205026	KC205043	KC205077	KC205060	-	-	Braganca et al. (2016)[7]
C. paxtonii	IMI165753*	*Musa* sp.	Saint Lucia	JQ948285	JQ948615	JQ948946	JQ949606	JQ949936	-	-	Damm et al. (2012)[6]
C. phormii	CBS 118194*	*Phormium* sp.	Germany	JQ948446	JQ948777	JQ949107	JQ949767	JQ950097	-	-	Fu et al. (2019)[1]
C. pyricola	CBS 128531*	*Pyrus communis*, fruit	New Zealand	JQ948445	JQ948776	JQ949106	JQ949766	JQ950096	-	-	Fu et al. (2019)[1]
C. rhombiforme	CBS 129953*	*Olea europaea*	Portugal	JQ948457	JQ948788	JQ949118	JQ949778	JQ950108	-	-	Fu et al. (2019)[1]
C. salicis	CBS 607.94*	*Salix* sp., leaf.	Netherlands	JQ948460	JQ948791	JQ949121	JQ949781	JQ950111	-	-	Fu et al. (2019)[1]
C. scovillei	CBS 126529*	*Capsicum* sp.	Indonesia	JQ948267	JQ948597	JQ948928	JQ949588	JQ949918	-	-	Diao et al. (2017)[4]
C. simmondsii	CBS 122122*	*Carica papaya*	Australia	JQ948276	JQ948606	JQ948937	JQ949597	JQ949927	-	-	Fu et al. (2019)[1]
C. sloanei	IMI 364297*	*Theobroma cacao*, leaf	Malaysia	JQ948287	JQ948617	JQ948948	JQ949608	JQ949938	-	-	Fu et al. (2019)[1]
C. tamarilloi	CBS 129814*	*Solanum betaceum*, fruit	Colombia	JQ948184	JQ948514	JQ948845	JQ949505	JQ949835	-	-	Fu et al. (2019)[1]
C. walleri	CBS 125472*	*Coffea* sp., leaf	Vietnam	JQ948275	JQ948605	JQ948936	JQ949596	JQ949926	-	-	Fu et al. (2019)[1]
C. wanningense	CGMCC 3.18936*	*Hevea brasiliensis*	China	MG830462	MG830318	MG830302	MG830270	MG830286	-	-	Cao et al. (2019)[8]
C. boninensis species complex											
C. annellatum	CBS 129826*	*Hevea brasiliensis*, leaf	Colombia	JQ005222	JQ005309	JQ005396	JQ005570	JQ005656	JQ005743	-	Fu et al. (2019)[1]
C. beeveri	CBS 128527*	*Brachyglottis repanda*	New Zealand	JQ005171	JQ005258	JQ005345	JQ005519	JQ005605	JQ005692	-	Fu et al. (2019)[1]
C. boninense	CBS 123755*	*Crinum asiaticum* var. sinicum	Japan	JQ005153	JQ005240	JQ005327	JQ005501	JQ005588	JQ005674	-	Fu et al. (2019)[1]
C. boninense	CBS 128506	*Solanum lycopersicum*,fruit	New Zealand	JQ005157	JQ005244	JQ005331	JQ005505	JQ005591	JQ005678	-	Fu et al. (2019)[1]
C. boninense	CBS 128549	*Solanum betaceum*, flowers	New Zealand	JQ005156	JQ005243	JQ005330	JQ005504	JQ005590	JQ005677	-	Damm et al. (2012)[9]
C. boninense	CBS 128526	Dacrycarpus dacrydioides, leaf	New Zealand	JQ005162	JQ005249	JQ005336	JQ005510	JQ005596	JQ005683	-	Douanla-Meli and Unger (2017)[10]
C. brasiliense	CBS 128501*	Passiflora edulis, fruit	Brazil	JQ005235	JQ005322	JQ005409	JQ005583	JQ005669	JQ005756	-	Fu et al. (2019)[1]
C. brassicola	CBS 101059*	*Brassica oleracea* var. gemmifera, leaf	New Zealand	JQ005172	JQ005259	JQ005346	JQ005520	JQ005606	JQ005693	-	Damm et al. (2012)[9]

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Species	Culture[a]	Host/Tissue	Country/Region	GenBank accession number[b]							Ref.
				ITS	*GAPDH*	*CHS1*	*ACT*	*TUB2*	*CAL*	ApMAT	
C. camelliae-japonicae	CGMCC 3.18118*	*Camellia japonica*	Japan	KX853165	KX893584	-	KX893576	KX893580	-	-	Fu et al. (2019)[1]
C. citricola	CBS 134228*	*Citrus unshiu*	China	KC293576	KC293736	KC293792	KC293616	KC293656	KC293696	-	Huang et al. (2013)[11]
C. colombiense	CBS 129818*	*Passiflora edulis*, leaf	Colombia	JQ005174	JQ005261	JQ005348	JQ005522	JQ005608	JQ005695	-	Fu et al. (2019)[1]
C. constrictum	CBS 128504*	*Citrus limon*, fruit	New Zealand	JQ005238	JQ005325	JQ005412	JQ005586	JQ005672	JQ005759	-	Fu et al. (2019)[1]
C. cymbidiicola	IMI 347923*	*Cymbidium* sp., leaf	Australia	JQ005166	JQ005253	JQ005340	JQ005514	JQ005600	JQ005687	-	Fu et al. (2019)[1]
C. dacrycarpi	CBS 130241*	*Dacrycarpus dacrydioides*, leaf	New Zealand	JQ005236	JQ005323	JQ005410	JQ005584	JQ005670	JQ005757	-	Fu et al. (2019)[1]
C. gloeosporioides	IMI 356878*	*Citrus sinensis*	Italy	JX010152	JX010056	JX009818	JX009531	JX010445	JX009731	-	Fu et al. (2019)[1]
C. hippeastri	CBS 125376*	*Hippeastrum vittatum*, leaf	China	JQ005231	JQ005318	JQ005405	JQ005579	JQ005665	JQ005752	-	Fu et al. (2019)[1]
C. karstii	CGMCC 3.14194*	*Vanda sp.*	China	HM585409	HM585391	HM582023	HM581995	HM585428	HM582013		Youlian et al. (2011)[12]
C. karstii	CBS 129833	*Musa* sp.	Mexico	JQ005175	JQ005262	JQ005349	JQ005523	JQ005609	JQ005696	-	Guarnaccia et al. (2017)[2]
C. karstii	CBS 129829	*Gossypium hirsutum*	Germany	JQ005189	JQ005276	JQ005363	JQ005537	JQ005623	JQ005710	-	Guarnaccia et al. (2017)[2]
C. karstii	CBS 126532	*Citrus* sp.	South Africa	JQ005209	JQ005296	JQ005383	JQ005557	JQ005643	JQ005730	-	Guarnaccia et al. (2017)[2]
C. karstii	CBS 128550	*Annona cherimola*, fruit	Mexico	JQ005219	JQ005306	JQ005393	JQ005567	JQ005653	JQ005740	-	Damm et al. (2012)[9]
C. karstii	CBS 129824	*Musa* sp., fruit	Colombia	JQ005215	JQ005302	JQ005389	JQ005563	JQ005649	JQ005736	-	Damm et al. (2012)[9]
C. novae-zelandiae	CBS 128505*	Capsicum annuum, fruit	New Zealand	JQ005228	JQ005315	JQ005402	JQ005576	JQ005662	JQ005749	-	Fu et al. (2019)[1]
C. oncidii	CBS 129828*	*Oncidium* sp., leaf	Germany	JQ005169	JQ005256	JQ005343	JQ005517	JQ005603	JQ005690	-	Douanla-Meli and Unger (2017)[10]
C. parsonsiae	CBS 128525	*Parsonsia capsularis*, leaf	New Zealand	JQ005233	JQ005320	JQ005407	JQ005581	JQ005667	JQ005754	-	Fu et al. (2019)[1]
C. petchii	CBS 378.94*	*Dracaena marginata*, leaf	Italy	JQ005223	JQ005310	JQ005397	JQ005571	JQ005657	JQ005744	-	Fu et al. (2019)[1]
C. philodendricola	CGMCC 3.19290*	*Philodendron tatei*	China	MH105257	MH105261	MH105265	MH105273	MH105277	MH105281	-	Xue et al. (2020)[13]
C. phyllanthi	CBS 175.67*	*Phyllanthus acidus*	India	JQ005221	JQ005308	JQ005395	JQ005569	JQ005655	JQ005742	-	Damm et al. (2012)[9]
C. pseudoboninense	CGMCC 3.19755*	*Philodendron. tatei*	China	MK796540	MK796573	-	MK796547	MK796554	-	-	Xue et al. (2020)[13]
C. pseudoboninense	CBS 123921	*Dendrobium kingianum*	Japan	JQ005163	JQ005250	JQ005337	JQ005511	JQ005597	JQ005684	-	Xue et al. (2020)[13]

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Species	Culture[a]	Host/Tissue	Country/Region	GenBank accession number[b]							Ref.
				ITS	*GAPDH*	*CHS1*	*ACT*	*TUB2*	*CAL*	ApMAT	
C. torulosum	CBS 128544*	*Solanum melongena*	New Zealand	JQ005164	JQ005251	JQ005338	JQ005512	JQ005598	JQ005685	-	Fu et al. (2019)[1]
C. gloeosporioides sepcies coomplex											
C. aenigma	ICMP 18608*	*Persea americana*	Israel	JX010244	JX010044	JX009774	JX009443	JX010389	JX009683	KM360143	Vieira et al. (2017)[14]
C. aeschynomenes	ICMP 17673*	*Aeschynomene virginica*	USA	JX010176	JX009930	JX009799	JX009483	JX010392	JX009721	KM360145	Wang et al. (2016)[15]
C. alatae	CBS 304.67*	*Dioscorea alata*	India	JX010190	JX009990	JX009837	JX009471	JX010383	JX009738	KC888932	Wang et al. (2016)[15]
C. alienum	ICMP 12071*	*Malus domestica*	New Zealand	JX010251	JX010028	JX009882	JX009572	JX010411	JX009654	KC888927	Vieira et al. (2017)[14]
C. aotearoa	ICMP 18537*	*Coprosma* sp.	New Zealand	JX010205	JX010005	JX009853	JX009564	JX010420	JX009611	KC888930	Wang et al. (2016)[15]
C. asianum	CBS 130418*	*Coffea arabica*	Thailand	FJ972612	JX010053	JX009867	JX009584	JX010406	FJ917506	FR718814	Vieira et al. (2017)[14]
C. boninense	CBS 123755*	*Crinum asiaticum* var. sinicum	Japan	JQ005153	JQ005240	JQ005327	JQ005501	JQ005588	JQ005674	-	Fu et al. (2019)[1]
C. camelliae	ICMP 18542	*Camellia sasanqua*	USA	JX010223	JX009994	JX009857	JX009488	JX010429	JX009628	KJ954627	Wang et al. (2016)[15]
C. chrysophilum	CMM 4268*,	*Musa* sp.	Brazil	KX094252	KX094183	KX094083	KX093982	KX094285	KX094063	KX094325	Vieira et al. (2017)[14]
C. clidemiae	ICMP 18658*	*Clidemia hirta*	USA	JX010265	JX009989	JX009877	JX009537	JX010438	JX009645	KC888929	Wang et al. (2016)[15]
C. communis	MTCC 11599*	*Mangifera indica*	India	JQ894681	JQ894632	JQ894617	JQ894546	JQ894602	KC790791	JQ894582	Vieira et al. (2017)[14]
C. conoides	CGMCC 3.17615*	*Capsicum annuum*	China	KP890168	KP890162	KP890156	KP890144	KP890174	KP890150	-	Fu et al. (2019)[1]
C. cordylinicola	ICMP 18579*	*Cordyline fruticosa*	Thailand	JX010226	JX009975	JX009864	HM470235	JX010440	HM470238	JQ899274	Wang et al. (2016)[15]
C. dianesei	CMM 4085*	*Mangifera indica*	Brazil	KC329813	KX094158	KX094096	KC533740	KX094270	KX094051	KX094306	Vieira et al. (2017)[14]
C. endomangiferae	CMM 3814*	*Mangifera indica*	Brazil	KC702994	KC702955	KC598113	KC702922	KM404170	KC992372	KJ155453	Vieira et al. (2017)[14]
C. endophytica	CGMCC 3.17887	*Camellia sinensis*	China	KU251561	KU252015	KU251909	KU251642	KU252169	KU251804	KU251734	Wang et al. (2016)[15]
C. fructicola	CBS 130416*	*Coffea arabica*	Thailand	JX010165	JX010033	JX009866	FJ907426	JX010405	FJ917508	JQ807838	Vieira et al. (2017)[14]
C. fructicola	CBS 125397	*Tetragastris panamensis*	Panama	JX010173	JX010032	JX009874	JX009581	JX010409	JX009674	JQ807839	Vieira et al. (2017)[14]
C. fructicola	CGMCC3.17889	*Camellia sinensis*	China	KU251520	KU251974	KU251868	KU251601	KU252152	KU251763	KU251706	Wang et al. (2016)[15]
C. gloeosporioides	CBS 112999*	*Citrus sinensis*	Italy	JX010152	JX010056	JX009818	JX009531	JX010445	JX009731	JQ807843	Vieira et al. (2017)[14]
C. grossum	CAUG7*	*Capsicum* sp.	China	KP890165	KP890159	KP890153	KP890141	KP890171	KP890147	-	Diao et al. (2017)[4]
C. henanense	CGMCC 3.17354*	*Camellia sinensis*	China	KJ955109	KJ954810	-	KM023257	KJ955257	KJ954662	KJ954524	Wang et al. (2016)[15]
C. horii	ICMP 10492*	*Diospyros kaki*	Japan	GQ329690	GQ329681	JX009752	JX009438	JX010450	JX009604	JQ807840	Vieira et al. (2017)[14]
C. hymenocallidis	ICMP 18642*	*Hymenocallis americana*	China	JX010278	JX010019	GQ856730	GQ856775	JX010410	JX009709	JQ807842	Vieira et al. (2017)[14]
C. jasmini-sambac	ICMP 19118*	*Jasminum sambac*	Vietnam	HM131511	HM131497	JX009895	HM131507	JX010415	JX009713	JQ807841	Vieira et al. (2017)[14]

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Species	Culture[a]	Host/Tissue	Country/Region	GenBank accession number[b]							Ref.
				ITS	*GAPDH*	*CHS1*	*ACT*	*TUB2*	*CAL*	ApMAT	
C. jiangxiense	CGMCC 3.17363*	*Camellia sinensis*	China	KJ955201	KJ954902	-	KJ954471	KJ955348	KJ954752	KJ954607	Wang et al. (2016)[15]
C. kahawae subsp. ciggaro	ICMP 18539*	*Olea europaea*	Australia	JX010230	JX009966	JX009800	JX009523	JX010434	JX009635	-	Weir et al. (2012)[16]
C. kahawae subsp. kahawae	ICMP 17816*	*Coffea arabica*	Kenya	JX010231	JX010012	JX009813	JX009452	JX010444	JX009642	JQ894579	Weir et al. (2012)[16]
C. ledongense	CGMCC 3.18888*	*Hevea brasiliensis*	China, Hainan	MG242009	MG242017	MG242019	MG242015	MG242011	MG242013	-	Liu et al. (2018)[3]
C. melanocaulon	CBS 133251*	*Vaccinium macrocarpon*	USA	JX145144	KX094187	KX094110	KX093987	KX094290	KX094036	JX145313	Vieira et al. (2017)[14]
C. murrayae	CBS 133239*	*Murraya* sp.	China	JQ247633	JQ247609	-	JQ247657	JQ247644	JQ247596	-	Vieira et al. (2017)[14]
C. musae	CBS 116870*	*Musa* sp.	USA	JX010146	JX010050	JX009896	JX009433	HQ596280	JX009742	KC888926	Vieira et al. (2017)[14]
C. nupharicola	CBS 470.96*	*Nuphar lutea*	USA	JX010187	JX009972	JX009835	JX009437	JX010398	JX009663	JX145319	Wang et al. (2016)[15]
C. proteae	CBS 132882*	*Protea* sp.	South Africa	KC297079	KC297009	KC296986	KC296940	KC297101	KC296960	-	Wang et al. (2016)[15]
C. psidii	CBS 145.29*	*Psidium* sp.	Italy	JX010219	JX009967	JX009901	JX009515	JX010443	JX009743	KC888931	Weir et al. (2012)[16]
C. queenslandicum	ICMP 1778*	*Carica papaya*	Australia	JX010276	JX009934	JX009899	JX009447	JX010414	JX009691	KC888928	Vieira et al. (2017)[14]
C. rhexiae	CBS 133134*	*Rhexia virginica*	USA	JX145128	-	-	-	JX145179	-	JX145290	Wang et al. (2016)[15]
C. salsolae	ICMP 19051*	*Salsola tragus*	Hungary	JX010242	JX009916	JX009863	JX009562	JX010403	JX009696	KC888925	Vieira et al. (2017)[14]
C. siamense	CBS 130417*	*Coffea arabica*	Thailand	JX010171	JX009924	JX009865	FJ907423	JX010404	FJ917505	JQ899289	Vieira et al. (2017)[14]
C. syzygicola	MFLUCC 10-0624*	*Syzygium samarangense*	Thailand	KF242094	KF242156	-	KF157801	KF254880	KF254859	-	Wang et al. (2016)[15]
C. temperatum	CBS 133122*	*Vaccinium macrocarpon*	USA	JX145159	-	-	-	JX145211	-	JX145298	Wang et al. (2016)[15]
C. theobromicola	CBS 124945*	*Theobroma cacao*	Panama	JX010294	JX010006	JX009869	JX009444	JX010447	JX009591	KC790726	Vieira et al. (2017)[14]
C. ti	ICMP 4832*	*Cordyline* sp.	New Zealand	JX010269	JX009952	JX009898	JX009520	JX010442	JX009649	KM360146	Wang et al. (2016)[15]
C. tropicale	CBS 124949*	*Theobroma cacao*	Panama	JX010264	JX010007	JX009870	JX009489	GU994454	JX009719	GU994425	Vieira et al. (2017)[14]
C. viniferum	GZAAS 5.08601*	*Vitis vinifera*	China	JN412804	JN412798	-	JN412795	JN412813	JQ309639	-	Vieira et al. (2017)[14]
C. wuxiense	CGMCC 3.17894*	*Camellia sinensis*	China	KU251591	KU252045	KU251939	KU251672	KU252200	KU251833	KU251722	Wang et al. (2016)[15]
C. xanthorrhoeae	CBS 127831*	*Xanthorrhoea preissii*	Australia	JX010261	JX009927	JX009823	JX009478	JX010448	JX009653	KC790689	Weir et al. (2012)[16]

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[a]ICMP, International Collection of Microorganisms from Plants (New Zealand); CBS, Centraalbureau voor Schimmelcultures (Netherlands); COAD: Coleção Octávio Almeida Drummond, Viçosa, Brazil; CGMCC, China General Microbiological Culture Collection Center (China); MFLUCC: Mae Fah Luang University Culture Collection, Chiang Rai, Thailand; CMM: Culture Collection of Phytopathogenic Fungi, Prof. Maria Menezes, Federal Rural University of Pernambuco, Brazil; GZAAS, Guizhou Academy of Agricultural Sciences (China); BCRC, Bioresource Collection and Research Center, Hsinchu, Taiwan; *indicates the ex-type cultures.

[b]ITS: internal transcribed spacer; *GAPDH*: glyceraldehyde 3-phosphate dehydrogenase; *CHS-1*: chitin synthase; *ACT*: actin; *TUB2*: beta-tubulin; *CAL*: calmodulin; ApMAT: intergenic sequence between *Apn2* DNA lyase and *MAT1-2-1*.

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Supplemental Table 2.2. Conidia and appressoria measurements of five *Colletotrichum* spp. isolated in this study and type strains.

		Conidia			Appressoria			
Species	Accession No.[a]	Length x Width (μm)[b]	L/W ratio	Medium	Length x Width (μm)[b]	L/W ratio	Medium	Ref.
C. acutatum species complex								
C. miaoliense sp. nov.	This study	14.2 ± 1.1 × 4.1 ± 0.3	3.5	1/4 PDA	7.5 ± 1.1 × 5.0 ± 0.6	1.5	dH_2O[c]	This study
C. acutatum	CBS 112996*	12.6 ± 1.8 × 3.9 ± 0.3	3.2	SNA	7.3 ± 2.0 × 5.4 ± 1.2	1.3	SNA	Damm et al. (2012)[1]
C. *nymphaeae*	CBS 515.78*	16.1 ± 2.3 × 4.9 ± 0.7	3.3	SNA	8.7 ± 2.5 × 5.5 ± 1.0	1.6	SNA	Damm et al. (2012)[1]
C. simmondsii	CBS 122122*	8.1 ± 1.7 × 2.9 ± 0.4	2.7	SNA	7.8 ± 1.9 × 5.3 ± 1.1	1.5	SNA	Damm et al. (2012)[1]
C. boninensis species complex								
C. boninense	This study	15.2 ± 1.1 × 6.8 ± 0.5	2.3	PDA	8.9 ± 1.3 × 6.1 ± 0.7	1.5	dH_2O[c]	This study
C. boninense	CBS 123755*	12.8 ± 1.6 × 5.4 ± 0.4	2.4	SNA	10.5 ± 3.3 × 6.4 ± 1.5	1.6	SNA	Damm et al. (2012)[2]
C. karstii	This study	15.5 ± 1.2 × 6.5 ± 0.4	2.4	1/4 PDA	8.7 ± 1.2 × 6.4 ± 0.8	1.4	dH_2O[c]	This study
C. karstii	CGMCC3.14194*	15.4 ± 1.3 × 6.5 ± 0.5	2.4	PDA	9.3 ± 1.6 × 6.4 ± 0.9	1.5	PDA	Youlian et al. (2011)[3]
C. gloeosporioides sepcies coomplex								
C. fructicola	This study	13.0 ± 0.7 × 5.3 ± 0.4	2.5	1/4 PDA	8.1 ± 0.9 × 6.3 ± 1.0	1.3	dH_2O[c]	This study
C. fructicola	CBS 130416*	11.5 ± 1.0 × 3.6 ± 0.3	3.2	PDA	7.4 ± 1.3 × 4.5 ± 0.9	1.6	PDA	Prihastuti et al. (2009)[4]; Weir et al. (2012)[5]
C. siamense	This study	14.5 ± 0.9 × 4.9 ± 0.3	3.0	1/4 PDA	8.4 ± 0.9 × 5.7 ± 0.5	1.5	dH_2O[c]	This study
C. siamense	CBS 130417*	10.2 ± 1.7 × 3.5 ± 0.4	2.9	PDA	6.7 ± 1.1 × 4.1 ± 0.4	1.6	PDA	Prihastuti et al. (2009)[4]; Weir et al. (2012)[5]

[a]* indicates type strains.

[b]Data are mean ± standard deviation.

[c]Appressoria were induced in dH_2O on a microscopic slide.

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Supplemental Table 2.3. Mycelial growth rates (mm day^{-1}) of *Colletotrichum* spp. under different temperatures.

Temperature (°C)	*C. siamense* ML133	*C. fructicola* ML348	*C. karstii* ML351	*C. boninense* ML521	*C. miaoliense* ML1040
18	6.5 ± 0.1 a	6.1 ± 0.0 b	5.6 ± 0.1 c	6.0 ± 0.0 b	3.9 ± 0.0 d
22	6.5 ± 0.2 a	7.2 ± 0.4 a	5.3 ± 0.2 b	7.2 ± 0.1 a	4.5 ± 0.2 b
25	9.1 ± 1.0 a	9.4 ± 0.6 a	8.2 ± 0.3 a	9.1 ± 0.3 a	6.0 ± 0.2 b
28	10.3 ± 0.6 a	8.7 ± 0.2 b	7.4 ± 0.4 b	8.4 ± 0.2 b	5.7 ± 0.3 c
30	10.0 ± 0.3 a	7.7 ± 0.2 b	7.5 ± 0.3 b	6.4 ± 0.6 bc	5.6 ± 0.4 c
32	7.5 ± 0.8 a	5.6 ± 0.2 b	5.8 ± 0.3 b	1.5 ± 0.2 d	3.6 ± 0.4 c

The results from the same temperature were analyzed together. Data (mean ± standard error) with different letters are significantly different based on Tukey's range test at $P < 0.05$.

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Supplemental Table 2.4. Mycelial growth rates (mm day^{-1}) of *Colletotrichum* spp. at 25°C and 30°C.

Temp. (°C)	*C. siamense*			*C. fructicola*		*C. karstii*		*C. miaoliense*	
	ML133	ML540	ML612	ML356	ML368	ML442	ML1792	ML1042	ML1794
25	11.9 ± 0.2 a	12.0 ± 0.2 a	11.4 ± 0.3 ab	10.6 ± 0.2 bcd	10.7 ± 0.2 bc	9.7 ± 0.2 d	9.7 ± 0.2 cd	7.7 ± 0.1 e	7.5 ± 0.1 e
30	11.8 ± 0.2 a	12.0 ± 0.3 a	10.0 ± 0.6 bc	8.6 ± 0.2 cd	10.5 ± 0.2 b	8.3 ± 0.3 d	8.9 ± 0.3 cd	7.0 ± 0.2 e	6.6 ± 0.2 e

The results from the same temperature were analyzed together. Data (mean ± standard error) with different letters are significantly different based on Tukey's range test at $P < 0.05$.

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Supplemental Table 2.5. Primers used in this study.

Gene/Locus	Primer name	Sequence	Reference
Internal transcribed spacer (ITS)	ITS1	5'- TCC GTA GGT GAA CCT GCG G -3'	White et al (1990)[1]
	ITS4	5'- TCC TCC GCT TAT TGA TAT GC -3'	White et al (1990)[1]
Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*)	GDF3	5'- GCC GTC AAC GAC CCC TTC ATT GA -3'	This study
	GDR3	5'-TTC TCG TTG ACA CCC ATC ACG TAC ATG -3'	This study
Chitin synthase (*CHS-1*)	CHS-79F	5'- TGG GGC AAG GAT GCT TGG AAG AAG -3'	Carbone and Kohn (1999)[2]
	CHS-345R	5'- TGG AAG AAC CAT CTG TGA GAG TTG -3'	Carbone and Kohn (1999)[2]
Actin (*ACT*)	ACT-512F	5'- ATG TGC AAG GCC GGT TTC GC -3'	Carbone and Kohn (1999)[2]
	ACT-783R	5'- TAC GAG TCC TTC TGG CCC AT -3'	Carbone and Kohn (1999)[2]
β-Tubulin 2 (*TUB2*)	T1	5'- AAC ATG CGT GAG ATT GTA AGT -3'	O'Donnell and Cigelnik (1997)[3]
	T2	5'- TAG TGA CCC TTG GCC CAGT TG -3'	O'Donnell and Cigelnik (1997)[3]
Calmodulin (*CAL*)	CL1C	5'- GAA TTC AAG GAG GCC TTC TC -3'	Weir et al (2012)[4]
	CL2C	5'- CTT CTG CAT CAT GAG CTG GAC -3'	Weir et al (2012)[4]
Intergenic sequence between *Apn2* DNA lyase and *MAT1-2-1* (ApMAT)	APF-long	5'- TCA TTC TAC GTA TGT GCC CGC CCG TTG -3'	This study; Silva et al. (2012)[5]
	APR-long	5'- CCA GAA ATA CAC CGA ACT TGC AAA GAT -3'	This study; Silva et al. (2012)[5]

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Supplemental Table 2.6. Nucleotide substitution models used in phylogenetic analyses.

Gene/Locus[a]	Acutatum clade[b]	Boninenses clade[b]	Gloeosporioides clade[b]
ITS	HKY+I	SYM+I+G	GTR+I
GAPDH	HKY+G	HKY+I	HKY+I
CHS-1	K80+I	K80+G	K80+G
ACT	GTR+G	GTR+G	HKY+G
TUB2	GTR+G	HKY+I	GTR+G
CAL	-	GTR+G	GTR+G
ApMAT	-	-	HKY+G

[a]ITS: internal transcribed spacer; *GAPDH*: glyceraldehyde 3-phosphate dehydrogenase; *CHS-1*: chitin synthase; *ACT*: actin; *TUB2*: beta-tubulin; *CAL*: calmodulin; ApMAT: intergenic sequence between *Apn2* DNA lyase and *MAT1-2-1*.

[b]K80**:** Kimura 2-parameter; HKY: Hasegawa-Kishino-Yano; SYM: symmetrical model; GTR: general time reversible; G: gamma distribution; I: proportion of invariable sites.

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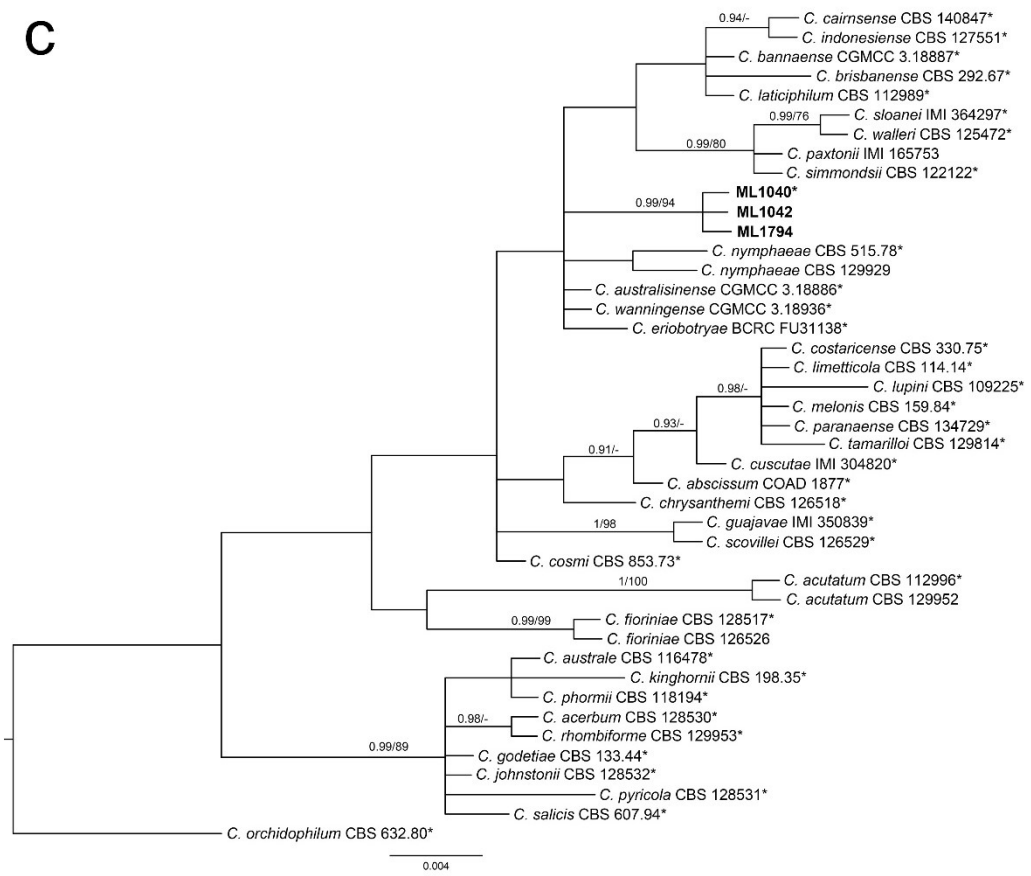

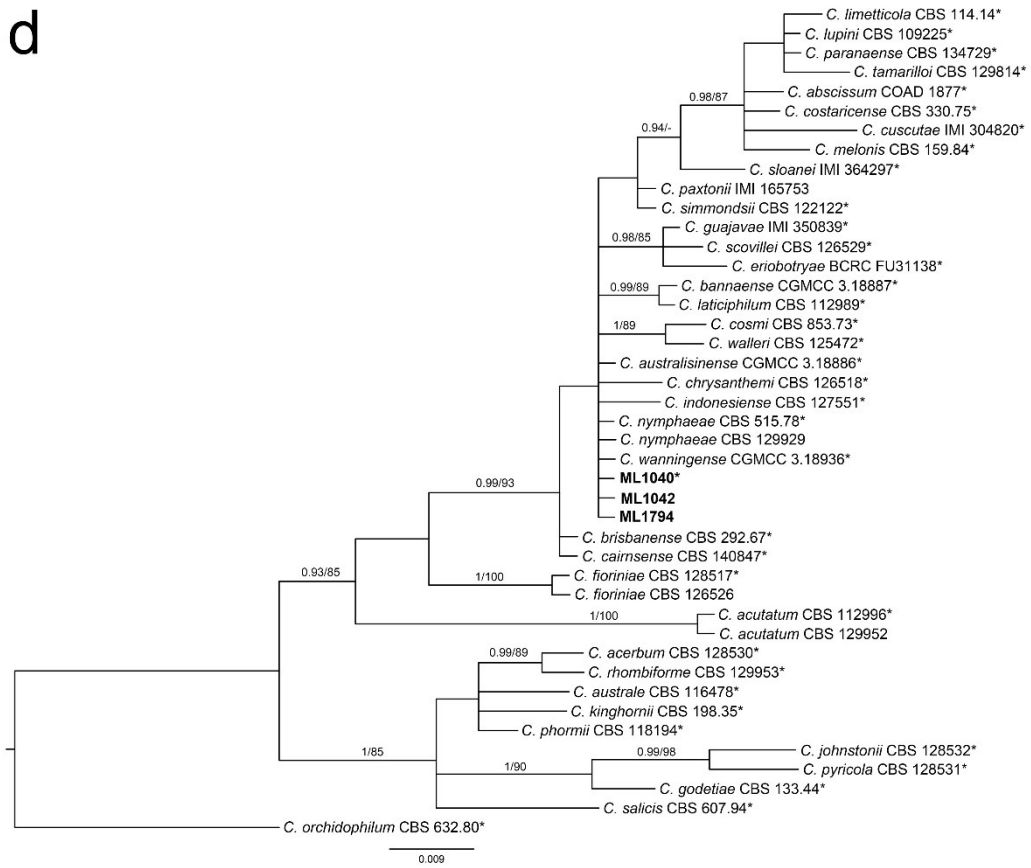

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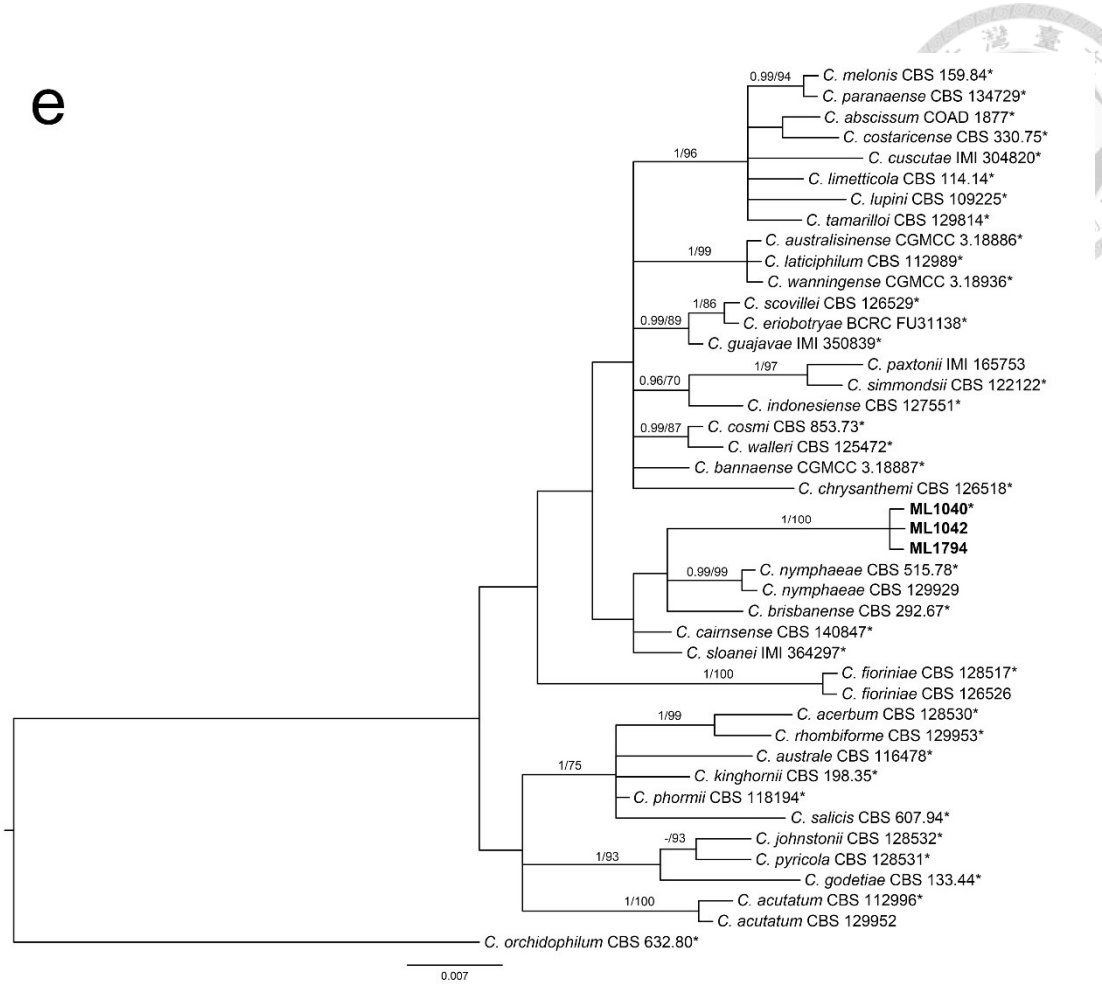

Supplemental Fig. 2.1. Single-locus Bayesian inference phylogenetic trees of the *C. acutatum* species complex.

The phylogenetic trees were built using the sequences of (a) ITS, (b) *GAPDH*, (c) *CHS-1*, (d) *ACT* and (e) *TUB2*. Bayesian inference (BI) posterior values above 0.9 and bootstrap support values from maximum likelihood (ML) above 70% are shown at each node (BI/ML). *C. orchidophilum* CBS 632.80 was used as the outgroup. * indicates the ex-type strains. Strains isolated in this study are shown in bold.

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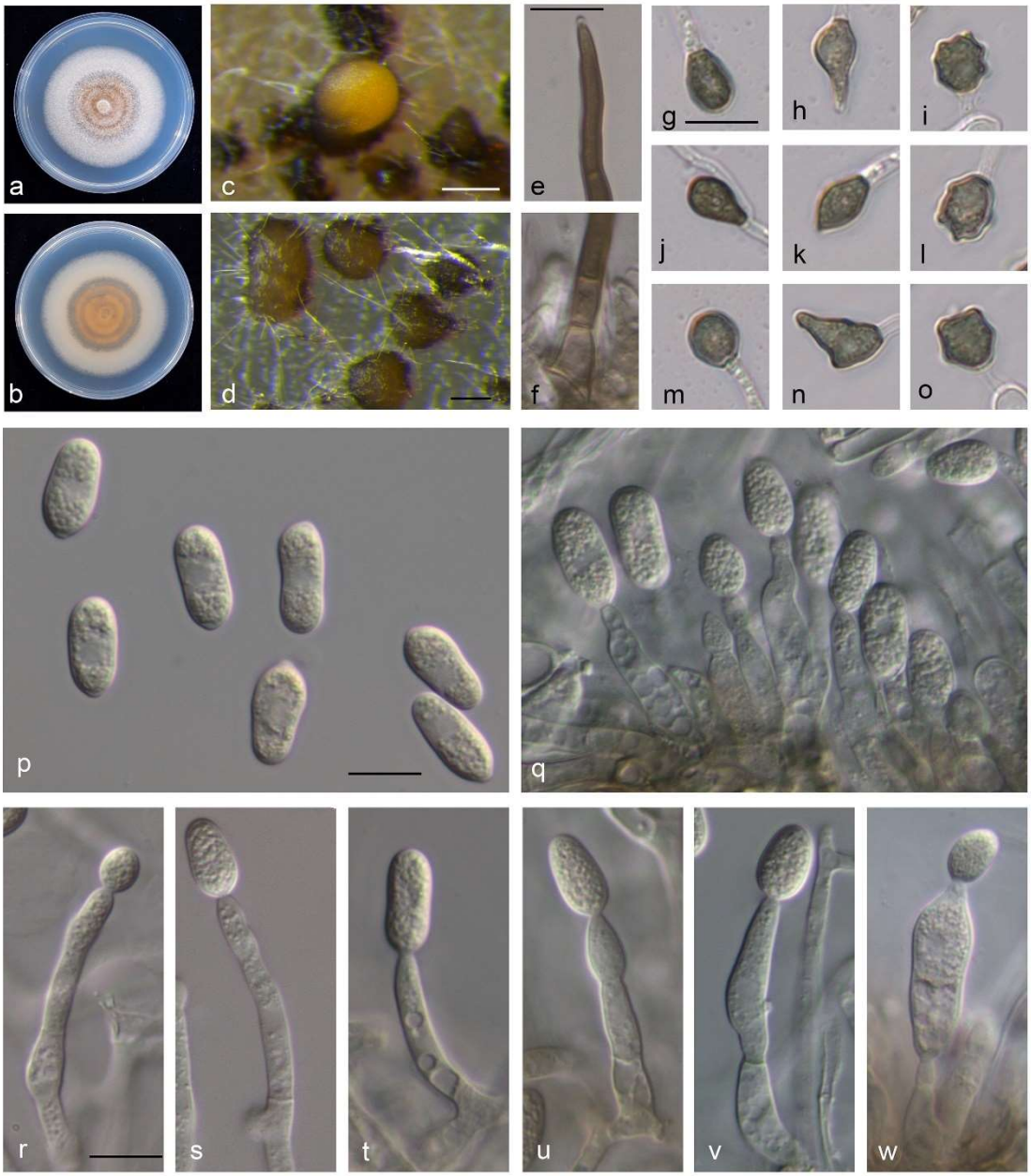

Supplemental Fig. 2.2. *Colletotrichum boninense* ML521.

(a) Upper side of colony; (b) reverse side of colony; (c-d) conidiomata; (e) tip of seta; (f) base of seta. (g-o) appressorium (induced in dH_2O on a microscope slide) [8.88 ± 1.33 × 6.12 ± 0.71 µm (n = 30)]; (p) conidia [18.23 ± 1.12 × 6.77 ± 0.54 µm (n = 100)]; (q-w) conidiophores. a-b on potato dextrose agar (PDA); c-f and p-w on 1/4-strength PDA. Scale bars: c, d = 0.2 mm; e, g, p, r = 10 µm, applies to e-w.

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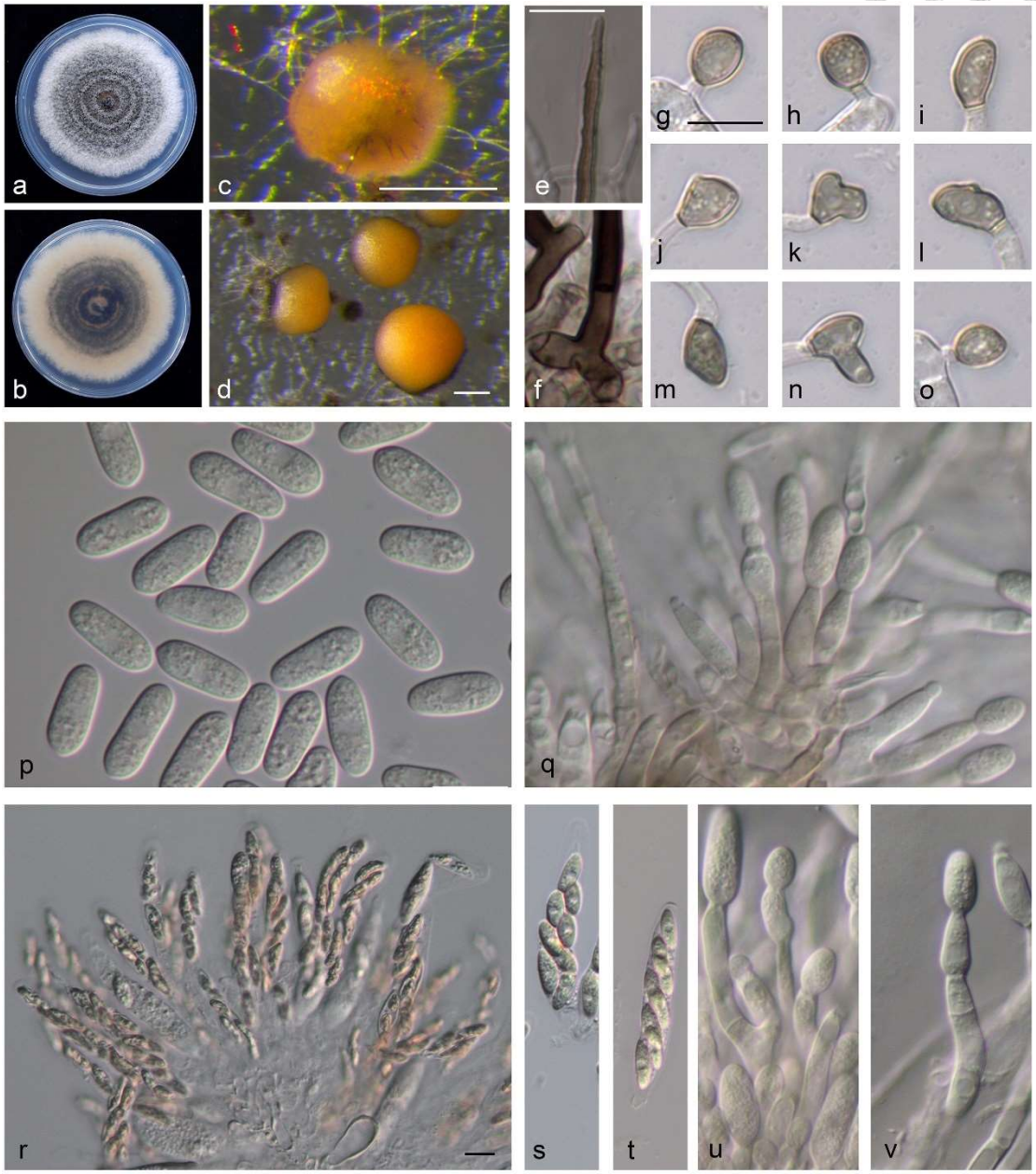

Supplemental Fig. 2.3. *Colletotrichum fructicola* ML348.

(a) Upper side of colony; (b) reverse side of colony; (c-d) conidiomata; (e) tip of seta; (f) base of seta; (g-o) appressorium (induced in dH_2O on a microscope slide) [8.09 ± 0.89 × 6.31 ± 1.03 µm (n = 30)]; (p) conidia [13.03 ± 0.69 × 5.29 ± 0.4 µm (n = 100)]; (q, u-v) conidiophores; (r-t) asci. a-b on potato dextrose agar (PDA); c-f and p-v on 1/4-strength PDA. Scale bars: c, d = 0.2 mm; e, g = 10 µm, applies to e-q, u-v; r = 10 µm, applies to r-t.

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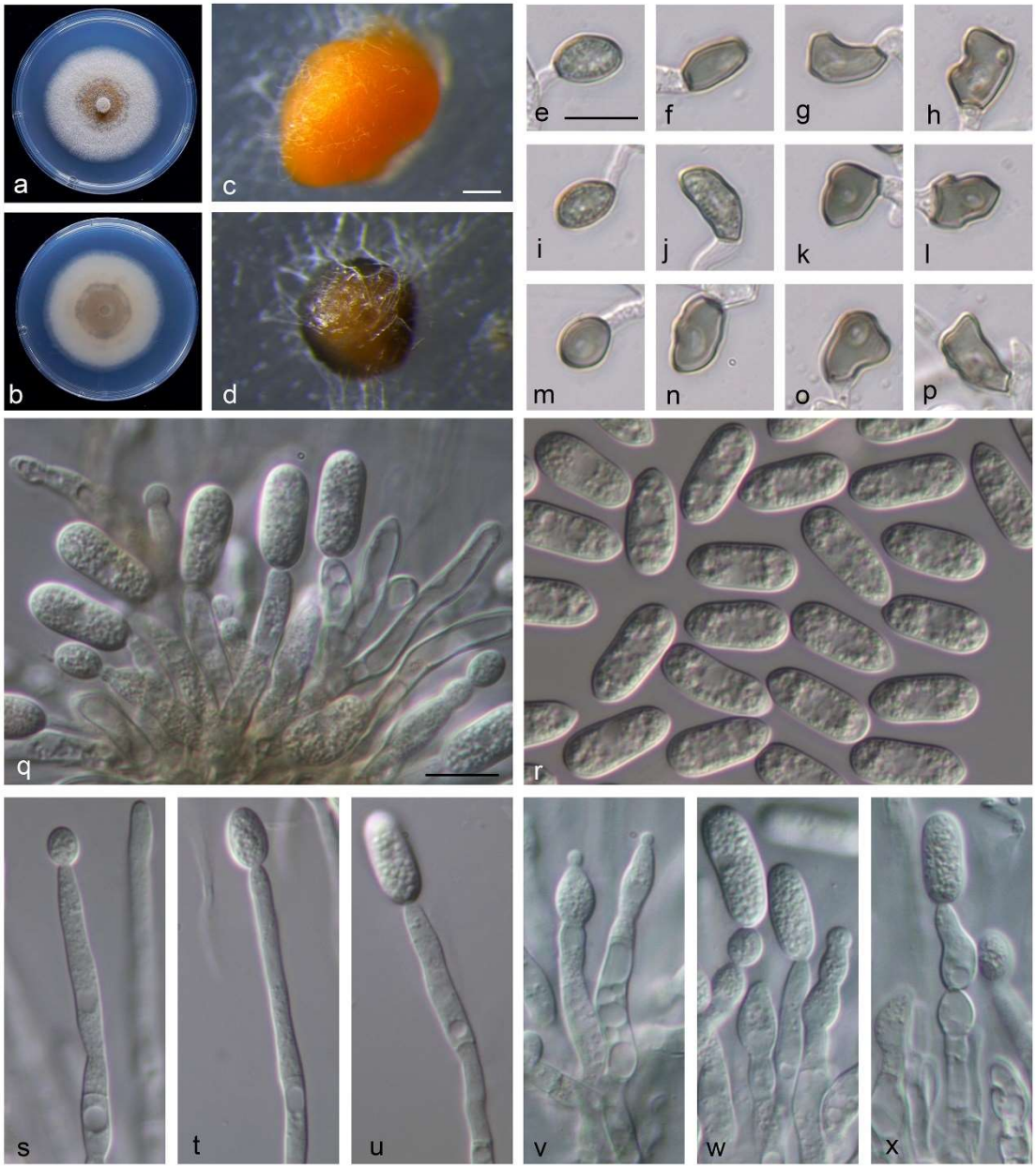

Supplemental Fig. 2.4. ***Colletotrichum karstii*** **ML351.**

(a) Upper side of colony; (b) reverse side of colony; (c-d) conidiomata; (e-p) appressorium (induced in dH_2O on a microscope slide) [8.73 ± 1.24 × 6.40 ± 0.75 µm (n = 30)]; (q, s-x) conidiophore; (r) conidia [15.53 ± 1.23 × 6.49 ± 0.36 µm (n = 100)]. a-b on potato dextrose agar (PDA); c-d and q-x on 1/4-strength PDA. Scale bars: c = 0.2 mm, applies to d; e, q = 10 µm, applies to e-x.

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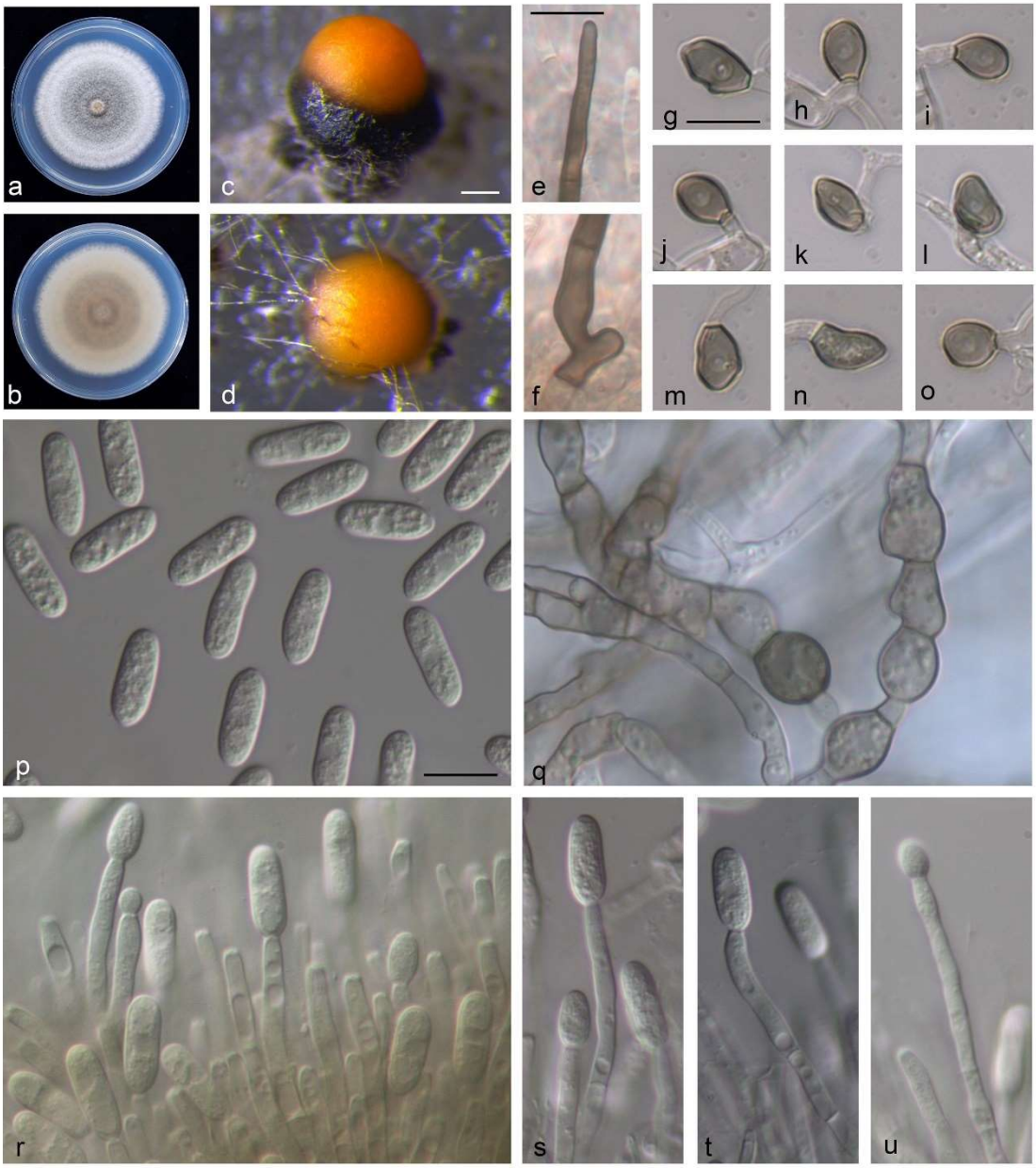

Supplemental Fig. 2.5. *Colletotrichum siamense* ML133.

(a) Upper side of colony; (b) reverse side of colony; (c-d) conidiomata; (e) tip of seta; (f) base of seta; (g-o) appressorium (induced in dH_2O on a microscope slide) [8.44 ± 0.91 × 5.73 ± 0.47 (n = 30)]; (p) conidia [14.47 ± 0.88 × 4.88 ± 0.27 μm (n = 100)]; (q) thick-walled mycelium; (r-u) conidiophore. a-b on potato dextrose agar (PDA); c-f and p-u on 1/4-strength PDA. Scale bars: c = 0.2 mm, applies to d; e, g, p = 10 μm, applies to e-u.

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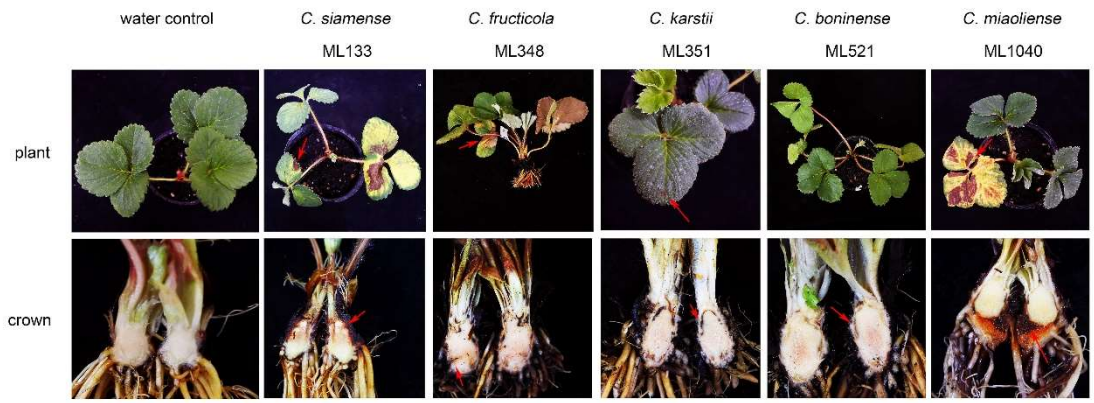

Supplemental Fig. 2.6. Leaf necrosis and crown rot symptoms caused by *Colletotrichum* spp.

Strawberry seedlings were spray-inoculated with spore suspension on the leaves and treated with spore suspension on the crown after removal of old leaves. Water was used as a control. The photos were taken at 14 days post inoculation.

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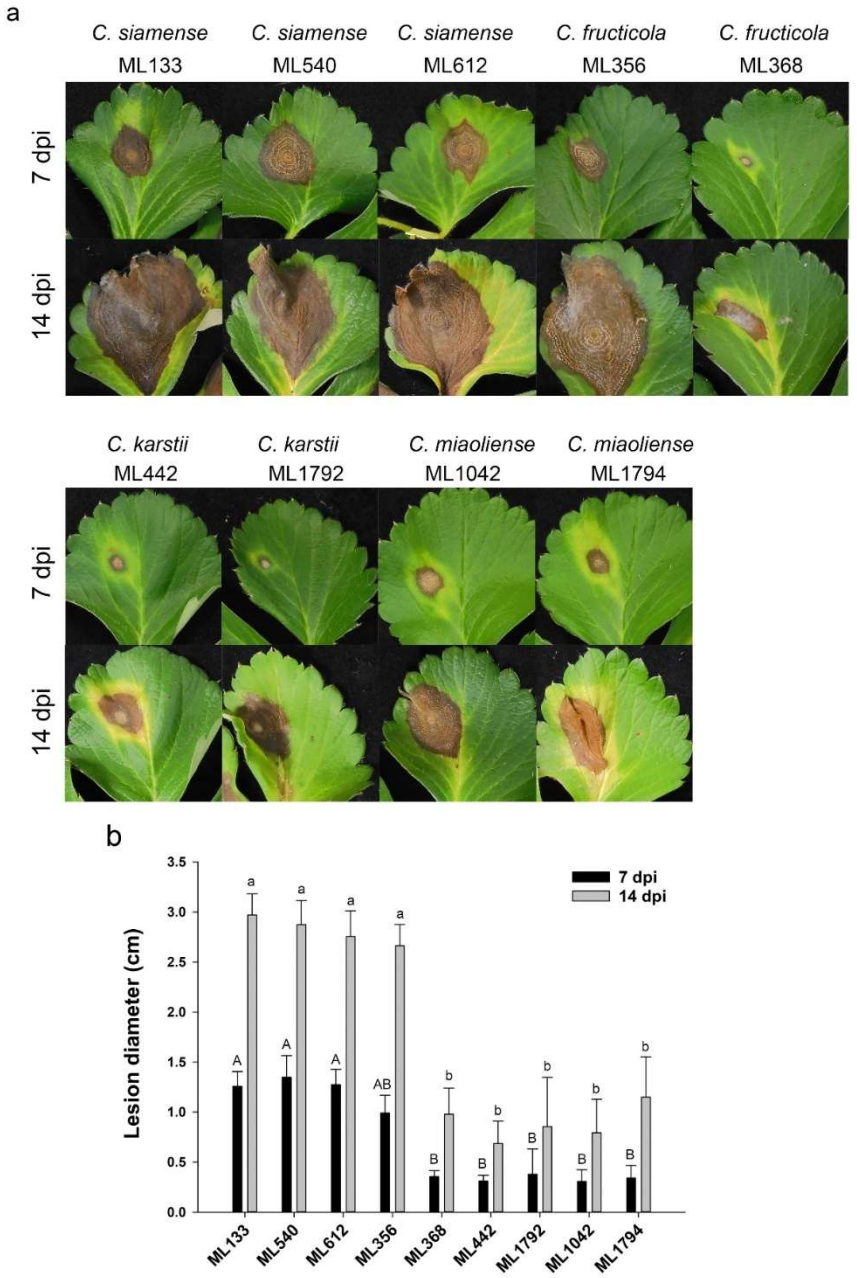

Supplemental Fig. 2.7. Inoculation of representative isolates of different *Colletotrichum* spp. on detached strawberry leaves at 30°C.

(a) Lesions at 7 and 14 days post inoculation (dpi) on wounded leaves. The left side of each leaflet was inoculated with 10 µl spore suspension (10^6 spores/ml) and the right side with water (control). (b) Lesion sizes. The results from the same day were analyzed together. Data (mean ± standard error) with different letters are significantly different based on Tukey's range test at $P < 0.05$ (n = 8).

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Chapter 3

Development of a nested PCR assay for detecting *Colletotrichum siamense* and *Colletotrichum fructicola* on symptomless strawberry plants

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鐘珮哲、吳竑毅、洪挺軒、鍾嘉綾。2021。臺灣草莓炭疽病菌族群分析與檢測技術開發。2021 草莓研發成果與產業應用研討會專輯 66-74。

鐘珮哲、吳竑毅、曹嘉惠。2020。偵測潛伏感染期草莓炭疽病病原菌技術之研發。苗栗區農業改良場研究彙報 9:46-57。

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3.1 Abstract

Anthracnose is a major disease of strawberry that seriously impacts the strawberry industry. To prevent the spread of anthracnose through symptomless plants, it is important to detect pathogenic *Colletotrichum* spp. at the latent infection stage in the nursery. Previous PCR-based methods developed for the diagnosis or detection of *Colletotrichum acutatum* and *Colletotrichum gloeosporioides* have used primers targeting the internal transcribed spacer region of ribosomal DNA, β-tubulin gene, or mating type gene. In this study, to specifically detect *Colletotrichum siamense* and *Colletotrichum fructicola*, the most predominant and virulent *Colletotrichum* species causing strawberry anthracnose in Taiwan, we conducted a comparative genomics analysis of 29 *Colletotrichum* spp. and identified a non-conserved 1157-bp intergenic region suitable for designing specific primers for a nested PCR assay. *In silico* analysis and actual tests suggested that the new nested PCR assay could detect pathogenic *C. siamense* and *C. fructicola*, but not other strawberry pathogens (*Botrytis* sp., *Fusarium* spp., *Neopestalotiopsis rosae*, and *Phytophthora* sp.) or ubiquitous saprophytes (*Fusarium* spp. and *Trichoderma* spp.). The outer to inner primer ratio was optimized to 1:10 to eliminate unexpected bands and enhance the signal. The assay could detect as little as 1 pg of *C. siamense* genomic DNA, which corresponds to ~15 cells. Application of the new detection assay on 747 leaf samples collected from 18 strawberry nurseries in 2019 and 2020 showed that an average of 20% of strawberry mother plants in Taiwan were latently infected by *C. siamense* or *C. fructicola*. The newly developed assay is being applied to facilitate the production of healthy strawberry runner plants in Taiwan.

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3.2 Introduction

Strawberry (*Fragaria* × *ananassa* Duch.) is an economically important small fruit crop that can grow in temperate, sub-tropical, and tropical regions around the world. The average strawberry cultivation area in Taiwan was about 514 ha from 2016 to 2020 (FAOSTAT 2020), and Miaoli County is the predominant strawberry production region (~90% of the total cultivated area), where the major cultivars are 'Taoyuan No. 1,' and 'Xiang-Shui'. Farmers in Taiwan usually propagate strawberry runner plants from mother plants in the spring and summer. The high temperature and intermittent heavy rains during the monsoon and typhoon seasons create environmental conditions conducive to infectious disease and epidemics. In recent years, strawberry anthracnose has become a more serious problem, causing the death of about 20%–40% of plants (Chung et al. 2019). A high percentage of severely diseased plants are removed during the nursery stage or within 1–2 months after transplanting, which eventually leads to considerable economic loss.

Colletotrichum is a fungal genus containing important plant pathogens causing anthracnose diseases of various economically valuable crops (Cannon et al. 2012). Several *Colletotrichum* species can infect strawberry, causing leaf spot, crown rot, stolon spot, petiole spot, and fruit rot (Howard et al. 1992). Based on traditional morphological characteristics, the major pathogens of strawberry anthracnose are considered to be *C. fragariae*, *C. gloeosporioides*, and *C. acutatum* (Denoyes and Baudry 1995; Smith 2008). Recent advances in molecular phylogenetics revealed that *C. gloeosporioides* and *C. acutatum* are highly diverse species complexes and can be classified into several species (Damm et al. 2012; Weir et al. 2012). *C. nymphaeae* in the *C. acutatum* species complex was identified as the major pathogen of strawberry anthracnose in the UK and USA (Baroncelli et al. 2015; Wang et al. 2019); *C. fructicola* (*C. gloeosporioides* species

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complex) in Japan (Chiba Prefecture) and China (Zhejiang province) (Chen et al. 2020; Gan et al. 2017); and *C. siamense* (*C. gloeosporioides* species complex) in China (Hubei province) (Han et al. 2016). In Taiwan, a recent investigation of the diversity of infectious agents causing strawberry anthracnose in Miaoli, Hsinchu, Nantou, and Chiayi Counties from 2010 to 2018 revealed five *Colletotrichum* spp., i.e., *C. siamense* (75% of all isolates), *C. fructicola* (11%), *C. karstii* (6%), *C. miaoliense* (6%), and *C. boninense* (2%) (Chung et al. 2020).

Colletotrichum is a hemibiotrophic pathogen that can remain in a latent state in the host tissues. Its conidium germinates to form an appressorium on the plant surface, which is followed by penetration peg invasion of the epidermal cells and the production of primary hyphae (biotrophic phase). The time required from the adherence of the conidia to successful infection can be within 48 hours (Jayawardena et al. 2021; O'Connell et al. 2012). At this stage, no visible difference can be observed between healthy and infected tissues (De Silva et al. 2017; Peres et al. 2005). However, *Colletotrichum* is capable of producing secondary conidia (Leandro et al. 2003; Leandro et al. 2001), which makes the latently infected but asymptomatic mother plants an important inoculum source for runner plants in the nursery. Latently infected runner plants can also be an inoculum source in production fields (Debode et al. 2009; Debode et al. 2015; Parikka and Lemmetty 2004). After the lifestyle transition from biotrophy to necrotrophy, this polycyclic pathogen produces masses of conidia, spreads rapidly by rain splash, and causes epidemics under suitable conditions in the field (Debode et al. 2015).

The use of pathogen-free strawberry mother plants and their propagules is a critical step for disease control in the nursery and production fields and thus the reduction of losses caused by anthracnose (Debode et al. 2015). Several methods for diagnosing latent infection or distinguishing *Colletotrichum* spp. infecting strawberry have been developed

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(Cerkauskas 1987; Debode et al. 2009; Freeman et al. 2001; Freeman and Katan 1997; Furuta et al. 2017; Garrido et al. 2009; Ishikawa 2003; Ishikawa 2004; Martinez-Culebras et al. 2000; Mertely and Legard 2004; Parikka and Lemmetty 2004; Sreenivasaprasad et al. 1996). For instance, culture-based methods involve incubation of strawberry tissues after surface sterilization with ethanol or killing with paraquat or freezing (Cerkauskas 1987; Ishikawa 2003; Ishikawa 2004; Mertely and Legard 2004). These treatments promote the necrotrophic growth of the latently infected pathogens, resulting in the formation of yellow to orange conidiomata after 1–2 weeks of incubation. Culture-based methods are simple and convenient but a long time is required for sporulation; in addition, a great deal of experience is required to correctly differentiate *Colletotrichum* spp. from other fast-growing fungi with similar morphology (Ishikawa 2003; Ishikawa 2004). Faster and more accurate PCR-based detection methods have also been developed, using specific primers targeting the internal transcribed spacer (ITS) region of ribosomal DNA (Freeman et al. 2001; Garrido et al. 2009; Martinez-Culebras et al. 2000; Sreenivasaprasad et al. 1996), β-tubulin (*TUB2*) gene (Debode et al. 2009), or mating type gene *MAT1-2* (Furuta et al. 2017). During latent infection with *Colletotrichum* spp., the number of pathogen cells at the biotrophic phase is very low (Munch et al. 2008; Ohm et al. 2012), and only methods with high sensitivity (e.g., nested PCR or qPCR) can be applied to detect such a low amount of target DNA.

PCR-based detection methods were previously designed to detect the anthracnose pathogens *C. acutatum* and *C. gloeosporioides* in strawberry (Debode et al. 2009; Freeman et al. 2001; Furuta et al. 2017; Garrido et al. 2009; Parikka and Lemmetty 2004; Perez-Hernandez et al. 2008; Sreenivasaprasad et al. 1996). However, *C. acutatum* and *C. gloeosporioides* are now considered a species complex based on recent evidence from multilocus molecular phylogenetic analyses (Damm et al. 2012; Weir et al. 2012), so the

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previous methods may not be able to distinguish current taxonomic species. This study aimed to develop a highly sensitive and specific method applicable for detecting the anthracnose pathogens on symptomless strawberry plants in Taiwan (the workflow of this study is shown in Fig. 3.1). Due to the short period of time required for pathogenic *Colletotrichum* spp. to invade host tissues (Jayawardena et al. 2021; O'Connell et al. 2012), the assay mainly targets the pathogens in the latent infection stage, although a small number of pathogens present on the host surface cannot be excluded. *C. siamense* and *C. fructicola*, the most predominant and virulent *Colletotrichum* species causing strawberry anthracnose in Taiwan (Chung et al. 2020), were targeted. Comparative genomics analysis of 29 available *Colletotrichum* spp. was conducted to search for a non-conserved region suitable for designing primers for a nested PCR assay (Fig. 3.1A). *In silico* analysis and specificity tests were conducted to rule out detection of other pathogenic and saprophytic fungi frequently isolated from strawberries (Fig. 3.1B), and the ratio of outer and inner primers used in the nested PCR were optimized to eliminate unexpected PCR products. To verify the new method and investigate the latent infection of strawberry plants by *Colletotrichum* spp. in Taiwan, a field survey was conducted on 747 asymptomatic mother plants in 18 strawberry nurseries (Fig. 3.1C). As the production of strawberry runner plants is moving from propagation by small farmers toward professional propagation, it is expected that the highly specific and sensitive new method developed here will help reduce the disease incidence in mother plants, thereby increasing the rate of healthy runner plants in Taiwan.

3.3 Materials and Methods

3.3.1 Fungal isolation and cultivation

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The isolates of *Botrytis* sp. and *Colletotrichum* spp. (Chung et al. 2020), *Fusarium* spp., *Phytophthora* sp., and *Neopestalotiopsis rosae* (Wu et al. 2021), and *Trichoderma* spp. (Chou et al. 2019) used in this study are listed in Table S7. In addition to five species causing strawberry anthracnose (*C. siamense*, *C. fructicola*, *C. karstii*, *C. miaoliense*, and *C. boninense*) (Chung et al. 2020) and *N. rosae*, which causes strawberry leaf blight and crown rot (Wu et al. 2021), we isolated *Botrytis* sp. from strawberry fruit showing gray mold, *Fusarium* spp. from the root, crown, and nearby soil of diseased plants showing typical Fusarium wilt symptoms, and *Phytophthora* sp. from the root of a wilted strawberry plant. A *Fusarium* sp. and *Trichoderma* sp. isolated from the symptomless runner and petiole of a strawberry plant and a *Trichoderma asperellum* isolate collected from the rhizosphere soil of grape were also included. Fungal isolation from host tissues was conducted as described by Chung et al. (2020) (Chung et al. 2020). Tissues approximately 3 x 3 mm in area were surface sterilized with 0.5%–1% sodium hypochlorite, rinsed with sterile deionized water three times, then placed onto 1.5% water agar at 25°C. *Fusarium* isolation from soil was carried out by mixing 10 g of soil with 90 ml of 0.05% agar solution, then evenly spreading 200 μl of 10-fold serial dilutions on FoG1 medium (*Fusarium* colonies are purple on FoG1 medium) (Nishimura 2007). After 2 to 3 days of incubation, extended single hyphal tips from tissues were transferred to potato dextrose agar (PDA, BD Difco) and incubated for 5–7 days at 25°C under a 12-h/12-h light/dark photoperiod. Fungal isolates were identified to the genus level by morphological characteristics and ITS sequences (as described below).

3.3.2 DNA extraction and sequence alignment

Genomic DNA was extracted from strawberry leaves or petioles using a plant genomic DNA extraction mini-prep system (VIOGENE) according to the procedures

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provided by the manufacturer. For extraction of fungal genomic DNA, the mycelium collected from a 7-day-old colony grown on PDA was frozen in liquid nitrogen and ground to a fine powder with a sterile mortar and pestle. The ITS was amplified with ITS1/ITS4 primers (White et al. 1990). Amplicons were bidirectionally sequenced on an ABI 3730 DNA analyzer (Tri-I Biotech, Taiwan), and the sequences were used as queries in blast searches against the NCBI GenBank nr/nt database (blast.ncbi.nlm.nih.gov).

3.3.3 Target region selection and primer design

To identify ideal regions for primer design, we searched for non-conserved regions located in between two conserved regions in the genomes of *Colletotrichm* spp. The conserved regions were used to design primers to sequence the internal non-conserved regions, which are highly diverse and can be used to distinguish *Colletotrichum* species. Based on the initial results of blastn searches against 29 *Colletotrichum* genome sequences (Table S8) using the ITS, chitin synthase (*CHS-1*), actin (*ACT*), *TUB2*, calmodulin (*CAL*), and intergenic region between the *Apn2* DNA lyase and *MAT1-2* (ApMAT) genes (sequences obtained from our previous study [2]) as queries, *C. gloeosporioides* strain 30206, *C. gloeosporioides* Cg-14, and *C. fructicola* Nara gc5 (which was designated *C. gloeosporioides* before 2018) were the closest strains to *C. siamense* ML133. Note that the primer design for this study was conducted in 2017, at a time when the genome sequence of *C. siamense* was not available (*C. siamense* ICMP18578 was released in 2019). Therefore, *C. gloeosporioides* 30206 was used as a query to search against the genomes of 26 *Colletotrichum* species (all strains in Table S8 except *C. gloeosporioides* Cg-14, *C. gloeosporioides* 30206, and *C. fructicola* Nara gc5). All genome sequences were downloaded from the NCBI genome database [https://www.ncbi.nlm.nih.gov/genome/], and the genome blast was performed using

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BLAST Command Line Applications (Altschul et al. 1990) following the user manual [https://www.ncbi.nlm.nih.gov/books/NBK279690/]. The blastn parameters were set to word size 28, e value $<10^{-5}$, and output format 5. The output file was parsed using Python (Van and Drake 2009). The 500- to 2500-bp non-conserved regions between conserved hit regions (hereafter referred to as 'spacers') were selected. The 1000-bp upstream and 1000-bp downstream sequences of each selected spacer were blasted against the genome sequences of 29 *Colletotrichum* spp. (blastn word size 28, e value $<10^{-100}$). Spacers of 1000–1500 bp in length were selected from among those with upstream and downstream sequence hit numbers ≥ 50. Candidate spacers were checked manually and a region suitable for designing high-quality primers for a nested PCR assay was selected. The identified spacer region in *C. siamense* ML133 was sequenced by the primer pair 5'-TTGGCCTGCGCTTCAACGAC-3' (forward) and 5'-AACTCACCCGCAAACACCAGT-3' (reverse). Primers for the first PCR (outer primers) and second PCR (inner primers) were designed based on the spacer sequences. Primers with high scores and that were compatible with each other were chosen using Oligo 7 software (Rychlik 2007). Furthermore, nested PCR primer candidates were blasted against *Fragariae* x *ananassa* (NCBI accession No. PRJDB1477) and other pathogen/microbial genomes, including *Fusarium* spp. (Table S9), *Trichoderma* spp. (Table S10), and strawberry pathogens *Botrytis cinerea*, *Phytophthora cactorum*, and *Xanthomonas fragariae*, to rule out possible non-target reactions *in silico*. The primers with lower hit numbers and lower e values were chosen.

3.3.4 Specificity and sensitivity of the nested PCR assay

Fifteen fungal isolates including pathogens and saprophytes isolated from strawberry or soil (Table S7) and three strawberry cultivars (i.e., 'Taoyuan No. 1',

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‘Xiang-Shui,’ and ‘Miaoli No. 1’) were used for evaluation of the specificity of the nested PCR assay. The primers targeting the ITS (ITS1/ITS4 (White et al. 1990)) and *ACTIN* gene (Actin-F/Actin-R (Zhou et al. 2015)) were used to test the quality of fungal and strawberry DNA, respectively. Each PCR reaction was performed in a 50-μl mixture containing 2.5 U Taq polymerase (Prime Taq, GenetBio). For ITS and *ACTIN*, each reaction contained 1–20 ng DNA and 0.2 μM of each primer. For the nested PCR assay, the first PCR reaction contained 1–20 ng DNA and 0.02 μM of each outer primer (Col_nest-1F/Col_nest-1R), and the second PCR reaction contained 1 μl of the first PCR product and 0.2 μM of each inner primer (Col_nest-2F/Col_nest-2R) (primer sequences in Table 3.1). Different ratios of outer primers to inner primers (1:1, 1:2, 1:5, 1:10, 1:20, 1:50, and 1:100) were tested and 1:10 was found to be optimal. The conditions for the first PCR were an initial denaturation at 94°C for 5 min, followed by 40 cycles of 94°C denaturation for 30 sec, 55°C annealing for 30 sec, and 72°C extension for 30 sec with a final cycle of 72°C for 5 min. The conditions for the second PCR were an initial denaturation at 94°C for 1 min, followed by 30 cycles of 94°C denaturation for 30 sec, 55°C annealing for 30 sec, and 72°C extension for 30 sec with a final cycle of 72°C for 5 min. The PCR products were analyzed by electrophoresis on a 1.5% agarose gel in TAE buffer. Images were captured using a Fluorescent Gel Image System (FGIS-3, TopBio). The expected sizes of the first and second PCR products were 490 bp and 151 bp, respectively. To test the detection limit, genomic DNA of *C. siamense* was 10-fold serially diluted from 1 ng/μl to 10 fg/μl. The first and second PCR reactions were performed as described above.

3.3.5 Detection of *Colletotricum* spp. on symptomless strawberry plants in nurseries

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From 2019 to 2020, 747 asymptomatic leaf samples (747 mother plants) collected from 18 strawberry nurseries in Hsinchu City, Miaoli County, Taichung City, and Nantou County were tested using the nested PCR and simple diagnosis by ethanol immersion (SDEI) methods (Ishikawa 2004) (Table 3.2). In these nurseries, the mother plants were reproduced from field plants by the farmers themselves. Previous studies showed that latent infection with *C. acutatum* and *C. gloeosporioides* was more frequently detected in the older leaves and petioles (Debode et al. 2015; Ishikawa 2003). In this study, the oldest leaf was removed from the crown of each tested plant. An approximately 1-cm segment of the basal petiole was used for the nested PCR assay. The remaining leaf and petiole were used for the SDEI assay. The SDEI assay was conducted following the procedures in Ishikawa (2004) with modification (Ishikawa 2004). In brief, the collected leaf samples were washed with tap water, rinsed with deionized water, and blotted dry on tissue paper. The abaxial and adaxial surfaces of the leaves were sprayed thoroughly with 75% ethanol. At 30–60 sec after spraying, the leaves were washed with deionized water once, rinsed with sterile water, and blotted dry on sterile tissue paper. The leaves were put into a plastic bag with a wetted cotton pad to maintain high humidity (> 90%). Leaves were incubated for 7–14 days at 28–30°C under a 12-h/12-h light/dark photoperiod.

3.4 Results

3.4.1 Identification of a highly diverse intergenic region for primer design

Through comparative genomics analysis of 29 *Colletotrichum* spp. isolates, 19 non-conserved regions (1000–1500 bp in length) located between conserved regions were identified. After manually checking the sequences, a non-coding region was selected for primer design. This region in *C. siamense* ML133 was 1157 bp in length (sequences uploaded to GenBank under accession number ON350970), which has 95.77% and

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94.65% identity to the corresponding regions in *C. gloeosporioides* 30206 and *C. siamense* Cg363, respectively. An alignment of the sequences of different *Colletotrichum* spp. is shown in Fig. S8. In the genome of *C. siamense* Cg363, the region is located between the L-arabinitol 4-dehydrogenase (*ladA*) and NAD(P)H-dependent D-xylose reductase (*xyl1*) genes. Using this region as template, 79 pairs of primers were designed. After performing blast searches against the sequences of *Fragariae* x *ananassa*, strawberry pathogens, and saprophytes, two primer pairs suitable for nested PCR were selected. The sizes of the first and second PCR products were 490 bp and 151 bp, respectively.

3.4.2 Specificity and sensitivity of the nested PCR assay

Five *Colletotrichum* spp. causing strawberry anthracnose in Taiwan and a selected set of microorganisms commonly isolated from strawberry or soil were used for the specificity test. Among the five pathogenic *Colletotrichum* spp., *C. siamense* ML133 and *C. fructicola* ML348 but not *C. karstii* ML351, *C. boninense* ML521, or *C. miaoliense* ML1040 were detectable (Fig. 3.2A). The first and second PCR resulted in specific bands of the expected sizes (490 bp and 151 bp, respectively). The pathogenic fungi *Neopestalotiopsis rosae* ML2147, *Fusarium* spp., *Botrytis* sp., and *Phytophthora* sp. and saprophyte fungi *Fusarium* spp. and *Trichoderma* spp. were not detectable (Fig. 3.2B). No signal was detected from three strawberry cultivars, ‘Taoyuan No. 1’, ‘Xiang-Shui’, and ‘Miaoli No. 1’ (Fig. 3.2C). PCR products of the expected sizes were observed from the controls (fungal ITS and strawberry *ACTIN*) (Fig. 3.2).

In the sensitivity test, a bright and specific band was observed from the reactions using 1 ng, 100 pg, 10 pg, 1 pg, and 100 fg of the genomic DNA of *C. siamense* ML133. The product sometimes failed to be amplified when using 100 fg. The results showed that

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performing the first PCR with 40 cycles followed by the second PCR with 30 cycles can reliably detect as little as 1 pg genomic DNA (Fig. 3.3), which corresponds to the DNA contents of ~15 cells of *C. siamense* (based on the genome size of *C. siamense* Cg363: ~62.9 Mb) (Gan et al. 2020).

3.4.3 Optimization of the nested PCR assay by changing outer and inner primer ratio

It was observed that the nested PCR often resulted in four bands of approximately 500 bp, 400 bp, 250 bp, and 150 bp. The sizes of these bands suggested that they may have come from amplification directed by different combinations of the outer primers and inner primers. To reduce the non-target signals, we tested different ratios of the outer and inner primers ranging from 1:1 to 1:100. When the ratio was 1:1 or 1:2, all four bands appeared. A single band of the expected size (151 bp) was observed for ratios 1:5, 1:10, and 1:20 (Fig. 3.4). Notably, the signal was stronger with ratios of 1:5 and 1:10.

3.4.4 Field survey of *Colletotricum* spp. on symptomless strawberry plants in nurseries

From 2019 to 2020, 747 asymptomatic leaf samples collected from 18 strawberry nurseries were tested for strawberry anthracnose pathogens. The number of samples per nursery ranged from 6 to 70 (average 42 samples/nursery) (Table 3.2). Using our nested PCR assay, the detection rates ranged from 0% to 100% (average 20%); using the SDEI method (Ishikawa 2004), the detection rates ranged from 5% to 90% (average 45%) (Table 3.2). For the samples from 17 out of 18 nurseries, the detection rates from the nested PCR assay were lower than those from the SDEI method.

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3.5 Discussion

Symptomless runner plants carrying the inoculum of *Colletotrichum* spp. is an important route for the spread of strawberry anthracnose from the nursery to the field (Furuta et al. 2017; Parikka and Lemmetty 2004). In Taiwan, *C. siamense* and *C. fructicola* are the most prevalent and virulent strawberry anthracnose pathogens (Chung et al. 2020). Several PCR-based methods (conventional PCR, nested-PCR, and quantitative PCR) and culture-based methods (incubation of leaves treated with ethanol, herbicide, or freezing) have been developed for the diagnosis or detection of strawberry anthracnose (Cerkauskas 1987; Debode et al. 2009; Freeman et al. 2001; Freeman and Katan 1997; Furuta et al. 2017; Garrido et al. 2009; Ishikawa 2003; Ishikawa 2004; Martinez-Culebras et al. 2000; Mertely and Legard 2004; Parikka and Lemmetty 2004; Sreenivasaprasad et al. 1996). However, a highly sensitive PCR-based detection method was previously not available for *C. siamense* and *C. fructicola.* In previous studies, PCR primers for detecting *Colletotrichum* spp. associated with strawberry were designed using the ITS, *TUB2*, or *MAT1-2* as the template (Debode et al. 2009; Freeman et al. 2001; Furuta et al. 2017; Garrido et al. 2009; Martinez-Culebras et al. 2000; Sreenivasaprasad et al. 1996). However, among *Colletotrichum* spp., there is a high degree of sequence similarity between phylogenetic markers (ITS, *CHS-1*, *ACT*, *TUB2, CAL*, and ApMAT). The non-coding regions of *CHS-1*, *ACT*, *TUB2*, and *CAL* are more variable but too short (mostly < 100 bp) for designing highly specific nested PCR primers. In this study, we conducted comparative genomic analysis and identified an intergenic region between *ladA* and *xyl1* that was ideal for distinguishing *C. siamense* and *C. fructicola* from the other 16 *Colletotrichun* spp. *In silico* analysis and actual tests suggested that our newly developed nested PCR assay could detect pathogenic *C. siamense* and *C. fructicola*, but not other strawberry pathogens (*Botrytis* sp., *Fusarium* spp., *Neopestalotiopsis rosae*, and

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Phytophthora sp.) or ubiquitous saprophytes (*Fusarium* spp. and *Trichoderma* spp.). Although *C. boninense*, *C. karstii*, and *C. miaoliense* (the other three *Colletotrichun* spp. causing strawberry anthracnose in Taiwan) were not detectable, the assay is expected to detect most cases of latent infection that can lead to serious disease. *C. boninense*, *C. karstii*, and *C. miaoliense* are present in Taiwan at low percentage (total 14%) and cause tiny lesions (0.07–0.35 cm in diameter) only on wounded leaves even under a conducive high temperature (30 °C) condition (Chung et al. 2020). The assay can detect as low as 1 pg genomic DNA, which corresponds to ~15 cells of the pathogen. The ratio of the concentrations of nested primer pairs is critical for specificity (Deepachandi et al. 2019; Yang and Marchand 2002), and the optimal outer and inner primer ratio for our nested PCR assay is 1:10. The high sensitivity and specificity of this assay allows the detection of trace amounts of pathogenic *C. siamense* and *C. fructicola*, without the problem of unexpected PCR products amplification.

Anthracnose spores are mainly disseminated by rain and overhead irrigation water. Older leaves at lower positions have more chances to be exposed to the pathogen inoculum; therefore, they are more likely to be infected than younger leaves at higher positions. In previous studies, old strawberry leaves/petioles were used as materials for detecting latent anthracnose infection (Debode et al. 2015; Ishikawa 2003). Since older leaves are often removed by farmers for pest control purposes, they are good materials available all year round for detecting the source of pathogen inoculum.

Strawberry is propagated from stolons (runners) and transplanted in the form of runner plants. In our field survey conducted from 2019 to 2020, the nested PCR assay detected *C. siamense* and *C. fructicola* in an average of 20% of symptomless mother plants (Table 3.2). The percentage of plants latently infected or carrying the pathogen inoculum on surface was > 20% in 6 out of 18 nurseries. This reflects the severe epidemic

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of strawberry anthracnose in recent years (Chung et al. 2019; Chung et al. 2020) and indicates the importance of early detection and removal of latently infected mother plants before they are used for propagation. In 16 out of 18 nurseries, higher detection rates were observed using the culture-based SDEI method, perhaps because the nested PCR assay targets only two of five known pathogenic *Colletotrichum* spp. and only the basal petiole was assayed, whereas the SDEI method nonspecifically detects any viable *Colletotrichum* spp. that forms conidial masses on the whole leaf. In the remaining two nurseries (sites 6 and 10), higher detection rates were observed using the nested PCR assay than the SDEI method. This could be due to more frequent usage of fungicides or the farmers just sprayed fungicides before our sampling. When most *Colletotrichum* spp. were killed, the dead cells could only be detected by the nested PCR assay.

The use of overhead irrigation in strawberry nurseries or open field cultivation often increases the latent *Colletotrichum* spp. infection rate in strawberry runner plants, which leads to disease outbreaks in fruit-producing fields (Daugovish et al. 2012; Yonemoto et al. 2008). In some strawberry nurseries in Taiwan, the frequency of spraying fungicides can be as high as once every three days during the six-month nursery period. Frequent application of fungicides not only increases production costs but also causes the emergence of fungicide resistance in the pathogen population (Daugovish et al. 2012; Debode et al. 2015). To prevent the spread of diseases and improve the health of runner plants produced by strawberry nurseries, the Council of Agriculture (COA) in Taiwan has established a voluntary pathogen-free certification system for strawberry propagation in 2018. According to the guidelines, anthracnose is one of the key diseases required to be tested and excluded from strawberry propagation. The nested PCR assay developed in this study has been applied for certification of ‘pathogen-free’ strawberry plants, from which healthy mother plants and runner plants can be supplied to farmers. The use of

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healthy plant materials combined with integrated control measures will contribute to the production of safe and high-quality strawberries, which is a win-win situation for both producers and consumers.

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3.7 Tables

Table 3.1. Nested PCR primers used in this study.

Name	Sequence	Product size (bp)	Note
Col_nest-1F	5’- ACAAACGGTGATCCTTTCGTC -3’	490	Outer primers for the first PCR
Col_nest-1R	5’- GGTGCCCCTCAACACGAAC -3’		
Col_nest-2F	5’- CTCCCAACCGGATAATCTGC -3	151	Inner primers for the second PCR
Col_nest-2R	5’- ACCGACCGGAACATAGATCACA -3’		

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Table 3.2. Field survey of *Colletotrichum* spp. on symptomless strawberry plants using the nested PCR and SDEI (simple diagnosis by ethanol immersion) assays.

Nursery site	Collection date	County/City	No. of leaf samples	Method	No. of positive samples	Detection rate (%)
1	2019/01/14	Miaoli County	50	Nested PCR	10	20
			50	SDEI	20	40
2	2019/03/08	Miaoli County	60	Nested PCR	9	15
			60	SDEI	16	27
3	2019/03/22	Hsinchu City	16	Nested PCR	2	13
			16	SDEI	5	31
4	2019/03/25	Taichung City	22	Nested PCR	6	27
			22	SDEI	14	64
5	2019/04/01	Miaoli County	50	Nested PCR	7	14
			50	SDEI	23	46
6	2019/04/09	Hsinchu City	6	Nested PCR	6	100
			6	SDEI	1	17
7	2019/05/01	Miaoli County	40	Nested PCR	1	3
			40	SDEI	2	5
8	2019/06/04	Miaoli County	41	Nested PCR	12	29
			41	SDEI	23	56
9	2019/06/21	Hsinchu City	7	Nested PCR	1	14
			7	SDEI	2	29
10	2019/07/04	Miaoli County	50	Nested PCR	19	38
			50	SDEI	11	22

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11	2019/10/02	Nantou County	35	Nested PCR	1	3
			35	SDEI	6	17
12	2020/02/06	Miaoli County	50	Nested PCR	8	16
			50	SDEI	30	60
13	2020/02/13	Miaoli County	50	Nested PCR	2	4
			50	SDEI	21	42
14	2020/02/19	Miaoli County	50	Nested PCR	11	22
			50	SDEI	42	84
15	2020/03/04	Miaoli County	50	Nested PCR	0	0
			50	SDEI	14	28
16	2020/03/11	Miaoli County	50	Nested PCR	7	14
			50	SDEI	42	84
17	2020/04/08	Miaoli County	50	Nested PCR	11	22
			50	SDEI	45	90
18	2020/04/21	Miaoli County	70	Nested PCR	4	6
			70	SDEI	49	70
Total			747	Nested PCR	117	16
			747	SDEI	366	49
Average (per nursery)			41.5	Nested PCR	6.5	20
			41.5	SDEI	20.3	45

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3.8 Figures

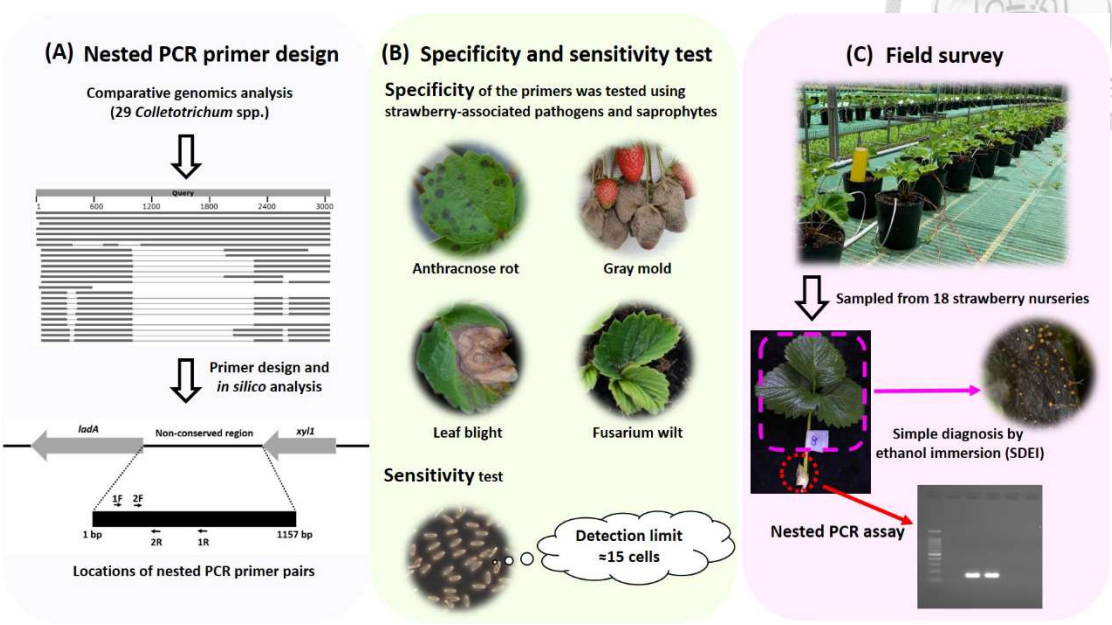

Fig. 3.1. Workflow diagram for the nested PCR primer design, specificity and sensitivity tests, and field survey in this study.

(A) Comparative genomics analysis and *in silico* analysis were conducted to identify non-conserved regions suitable for designing nested PCR primers. (B) Specificity and sensitivity tests of nested primers. Specificity was determined by testing the ability of primers to amplify strawberry-associated pathogens and saprophytes. The detection limit was ~15 cells of *C. siamense*. (C) Samples collected from 747 mother plants in 18 strawberry nurseries were assayed by the nested PCR and simple diagnosis by ethanol immersion (SDEI) methods.

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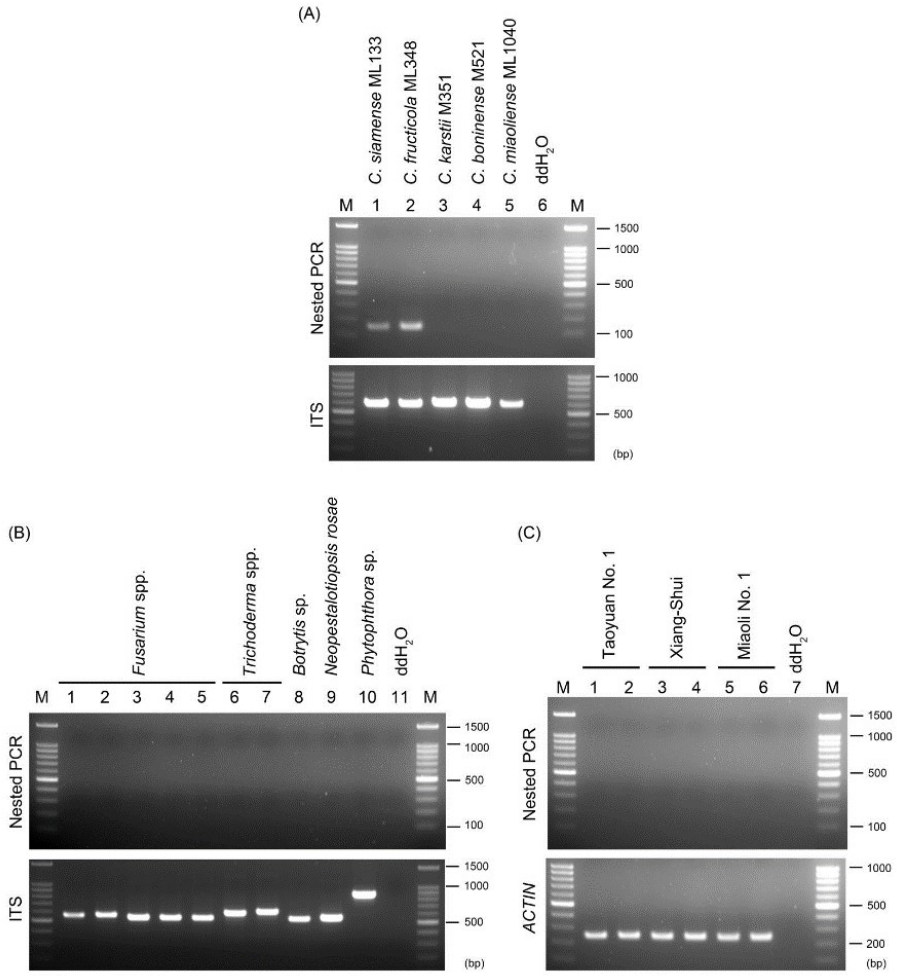

Fig. 3.2. Specificity test of the nested PCR assay.

(A) DNA of *Colletotrichum* spp. associated with strawberry anthracnose in Taiwan were used as template. *C. siamense* and *C. fructicola* are the most prevalent and virulent species, and *C. boninense*, *C. karstii*, and *C. miaoliense* are lowly pathogenic and present in a low percentage of strawberry plants. (B) DNA of pathogenic or saprophytic fungi isolated in the field were used as template. (C) DNA of different strawberry cultivars were used as template. The nested PCR assay was performed using primers Col_nest-1F/Col_nest-1R for the first PCR, and Col_nest-2F/Col_nest-2R for the second PCR. The quality of fungal and strawberry DNA was tested using primers targeting the ITS (ITS1/ITS4) and *ACTIN* (Actin-F/Actin-R), respectively. M, 100-bp DNA ladder (Faith Biotechnology Co., Ltd).

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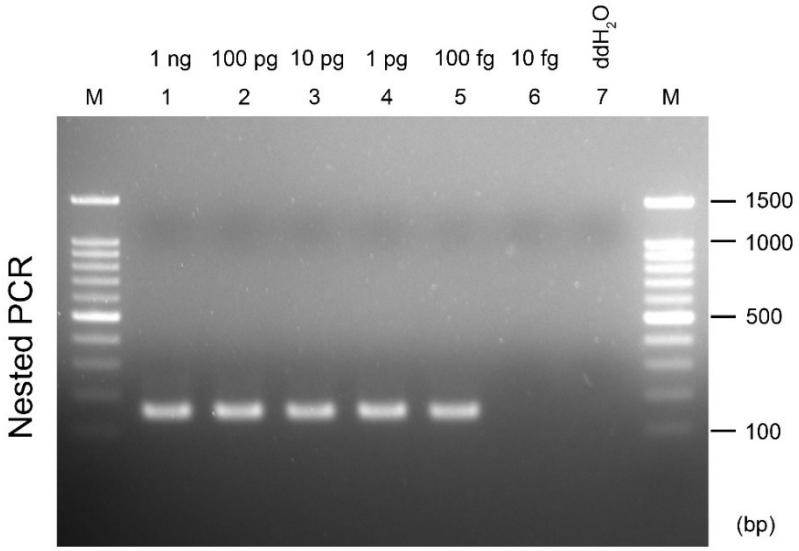

Fig. 3.3. Sensitivity test of the nested PCR assay.

Ten-fold dilutions (1 ng to 10 fg) of *C. siamense* ML133 genomic DNA were used as the template. The nested PCR assay was performed using primers Col_nest-1F/Col_nest-1R for the first PCR, and Col_nest-2F/Col_nest-2R for the second PCR. M, 100-bp ladder (Faith Biotechnology Co., Ltd).

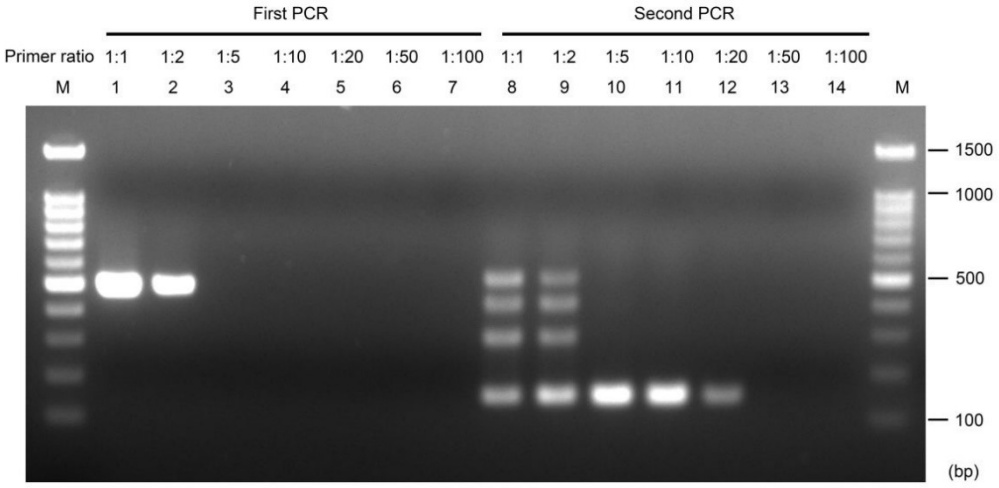

Fig. 3.4. Optimization of the primer ratio for the nested PCR assay.

Lanes 1–7, first PCR product; lanes 8–14, second PCR product. The nested PCR assay was performed using primers Col_nest-1F/Col_nest-1R for the first PCR, and Col_nest-2F/Col_nest-2R for the second PCR. Primer ratio represents the ratio of the concentrations of outer primers (first PCR) to inner primers (second PCR). The genomic DNA of *C. siamense* ML133 was used as the template. M, 100-bp ladder (Faith Biotechnology Co., Ltd).

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3.9 Supplemental Materials

Supplemental Table 3.1. Fungal isolates used in this study.

Species	Isolate	Collection date	Host plant / source	Sampling site	Reference
Botrytis sp.	ML2145	2019/02/13	Strawberry / diseased fruit	Dahu Township, Miaoli County	This study
Colletotrichum boninense	ML521	2013/01/21	Strawberry / diseased leaf	Taian Township, Miaoli County	Chung et al. (2020)[1]
Colletotrichum fructicola	ML348	2012/07/06	Strawberry / diseased leaf	Shitan Township, Miaoli County	Chung et al. (2020)[1]
Colletotrichum karstii	ML351	2012/07/06	Strawberry / diseased leaf	Shitan Township, Miaoli County	Chung et al. (2020)[1]
Colletotrichum miaoliense	ML1040	2016/10/28	Strawberry / diseased crown	Shitan Township, Miaoli County	Chung et al. (2020)[1]
Colletotrichum siamense	ML133	2011/10/28	Strawberry / diseased crown	Dahu Township, Miaoli County	Chung et al. (2020)[1]
Fusarium sp.	ML292	2012/06/27	Strawberry / diseased root	Taian Township, Miaoli County	This study
Fusarium sp.	ML432	2012/08/22	Strawberry / diseased crown	Dahu Township, Miaoli County	This study
Fusarium sp.	ML653	2014/07/15	Strawberry / diseased crown	Dahu Township, Miaoli County	This study
Fusarium sp.	ML672	2015/04/02	Strawberry / nearby soil (diseased plant)	Dahu Township, Miaoli County	This study
Fusarium sp.	ML841	2016/07/21	Strawberry / runner (symptomless plant)	Tongluo Township, Miaoli County	This study
Neopestalotiopsis rosae	ML2147	2019/02/18	Strawberry / diseased leaf	Dahu Township, Miaoli County	Wu et al. (2021)[2]

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Phytophthora sp.	ML2640	2020/09/29	Strawberry / diseased root	Dahu Township, Miaoli County	This study
Trichoderma asperellum	ML01	2009/05/18	Grape / rhizosphere soil (symptomless plant)	Zhuolan Town, Miaoli County	Chou et al. (2019)[3]
Trichoderma sp.	ML1425	2017/03/15	Strawberry / petiole (symptomless plant)	Dahu Township, Miaoli County	This study

References

1. Chung, P. C., Wu, H. Y., Wang, Y. W., Ariyawansa, H. A., Hu, H. P., Hung, T. H., Tzean, S. S., and Chung, C. L. 2020. Diversity and pathogenicity of *Colletotrichum* species causing strawberry anthracnose in Taiwan and description of a new species, *Colletotrichum miaoliense* sp. nov. Sci. Rep. 10:14664.

2. Wu, H. Y., Tsai, C. Y., Wu, Y. M., Ariyawansa, H. A., Chung, C. L., and Chung, P. C. 2021. First report of *Neopestalotiopsis rosae* causing leaf blight and crown rot on strawberry in Taiwan. Plant Dis. 105:487.

3. Chou, H., Xiao, Y.T., Tsai, J. N., Li, T. T., Wu, H. Y., Liu, L. Y. D., Tzeng, D. S., and Chung, C. L. 2019. In vitro and in planta evaluation of *Trichoderma asperellum* TA as a biocontrol agent against *Phellinus noxius*, the cause of brown root rot disease of trees. Plant Dis. 103:2733-2741.

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Supplemental Table 3.2. List of *Colletotrichum* spp. genomes used for comparative genomics analysis in this study.

No.	Species	Strain	Size (Mb)	No. of scaffolds
1	*Colletotrichum acutatum*	1	52.13	34
2	*Colletotrichum acutatum*	C71	44.92	2614
3	*Colletotrichum chlorophyti*	NTL11	52.39	558
4	*Colletotrichum falcatum*	Cf671	48.19	4358
5	*Colletotrichum fioriniae*	PJ7	49	1096
6	*Colletotrichum fructicola*	Nara gc5	59.6	13
7	*Colletotrichum gloeosporioides*	Cg-14	53.21	4537
8	*Colletotrichum gloeosporioides*	30206	57.6	1564
9	*Colletotrichum godetiae*	C184	35.03	1819
10	*Colletotrichum graminicola*	M1.001	51.64	654
11	*Colletotrichum graminicola*	M5.001	59.91	8455
12	*Colletotrichum higginsianum*	IMI 349063	49.08	10235
13	*Colletotrichum higginsianum*	IMI 349063	50.72	25
14	*Colletotrichum incanum*	MAFF 238704	53.6	2896
15	*Colletotrichum incanum*	MAFF238712	53.25	1036
16	*Colletotrichum lindemuthianum*	89 A2 2-3	99.17	1276
17	*Colletotrichum lindemuthianum*	83.501	97.41	1857
18	*Colletotrichum nymphaeae*	SA-01	49.96	1884
19	*Colletotrichum orbiculare*	104-T	89.75	355
20	*Colletotrichum orchidophilum*	IMI 309357	48.56	321
21	*Colletotrichum salicis*	CBS 607.94	48.37	2776
22	*Colletotrichum simmondsii*	CBS122122	50.47	929
23	*Colletotrichum sublineola*	TX430BB	46.76	1625
24	*Colletotrichum sublineola*	CgSl1	64.85	548
25	*Colletotrichum tofieldiae*	CBS 127615	52.72	791
26	*Colletotrichum tofieldiae*	CBS 130851	53.22	476
27	*Colletotrichum tofieldiae*	CBS 168.49	52.96	1300
28	*Colletotrichum tofieldiae*	CBS 495.85	53.49	1647
29	*Colletotrichum tofieldiae*	861	52.84	1046

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Supplemental Table 3.3. List of *Fusarium* spp. genomes used in this study.

No.	Species	Strain	Size (Mb)	No. of scaffolds
1	*Fusarium agapanthi*	NRRL 54464	41.96	1842
2	*Fusarium agapanthi*	NRRL 31653	41.31	2401
3	*Fusarium avenaceum*	FaLH27	43.17	78
4	*Fusarium avenaceum*	FaLH03	42.71	105
5	*Fusarium avenaceum*	Fa05001	41.59	83
6	*Fusarium circinatum*	FSP 34	43.95	422
7	*Fusarium circinatum*	GL1327	42.54	909
8	*Fusarium commune*	JCM 11502	46.18	19
9	*Fusarium culmorum*		41.93	6
10	*Fusarium cuneirostrum*	NRRL 31157	49.01	18692
11	*Fusarium fujikuroi*	B14	43.81	333
12	*Fusarium fujikuroi*	IMI 58289	43.83	12
13	*Fusarium fujikuroi*	KSU X-10626	43.11	187
14	*Fusarium fujikuroi*	FGSC 8932	43.1	835
15	*Fusarium fujikuroi*	KSU 3368	43.5	2959
16	*Fusarium graminearum*	PH-1	36.46	31
17	*Fusarium graminearum*	CS3005	36.67	199
18	*Fusarium graminearum*	PH-1	38.05	6
19	*Fusarium graminearum*	241165	36.57	486
20	*Fusarium graminearum*	233423	36.49	869
21	*Fusarium langsethiae*	Fl201059	37.54	1586
22	*Fusarium metavorans*	FSSC_6	46.94	103
23	*Fusarium nygamai*	MRC8546	51.62	409
24	*Fusarium oxysporum*	Fo5176	54.77	7858
25	*Fusarium oxysporum*	Fo47	49.66	124
26	*Fusarium oxysporum*	NRRL 32931	47.91	168
27	*Fusarium oxysporum*	UASWS AC1	50.55	1166
28	*Fusarium oxysporum f. sp. conglutinans*	1	52.54	13202
29	*Fusarium oxysporum f. sp. conglutinans race 2*	54008	53.58	2552
30	*Fusarium oxysporum f. sp. cubense race 1*	race 1	47.66	1341
31	*Fusarium oxysporum f. sp. cubense race 4*	race 4	52.93	840
32	*Fusarium oxysporum* f. sp. *cubense tropical* race 4	54006	46.55	418
33	*Fusarium oxysporum* f. sp. *lycopersici*	4287	61.39	114
34	*Fusarium oxysporum f. sp. lycopersici*	MN25	48.64	388
35	*Fusarium oxysporum f. sp. medicaginis*	Fom-5190a	51.14	4034

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36	*Fusarium oxysporum f. sp. melonis*	26406	54.03	1146
37	*Fusarium oxysporum f. sp. pisi*	HDV247	55.19	472
38	*Fusarium oxysporum f. sp. radicis-lycopersici*	26381	49.36	418
39	*Fusarium oxysporum f. sp. raphani*	54005	53.5	1,218
40	*Fusarium oxysporum f. sp. vasinfectum*	25433	52.91	985
41	*Fusarium poae*	2516	46.48	181
42	*Fusarium pseudograminearum*	CS3096	36.97	281
43	*Fusarium pseudograminearum*	CS3270	37.07	26
44	*Fusarium sambucinum*	F-4	37.78	961
45	*Fusarium sp.*	JS626	42.87	63
46	*Fusarium sp.*	JS1030	53.75	107
47	*Fusarium temperatum*	CMWF389	45.46	43
48	*Fusarium verticillioides*	7600	41.84	37
49	*Fusarium virguliforme*	Mont-1	50.45	3098

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Supplemental Table 3.4. List of *Trichoderma* spp. genomes used in this study.

No.	Species	Strain	Size (Mb)	No. of scaffolds
1	*Trichoderma asperellum*	B05	37.69	1,439
2	*Trichoderma atroviride*	IMI 206040	36.14	29
3	*Trichoderma atroviride*	XS2015	36.4	131
4	*Trichoderma atroviride*	JCM 9410	37.32	23
5	*Trichoderma gamsii*	T6085	37.91	172
6	*Trichoderma hamatum*	GD12	38.43	745
7	*Trichoderma harzianum*	T6776	39.72	1,572
8	*Trichoderma harzianum*	B97	40.68	1,054
9	*Trichoderma koningii*	JCM 1883	32.32	13
10	*Trichoderma longibrachiatum*	SMF2	31.74	194
11	*Trichoderma parareesei*	CBS 125925	32.07	885
12	*Trichoderma pleuroti*	TPhu1	38.14	2,355
13	*Trichoderma reesei*	QM6a	33.4	77
14	*Trichoderma reesei*	RUT C-30	32.68	177
15	*Trichoderma reesei*	CBS 999.97	32.47	167
16	*Trichoderma reesei*	QM6a	34.92	7
17	*Trichoderma* sp.	IMV 00454	42.03	197
18	*Trichoderma virens*	Gv29-8	39.02	93
19	*Trichoderma virens*	FT-333	38.63	586
20	*Trichoderma virens*	IMI 304061	45.82	107

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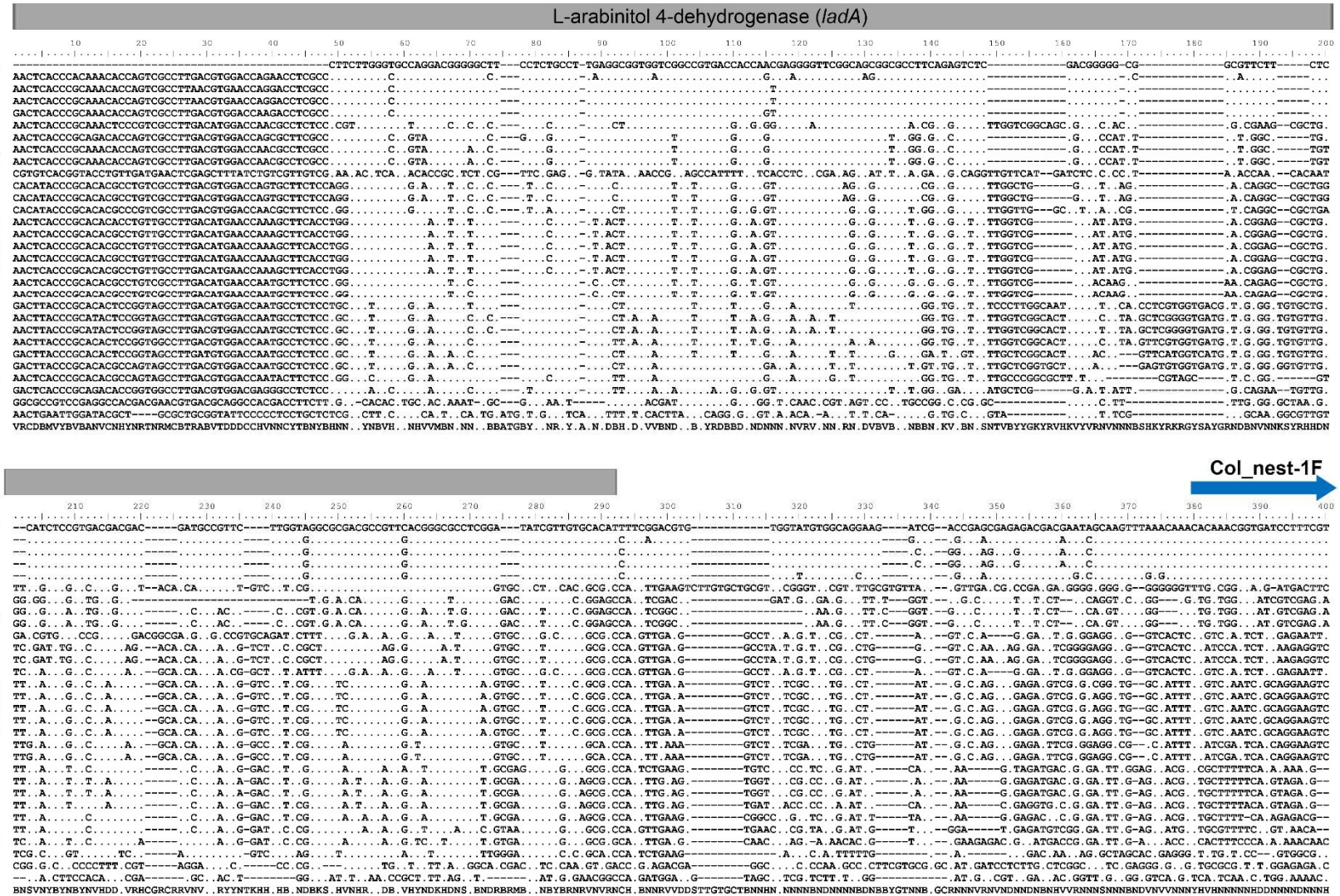

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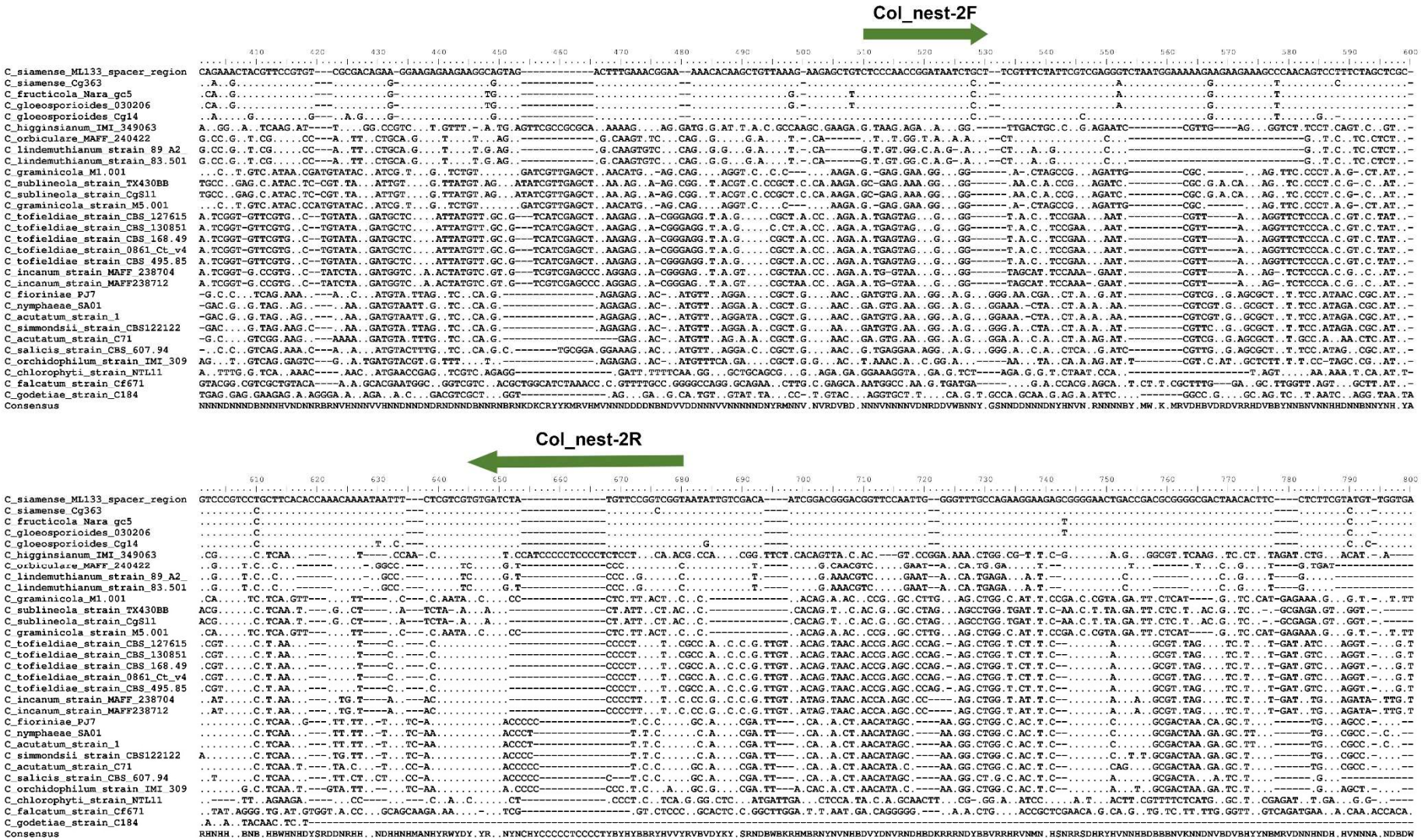

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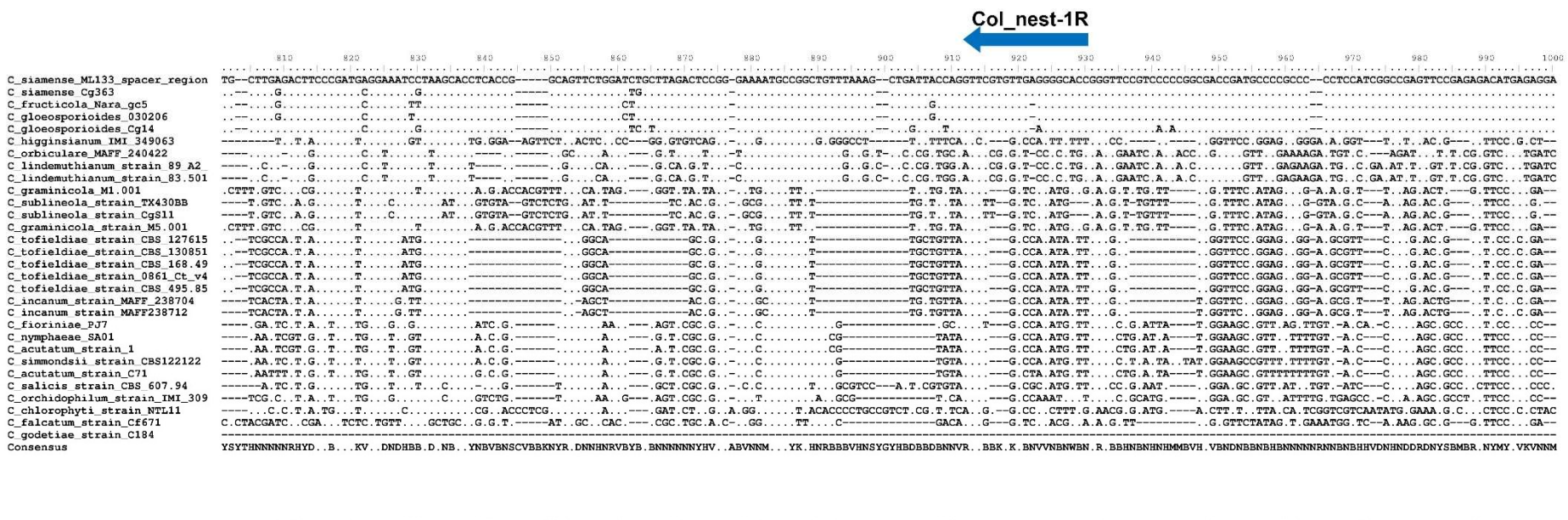

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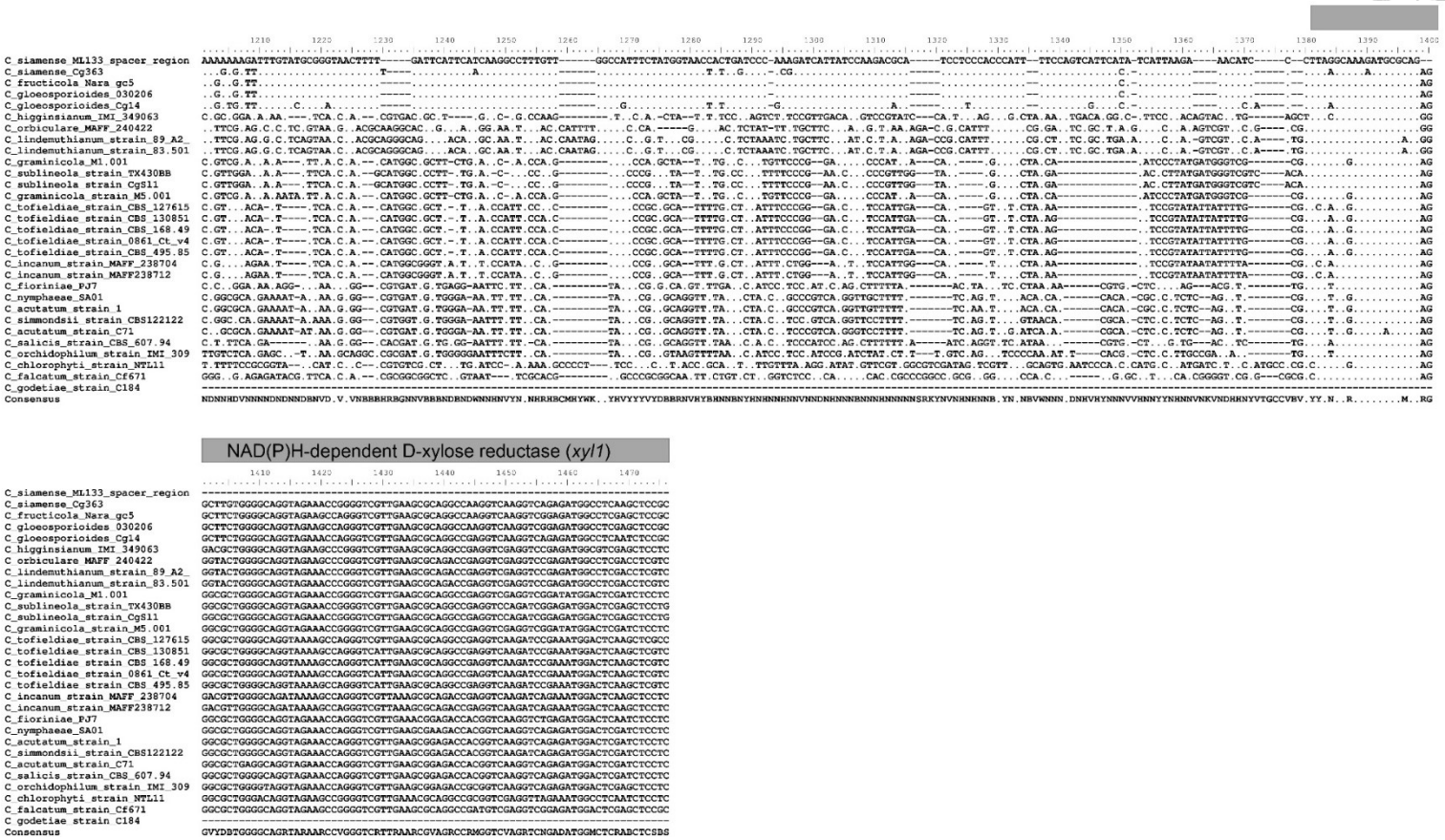

Supplemental Fig. 3.1. Alignment of the spacer region sequences from different *Colletotrichum* spp.

The locations of the nested PCR primers in the alignment region (indicated by arrows) are 381–401 bp (Col_nest-1F), 912–930 bp (Col_nest-1R), 510–529 bp (Col_nest-2F), and 645–680 bp (Col_nest-2R). Parts of the sequences of the L-arabinitol 4-dehydrogenase (*ladA*) and NAD(P)H-dependent D-xylose reductase (*xyl1*) genes are also shown.

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Chapter 4

Conclusions and future prospects

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Strawberry is a popular fruit crop native to the temperate regions. Strawberry cultivation in tropical and subtropical regions is often threatened by diseases. In recent years, because of global warming and severe weather events, farmers have to face more challenges from different diseases (Coakley et al. 1999; Hunjan and Lore 2020; Velásquez et al. 2018). Fungicides are often the first choice that farmers use to effectively prevent and control the diseases. Nowadays, due to the arising awareness of consumers, food safety issues such as pesticide residues have become important livelihood issues that everyone cares about. The fundamental solution lies in the development and implementation of integrated pest management (IPM) strategies. IPM is a procedure which combines cultural, physical, chemical, and biological strategies to manage pests in a way that minimizes overall environmental, human health, and economic losses (Dara 2019). The integrated management of strawberry anthracnose includes three stages, i.e., prevention, monitoring, and control. The use of healthy strawberry runner plants without latent infection is the first priority. In strawberry nurseries with rain shelter and drip irrigation system, the time intervals between fungicide sprayings were extended from 3-5 days to 10 days (鐘 and 吳，2021). Compared with the open field sprinkling irrigation method, the frequency of fungicide application was reduced by 50%-70%, and the percentage of replanting (due to the loss of runner plants caused by anthracnose) was less than 1%, which is significantly lower than the average (20%). In Japan and USA, because of the difficulty in managing anthracnose in the nursery stage, the water supply method is changed to reduce the spread of the disease caused by the overhead sprinkler water supply (Daugovish et al. 2012; Yonemoto et al. 2008). In the rain shelter and nonwoven fabric single-plant irrigation, even if there were several diseased plants, the disease incidence was only 2.1% after 34 days; on the contrary, when the overhead sprinkler irrigation system was used in the rain shelter, the disease incidence increased to 85.9%.

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Therefore, as long as strawberry runner plants are cultivated in nurseries with rain shelter and under the avoidance of sprinkler irrigation, the spread of the conidia of *Colletotrichum* spp. by splashing water can be largely reduced.

The research in Chapter 2 was the systematic collection and identification of strawberry anthracnose strains in Taiwan. Members of *Colletotrichum* are known to have similar morphological characteristics. Based on the multi-gene phylogenetic analysis and morphology, five *Colletotrichum* species were identified. When comparing the length of their conidia, *C. miaoliense* (*C. acutatum* species complex) was the longest, *C. gloeosporioides* and *C. fructicola* (*C. gloeosporioides* species complex) in the middle, and *C. boninense* and *C. karstii* (*C. boninense* species complex) were the shortest. *C. siamense* isolates from this study formed a separate subclade within the *C. siamense* clade, which belongs to the *C. gloeosporioides* species complex, and some additional clusters were found within this subclade. This implies the richness of genetic diversity of *C. siamense*. A few previous studies have suggested that *C. siamense* is a species complex (Sharma et al. 2015; Udayanga et al. 2013; Vieira et al. 2014); however, a recent study implementing a polyphasic approach argued that *C. siamense* is a single species (Liu et al. 2016). Different approaches to delimit species may affect the estimation of species diversity within *Colletotrichum* communities (Veloso et al. 2018). Based on the phylogenetic tree constructed using combined five-locus (ITS, *GAPDH*, *CHS-1*, *ACT*, *TUB2*) data, the clade where the new species *C. miaoliense* resides was clearly separated from other species belonging to the *C. acutatum* species complex. The result of our analysis is consistent with the findings of the latest publications (Kennedy et al. 2022; Liu et al. 2022). Morphological differences also exist between *C. miaoliense* and two phylogenetically closely related strawberry anthracnose pathogens, *C. nymphaeae* and *C. simmondsii* (Baroncelli et al. 2015; Damm et al. 2012). The conidial length and width

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ratio of *C. miaoliense* was similar to that of *C. nymphaeae* but different from that of *C. simmondsii*. The appressoria of *C. miaoliense* and *C. simmondsii* are elliptical to clavate. The appressoria of *C. nymphaeae* are clavate or irregular in outline, entire, and have an undulate to lobate margin (Chung et al. 2020). *C. miaoliense* was isolated from strawberry runner plants in the Ren'ai Township in Nantou, where other flower and vegetable crops were also present. The geographical distribution and host range of this new species are worthy of further exploration.

The use of healthy runner plants is the key to preventing anthracnose. The voluntary pathogen-free certification system for strawberry propagation was announced in 2018 in Taiwan. There have been certified cases for different stages of runner plants propagation for strawberry cultivars including ‘Taoyuan No. 1’, ‘Miaoli No. 1’, ‘Tainong No. 1’, and ‘Changzhi No. 1’. In order to ensure that the verification target does not carry the anthracnose pathogen, an 100% sampling rate in G0 and 1/1,000 sampling rate and visual inspection of the whole area are required in G1. The nested PCR assay developed in Chapter 3 has been applied in the certification system. The tissue culture strawberry plants were provided to farmers who propagated runner plants in the open field. With the certified plants as mother plants, the incidence of anthracnose could be reduced to as low as 0.1% (吳及鐘 2021). While the tissue culture plants were propagated in the greenhouse, no symptoms were observed, and the interval of preventive fungicide spraying were extended from one to two weeks. This significantly reduced the frequency of fungicide application and improved the healthy rate of runner plants. Although the current certification system is voluntary for farmers, it is recommended that tissue culture and pathogen detection techniques should be established before the release of a new variety (石川 2012; FPS 2008; Moyer 2019).

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Early, accurate and specific detection methods for plant pathogens are necessary for effective management of plant diseases (Sankaran et al. 2010). Due to the multi-copy characteristic of the ITS region, it has higher sensitivity for detecting *C. acutatum*, and is more suitable for the design of real-time PCR primers than the single-copy *β-tubulin* region (Debode et al. 2009). In our study in Chapter 3, considering the low cost of general PCR equipment and reagents, we performed a comparative genomics analysis of 29 *Colletotrichum* spp. and distinguished a non-conserved 1157-bp intergenic region located between *ladA* and *xyl1* genes suitable for designing specific primers for a nested PCR assay (Chung et al. 2022). Since nested PCR performs two rounds of PCR, cross-contamination is easy to occur during the process. In order to avoid this problem, the whole process was carried out in two rooms. The preparation of the mixtures of DNA and PCR reagents for both first and second PCR were performed in the room 1, and DNA extraction and electrophoresis were performed in the room 2. It has also been pointed out that the reaction of two-step nested PCR could be changed to single-tube nested PCR (STNPCR). The inner primers were immobilized on internal interface of microtube and then dissolved before the second reaction. This procedure could skip the step of opening the lid which might cause false-positive results by cross-contamination, but the sensitivity would be 10 times less than the original method (da Silva et al. 2013). On-site detection method would contribute faster and efficient decisions on disease prevention and control strategy. Rapid sample preparation method, LAMP assay or a portable device were applied for on-site detection of diseases in grape, potato, and cotton (Kogovšek et al. 2015; Kong et al. 2020; Rafiq et al. 2021). To make strawberry anthracnose detection easier, our cooperative team has developed a non-powered nucleic acid extraction kit, which mainly consisted of 5 bottles of buffer/reagents and two sets of filter syringes. There was no need to use a high-speed centrifuge, which could better meet the needs of on-site

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operations. Furthermore, the non-conserved 1157-bp intergenic region found in this study was used as the region for designing LAMP primers, and then hydroxy naphthol blue (HNB) was used for visualization. In the future, there is an opportunity to directly detect the latent infection of strawberry anthracnose on-site in the field.

Fungicides have been used to control strawberry anthracnose routinely, but frequent usage can lead to the emergence of fungicide-resistant strains and ineffective control. Planting resistant varieties can reduce the usage of fungicides (Forcelini and Peres 2018; Forcelini et al. 2016; Smith et al. 2013). Based on traditional or molecular-assisted breeding methods, disease-resistant germplasms or resistance genes have been found for strawberry anthracnose and Fusarium wilt (Bai et al. 2022; Miller-Butler et al. 2018; Miller-Butler et al. 2019; Pincot et al. 2022). This study has identified the predominant *Colletotrichum* species that caused strawberry anthracnose in Taiwan, our collaborative team has used *C. siamense* ML133 and *C. fructicola* ML356 to screen the strawberry germplasm resources which are preserved in Wufong Branch Station at Taoyuan District Agricultural and Research Extension Stations and Biological Control Branch at Miaoli District Agricultural and Research Extension Station (吳等 2022). According to the inoculation tests, we found that 'TS4', 'Rassen', 'TS13', '久留米 103' and 'TYS16109' showed good resistance performance. In addition, we found that 'Taoyuan No. 1' and 'Miaoli No. 1' showed resistance to the leaf blight disease (caused by *Neopestalotiopsis rosae*) of strawberry after inoculation testing (鐘等 2021). This will be a valuable source of germplasms for disease resistance breeding in the future.

In the face of climate change and the emergence of diseases, it is necessary to use modern technology to assist farmers in preventing and controlling the occurrence of diseases. For example, the strawberry advisory system (SAS) was developed by the University of Florida for strawberry anthracnose and gray mold (Hu et al. 2021;

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MacKenzie and Peres 2012; Pavan et al. 2011). Through the meteorological monitoring station, the temperature of the microenvironment and the leaf wetness duration are automatically collected, and the monitoring values are substituted into the formula and converted into an infection index. Based on the values of infection index, recommended control strategies are provided to farmers. The Lithuania's iMETOS® and Korea's Farm as a Service (FaaS) integrated system use similar concepts to provide farmers with early warning and control strategies for strawberry gray mold (Kim et al. 2018; Rasiukevičiūtė et al. 2012). Smart agriculture has become the trend of agricultural development around the world. The development of intelligent disease monitoring and warning systems can not only save labor and time, but also reduce the usage of pesticides and improve the food safety of crops. In recent years, our collaborative team has been working on developing an integrated advisory system that can provide three types of services including image recognition of strawberry diseases, prediction of disease risk based on weather factors, and disease control recommendations optimal for different cultivation modes. In the future, we will try to use intelligent chatbots as the user interface for farmers, and expect that it could reduce the usage of chemical pesticides and improve control efficacies. By understanding the characteristics of the anthracnose pathogens, combined with IPM control strategies including healthy runner plants, rain shelter, disease resistance breeding and early warning system etc., the incidence of anthracnose and the usage of fungicides can be effectively reduced, thereby improving the incomes of farmers and enhancing the environmental and consumers' safety.

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Appendices

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Publications

1. **Chung, P. C.**, Wu, H. Y., Ariyawansa, H. A., Tzean, S. S., and Chung, C. L. 2019. First report of anthracnose crown rot of strawberry caused by *Colletotrichum siamense* in Taiwan. Plant Dis. 103:1775-1776.

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Previous Next

DISEASE NOTES

First Report of Anthracnose Crown Rot of Strawberry Caused by *Colletotrichum siamense* in Taiwan

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In Taiwan, strawberry (*Fragaria* × *ananassa* Duch.) is a high-value crop with an average annual cultivated area of ~500 ha in the last 5 years. Over 90% of strawberry cultivation is in Miaoli County, with 'Taoyuan No. 1' as the predominant cultivar for more than 30 years. Anthracnose has become more destructive over the past decade. Although *Colletotrichum gloeosporioides*, *C. dematium*, *C. fragariae*, and *C. acutatum* were mentioned as the causal agents of strawberry anthracnose in Taiwan (Plant Protection Information System; https://otserv2.tactri.gov.tw/ppm/), we lack information on the isolation, pathogenicity, and morphological or molecular identification of the pathogen. From 2010 to 2016, we surveyed anthracnose in strawberries in Miaoli County; more than 50% of diseased plants showed typical anthracnose crown rot (ACR) symptoms (**McInnes et al. 1992**). ACR caused up to 30 to 40% plant loss during the seedling stage and ~20% after transplanting. Infected crown tissue initially showed red and white marbling and then gradually brown rot, followed by rapid wilting of the entire plant. Anthracnose symptoms were observed in other parts of the plant, including leaves, petioles, runners, fruits, and roots. Symptoms appeared as circular

black spots on the leaves and withering and girdling on runners. To isolate the causal agent, approximately 0.2 × 0.2-cm fragments of diseased crowns were surface disinfested with 1% sodium hypochlorite, triple rinsed with sterile water, and then placed onto 1.5% water agar. After 2 to 3 days, extended single hyphal tips from tissues were transferred to potato dextrose agar and incubated for 7 days at 25°C under a 12-h/12-h photoperiod. Colonies were initially white, later became somewhat zonate, velvety, light gray on the upper side and gray on the reverse side of plates, with concentric rings of salmon sporodochia. Conidia were 9.68 to 17.95 × 3.88 to 5.84 µm (14.53 ± 0.31 × 4.99 ± 0.08 µm, *n* = 90), hyaline, oblong to cylindrical, with round obtuse ends. Morphological characteristics of the causal agent resembled species belonging to the *C. gloeosporioides* species complex (**Weir et al. 2012**). To confirm the species identification, we extracted genomic DNA from 10 isolates by using the Plant Genomic DNA Extraction Miniprep System (Viogene, Taipei) and polymerase chain reaction–amplified the internal transcribed spacer (ITS) region, chitin synthase (*CHS-1*), actin (*ACT*), β-tubulin 2 (*TUB2*), calmodulin (*CAL*), and intergenic region of *Apn2* and *MAT1-2-1* (ApMAT) with published primers (**Carbone and Kohn 1999**; **O'Donnell and Cigelnik 1997**; **Silva et al. 2012**; **Weir et al. 2012**; **White et al. 1990**). Sequences were submitted to GenBank (accession nos. MK174223 [ITS], MK174224 [*CHS-1*], MK174225 [*ACT*], MK174226 [*TUB2*], MK174227 [*CAL*], and MK174228 [ApMAT]). The ITS, *CHS-1*, *ACT*, *TUB2*, *CAL*, and ApMAT sequences were compared with the GenBank nr database, restricted to type material. Results showed 98 to 99% identity to *C. siamense* (syn. *C. dianesei* and *C. melanocaulon*) (**Liu et al. 2016**; **Prihastuti et al. 2009**), which belongs to the *C. gloeosporioides* species complex, with the corresponding sequences (*ITS*: NR_144800; *CHS-1*: KX094094; ApMAT: KX094304 [**Lima et al. 2013**]; *ACT*: KX093987; *TUB2*: KX094290; and *CAL*: KX094036 [**Doyle et al. 2013**]). Koch's postulates were fulfilled for two isolates (ML133 and ML612) by spraying 1 × 10^6 conidia/ml suspension on seedlings until run-off at the four- to five-leaf stage (two trials per isolate, *n* = 5 seedlings per trial). Inoculated plants were covered with plastic bags (>90%

typical necrotic leaf spots and wilt symptoms similar to those in the field. Control plants sprayed with sterile water had no symptoms (*n* = 5 per trial). Longitudinal sections of the inoculated crown showed reddish-brown and white-marbled necrosis. The fungi were reisolated from lesions of diseased leaves or crowns with 100% frequency (*n* ≥ 3 isolates per trial), and morphological characteristics and gene sequences were identical to the original isolates. To our knowledge, this is the first report of *C. siamense* causing ACR of strawberry in Taiwan. The disease has the potential for causing serious losses to the strawberry industry in Taiwan, and research is needed on management strategies to minimize losses.

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The author(s) declare no conflict of interest.

APS Publications

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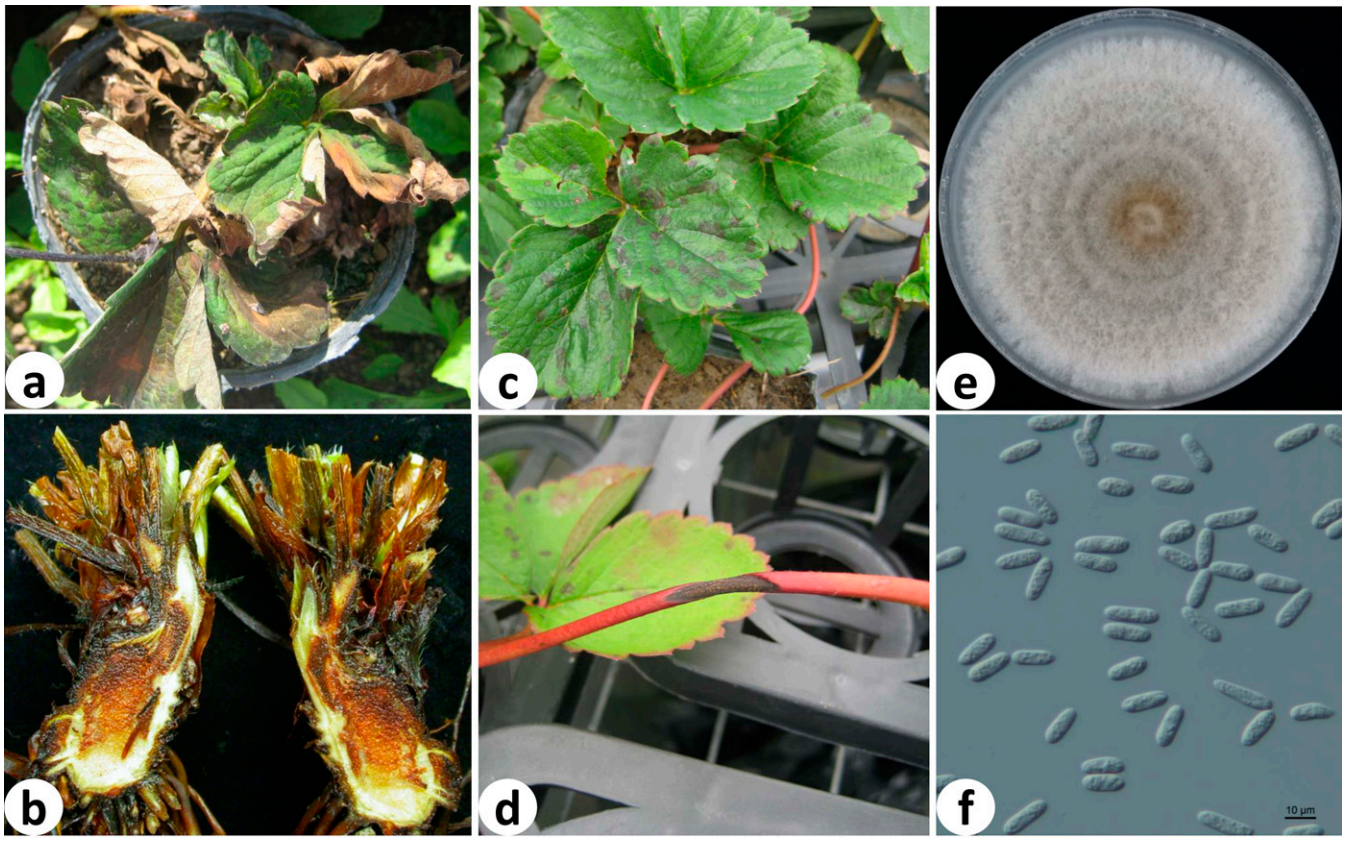

Supplementary Figure S1. Anthracnose symptoms on strawberry and morphological characters of *Colletotrichum siamense*. (a) A strawberry plant with anthracnose crown rot showing chlorotic to blighted leaves and wilting symptoms. (b) Longitudinal section of an infected crown showing marbled reddish-brown necrosis. (c) Black necrotic leaf spots. (d) Withering and girdling on a runner. (e) The fungal colony was initially white, later became light gray on the upper side. (f) Conidia hyaline, oblong to cylindrical, with round obtuse ends. Scale bar = 10 μm.

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The morphological characteristics and phylogenetic analysis of *Pratylenchus vulnus* Taiwan strawberry isolate

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Abstract

Pratylenchus vulnus was discovered in nematode-distributing fields from symptomatic seedling roots and corresponding rhizosphere soil on strawberry farms in Taiwan. Microscopic measurements and scanning electron microscope observations of both sexes of the nematode coincided with the general morphological descriptions of the species. Four different types of female tail termini were observed, including pointed, digitate, smooth and tapering. Molecular analysis of the ribosomal RNA sequence (SSU, ITS and LSU regions) and the mitochondria COI gene sequences confirmed the species identification. Phylogenetic analysis suggested no specific geographic linkage of the Taiwan population to other previously reported populations.

Keywords

Pratylenchus vulnus, Morphology, SEM, Phylogenetic analysis, Strawberry, Root lesion nematode.

Root lesion nematodes (*Pratylenchus* sp.) are among the most economically damaging phytoparasitic nematodes on fruits, tree rootstocks and vegetables (Yu et al., 2012). *Pratylenchus vulnus* was first reported in 1951 as a pathogen of multiple trees and vines in California, USA (Allen and Jensen, 1951) and was later found to infect over 80 plant species (Castillo and Vovlas, 2007). In Uruguay, Sri Lanka and Australia, serious damage in strawberry (*Fragaria* × *ananassa*) fields caused by this nematode have been reported (Colbran, 1974; Minagawa and Maeso-Tozzi, 1990; Mohotti et al., 1997). We here report the discovery of one *Pratylenchus* sp. population in strawberry fields in Dahu township of Miaoli, Taiwan in 2017. The strawberry crops growing in the nematode-distributing fields GS and KDL were obviously stunted when discovered. Both fields had been cropping strawberry for over 10 years. The soil composition of the area was characterized as sandy loam by hydrometer method (Bouyoucos, 1951). Nematodes were extracted from 100 g ± 5% soil with modified Baermann funnel technique (Hooper, 1986). Morphological observations and measurements of adults (30 females and 30 males) were conducted with a compound microscope at magnification of up to 1000× (Table 2). The dorsal gland orifice of the female of our isolates fit the description of *P. vulnus* perfectly, with a range between 3.0 and 4.42 (means = 3.55), rather than 1.9 to 3.0 characteristic of *P. penetrans* (Roman and Hirschmann, 1969). All nematodes observed had a lateral field composed of four incisures with wider inner band but two types of lateral field were observed. The first type had an evenly uplifted lateral field (Fig. 1E), while the second type carried uplifted and thinner outer bands (Fig. 1F). Four different types of female tail termini were observed, including pointed, digitate,

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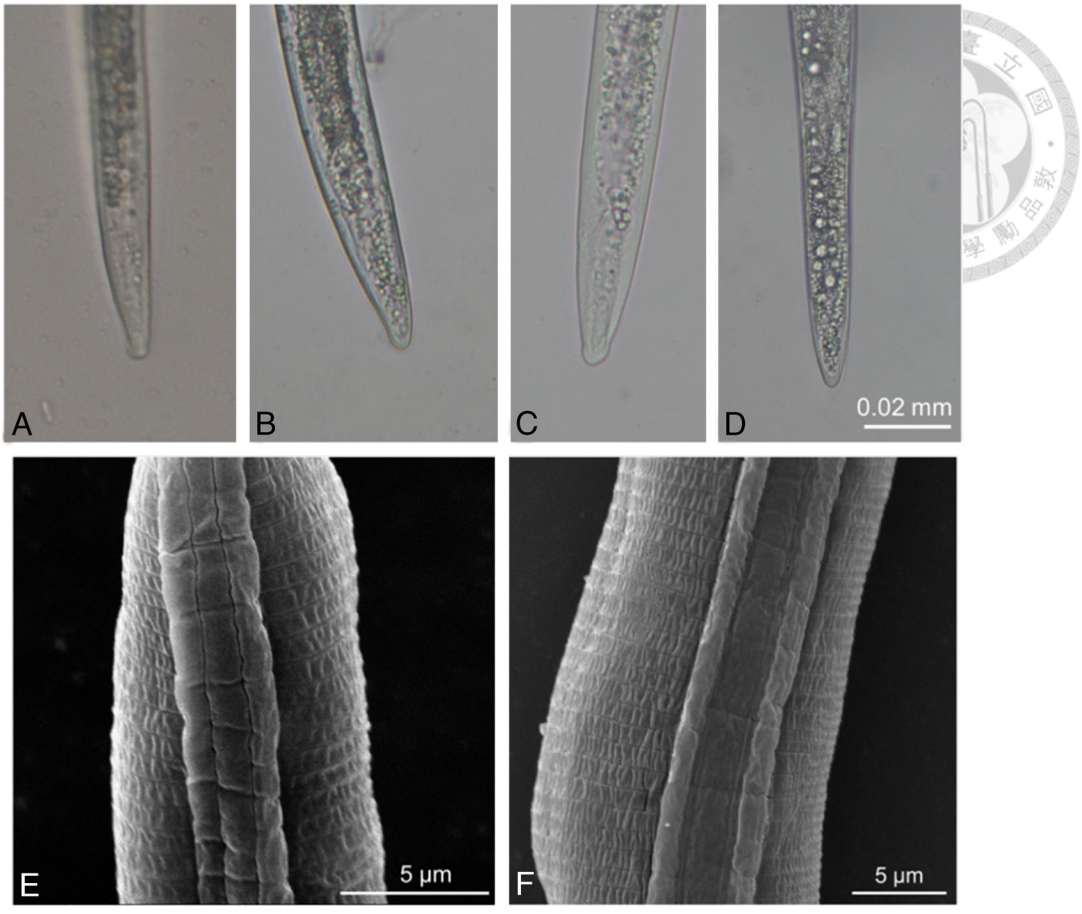

Figure 1: The morphological variations within the *P. vulnus* Taiwan strawberry population. Two lateral field types and four tail termini were observed. Photographs were taken at 1000X magnification with compound microscope and SEM.

smooth and tapering (Fig. 1A-D). *P. vulnus* is known to express morphological differences when cultured under different temperature conditions (Doucet et al., 2001). A previous report also characterized five *P. vulnus* groups in Japan by tail morphology (Mizukubo 1990). The fact that four different tail terminus types were observed in the *P. vulnus* Taiwan population implies this population may have been established in the region rather than being recently imported.

Molecular analysis of the ribosomal RNA and mitochondrial gene sequences of the extracted nematodes confirmed their species as *P. vulnus* (Table 1). The rDNA LSU region (D2A/D3B: ACAAGTA CCGTGAGGGAAAGTTG/TCGGAAGGAACCAGCT ACTA) (Nunn, 1992), rDNA ITS region (TW81/AB28: GTTTCCGTAGGTGAACCTGC/ATATGCTTAAGTTCA GCGGGT) (Amiri et al., 2002; Subbotin et al., 2001), rDNA SSU region (SSU18A/SSU26R: AAAGATTAAG CCATGCATG/CATTCTTGGCAAATGCTTTCG) (Eyualem and Blaxter, 2003) and mtDNA COI region (JB3/JB4.5: TTTTTTGGGCATCCTGAGGTTTAT/TAAA GAAAGAACATAATGAAAATG) (Derycke et al., 2010) of the Pv-GS and Pv-KDL are 99.04, 95.96, 99.66 and 99.24% identical, respectively. Both Pv-GS and Pv-KDL isolates had maximum 100% similarity of their rDNA LSU sequences to *P. vulnus* (GenBank accession: U47547.1). The Pv-KDL mtDNA COI region sequences of isolates were 99.74 to 100% similar to other sequences of *P. vulnus* available in the database (GenBank accessions KY424094, KY424095, KY828312, KY828317, KY424096-7 and KX349427), and only 81.08% identical to the second most closely related species, *P. scribneri* (GenBank accession: KY424089.1). The rDNA SSU region sequence analysis provided a similar result as Pv-KDL and are 99.42% (GenBank accession: KY424163) to 99.88% (GenBank accession: KY424164) identical to all available *P. vulnus* sequences in GenBank, and only 94.48%

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Table 1. GenBank sequence deposit information of multiple gene regions of *P. vulnus* Taiwan isolates from strawberry fields GS and KDL.

P. vulnus sequences GenBank deposit information				
		rDNA		mtDNA
Isolate location	LSU (730 bp)	ITS (669 bp)	SSU (883 bp)	COI (441 bp)
GS	MG372808	MG372806.1	MG372807	MN431203
KDL	MK713641	MK713613	MK713614	MK764689

similar to *P. kumamotoensis* (GenBank accession: AB905295.1). The rDNA ITS sequences also separated the Pv-KDL from *P. kumamotoensis* (GenBank accession: KT175521.1) clearly with a very low 86.22% similarity. Phylogenetic analysis of the rDNA ITS and LSU regions combined suggested the nematodes from the two fields belong to one population (Fig. 2) and are isolated from the rest of the previous populations from other geographic regions (Table 2).

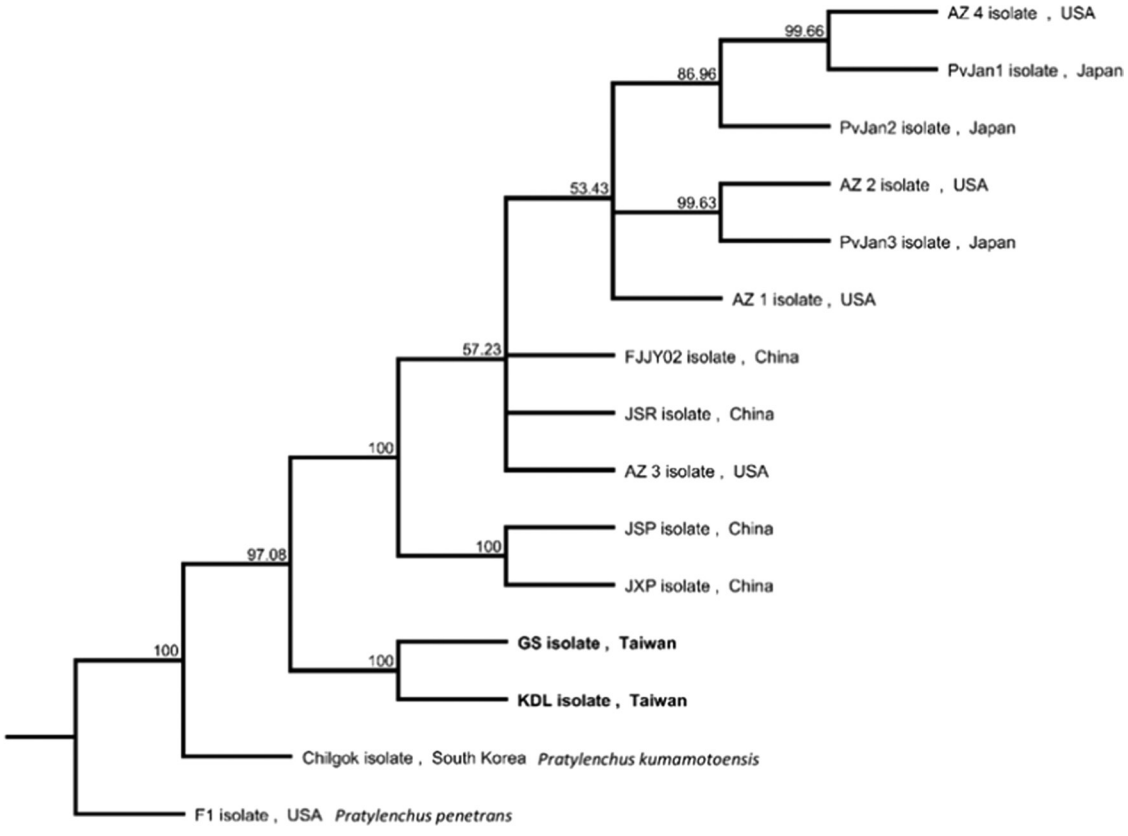

Figure 2: The phylogenetic tree of the combined partial 28S D2-D3 region and complete ITS region of the *P. vulnus* rDNA sequences. Morphologically similar species, *Pratylenchus penetrans* F1 isolate and of *P. kumamotoensis* Chilgok isolate, were used as out groups. Each node is marked with the isolate code, species and origin. The number at the fork represents the percentage of the bootstrap tested with 10,000 times for the indicating result.

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Table 2. Measurements of the Taiwan *P. vulnus* population morphological characteristics.

	Female (*n* = 30)			Male (*n* = 30)		
Characteristics	**Mean**	**SD m.**	**Range**	**Mean**	**SD m.**	**Range**
L (µm)	704.01	74.85	573.46–839.91	587.02	46.83	492.76–678.1
K (µm)	25.97	4.06	17.76–36.18	18.47	1.47	15.49–21.50
Stylet (µm)	14.5	1.1	12.6–16.5	13.76	0.73	12.18–15.40
Tail (µm)	32.87	3.93	24.35–41.10	28.74	4.07	22.28–39.80
a (ratio)	27.47	3.15	22.74–34.30	31.85	2.53	26.83–36.62
b (ratio)	6.1	0.6	4.87–7.16	5.7	0.47	4.88–6.95
b' (ratio)	4.86	0.42	4.07–5.59	4.51	0.37	3.92–5.26
c (ratio)	21.57	2.38	17.83–27.46	20.71	2.68	13.51–23.99
c' (ratio)	2.29	0.31	1.59–2.86	2.29	0.38	1.77–3.83
V (%)	78.34	1.67	73.84–81.09	–	–	–
Spicules (µm)	–	–	–	16.88	1.89	12.06–20.15
DGO (µm)	3.55	0.32	3.00–4.42	3.41	0.33	2.77–4.31

Our discovery of the existence and population dynamics of *P. vulnus* in Taiwan implies a new threat to the 1,639 million NTD (ca. $52 million) strawberry industry. Currently, no nematicide is registered for root-lesion nematode suppression in strawberry. Related cultural and physical control options are currently undergoing evaluation by agricultural extension agencies to prevent serious damage.

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Diversity and pathogenicity of *Colletotrichum* species causing strawberry anthracnose in Taiwan and description of a new species, *Colletotrichum miaoliense* sp. nov.

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Strawberry is a small fruit crop with high economic value. Anthracnose caused by *Colletotrichum* spp. poses a serious threat to strawberry production, particularly in warm and humid climates, but knowledge of pathogen populations in tropical and subtropical regions is limited. To investigate the diversity of infectious agents causing strawberry anthracnose in Taiwan, a disease survey was conducted from 2010 to 2018, and *Colletotrichum* spp. were identified through morphological characterization and multilocus phylogenetic analysis with internal transcribed spacer, glyceraldehyde 3-phosphate dehydrogenase, chitin synthase, actin, beta-tubulin, calmodulin, and the intergenic region between *Apn2* and *MAT1-2-1* (ApMAT). Among 52 isolates collected from 24 farms/nurseries in Taiwan, a new species, *Colletotrichum miaoliense* sp. nov. (6% of all isolates), a species not previously known to be associated with strawberry, *Colletotrichum karstii* (6%), and three known species, *Colletotrichum siamense* (75%), *Colletotrichum fructicola* (11%), and *Colletotrichum boninense* (2%), were identified. The predominant species *C. siamense* and *C. fructicola* exhibited higher mycelial growth rates on potato dextrose agar and caused larger lesions on wounded and non-wounded detached strawberry leaves. *Colletotrichum boninense*, *C. karstii*, and *C. miaoliense* only caused lesions on wounded leaves. Understanding the composition and biology of the pathogen population will help in disease management and resistance breeding.

Strawberry (*Fragaria* × *ananassa* Duch.) is a popular small fruit crop with high economic and nutritive value. Strawberry is in high demand globally. From 2008 to 2018, the annual worldwide cultivation of strawberries increased from approximately 400 to 483 thousand hectares[1]. Although strawberries are native to temperate regions, they can also be grown in tropical and subtropical regions (sometimes under high-altitude conditions). The land areas devoted to strawberry cultivation in Colombia, Peru, Guatemala, Bolivia, and Taiwan in 2018 were 1,482 ha, 1,453 ha, 690 ha, 522 ha, and 506 ha, respectively[1].

Anthracnose caused by *Colletotrichum* spp. is a serious threat to strawberry production, especially in warm and humid climates[2]. Rain-splashed conidia of *Colletotrichum* spp. serve as the major inoculum causing epidemics of strawberry anthracnose disease[3]. After landing on the plant surface, the conidia germinate, form appressoria, then penetrate the epidermal cells[4]. *Colletotrichum* spp. can infect various strawberry tissues, causing black spots or irregular spots on leaves, sunken black spots or necrosis lesions on petioles, stolons, and fruits, and wilting of the whole plant due to crown rot[2]. Under high humidity, concentric rings of acervuli with orange conidial masses can be observed on necrotic tissues. In the US state of Florida, anthracnose causes the death of up to 80% of seedlings in the nursery and yield losses of over 50% in the field[2]. In Taiwan, strawberry seedlings are propagated from March to September, and the high temperature, high humidity and heavy rainfall during

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this period provide a suitable environment for epidemics. From 2010 to 2016, anthracnose crown rot caused the loss of 30–40% of seedlings and ~ 20% of plants after transplanting[5].

Colletotrichum spp. have traditionally been classified based on the shape of the conidia and appressorium, the presence of a seta or perithecium, and culture characteristics[6,7]. Using these criteria, early studies reported *C. acutatum*, *C. gloeosporioides*, and *C. fragariae* as strawberry anthracnose pathogens[2,8]. However, *Colletotrichum* spp. share similar features, and morphological characteristics can be influenced by environmental factors including culture media, light, and temperature[9–11]. Therefore, a polyphasic approach based on morphology and genetic characteristics was proposed for identification of *Colletotrichum* species[9]. A combination of multiple gene sequences, including internal transcribed spacer (ITS), glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*), chitin synthase (*CHS-1*), actin (*ACT*), beta-tubulin (*TUB2*), calmodulin (*CAL*), and the intergenic region between *Apn2* and *MAT1-2-1* (ApMAT), can provide more molecular features to resolve different species in a *Colletotrichum* species complex[12,13]. Through multilocus sequence analysis coupled with morphological characterization, recent studies have identified many additional *Colletotrichum* species associated with strawberry, namely *C. acutatum*, *C. fioriniae*, *C. godetiae*, *C. nymphaeae*, *C. salicis* and *C. simmondsii* (*C. acutatum* species complex), *C. aenigma*, *C. changpingense*, *C. fructicola*, *C. gloeosporioides*, *C. siamense* and *C. theobromicola* (syn. *C. fragariae*) (*C. gloeosporioides* species complex) and *C. boninense* (*C. boninense* species complex)[6,12–18].

Although strawberry is of great economic importance in Taiwan and anthracnose has become more destructive in the past decade, the pathogen population in Taiwan has not been systematically investigated. The causal agents of strawberry anthracnose were previously reported to be *C. gloeosporioides*[19], *C. dematium*, *C. fragariae*, and *C. acutatum* (Plant Protection Information System; https://otserv2.tactri.gov.tw/ppm/), but information about the isolation, pathogenicity, morphology, and sequences of these species is not sufficient for species identification. Recently, based on analysis of multiple gene sequences, we identified *C. siamense* as the pathogen causing anthracnose crown rot[5]. To provide accurate information for disease control and resistance breeding, in this study we aimed to reveal the population composition of the infectious agents associated with strawberry anthracnose in Taiwan. Samples collected from the major strawberry-producing areas of Taiwan from 2010 to 2018 were subjected to morphological and multi-gene phylogenetic analyses. To further understand the in vitro and *in planta* aggressiveness of different *Colletotrichum* spp. at different temperatures, multiple representative isolates of each species were tested for mycelial growth rates in an artificial medium as well as the ability to cause lesions on wounded or non-wounded strawberry leaves. Since population analysis of *Colletotrichum* spp. causing strawberry anthracnose has only been reported for species from the UK[14] and China [Anhui, Hainan (only one isolate), Hebei, Hubei, Liaoning, Shandong, and Zhejiang Provinces and Beijing and Shanghai cities][16–18], which, with the exception of Hainan, are geographical regions located at higher latitudes (30–53°N) relative to Taiwan (24.5°N), this study will provide insights into the biology of strawberry anthracnose disease in subtropical regions.

Results

Molecular identification and phylogenetic analysis. *Colletotrichum* spp. isolates were first identified at the species complex level. Among 52 *Colletotrichum* spp. isolates sampled from the major strawberry-producing areas of Taiwan, 45 (86.5%) isolates belonged to the *C. gloeosporioides* species complex, 4 (7.7%) belonged to the *C. boninense* species complex, and 3 (5.8%) belonged to the *C. acutatum* species complex (Table 1).

To further analyze the *C. acutatum* species complex, 3 isolates together with 40 reference isolates, including the outgroup *C. orchidophilum* (CBS 632.80), were used to construct phylogenetic trees with five gene sequences (ITS, *GAPDH*, *CHS-1*, *ACT*, and *TUB2*) (Table 1 and Supplementary Table S1) following Damm et al.[12] and Fu et al.[20]. The final data matrix contained a total of 1,821 characters with gaps (ITS: 1–540, *GAPDH*: 541–799, *CHS-1*: 800–1,081, *ACT*: 1,082–1,329, *TUB2*: 1,330–1,821), of which 237 characters were parsimony informative, 174 parsimony uninformative, and 1,410 constant. After 2,000,000 generations of topological convergence via Bayesian inference (BI) analysis, 2,378 trees were obtained. The first 25% of the trees were discarded, representing the burn-in phase of the analyses, and the remaining trees were used to calculate the Bayesian posterior probabilities in the majority rule consensus tree (Fig. 1). The maximum likelihood (ML) analysis resulted in a best scoring RAxML tree with a final optimized likelihood value of − 6,726.174303. The most parsimonious tree resulted from the maximum parsimony (MP) analysis received tree length = 692, consistency index (CI) = 0.714, and retention index (RI) = 0.843. All three isolates (ML1040, ML1042, ML1794) were grouped in a distinct clade with significant statistical support in the multilocus phylogenetic analysis (1/100/100, BI/ML/MP) (Fig. 1) and the single gene trees of *GAPDH*, *CHS-1*, and *TUB2* (Supplementary Fig. S1). This clade was distinct from all other known species, and is herein described as a new species, *C. miaoliense* sp. nov.

To analyze the phylogeny of the *C. boninense* species complex, six gene sequences (ITS, *GAPDH*, *CHS-1*, *ACT*, *TUB2*, and *CAL*) from 4 isolates together with 31 reference isolates, including the outgroup sequence of *C. gloeosporioides* (IMI 356878), were used to construct phylogenetic trees (Table 1 and Supplementary Table S1). The final data matrix contained a total of 2,363 characters with gaps (ITS: 1–558, *GAPDH*: 559–852, *CHS-1*: 853–1,132, *ACT*: 1,133–1,411, *TUB2*: 1,412–1,914, *CAL*: 1,915–2,363), of which 365 characters were parsimony informative, 407 parsimony uninformative, and 1,591 constant. After 1,187,000 generations of topological convergence via BI analysis, 492 trees were obtained. The first 25% of the trees were discarded, representing the burn-in phase of the analyses, and the remaining trees were used to calculate the Bayesian posterior probabilities in the majority rule consensus tree (Fig. 2). The ML analysis resulted in a best scoring RAxML tree with a final optimized likelihood value of − 10,025.941645. The most parsimonious tree resulted from the MP analysis received tree length = 1,281, CI = 0.774, and RI = 0.839. In single gene trees of *GAPDH*, *CHS-1*, and *TUB2* and the multilocus phylogenetic tree, three isolates (ML351, 442, 1792) clustered with strong statistical support in the clade containing the type strain CGMCC 3.14194 and other related isolates of *C. karstii* (Fig. 2; single gene trees

Species		Isolate[a]	Strawberry tissue	Collection date	Sampling site	GenBank accession number[b]						
						ITS	*GAPDH*	*CHS1*	*ACT*	*TUB2*	*CAL*	ApMAT
***C. acutatum* species complex**												
	***C. miaoliense* sp. nov**	**ML1040**	**crown**	**2016/10/28**	**Shitan Township, Miaoli County**	**MK908419**	**MK908470**	**MK908522**	**MK908573**	**MK908624**	-[c]	-
		ML1042	leaf	2016/11/24	Renai Township, Nantou County	MK908420	MK908471	MK908523	MK908574	MK908625	-	-
		ML1794	leaf	2018/07/04	Renai Township, Nantou County	MK908421	MK908472	MK908524	MK908575	MK908626	-	-
***C. boninense* species complex**												
	C. boninense	**ML521**	leaf	2013/01/21	Taian Township, Miaoli County	MK908424	MK908475	MK908527	MK908578	MK908629	MK908677	-
	C. karstii	**ML351**	leaf	2012/07/06	Shitan Township, Miaoli County	MK908422	MK908473	MK908525	MK908576	MK908627	MK908675	-
		ML442	leaf	2012/07/04	Nanchuang Township, Miaoli County	MK908423	MK908474	MK908526	MK908577	MK908628	MK908676	-
		ML1792	leaf	2018/05/16	Renai Township, Nantou County	MK908425	MK908476	MK908528	MK908579	MK908630	MK908678	-
***C. gloeosporioides* species complex**												
	C. fructicola	**ML348**	leaf	2012/07/06	Shitan Township, Miaoli County	MK908461	MK908513	MK908564	MK908615	MK908666	MK908714	MK908758
		ML353	root	2012/07/10	Shitan Township, Miaoli County	MK908462	MK908514	MK908565	MK908616	MK908667	MK908715	MK908759
		ML356	crown	2012/07/10	Shitan Township, Miaoli County	MK908463	MK908515	MK908566	MK908617	MK908668	MK908716	MK908760
		ML368	stolon	2012/07/24	Dahu Township, Miaoli County	MK908464	MK908516	MK908567	MK908618	MK908669	MK908717	MK908761
		ML818	crown	2016/07/06	Renai Township, Nantou County	MK908468	MK908520	MK908571	MK908622	MK908673	MK908721	MK908765
		ML1012	leaf	2016/11/09	Renai Township, Nantou County	MK908469	MK908521	MK908572	MK908623	MK908674	MK908722	MK908766
	C. siamense	ML040	fruit	2010/03/22	Gongguan Township, Miaoli County	MK908426	MK908477	MK908529	MK908580	MK908631	MK908679	MK908723
		ML041	fruit	2010/03/24	Dahu Township, Miaoli County	MK908427	MK908478	MK908530	MK908581	MK908632	MK908680	MK908724
		ML048	fruit	2010/05/12	Dahu Township, Miaoli County	MK908428	MK908479	MK908531	MK908582	MK908633	MK908681	MK908725
		ML076	stolon	2010/08/27	Dahu Township, Miaoli County	MK908429	MK908480	MK908532	MK908583	MK908634	MK908682	MK908726
		ML133	crown	2011/10/28	Dahu Township, Miaoli County	MK174223	MK908481	MK174224	MK174225	MK174226	MK174227	MK174228
		ML177	fruit	2012/03/09	Dahu Township, Miaoli County	MK908430	MK908482	MK908533	MK908584	MK908635	MK908683	MK908727
		ML275	crown	2012/06/04	Dahu Township, Miaoli County	MK908431	MK908483	MK908534	MK908585	MK908636	MK908684	MK908728
		ML284	crown	2012/06/04	Shitan Township, Miaoli County	MK908432	MK908484	MK908535	MK908586	MK908637	MK908685	MK908729

Continued

Species		Isolate[a]	Strawberry tissue	Collection date	Sampling site	GenBank accession number[b]						
						ITS	*GAPDH*	*CHS1*	*ACT*	*TUB2*	*CAL*	ApMAT
***C. acutatum* species complex**												
	***C. miaoliense* sp. nov**	**ML1040**	**crown**	**2016/10/28**	**Shitan Township, Miaoli County**	**MK908419**	**MK908470**	**MK908522**	**MK908573**	**MK908624**	-[c]	-
		ML293	root	2012/4/25	Guanxi Township, Hsinchu County	MK908433	MK908485	MK908536	MK908587	MK908638	MK908686	MK908730
		ML294	crown	2012/06/13	Dahu Township, Miaoli County	MK908434	MK908486	MK908537	MK908588	MK908639	MK908687	MK908731
		ML296	crown	2012/06/13	Dahu Township, Miaoli County	MK908435	MK908487	MK908538	MK908589	MK908640	MK908688	MK908732
		ML320	crown	2012/06/27	Taian Township, Miaoli County	MK908436	MK908488	MK908539	MK908590	MK908641	MK908689	MK908733
		ML328	crown	2012/07/02	Shitan Township, Miaoli County	MK908437	MK908489	MK908540	MK908591	MK908642	MK908690	MK908734
		ML372	crown	2012/07/26	Shitan Township, Miaoli County	MK908438	MK908490	MK908541	MK908592	MK908643	MK908691	MK908735
		ML393	stolon	2012/07/26	Shitan Township, Miaoli County	MK908439	MK908491	MK908542	MK908593	MK908644	MK908692	MK908736
		ML416	crown	2012/08/15	Dahu Township, Miaoli County	MK908440	MK908492	MK908543	MK908594	MK908645	MK908693	MK908737
		ML418	crown	2012/08/15	Dahu Township, Miaoli County	MK908441	MK908493	MK908544	MK908595	MK908646	MK908694	MK908738
		ML419	root	2012/08/06	Shitan Township, Miaoli County	MK908442	MK908494	MK908545	MK908596	MK908647	MK908695	MK908739
		ML443	crown	2012/07/26	Shitan Township, Miaoli County	MK908443	MK908495	MK908546	MK908597	MK908648	MK908696	MK908740
		ML458	crown	2012/08/15	Dahu Township, Miaoli County	MK908444	MK908496	MK908547	MK908598	MK908649	MK908697	MK908741
		ML461	leaf	2012/09/12	Gongguan Township, Miaoli County	MK908445	MK908497	MK908548	MK908599	MK908650	MK908698	MK908742
		ML462	crown	2012/09/12	Gongguan Township, Miaoli County	MK908446	MK908498	MK908549	MK908600	MK908651	MK908699	MK908743
		ML463	root	2012/09/12	Gongguan Township, Miaoli County	MK908447	MK908499	MK908550	MK908601	MK908652	MK908700	MK908744
		ML464	crown	2012/09/13	Shitan Township, Miaoli County	MK908448	MK908500	MK908551	MK908602	MK908653	MK908701	MK908745
		ML469	crown	2012/09/18	Dahu Township, Miaoli County	MK908449	MK908501	MK908552	MK908603	MK908654	MK908702	MK908746
		ML471	crown	2012/09/18	Dahu Township, Miaoli County	MK908450	MK908502	MK908553	MK908604	MK908655	MK908703	MK908747
		ML476	root	2012/09/18	Dahu Township, Miaoli County	MK908451	MK908503	MK908554	MK908605	MK908656	MK908704	MK908748
		ML477	crown	2012/09/18	Dahu Township, Miaoli County	MK908452	MK908504	MK908555	MK908606	MK908657	MK908705	MK908749
		ML485	crown	2012/10/04	Guoxing Township, Nantou County	MK908453	MK908505	MK908556	MK908607	MK908658	MK908706	MK908750

Continued

Species		Isolate[a]	Strawberry tissue	Collection date	Sampling site	GenBank accession number[b]						
						ITS	*GAPDH*	*CHS1*	*ACT*	*TUB2*	*CAL*	ApMAT
***C. acutatum* species complex**												
	***C. miaoliense* sp. nov**	**ML1040**	**crown**	**2016/10/28**	**Shitan Township, Miaoli County**	**MK908419**	**MK908470**	**MK908522**	**MK908573**	**MK908624**	-[c]	-
		ML490	crown	2012/11/13	Gongguan Township, Miaoli County	MK908454	MK908506	MK908557	MK908608	MK908659	MK908707	MK908751
		ML491	crown	2012/11/13	Gongguan Township, Miaoli County	MK908455	MK908507	MK908558	MK908609	MK908660	MK908708	MK908752
		ML494	crown	2012/12/10	Dahu Township, Miaoli County	MK908456	MK908508	MK908559	MK908610	MK908661	MK908709	MK908753
		ML513	crown	2013/01/11	Shitan Township, Miaoli County	MK908457	MK908509	MK908560	MK908611	MK908662	MK908710	MK908754
		ML540	stolon	2013/04/22	Gongguan Township, Miaoli County	MK908458	MK908510	MK908561	MK908612	MK908663	MK908711	MK908755
		ML608	stolon	2013/07/10	Shitan Township, Miaoli County	MK908459	MK908511	MK908562	MK908613	MK908664	MK908712	MK908756
		ML612	leaf	2013/07/10	Shitan Township, Miaoli County	MK908460	MK908512	MK908563	MK908614	MK908665	MK908713	MK908757
		ML617	crown	2013/07/18	Dahu Township, Miaoli County	MK908465	MK908517	MK908568	MK908619	MK908670	MK908718	MK908762
		ML754	leaf	2016/03/17	Chiayi County	MK908466	MK908518	MK908569	MK908620	MK908671	MK908719	MK908763
		ML762	fruit	2016/03/28	Dahu Township, Miaoli County	MK908467	MK908519	MK908570	MK908621	MK908672	MK908720	MK908764

Table 1. List of *Colletotrichum* spp. associated with strawberry anthracnose in Taiwan. [a]Isolates in bold are representatives of each *Colletotrichum* spp. selected for mycelial growth and pathogenicity assays. [b]ITS: internal transcribed spacer; *GAPDH*: glyceraldehyde 3-phosphate dehydrogenase; *CHS-1*: chitin synthase; *ACT*: actin; *TUB2*: beta-tubulin; *CAL*: calmodulin; ApMAT: intergenic sequence between *Apn2* DNA lyase and *MAT1-2-1*. [c]-: not available.

not shown). In single gene trees of *GAPDH* and *CAL* and the multilocus phylogenetic tree, the isolate ML521 clustered with strong statistical support in the clade containing the type strain CBS 123755 and other related isolates of *C. boninense* (Fig. 2; single gene trees not shown).

To identify species in the *C. gloeosporioides* species complex, a combination of seven gene sequences (ITS, *GAPDH*, *CHS-1*, *ACT*, *TUB2*, *CAL* and ApMAT) from 45 isolates together with 47 reference isolates, including the outgroup sequence of *C. boninense* (CBS 123755), were used to construct phylogenetic trees (Table 1 and Supplementary Table S1). The final data matrix contained a total of 3,571 characters with gaps (ITS: 1–553, *GAPDH*: 554–808, *CHS-1*: 809–1,051, *ACT*: 1,052–1,325, *TUB2*: 1,326–2,028, *CAL*: 2,029–2,728, ApMAT: 2,729–3,571), of which 655 characters were parsimony informative, 735 parsimony uninformative, and 2,181 constant. After 6,574,000 generations of topological convergence via BI analysis, 9,864 trees were obtained. The first 25% of the trees were discarded, representing the burn-in phase of the analyses, and the remaining trees were used to calculate the Bayesian posterior probabilities in the majority rule consensus tree (Fig. 3). The ML analysis resulted in a best scoring RAxML tree with a final optimized likelihood value of − 18,514.217014. The most parsimonious tree resulted from the MP analysis received tree length = 2,400, CI = 0.722, and RI = 0.876. In single gene trees of *TUB2*, *CAL*, and ApMAT and the multilocus phylogenetic tree, 39 isolates clustered with strong statistical support in the clade containing the type strain CBS 130417 and other related isolates of *C. siamense* (Fig. 3; single gene trees not shown). The 39 isolates formed a subclade with a high support value (1/100/98, BI/ML/MP) (Fig. 3). In single gene trees of *GAPDH*, *ACT*, *TUB2*, *CAL*, and ApMAT and the multilocus phylogenetic tree, six isolates clustered with strong statistical support in the clade containing the type strain CBS 130416 and other related isolates of *C. fructicola* (Fig. 3; single gene trees not shown).

Taxonomy

Based on morphological traits and multilocus phylogenetic analysis, the 52 isolates were assigned to five *Colletotrichum* spp. including one new taxon (*C. miaoliense* sp. nov.) (Fig. 4; described in detail below), one newly recorded taxon in strawberry (*C. karstii*), and three species known to be associated with strawberry anthracnose

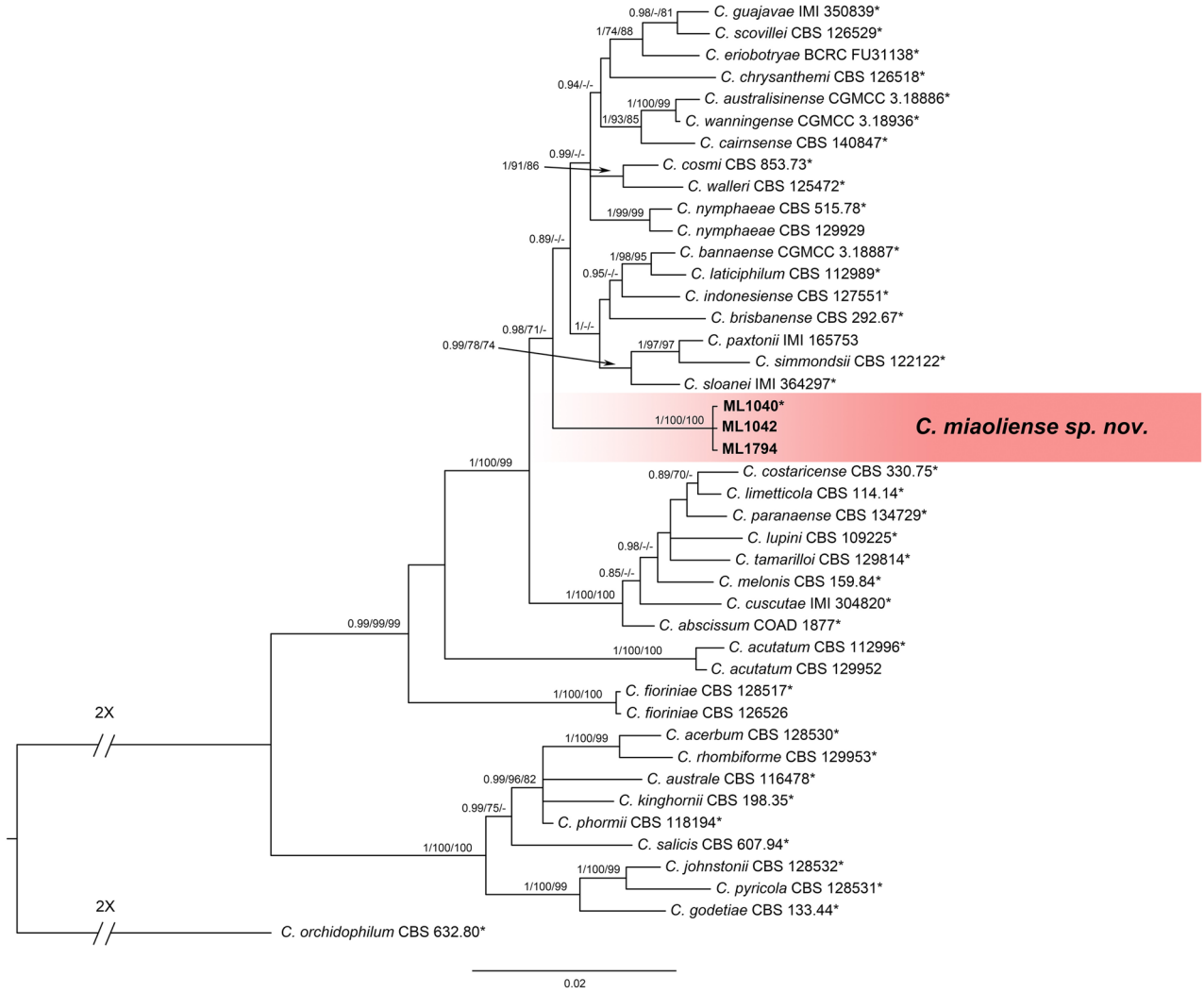

Figure 1. A Bayesian inference phylogenetic tree of the *C. acutatum* species complex. The phylogenetic tree was built using concatenated sequences of the ITS and the *GAPDH*, *ACT*, *CHS-1* and *TUB2* genes. Bayesian inference (BI) posterior values above 0.9 and bootstrap support values from maximum likelihood (ML) and maximum parsimony (MP) above 70% are shown at each node (BI/ML/MP). *C. orchidophilum* CBS 632.80 was used as the outgroup. *Indicates the ex-type strains. Strains isolated in this study are shown in bold.

(*C. boninense*, *C. fructicola* and *C. siamense*) (Supplementary Fig. S2–S5). The colony features that developed at 25 °C on PDA and 1/4 PDA were all white to grey, with orange conidia ooze. *C. siamense* ML133 and *C. karstii* ML351 produced abundant conidia when cultured on 1/4 PDA at 25 °C; *C. boninense* ML521 produced more conidia on PDA at 25 °C; *C. fructicola* ML348 and *C. miaoliense* ML1040 sporulated more abundantly on 1/4 PDA at 30 °C. The conidium and appressorium measurements of the five *Colletotrichum* spp. (isolates from this study and the type strains) are listed in Supplementary Table S2. The conidia produced by *C. miaoliense* ML1040 were longer [length to width (L/W) ratio = 3.4] (Fig. 4) than the conidia of the other four species in this study (L/W ratio = 2.3–3) (Fig. S2–S5; Supplementary Table S2).

Colletotrichum miaoliense sp. nov. P. C. Chung & H. Y. Wu. Figure 4. *MycoBank number MB835424.* Etymology: The epithet *miaoliense* specifically refers to Miaoli County, Taiwan, where the new taxon was discovered.

Sexual morph not observed. *Asexual morph* observed on 1/4 PDA [BCRC FU31304 (= NTUCC 20-001-1, ML1040)]. Vegetative hyphae 3–6 µm in diameter, hyaline, smooth-walled, septate, branched. Chlamydospores not observed. Sporodochia developed, conidiophores formed directly on hyphae. Conidiophores hyaline, smooth-walled, simple or branched. Conidiogenous cells hyaline, smooth-walled, cylindrical to ampulliform, often integrated, occasionally polyphialidic; phialides discrete, 5.9–26.4 µm ($\bar{x}$ = 13.3 ± 4.8, n = 55) in length, apical opening 1.1–2.6 µm in diameter (1.7 ± 0.3, n = 55). Conidia hyaline, smooth-walled, aseptate, straight, fusiform to cylindrical, acute ends, 11.2–17 × 3.3–5 µm ($\bar{x}$ = 14.2 ± 1.1 × 4.1 ± 0.3 µm, n = 100), L/W ratio = 3.4. Conidia from aerial hyphae varied in size (6.6–20 × 2.9–4.9 µm, $\bar{x}$ = 11.2 ± 2.5 × 3.8 ± 0.4 µm, n = 100), L/W ratio = 3.0.

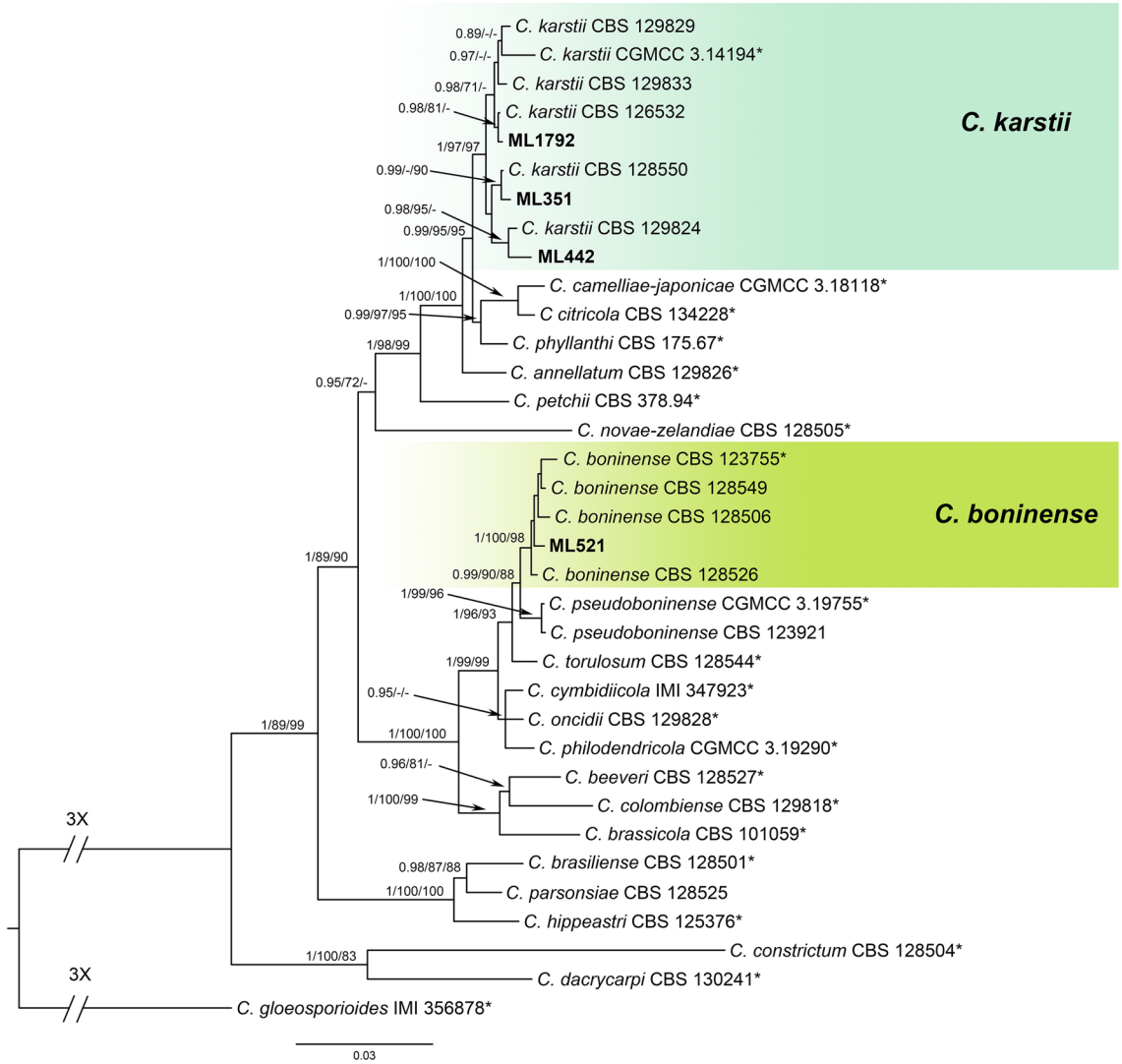

Figure 2. A Bayesian inference phylogenetic tree of the *C. boninense* species complex. A phylogram was built using concatenated sequences of the ITS and the *GAPDH*, *ACT*, *CHS-1*, *TUB2* and *CAL* genes. Bayesian inference (BI) posterior values above 0.9 and bootstrap support values from maximum likelihood (ML) and maximum parsimony (MP) above 70% are shown at each node (BI/ML/MP). *C. gloeosporioides* IMI 356878 was used as the outgroup. *Indicates the ex-type strains. Strains isolated in this study are shown in bold.

Seta absent. Appressoria single or in loose clusters, pale brown, smooth-walled, elliptical to clavate, entire edge, 5.9–9.1 × 4–6.0 µm ($\bar{x}$ = 7.5 ± 1.1 × 5 ± 0.6 µm, n = 27), L/W ratio = 1.5.

Culture features: Colonies on PDA flat to somewhat raised, margin entire; mycelium partly floccose, white to pale olivaceous grey; sporodochia orange, scattered in rings, reverse bright orange to orange; average 4.2 cm in diameter in 7 days at 25 °C. Conidia ooze was visible as an orange mass.

Material examined: Taiwan, Miaoli County, Shitan Township, from crown rot of *Fragaria* × *ananassa*, 28 Oct. 2016, P.-C. Chung; holotype NTUH 20-001-1, ex-holotype living culture BCRC FU31304 (= NTUCC 20-001-1, ML1040).

Additional materials examined: Taiwan, Nantou County, Renai Township, from leaf spot of *Fragaria* × *ananassa*, 24 Nov. 2016, P.-C. Chung, NTUH 20-001-2; living culture NTUCC 20-001-2 (= ML1042). Taiwan, Nantou County, Renai Township, from leaf spot of *Fragaria* × *ananassa*, 4 Jul. 2018, P.-C. Chung; NTUH 20-001-3, living culture NTUCC 20-001-3 (= ML1794). Known distribution: Miaoli and Nantou Counties, Taiwan.

Notes Three isolates of *C. miaoliense* were collected from Miaoli County and Nantou County, Taiwan. Multilocus analysis indicated that *C. miaoliense* forms a robust clade clearly distinct from all the other known species in the *C. acutatum* species complex. Of the six *Colletotrichum* species in this complex (*C. acutatum*, *C. fioriniae*, *C. godetiae*, *C. nymphaeae*, *C. salicis*, and *C. simmondsii*) that have been reported as anthracnose pathogens of strawberry, *C. miaoliense* is phylogenetically most closely related to *C. nymphaeae* and *C. simmondsii*. Morphologically, *C. miaoliense* differs from *C. nymphaeae* (CBS 515.78) in the size of conidia (16.1 ± 2.3 × 4.9 ± 0.7 µm versus 14.2 ± 1.1 × 4.1 ± 0.34 µm), the shape of conidia (one end round and one end rounded to acute in contrast to the new species, in which both ends are acute), the size of appressoria (8.7 ± 2.5 × 5.5 ± 1.0 versus 7.5 ± 1.

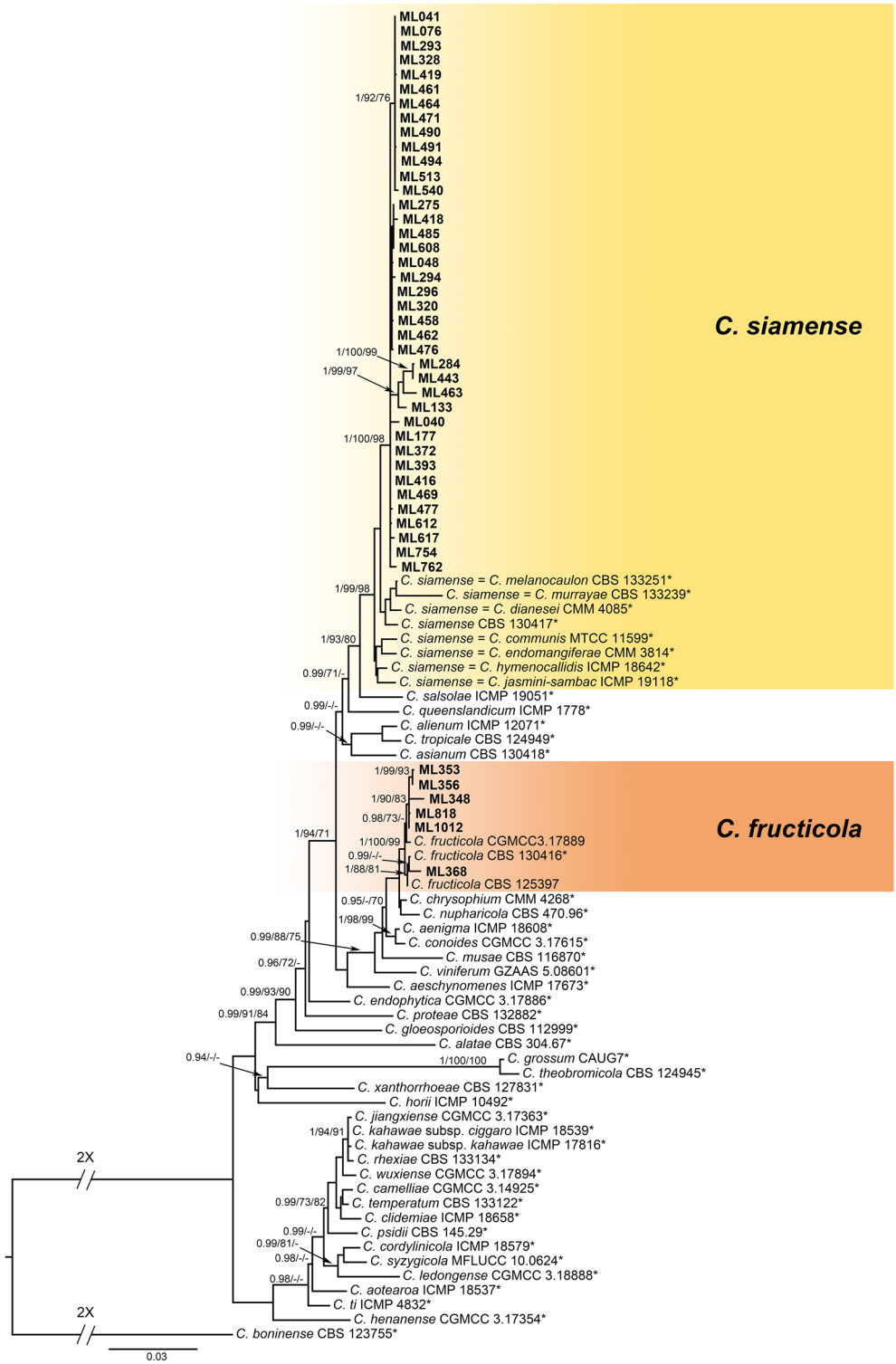

Figure 3. A Bayesian inference phylogenetic tree of the *C. gloeosporioides* species complex. The phylogenetic tree was built using concatenated sequences of the ITS, ApMAT, and the *GAPDH*, *ACT*, *CHS-1*, *TUB2*, *CAL* genes. Bayesian inference (BI) posterior values above 0.9 and bootstrap support values from maximum likelihood (ML) and maximum parsimony (MP) above 70% are shown at each node (BI/ML/MP). *C. boninense* CBS 123755 was used as the outgroup. *Indicates the ex-type strains. Strains isolated in this study are shown in bold.

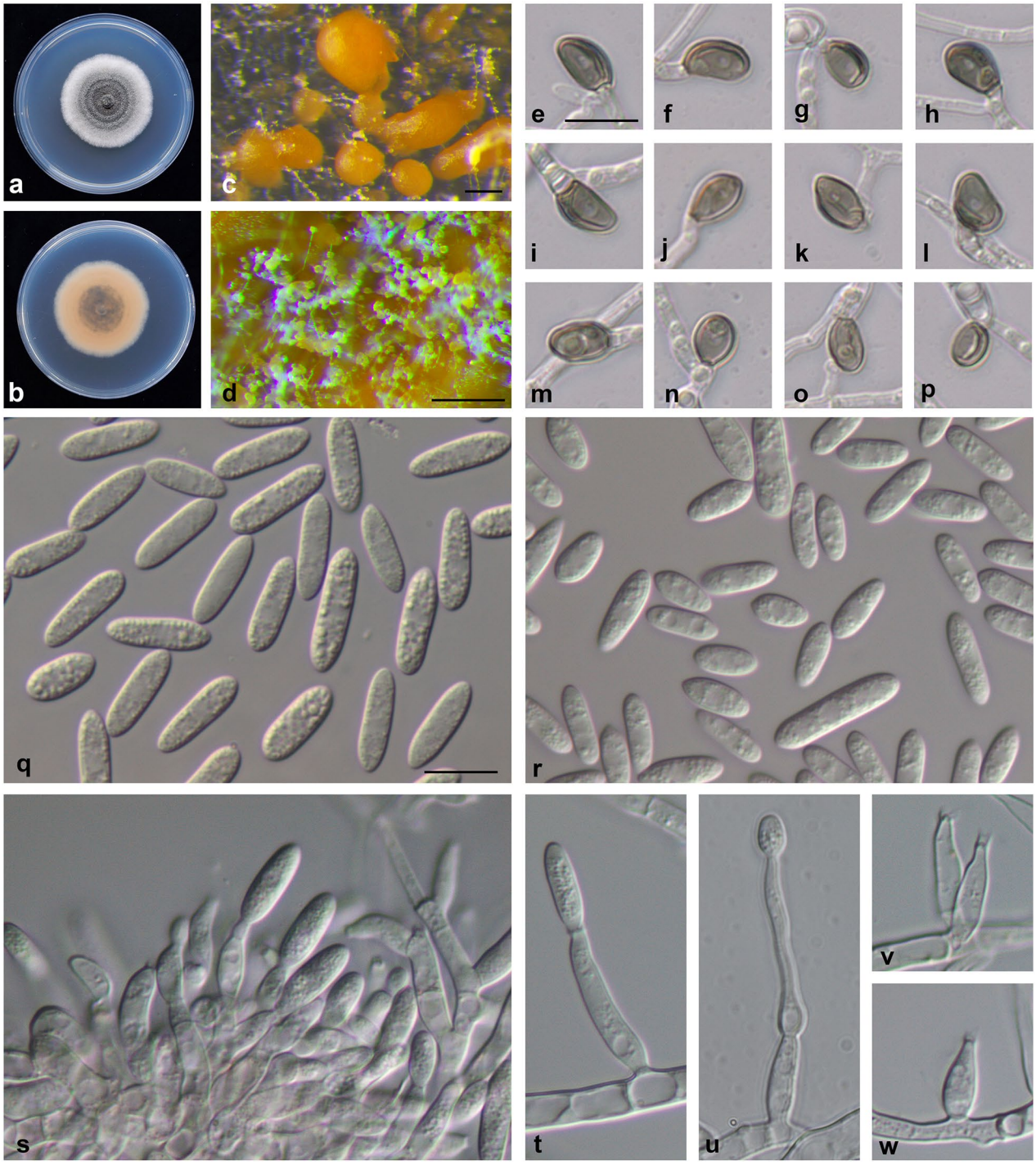

Figure 4. *Colletotrichum miaoliense* sp. nov. ML1040. (**a**) Upper side of colony; (**b**) reverse side of colony; (**c**, **d**) conidiomata; (**e**–**p**) appressorium (induced in dH_2O on a microscope slide); (**q**) conidia from conidiomata; (**r**) conidia from aerial mycelium; (**s**–**w**) conidiophores. (**a**, **b**) on potato dextrose agar (PDA); (**c**, **d**) and (**q**–**w**) on 1/4-strength PDA. Scale bars: (**c**, **d**) = 0.2 mm, (**e**) = 10 µm, applies to (**f**–**p**, **q**) = 10 µm, applies to (**r**–**w**).

$1 \times 5 \pm 0.6$ µm) (Supplementary Table S2), and the shape of appressoria (*C. miaoliense* and *C. simmondsii* appressoria are elliptical to clavate, whereas the appressoria of *C. nymphaeae* are clavate or irregular in outline, entire, and have an undulate to lobate margin[12]). Compared with *C. simmondsii*, the conidia of *C. miaoliense* are longer (mean length 14.2 µm versus 8.1 µm). In addition, the conidia of *C. simmondsii* are cylindrical with one end round and one end acute or both ends acute. Although the appressoria of *C. miaoliense* and *C. simmondsii* are similar in shape and L/W ratio, the appressoria of *C. simmondsii* are larger (Supplementary Table S2).

Effect of temperature on mycelial growth. A representative isolate selected from each of five *Colletotrichum* species was grown on PDA at 18 °C to 32 °C. The maximum growth rate of *C. siamense* ML133 was

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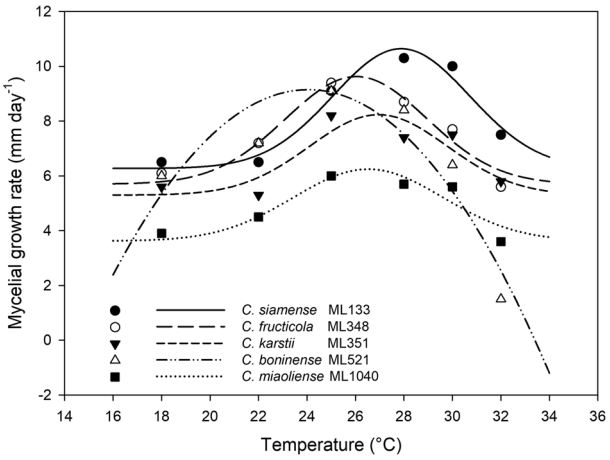

Figure 5. Mycelial growth rates of *Colletotrichum* spp. at different temperatures. Different symbols represent the mean growth rates of different species at the tested temperatures. Gaussian process regression was used to estimate the optimum temperature for mycelial growth.

estimated at 27.9 °C, whereas the maximum growth rates of *C. fructicola* ML348, *C. karstii* ML351, *C. boninense* ML521 and *C. miaoliense* ML1040 were at 26.0 °C, 26.9 °C, 24.0 °C, and 26.5 °C, respectively (Fig. 5 and Supplementary Table S3). The growth rate of *C. boninense* ML521 drastically decreased at 32 °C (Fig. 5). *C. miaoliense* ML1040 exhibited the slowest growth rate at all tested temperature regimes except 32 °C (Fig. 5). The ranking of species by growth rates at higher temperatures (28 °C, 30 °C and 32 °C) is as follows: *C. siamense* ML133 > *C. fructicola* ML348 > *C. karstii* ML351 and *C. boninense* ML521 (28 °C and 30 °C) > *C. miaoliense* ML1040 > *C. boninense* ML521 (32 °C) (Fig. 5 and Supplementary Table S3).

The growth rates of *C. siamense* ML133 were also compared with those of another two representative isolates selected from *C. siamense*, *C. fructicola*, *C. karstii*, and *C. miaoliense* (Supplementary Table S4). The ranking of isolates by mycelial growth rates at both 25 °C and 30 °C on PDA was as follows: *C. siamense* ML133 and ML540 > *C. siamense* ML612 and *C. fructicola* ML368 > *C. fructicola* ML356 and *C. karstii* ML1792 > *C. karstii* ML442 > *C. miaoliense* ML1042 and ML1794.

Pathogenicity assay. Pathogenicity was tested using Koch's postulates for *C. siamense* ML133, *C. fructicola* ML348, *C. karstii* ML351, *C. boninense* ML521, and *C. miaoliense* ML1040. These isolates all caused leaf and/or crown necrosis in strawberry seedlings (Fig. S6). *C. siamense* ML133 caused the most severe symptoms with 100% disease incidence. The disease incidences for *C. fructicola* ML348, *C. karstii* ML351, *C. boninense* ML521, and *C. miaoliense* ML1040 were only 30%, 30%, 30% and 50%, respectively. Notably, after spray inoculation of the seedlings with *C. karstii* ML351, leaf spots scarcely occurred, and no leaf lesions were observed for *C. boninense* ML521. Even though there were few visible symptoms, *C. boninense* ML521 could be re-isolated from surface-sterilized inoculated leaves.

Virulence of the five selected isolates was subsequently assayed using wounded and non-wounded detached leaves at 25 °C and 30 °C (Fig. 6). For all five isolates, inoculation of wounded leaves resulted in typical anthracnose lesions, which were first observed at 2–4 days post inoculation (dpi). *C. siamense* ML133 caused the largest brown necrotic lesions, sometimes with chlorotic or reddish margins (Fig. 6a). The necrotic lesions caused by *C. fructicola* ML348, *C. karstii* ML351, *C. boninense* ML521, and *C. miaoliense* ML1040 were significantly smaller (Fig. 6b; *C. fructicola* ML348 was slightly more virulent than *C. karstii* ML351, *C. boninense* ML521, and *C. miaoliense* ML1040). At 7 dpi, *C. siamense* ML133 caused significantly larger lesions at 30 °C (1.26 cm in diameter) than 25 °C (0.65 cm in diameter), whereas the sizes of lesions caused by other *Colletotrichum* species were similar (0.07–0.35 cm in diameter) at different temperatures (Fig. 6b).

In regard to inoculations of unwounded leaves, necrotic lesions caused by *C. siamense* ML133 and *C. fructicola* ML348 first appeared at 4–7 dpi, but no lesions occurred in the plants inoculated with the other three *Colletotrichum* spp. isolates (Fig. 6a). Inoculation of unwounded leaves with *C. siamense* ML133 resulted in larger lesions than inoculation with the other *Colletotrichum* spp., and the lesion sizes at 14 dpi were significantly larger at 30 °C (1.35 cm in diameter) than at 25 °C (0.35 cm in diameter) (Fig. 6c).

Inoculations of wounded leaves were conducted at 30 °C for *C. siamense* ML133, *C. boninense* ML521, and two additional representative isolates of *C. siamense*, *C. fructicola*, *C. karstii*, and *C. miaoliense* (Fig. S7). The results showed that *C. siamense* (ML133, ML540, ML612) and *C. fructicola* ML356 caused significantly larger lesions (1.21–1.74 cm in diameter at 7 dpi; 3.06–3.47 cm in diameter at 14 dpi) than *C. fructicola* ML368, *C. karstii* (ML442, ML1792), and *C. miaoliense* (ML1042, ML1794) (0.42–0.76 cm in diameter at 7 dpi; 1.06–2.15 cm in diameter at 14 dpi).

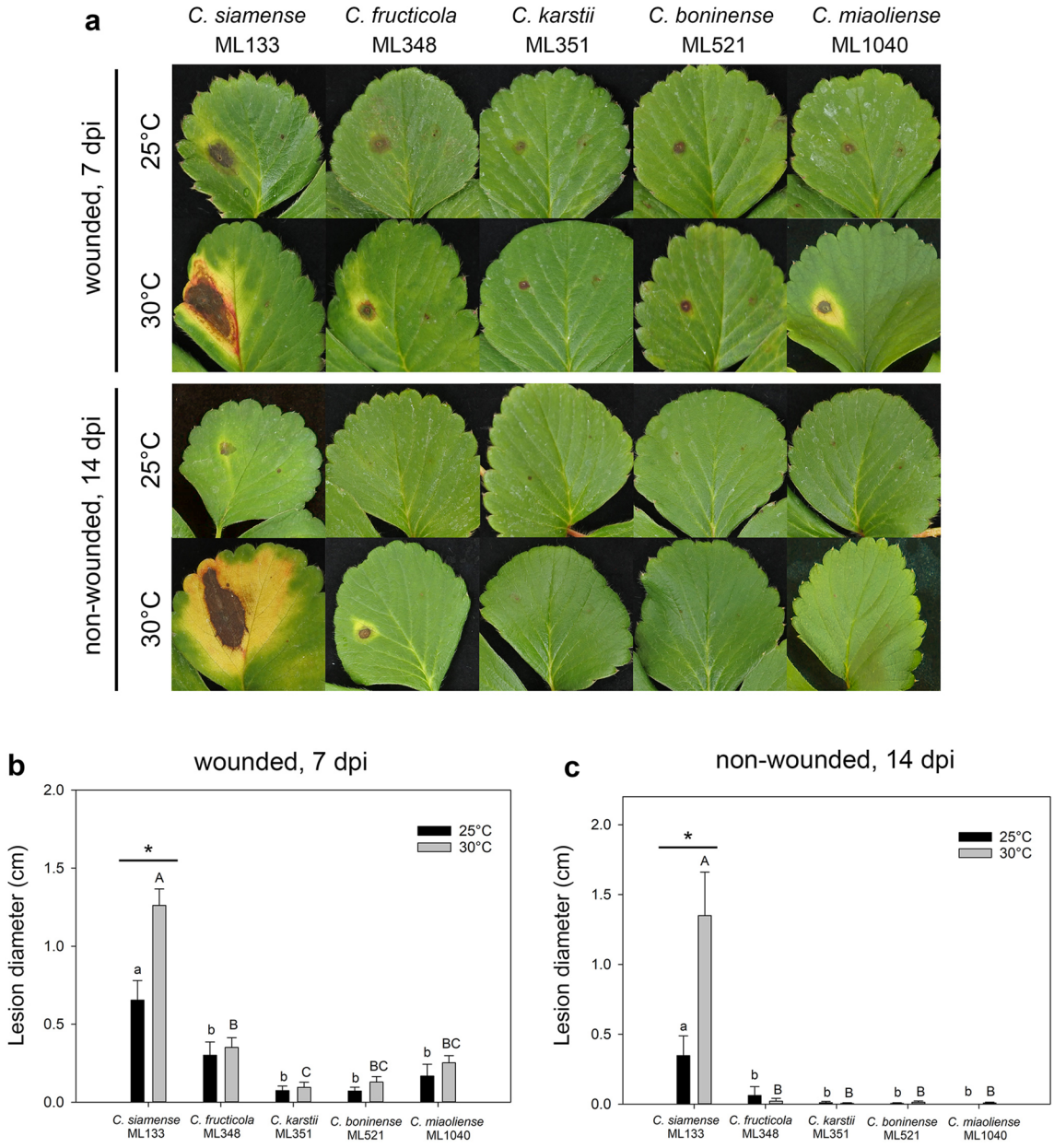

Figure 6. Inoculation of *Colletotrichum* spp. on detached wounded and non-wounded strawberry leaves at different temperatures. (**a**) Lesions at 7 days post inoculation (dpi) on wounded leaves or at 14 dpi on non-wounded leaves incubated at 25 °C or 30 °C. The left side of each leaflet was inoculated with 10 µl spore suspension (10^6 spores/ml) and the right side with water (control). (**b**–**c**) Lesion sizes resulting from inoculation of wounded and non-wounded leaves. The results from the same temperature were analyzed together. Data (mean ± standard error) with different letters are significantly different based on Tukey's range test at $P < 0.05$ (n = 12). For each species, the difference between 25 °C and 30 °C was analyzed by Student's *t* test (*denotes $P < 0.05$).

Discussion

Over the past decade, our knowledge of fungi and their relationships with plant hosts has seen an exponential growth due to the progress in bioinformatics and molecular phylogenetics. Cryptic taxa identification is progressing rapidly and groups of fungi, including important plant pathogens, are now mainly classified using molecular data-based phylogenetic inference. For instance, the *C. acutatum*, *C. boninense*, and *C. gloeosporioides* species complexes now each contain over 20 species[12,13,21]. Several *Colletotrichum* spp. with the capacity to cause strawberry anthracnose in temperate regions have been reported[12–15,17,22–30]; however, knowledge of the composition of the pathogen populations in tropical and subtropical regions is limited. Through morphological characterization, phylogenetic analyses involving five to seven loci (ITS, *GAPDH*, *CHS-1*, *ACT*, *TUB2*, *CAL*, and ApMAT), and inoculation tests on strawberry seedlings and detached leaves, the present study revealed that five *Colletotrichum* species cause strawberry anthracnose in Taiwan. In addition to the known strawberry anthracnose

pathogens *C. boninense*[15], *C. fructicola*[25,31], and *C. siamense*[13,16,18], one new species, *C. miaoliense*, and one species not previously known to infect strawberry, *C. karstii*, were identified. *C. karstii* was previously isolated from a wide range of plants such as anthurium, apple, citrus, and chili pepper[21,32,33], but not from strawberry. In this study, *C. siamense*, *C. fructicola*, and *C miaoliense* were isolated from different tissues, and all five *Colletotrichum* species were proved to be pathogenic to strawberry leaves and crowns (Fig. S6). The lack of tissue specificity is in agreement with previous observations of *C. acutatum* in strawberry[2,34].

The predominance of *C. siamense* (75%) and *C. fructicola* (11%) in the strawberry anthracnose pathogen population in Taiwan can be attributed to their higher levels of pathogenicity and aggressiveness. While all five *Colletotrichum* spp. were pathogenic to wounded leaves, only *C. siamense* and *C. fructicola* were able to cause lesions on non-wounded leaves. In addition, *C. siamense* (ML133, ML540, ML612) and *C. fructicola* (ML356) caused significantly larger lesions at 25 °C and 30 °C (Fig. 6 and Fig. S7). A similar phenomenon was observed for *Colletotrichum* spp. causing strawberry anthracnose in Zhejiang, China (latitude ~ 30°N)[18]: *C. fructicola* (53% of the isolates) and *C. siamense* (23%) dominated the population, and *C. fructicola* exhibited the highest level of pathogenicity (only 25 °C was tested)[18]. Although *C. boninense*, *C. karstii*, and *C. miaoliense* were much less virulent, wounds caused by natural agencies (wind, rain, insects and animals) as well as human activities (e.g., trimming old leaves, which is a common agricultural practice employed by most strawberry farmers in Taiwan) would provide potential infection sites allowing these pathogens to bypass the first line of defense (e.g., the cuticle)[35–37] in strawberry.

Among the five *Colletotrichum* species identified in this study, *C. siamense* exhibited greater mycelial growth rates on PDA, especially at higher temperatures. The fitness advantage of *C. siamense* in warm temperature weather may explain its current geographical distribution. In the literature, *C. siamense* was most reported in tropical and subtropical regions[13,16,38], whereas *C. boninense*, *C. fructicola*, and *C. karstii* were reported in regions across a wide range of latitudes[13,20,21,33,39–41]. Temperature is among the key environmental factors affecting a pathogen's survival[42]. A recent study based on published observations of 612 crop pests and pathogens from 1960 onwards revealed significant positive latitudinal shifts of many important pests and pathogens under climate change[43]. More research on the genetic and biological characteristics of different *Colletotrichum* species from diverse geographical areas will be needed to understand the emergence and spread of anthracnose diseases. With rising global temperatures, it will be particularly important to monitor the expansion of the heat-adapted *C. siamense* toward high latitudes.

C. boninense, *C. fructicola*, *C. siamense*, and *C. karstii* have been isolated from diverse plants other than strawberry in different countries/regions[13,21]. In Taiwan, *C. fructicola* has been reported as an anthracnose pathogen in mango, wax apple, and chili[44,45], *C. siamense* in lychee, star fruit, and mango[46,47], *C. karstii* in passion fruit[48], and *C. boninense* in pitaya[49]. Previous studies have demonstrated that *Colletotrichum* spp. from strawberry are pathogenic to other crops and even weeds. For example, *C. acutatum* could not only infect pepper, eggplant, tomato, and bean but also latently colonize weeds such as *Vicia* spp. and *Conyza* spp.[50]. In one study, *C. fructicola* was frequently isolated from leaves of *Amaranthus blitum*, and artificial inoculation of *C. fructicola* caused brown leaf spots on *A. blitum*[31]. To determine whether weed control is necessary to minimize the primary infection in the field, it is worth investigating whether the five *Colletotrichum* spp. we identified could colonize the weeds commonly present in and nearby strawberry fields in Taiwan. More sampling and artificial inoculation assays will be required to understand the host range of the new species *C. miaoliense*.

Anthracnose is a key limiting factor for strawberry production in Taiwan and many other areas. Outbreaks of anthracnose in strawberry nurseries and fields have caused yield losses of up to 50–80%[2,18,51,52]. This study demonstrated the diversity of pathogenic *Colletotrichum* species associated with strawberry in Taiwan. The findings offer precise information about pathogen identity, which is valuable for screening of resistant varieties and development of effective disease management strategies. Regardless of whether it was inoculated on wounded or non-wounded leaves, the predominant pathogen *C. siamense* caused larger lesions at 30 °C than 25 °C, which is meaningful in subtropical Taiwan and areas with a similar phenology. Because no significant difference was observed between the mycelial growth rates of *C. siamense* at 25 °C, 28 °C, and 30 °C, higher disease severity at 30 °C could be due to reduced resistance of strawberry against anthracnose at higher temperatures[53,54]. In Taiwan, the susceptible cultivar 'Taoyuan No. 1' has been widely cultivated for over 30 years. Development of temperature-independent resistant cultivars will be particularly important for strawberry breeding programs in Taiwan and other tropical and subtropical regions. Future work will focus on monitoring pathogen population changes, investigating the fungicide sensitivity levels of different *Colletotrichum* species, and developing molecular detection methods to aid the production of strawberry seedlings without latent infection of major *Colletotrichum* species.

Methods

Sample collection and pathogen isolation. From 2010 to 2018, different strawberry tissues (including the leaf, stolon, fruit, root and crown) showing anthracnose disease symptoms were collected from 24 farms and nurseries located in Miaoli, Hsinchu, Nantou, and Chiayi Counties in Taiwan. From 2009 to 2018, the strawberry-cultivated areas in Miaoli, Hsinchu, Nantou, and Chiayi accounted for approximately 89.6%, 2.8%, 2.6%, and 0.2% of the total strawberry-cultivated area in Taiwan, respectively. Pure cultures of all fungal isolates were obtained by the single hyphal tip isolation method. Approximately 2 × 2 mm fragments bordering healthy and necrotic zones in diseased tissues were surface-sterilized with 0.5–1% sodium hypochlorite, rinsed with sterile deionized water three times, then placed onto 1.5% water agar. After 2–3 days of incubation at 25 °C, single hyphal tips were transferred to potato dextrose agar (PDA, BD Difco) and cultured for further use. A total of 52 *Colletotrichum* spp. isolates were used in this study (Table 1): 26 (50%) isolated from crowns, 11 (21.2%) from leaves, 5 (9.6%) from fruits, 5 (9.6%) from roots, and 5 (9.6%) from stolons (Table 1). Type specimens in this

study were deposited in the herbarium of the Department of Plant Pathology and Microbiology, National Taiwan University (NTUH). Ex-type living cultures were deposited in the Culture Collection of the Department of Plant Pathology and Microbiology, National Taiwan University (NTUCC), Bioresource and Collection Research Center (BCRC), and Miaoli District Agricultural Research and Extension Station. Nomenclature and taxonomic information were deposited in MycoBank[55] (www.mycobank.org). *Colletotrichum* spp. were preserved as mycelium discs in ddH_2O at 4 °C for short-term storage and in 10% glycerol with 5% lactose at − 80 °C for long term storage. Before conducting experiments, each isolate was revived by culturing on PDA for 5–7 days at 25 °C under a 12-h/12-h photoperiod.

DNA extraction, PCR amplification, and sequencing. For each *Colletotrichum* spp. isolate, the mycelium was taken from a 7-day-old culture grown on PDA medium. The mycelium was frozen in liquid nitrogen and ground into a fine powder using a sterile mortar and pestle. Genomic DNA was extracted using the Plant Genomic DNA Extraction Miniprep System Kit (VIOGENE) according to the manufacturer's instructions. Seven genetic fragments, namely ITS, *GAPDH*, *CHS-1*, *ACT*, *TUB2*, *CAL*, and ApMAT, were amplified with the primers listed in Supplementary Table S5 using the Biometra Thermal Cycler (Biometra TRIO). Each PCR reaction contained 1 µl of genomic DNA (20–50 ng), 5 µl of 10X reaction buffer [with Tris–HCl (pH 9.0), PCR enhancers, $(NH_4)_2SO_4$, 20 mM $MgCl_2$], 2 µl of dNTPs (2.5 mM each), 1 µl of 10 µM forward primer, 1 µl of 10 µM reverse primer, 0.5 µl (2.5 U) of Prime Taq DNA Polymerase (GenetBio Inc.), and 39.5 µl of ddH_2O. The thermal cycling parameters were 1 cycle of 95 °C for 4 min and 30–35 cycles of 95 °C for 30 s, 52–62 °C for 30 s, and 72 °C for 60 s followed by a final extension step of 72 °C for 7 min. The optimal annealing temperatures for different genetic regions were: ITS: 58 °C, *GAPDH*: 52 °C, *CHS-1*: 58 °C, *ACT*: 58 °C, *TUB2*: 58 °C, *CAL*: 59 °C, and ApMAT: 62 °C. Amplicons were bidirectionally sequenced using the dideoxy termination method on the ABI 3730 DNA Analyzer (Tri-I Biotech, Taiwan). Raw sequence chromatograms were manually examined, and the sequences of each fragment were assembled in ApE v2.0.55 (A Plasmid Editor, M. Wayne Davis at the University of Utah, Salt Lake City, UT).

Multilocus phylogenetic analysis and species recognition. Newly generated sequences from each isolate were blasted against the GenBank nr database, and searches were restricted to type materials for initial determination of the closest matching species and species complex. Related gene sequences (ITS, *GAPDH*, *CHS-1*, *ACT*, *TUB2*, *CAL*, and ApMAT) of *Colletotrichum* spp. from recent publications were downloaded from GenBank[12,13,21]. For each gene, sequences from the isolates belonging to the same species complex were aligned using the MAFFT v7 online server (https://mafft.cbrc.jp/alignment/server/)[56]. The aligned sequences were manually edited using MEGA v10[57] to improve the alignment. The post-alignment sequences of multiple genes/loci were concatenated in a text editor.

BI, ML, and MP approaches for each individual locus and the concatenated sequences were used to identify closely related taxa. Best-fit models of nucleotide substitution were selected using the Akaike information criterion implemented in MrModeltest v.2.4[58] and run in PAUP v.4.0[59] (Supplementary Table S6). BI analyses were performed using MrBayes v.3.2.6[60]. Two independent analyses of four Markov Chain Monte Carlo (MCMC) chains (3 heated, 1 cold) were run from a random tree for 2×10^6 (for the *C. acutatum* species complex), 4×10^6 (for the *C. boninense* species complex), and 6×10^6 (for the *C. gloeosporioides* species complex) generations or until the average standard deviation of split frequencies was below 0.01. The analysis was sampled every 1,000 generations, and the first 25% of the generations were discarded as burn-in. The effective sample size and convergence were monitored with Tracer v1.7.1[61]. MP analyses were performed in PAUP v.4.0[59] using the heuristic search option with Tree Bisection Reconnection branch swapping and 100 random sequence addition. Maxtrees were set to 5,000 and bootstrap analysis was performed with 1,000 replicates. ML analyses were performed in RAxML v8.2.10[62] using the GTR-gamma substitution model with 1,000 bootstrap replicates. Phylogenetic trees were visualized in FigTree v1.4.3. The concatenated alignments and phylogenetic trees were deposited in TreeBASE (www.treebase.org) with the study ID 26665.

We applied the Genealogical Concordance Phylogenetic Species Recognition (GCPSR) method[63,64] for species delimitation of *Colletotrichum* taxa. A novel species was considered novel the clade was strongly supported as monophyletic by BI (posterior probability ≥ 0.95), ML (bootstrap ≥ 70%), and MP (bootstrap ≥ 70%) analyses in the multilocus phylogenetic tree and in the majority of individual gene trees.

Morphological characterization. Morphological characterization of one selected isolate from each *Colletotrichum* species was conducted following the procedures of Weir et al. (2012) and Damm et al. (2012)[12,13,21]. Cultures were grown on PDA and 1/4-strength PDA (1/4 PDA)[65] for 2–3 weeks at 25 °C and 30 °C under a 12-h/12-h photoperiod. (Our preliminary tests showed that compared with PDA, the low nutrient medium 1/4 PDA could stimulate sporulation without affecting the size and shape of conidia.) The experiment was performed in two independent trials, each consisting of two to three plates per isolate. Conidiomata were investigated using a dissecting microscope (Leica M125). Conidia, conidiophores, setae, asci, ascomata and appressoria were examined using a light microscope (Leica DM2500). To induce the formation of appressoria, 20–30 µl of conidial suspension (prepared using sterile dH_2O) was dropped onto a microscope slide, covered with a cover slip, then incubated in a moist chamber at 25 °C for 2–3 days[39]. The lengths and widths of 55 conidiogenous cells, 100 conidia and 30 appressoria were measured using ImageJ software[66].

Effect of temperature on mycelial growth. Mycelium-agar discs (6 mm in diameter) were cut with a sterilized cork borer from the advancing edge of 5- to 7-day-old *Colletotrichum* spp. colonies then placed (with the mycelium-side down) onto the center of a 90 mm petri dish containing 25 ml PDA. Colony diameters were

measured after 7 days of incubation at different temperatures under a 12-h/12-h photoperiod in a growth chamber (Firstek, GC-560H). The mycelial growth rate (mm/day) was calculated as "(the diameter of the colony—the diameter of the mycelium-agar disc) / 7". For one selected isolate from each *Colletotrichum* species, the mycelial growth rates at 18 °C, 22 °C, 25 °C, 28 °C, 30 °C, and 32 °C were determined in two to three independent trials, each consisting of three PDA plates per isolate per temperature. The optimum temperature for the mycelial growth rate was estimated based on the Gaussian process (4 parameter) for nonlinear regression in SigmaPlot 14 (Systat Software, San Jose, CA). Growth of an additional two representative isolates of each *Colletotrichum* species, selected from distinct subclades within the species based on the multilocus phylogenetic analysis, was measured at 25 °C and 30 °C in two independent trials, each consisting of four PDA plates per isolate per temperature.

Pathogenicity assay. The susceptible cultivar 'Taoyuan No. 1' was used for all inoculation tests in this study. The pathogenicity of *Colletotrichum* spp. (one isolate from each *Colletotrichum* species) was examined via Koch's postulates. Strawberry seedlings at the four- to five-leaf stage were inoculated by spraying a spore suspension on the leaves until runoff (10^6 spores/ml), and also applying 1 ml of spore suspension (10^6 spores/ml) on the crown region after removal of one to two old leaves. After inoculation, the seedlings were covered with plastic bags (>90% relative humidity) for 3 days at 30 °C and then incubated in a growth chamber for 11 days at 30 °C and 70% relative humidity under a 12-h/12-h photoperiod. The fungi were re-isolated from lesions of diseased tissues (as mentioned above using the single hypha tip isolation method), then identified based on morphological characteristics and ITS sequences as described above.

The virulence levels of different *Colletotrichum* spp. isolates were determined by inoculation of wounded and non-wounded detached strawberry leaves. Fully expanded healthy leaves were collected from strawberry seedlings at the four- to five-leaf stage. For inoculation of wounded leaves, each leaflet was punctured with a sterile syringe needle on the left and right sides of the midrib, and 10 µl of a spore suspension (1×10^6 spores/ml) was deposited on the left wound site, and sterile dH_2O was deposited on the right wound site as a control. Similarly, inoculations of non-wounded leaves were performed in the same way as mentioned above. After inoculation, the leaves were kept in a moist chamber (a plastic box with dH_2O at the bottom; the cut end of the petiole was submerged in the water) at 25 °C or 30 °C under a 12-h/12-h photoperiod. Lesion size was measured at 7 and 14 days post inoculation (dpi). Lesions smaller than 0.1 cm in diameter were considered unsuccessful infections. The same isolates for the "effect of temperature on mycelial growth" test were used for the pathogenicity assay. For one selected isolate from each *Colletotrichum* species, inoculations of wounded and non-wounded leaves were performed at 25 °C and 30 °C in three independent trials, each consisting of 12 leaflets (4 leaves from 4 seedlings) per isolate per treatment. An additional two representative isolates of each *Colletotrichum* species, selected from distinct subclades within the species based on the multilocus phylogenetic analysis, were used for wound inoculation at 30 °C. The experiment was performed in two independent trials, each consisting of 12 leaflets (4 leaves from 4 seedlings) per isolate per treatment.

Statistical analysis. Data were analyzed by analysis of variance (ANOVA) using the software SPSS v18. Tukey's range test or Student's *t*-test was used to test for significant differences among or between different treatments at a significance threshold of $P < 0.05$.

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Author contributions
P.C.C., H.Y.W., T.H.H., S.S.T. and C.L.C. designed the experiments, analyzed the data, and drafted the manuscript. P.C.C., H.Y.W., Y.W.W., and H.P.H. performed the experiments. P.C.C., H.Y.W. and H.A.A. conducted phylogenetic analysis. All authors read and approved the final manuscript.

Competing interests
The authors declare no competing interests.

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偵測潛伏感染期草莓炭疽病病原菌技術之研發

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摘　要

草莓為臺灣具高經濟價值之作物，栽培面積約為500公頃。近10年來炭疽病成為草莓頭號殺手，由於本病原菌能潛伏感染寄主，種植健康不帶菌的草莓苗，將可大幅降低本田期病害的發生，同時減少化學藥劑的使用。而為生產健康的草莓苗，準確、靈敏、快速又合乎成本效益的檢測技術便是其中最重要的關鍵，因此以現有的炭疽病菌基因體資料庫，搜尋適合的目標區域，並以臺灣草莓炭疽病菌的序列進行引子設計，開發巢式聚合酶連鎖反應技術。本技術可以偵測最主要的炭疽病菌*Colletotrichum. siamense*與*C. fructicola*，但不會偵測到其他草莓病原菌或土壤中常見的腐生菌，可偵測到低至100 fg之*C. siamense* DNA（約2個分生孢子），代表具有高度專一性及靈敏度。結合本場草莓炭疽病巢式PCR檢測技術，進行核酸探針修飾與快篩檢驗試紙製作。目前已開發完成之技術尚需配合本團隊正在設置之植物病害檢測工作站，才能達到田間快速檢測之效果，檢測工作站包含植物樣本核酸萃取、PCR及核酸檢測試紙判讀等部分，完整結合後將有助於田間病害檢測工作及病害之判斷。

關鍵詞: 巢式聚合酶連鎖反應、核酸檢驗試紙、健康種苗

前　言

草莓因外型漂亮討喜，具有獨特風味，且營養價值高，為全世界非常受歡迎的水果。臺灣草莓栽種面積約500~600公頃，每年產值超過18億元，平均每公頃產值超過300萬元，為一極具經濟價值之水果（108年度行政院農業委員會統計年報）。每年草莓大約於初秋（9月底至10月初）定植，約於11月底至12月初開始採收，一直至隔年3~4月結束。草莓主要藉由走蔓進行無性繁殖，臺灣草莓育苗的時間落在每年3~9月之間，這期間包括了5~6月的梅雨季節與7~9月的颱風季節，高溫高濕

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加上近年來氣候變遷所造成的強降雨現象，導致近年來草莓炭疽病的發生頻率居高不下（鐘及彭，2013）。臺灣每年對於草莓苗的需求高達2,750萬株，近年來平均因炭疽病造成植株死亡的比例高達20~30%，2016年更因病害，造成全臺嚴重缺苗，出現有錢也買不到苗的窘境，因此如何有效防治草莓炭疽病，實為一極待解決的重要課題（鐘及吳，2018）。

炭疽病(Anthracnose)是草莓最主要的病害之一，其病原菌屬於炭疽刺盤孢菌屬(*Colletotrichum*)的真菌，於臺灣，以*C. siamense*為主要的病原菌(Chung *et al*., 2019)。炭疽病菌可以感染草莓的各個部位，包括葉片、葉柄、走蔓、冠部、根與果實，於葉片出現黑色斑點，葉柄與走蔓出現黑褐色凹陷斑點，冠部若受感染地上部呈現萎凋狀，切開冠部組織可見紅褐色壞疽病徵。一般認為炭疽病菌是半生物營養性 (hemibiotrophic)，依它在不同寄主的不同組織部位而有生物營養性期（biotrophic，潛伏感染時期）或壞死性期（necrotrophic phase，植株顯現病徵最後枯萎）。有些炭疽病菌在部分作物造成之病徵具有組織專一性之特性 (tissue specific) (Peres *et al*., 2005)，以甜橙為例，花會遭炭疽病菌感染，並且產生壞死病徵，然而嫩葉受到感染卻不會有病徵。由於炭疽病菌可以藉由附著器 (appressoria) 於植物組織表面潛伏感染，並且可以產生二次分生孢子 (secondary conidia)，因此潛伏感染時仍舊可以產生分生孢子，造成田間感染源的增加與大量散播。Leandro等人的研究證實*C. acutatum*可潛伏感染於無病徵之草莓葉片，而這些二次分生孢子可能即為果實受炭疽病菌感染的主要來源，而又因為育苗區與本田區在地理位置上通常相距甚遠，因此推測草莓田之炭疽病感染來源可能是經由種苗帶入的(Leandro *et al*., 2001)。

經長期田間監測發現，炭疽病菌會潛伏於種苗，然後隨著種苗傳播至本田，因而偵測此病原菌潛伏感染情形，應有助於預防性大幅降低其散播。炭疽病潛伏感染之偵測，早期為使用除草劑(paraquat)殺死植物後有促進真菌的生長，再觀察潛伏感染情形，Dr. Mertely等人以冷凍法替代除草劑之使用，同樣可以使分生孢子盤較易產生(Mertely and Legard, 2004)，此方法是以-15℃至-20℃低溫冷凍殺死植物組織後，利用次氯酸鈉表面消毒，於生長箱培養5~7天後觀察分生孢子盤產生之情形。Dr. Ishikawa則將外觀無病徵之草莓葉片以酒精表面消毒後(simple diagnosis by ethanol immersion)，經10~14天可觀察潛伏感染之炭疽病的分生孢子堆產生情形(Ishikawa, 2004)。分子生物學發展後，最常用來做為炭疽病菌種間鑑定及偵測潛伏感染情形之方法為Polymerase Chain Reaction (PCR)，利用ribosomal DNA internal transcribed spacer 1 region

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(rDNA ITS1) (Martinez-Culebras *et al*., 2003; Parikka and Lemmetty, 2004; Sreenivasaprasad *et al*., 1996) 或*β*-tubulin gene (Talhinhas *et al*., 2005)之序列設計種間專一性之引子對。然而當目標DNA量非常低時（潛伏感染時期），一般PCR不見得適用，此時需以高靈敏度之技術（如real-time PCR等）才能偵測得到。Debode等人運用real-time PCR (qPCR) TaqMan 系統，以rDNA ITS1及*β*-tubulin 2 gene為偵測對象，發現以ITS引子對可以偵測50 fg genomic DNA、100 copies of target DNA或25個分生孢子。相較之下，*β*-tubulin僅達上述方法1 / 66之靈敏度，因此較不適合以偵測為目標之設計。qPCR可以有效偵測從田間採集之自然感染葉片及沒有病徵之葉片上之*C. acutatum*，證明此方法不僅侷限於研究，更可以應用於田間的檢測技術(Debode *et al*., 2009)。qPCR後來應用於偵測*C. acutatum*在草莓園之潛伏感染及擴散情形 (Debode *et al*., 2015)，篩檢草莓植株不同部位後發現*C. acutatum*似乎主要存在於走莖及老的葉柄上，田間試驗顯示從罹病葉片擴散之潛伏感染距離約為每7天向外擴散1.75 m。此外，利用qPCR偵測無病徵之葉片上的分生孢子數量，可以發現與採收期及採收後果腐之發生有明顯之相關性(Debode *et al*., 2015)。炭疽病菌可於受感染的組織產生大量分生孢子，並可藉由雨水或噴灌灑水系統將孢子飛濺傳播，在鄰近病株的草莓苗上，可以觀察到炭疽病典型的病徵出現，而病株附近看似健康的草莓苗，則大多受到炭疽病潛伏感染，此種植株，幾乎無法以肉眼與健康植株區別，因此，造成許多農民買進外表健康之草莓苗，但在種植一段時間之後，便因炭疽病發病而死亡，導致缺株與損失發生。因此本研究便嘗試利用分子技術來進行炭疽病菌的檢測，讓無法以肉眼分辨的潛伏感染也可以現形，未來更可用以篩選出不帶有炭疽病菌的健康草莓苗。

材料與方法

一、PCR引子設計目標區域搜尋與引子對設計

為提升檢測靈敏度，尋找巢式聚合酶連鎖反應引子對，其概念為將第一次的PCR產物作為第二次PCR循環的模版，以提高專一性及靈敏度。引子設計的目標區域設定在尋找於*C. gloeosporioides* species complex當中保守的區域，而在其他已解序的*Colletotrichum* spp.中為不保守的區域，且該區域的上下游序列亦是為保守的區域。為了得到這樣的片段作為設計引子的目標，首先於National Center for Biotechnology Information (NCBI) 的資料庫當中進行初次篩選，搜尋已經被解序的*Colletotrichum*屬之基因體序列，並以*C. gloeosporioides*作為目標，利用Basic Local Alignment Search Tool (BLAST)及Python程式語言，與其它已經解序的炭疽病菌基因體資料庫進

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行比對，找出在*C. gloeosporioides* species complex當中具有保守性，但是在其他炭疽病菌當中不具有保守性的區域片段，並同時設定序列篩選長度為500~2,500 bp。篩選出來的片段，再經過第二次篩選，於所有炭疽病菌屬的基因體中，以BLAST比對該片段的上下游區域，挑選出上下游片段在大部分*Colletotrichum* 屬的基因體當中呈現保守性，而中間目標片段則只有在*C. gloeosporioides* species complex當中具有保守性。第三次篩選，則挑選中間非保守區域長度在1,000~1,500 bp（以實際比對的數值為準），作為之後在*C. siamense* ML133當中用來解序的區域。該區域的PCR引子設計完成後，將以BLAST比對NCBI中的*Fusarium* spp.之基因體資料庫，以排除掉可能的非專一性引子。

二、菌株之基因體DNA純化

草莓炭疽病菌*C. siamense* ML133培養於馬鈴薯葡萄糖瓊脂(Potato dextrose agar, PDA) 培養基上，置於25°C恆溫生長箱，12小時光照/黑暗之條件下，約5~7天可長滿直徑9 cm的培養皿。*C. siamense* ML133的基因體DNA使用Plant Genomic DNA Extraction Miniprep System (VIOGENE) 進行分離與純化，步驟依廠商提供之說明書進行，簡述如下：以無菌刀片刮取PDA上之菌絲，於研缽倒入適量之液態氮後將菌絲研磨成粉狀，加入400 µl 之PX1 buffer與RNase A buffer混合均勻，於65°C之水浴槽中反應10分鐘，再加入130 µl之PX2 buffer翻轉混合後，置於冰上5分鐘，將上清液吸至Shearing Tube內，以13,200 rpm離心2分鐘，將收集管內之液體吸至新的1.5 ml微量離心管內並加入0.5倍體積之PX3 buffer與1倍體積之98%酒精，翻轉數次混合均勻後，倒入Mini Column內以10,000 rpm離心1分鐘，捨棄流出液體，再加入700 µl之WS buffer，以13,200 rpm離心30秒，重複一次，最後加入50 µl之65°C無菌水，靜置兩分鐘，以13,200 rpm離心2分鐘，即得到純化之基因體DNA，儲存於 -20°C。純化之DNA皆以分光光度計進行濃度與純度檢驗，260 / 280需接近1.8，濃度大於10 ng /µL。

三、巢式聚合酶鏈鎖反應 (nested-PCR) 引子對之專一性與靈敏度測定

在專一性測試方面，除了以*C. siamense* ML133之DNA當作正對照外，也以不同的炭疽病菌分離株如*C. fructicola* 與*C. karstii*及不同*Fusarium* spp.或木黴菌(*Trichoderma* sp.)分離株之DNA進行PCR反應，以檢視有無非專一性的反應發生，並以ITS1/ITS4引子作為PCR反應的正對照組。在靈敏度測試方面，*C. siamense* ML133的DNA經由序列稀釋後(1 ng、100 pg、10 pg、1 pg、100 fg、10 fg per µl)，作為模板進行PCR反應，以便確認偵測的極限濃度。

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四、核酸檢測試劑之開發

以巢式聚合酶連鎖反應(Nested PCR)引子對為基礎，將其進行修飾，並在必要時進行序列的設計與修改，以避免因為修飾而造成之二級結構產生。修飾後之分子探針將作為草莓炭疽病巢式PCR反應的引子，用來進行巢式PCR反應，反應後直接將檢測試紙放入反應管內，藉由ICT (Immuno Chromatographic Testing)之原理，反應產物會由試紙底部往上移動，並與試紙內之SRGold (Specific Receptor-labeled Gold particle)、AHA (anti-hapten antibody)與IL (immobilized ligand)進行具專一性之反應，若增幅出目標產物，則出現2個條帶，若無則出現一個條帶。核酸檢測試之紙測試部分，DNA標準樣品稀釋倍率分別為20倍與10倍。樣本經核酸試紙檢驗結果與電泳法呈現之結果，進行資料比對分析。

五、田區樣本檢測

產銷班班員及育苗業者於2019年送檢母株樣本，藉由新開發的巢式PCR引子對進行檢測。樣本為每棵植株的最老葉（最下位葉），包含完整的葉片、葉柄與葉鞘，送檢測的葉片先將葉鞘組織，以Plant Genomic DNA Extraction Miniprep System (VIOGENE)進行DNA的純化（如上述），再以PCR進行偵測。進行PCR時，負對照組 (negative control, NC)以ddH_2O取代樣本DNA，以確認操作過程無人為汙染，而為確保抽取之DNA質量，以草莓housekeeping gene (*ACTIN*)做為正對照。

結　果

一、目標區域搜尋與引子對設計

藉由在NCBI的資料庫內，以*C. gloeosporioides*作為目標，搜尋在*C. gloeosporioides* species complex當中具保守性，但在其他種炭疽病菌不具保守性的區域片段，作為理想的引子設計區域，初次篩選搜尋出大約八千個片段符合初步篩選的條件，而後利用BLAST進行序列比對與第二次篩選，篩選出其上下游延伸的片段在大部分*Colletotrichum*屬的基因體當中呈現保守性，而中間目標片段則只有在*C. gloeosporioides* species complex當中具保守性，結果顯示大約有100個片段符合第二次篩選的條件，第三次篩選限制長度在1,000~1,500 bp，結果則約有20個序列區域符合條件，並挑選其中一個適合用於設計引子序列區域，於*C. siamense* ML133中進行解序，解序的結果得到一個約1,100 bp的片段，GC content約52.3%，並以此片段序列設計出多組PCR引子對組合，並藉由BLAST比對*Fusarium* spp.之基因體資料庫，挑選出一組出現非專一性反應可能性最低之引子組合，作為偵測炭疽病菌的PCR引子對。

二、引子對之專一性與靈敏度測定

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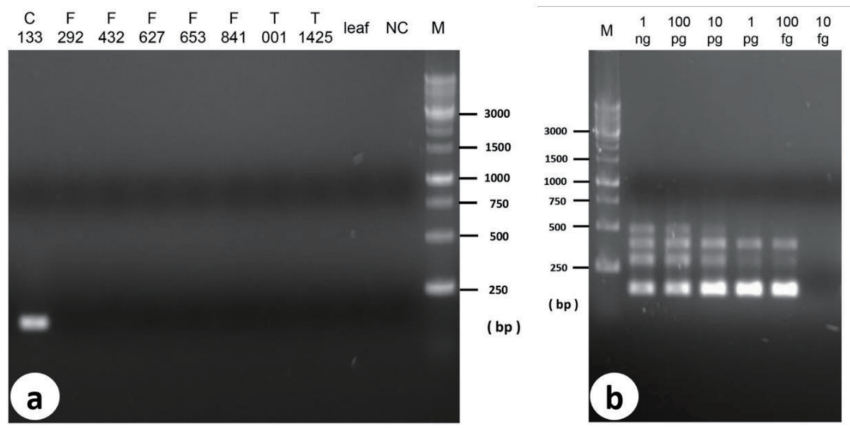
針對設計之巢式PCR引子對進行專一性分析，分別以*C. siamense* ML133的DNA（為正對照組），比較對於其他*C. siamense*分離株或是不同種類炭疽病菌菌株，如*C. fructicola*與*C. karstii*的專一性，結果顯示Nested-PCR引子可以偵測到*C. siamense*與*C. fructicola*但不會偵測到*C. karstii*，其中*C. siamense*與*C. fructicola*屬於*C. gloeosporioides species complex*而*C. karstii*屬於*C. boninense* species complex。再者，為求實際應用於田間樣本檢測時，不會偵測到其他草莓病原菌或土壤中常見的腐生菌，因此亦以於草莓或土壤中分離出來之*Fusarium* spp.或木黴菌(*Trichoderma* sp.)進行專一性測試，結果顯示沒有非專一性反應，顯示有較佳之專一性（圖一）。靈敏度測定的實驗方面，將*C. siamense* ML133的DNA序列稀釋後進行測試，結果顯示可以偵測低至100 fg之*C. siamense* ML133 DNA（約2個病原菌細胞），代表具有高靈敏度（圖一）。



圖一、巢式聚合酶鏈鎖反應之專一性及靈敏度測試。(a)DNA樣本包含*Colletotrichum siamense* ML133 (C133)、*Fusarium* spp. (F292、F432、F627、F653、F841)、*Trichoderma* sp. (T001、T1425)、草莓葉片(leaf)及負對照(NC)。M: 1kb DNA分子標記。(b) 測試樣本為序列稀釋之*Colletotrichum siamense* ML-133的DNA: 1 ng / μL、100 pg / μL、10 pg / μL、1 pg / μL、100 fg / μL、10 fg / μL。M: 1 kb DNA分子標記。

Fig. 1. Specificity and sensitivity test of the nested-PCR assay. (a) Samples included the DNA of *Colletotrichum siamense* ML133 (C133), *Fusarium* spp. (F292, F432, F627, F653、F841), *Trichoderma* sp. (T001, T1425), strawberry leaf (leaf), and negative control (NC). M: 1 kb DNA ladder. (b) Samples included serial dilutei-ons of C. *siamense* ML133 DNA: 1 ng / μL, 100 pg / μL, 10 pg / μL, 1 pg / μL, 100 fg / μL, 10 fg / μL. M: 1 kb DNA ladder.

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三、核酸檢測試劑之開發

核酸檢測試紙測試部分，利用不同PCR黏合溫度進行PCR反應，皆不會產生非專一性片段（圖二）。使用檢測試紙時，DNA標準樣品稀釋倍率分別為20倍與10倍。在10倍稀釋率下，檢驗試紙反應速度快，幾乎2分鐘內即可呈現反應結果，是以建議稀釋倍率為10。完成150個Nested PCR有專一性訊號的樣本及50個Nested PCR呈陰性反應的樣本進行核酸放大，將此產物進行檢測試紙的試驗，檢測結果與電泳結果相符。

圖 二 、核酸檢驗試劑測試結果。試劑1為負對照組，試劑2至9為分別在不同PCR黏合溫度下 (53.6℃、54.2℃、55.4℃、56.8℃、58.2℃、59.7℃、61.6℃、62.6℃)產生之產物反應結果。

Fig. 2. The results of nucleic acid strip tests. Strip 1 was negative control, Strip 2-9 were the results of the PCR products under different anneal temperature (53.6℃, 54.2℃, 55.4℃, 56.8℃, 58.2℃, 59.7℃, 61.6℃, 62.6℃).

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四、田區樣本檢測

田間檢測之樣本DNA，在利用專一性引子經過巢式PCR增幅後，有呈現150 bp大小條帶之樣本即視為有潛伏感染之植株，沒有出現該大小條帶之樣本即視為健康（沒有潛伏感染）之植株，所有的植株樣本皆需比對ACTIN基因增幅之結果，以作為PCR反應之正對照組（圖三）。在2019年受檢測之樣本當中，總共檢測377棵植株，分別由11個田區或單位送樣，其中74株經由巢式PCR檢測出帶有炭疽病菌，檢出率約為兩成(19.6%)。

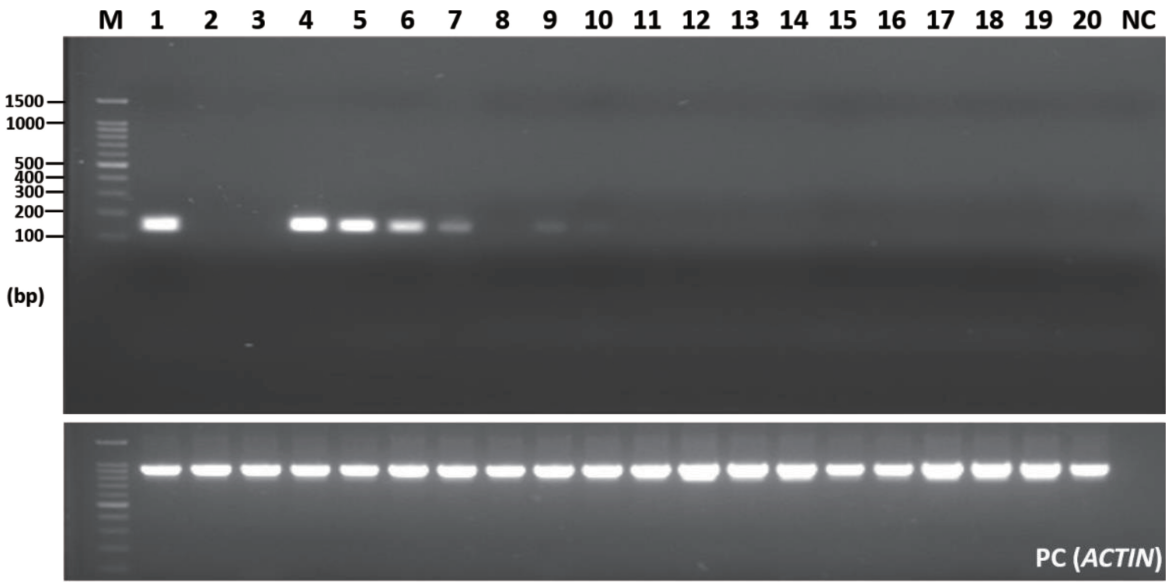

圖 三 、巢式聚合酶連鎖反應測試結果。編號1至20為送檢草莓母株編號，萃取葉柄DNA後進行巢式聚合酶鏈鎖反應，出現專一性片段之編號，該母株汰除不進行後續留苗。負對照 (NC)。M：100 bp DNA分子標記。正對照 (PC, *ACTIN*)。

Fig. 3. Results of nested-PCR tests. Numbers 1 to 20 are the number of the strawberry mother plants for inspection. After extracting petiole DNA, nested-PCR was performed, and the mother plants would be eliminated once the specific fragment appeared. Negative control (NC). M: 100 bp DNA marker. Positive control (PC, *ACTIN*)。

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討　論

炭疽病為草莓重要病害之一，因其具有潛伏感染寄主之特性，種植健康不帶炭疽病菌的草莓苗將可大幅降低本田期炭疽病的發生，並且同時減少化學藥劑的使用，而若要生產健康的草莓苗，準確、靈敏、快速又合乎成本效益的檢測技術便是其中最重要的關鍵，因此本研究利用現有的炭疽病菌基因體資料庫，搜尋適合的目標區域，並以臺灣草莓炭疽病菌之序列進行引子設計，得到一組巢式PCR引子對，適合用於檢測臺灣草莓炭疽病菌。

巢式PCR檢測技術可以偵測最主要的炭疽病菌*C. siamense*與*C. fructicola*，但不會檢測出別種炭疽病菌，且於土壤中常見的*Fusarium* spp.及木黴菌亦不會被檢測出來，代表該檢測技術具有一定的專一性，能準確分辨草莓炭疽病菌與其他不同的真菌。本檢測技術除具有良好專一性外，亦具有高度靈敏度，只要有100 fg的炭疽病菌DNA（約2個分生孢子）即可被偵測，適合檢測在草莓上潛伏感染的少量炭疽病菌。圖一(b)出現非專一性的多條帶現象，推測可能與兩次PCR的引子對含量比例相關，將視著調整引子對的比例以改善此現象。巢式PCR反應，相較於一般的PCR反應，其靈敏度與專一性皆較高，到達與qPCR相當的靈敏度，且每次反應的成本較qPCR低，唯有其反應所需要的時間稍長，但相較於需要10~14天才能完成的傳統酒精檢測法，整個檢測流程可在1~2天之內完成，已經符合快速檢測所需。

從田間檢測樣本的結果顯示，外觀看似健康的植株裡面，約有兩成至三成帶有炭疽病菌，顯示草莓炭疽病的潛伏感染比例不低，這些受檢驗的植株大多是草莓苗，未來將種植至本田，發病後成為田間草莓缺株的主要原因，並且炭疽病藉由草莓苗帶入本田區，因為病原菌已經成功感染進入植物體內，將使本田區的防治工作不易看到成效，並造成化學藥劑過量使用，且罹病株將再次成為感染其它健康植株的來源，於氣候適合發病的條件下，極易造成炭疽病大流行。未來，將持續監測經檢出潛伏感染的母株，實際在田間的發病率，以確認相關性，並藉由栽培管理降低發病率。

此外，核酸檢測試劑係依循免疫層析技術，運用毛細管層流、奈米金粒子、與共軛物(conjugate)親和作用，達到快篩檢驗試紙表現。核酸檢測試劑開發依據亞洲基因生技公司已開發技術，結合本場草莓炭疽病巢式PCR檢測技術，進行核酸探針修飾與快篩檢驗試紙製作。針對檢驗穩定性探討中，檢視試紙製作製程、反應試劑與條件，發現下列因素為關鍵因素：鋁箔封存避免潮濕；膠體金來源穩定性；提升膠體金濃度，可增加穩定性。目前已開發完成之技術尚需配合本團隊正在研發之植物病害檢測工作站，才能達到田間快速檢測之效果

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，檢測工作站包含植物樣本核酸萃取、PCR及核酸檢測試紙判讀等部分，完整結合後將有助於田間病害檢測工作及病害之判斷。

本研究所開發的檢測技術，可望應用於生產無炭疽病菌的健康草莓苗，國內目前正在積極推動草莓種苗三級繁殖制度，本檢測技術可以實際應用或支援種苗病害驗證作業（鐘，2017）。而依據草莓產業的狀態，可能的供苗型態包含農民自行育苗或專業育苗場產苗等，本技術將可針對不同供苗方式而調整抽檢比例。除了炭疽病，隨著草莓品種的更替，面臨不同病害的威脅，未來將持續開發其他草莓病害檢測技術，以提高國內健康草莓苗的品質，減少病害發生與降低化學藥劑使用。

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Development of technology for detecting pathogens of strawberry anthracnose during latent infection

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ABSTRACT

Strawberry (*Fragaria* × *ananassa* Duch.) is a high economic-value crop with an average annual cultivated area of 500 ha in Taiwan. Over the past decade, anthracnose has become the most destructive disease because of the latent infectious characteristics of the pathogens. It would reduce not only the occurrence of anthracnose rot but also the usage of fungicides in the fields by healthy and pathogen-free strawberry runner plants. To produce healthy runner plants, it is important to diagnose anthracnose at the stage of latent infection. We conducted comparative genomics analysis of known *Colletotrichum* spp. genomes to search for ideal regions suitable for design of specific primers, and developed a nested-PCR assay. The predominant pathogens of strawberry anthracnose in Taiwan include *C. siamense* and *C. fructicola* could be specifically detected, but not other pathogens or saprophytes associated with strawberry plants. This new detection technique with high sensitivity could detect as low as 100 fg genomic DNA of *C. siamense*, which corresponds to 2 cells of *C. siamense*. The detection techniques, however combined with the nested-PCR assay, nucleic acid probe modification and nucleic acid strip, but also needs to cooperate with the plant disease detection workstation being developed by our team to achieve the effect of rapid field detection. The detection workstation includes nucleic acid extraction of plant sample, PCR assay, and interpretation of nucleic acid test strip. The complete combination will held disease detection applied in the field and disease analysis.

Key words: nested-PCR, nucleic acid strip, healthy runner plants

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臺灣草莓炭疽病菌族群分析與檢測技術開發

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摘　要

草莓為臺灣具高經濟價值之作物，栽培面積為 500 公頃左右。近 10 年來炭疽病成為草莓頭號殺手，自 2010~2018 年間調查草莓主要產區，高達 50% 以上的病株呈現典型冠腐病徵，經過型態及多基因分析 52 株菌，確認造成臺灣草莓炭疽病之病原菌包含 *Colletotrichum siamense*、*C. fructicola* (*C. gloeosporioides* species complex) 、*C. karstii*、*C. boninense* (*C. boninense* species complex) 及一種歸類於 *C. acutatum* species complex 的新種 *Colletotrichum miaoliense* sp. nov.。其中又以 *C. siamense* 為最主要且生長速度快、病原性強之病原菌，瞭解炭疽病菌種類將有助於後續防治策略之開發。由於炭疽病菌能潛伏感染寄主，種植健康不帶菌的草莓苗，將可大幅降低本田期病害的發生，同時減少化學藥劑的使用。而為生產健康的草莓苗，準確、靈敏、快速又合乎成本效益的檢測技術便是其中最重要的關鍵，因此以現有的炭疽病菌基因體資料庫，搜尋適合的目標區域，並以臺灣草莓炭疽病菌的序列進行引子設計，開發巢式聚合酶鏈鎖反應技術。本技術可以偵測最主要的炭疽病菌 *C. siamense* 與 *C. fructicola*，但不會偵測到其他草莓病原菌或土壤中常見的腐生菌，可偵測到低至 100 fg 之 *C. siamense* DNA (約 2 個病原菌細胞)，代表具有高度專一性及靈敏度。

關鍵詞：潛伏感染、健康種苗、巢式聚合酶鏈鎖反應

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臺灣草莓炭疽病菌族群分析

臺灣草莓栽種面積約 500-600 公頃，每年產值超過 18 億元，平均每公頃產值超過 300 萬元，為一極具經濟價值之水果。每年草莓大約於初秋 (9 月底至 10 月初) 定植，約於 11 月底至 12 月初開始採收，一直至隔年 3 至 4 月結束。草莓主要藉由走蔓進行無性繁殖，育苗的時間落在每年 3 月至 9 月之間，這期間包括了 5 至 6 月的梅雨季節與 7 至 9 月的颱風季節，高溫高濕加上氣候變遷所造成的強降雨現象，導致近年來草莓炭疽病的發生頻率居高不下 (鐘及彭，2013)。臺灣每年對於草莓苗的需求高達 2500 萬株，近年來平均因炭疽病造成植株死亡的比例高達 20-30% (育苗期 30%，本田期 20%) 有時更因病害發生嚴重，造成全臺缺苗嚴重，出現有錢也買不到苗的現象發生，因此如何有效防治草莓炭疽病實為一亟待解決的重要問題。炭疽病 (anthracnose) 是草莓最主要的病害之一，其病原菌屬於炭疽刺盤孢菌屬 (*Colletotrichum*) 之真菌，近年來因分子生物學發展之影響，以往用傳統型態與寄主範圍做為主要鑑定病原真菌的方法，已逐漸轉變為以分子特徵為主要鑑定依據，特別是炭疽病菌屬之真菌，許多種皆無法以型態特徵來鑑定，需要以分子特徵才能準確鑑定。

為進行草莓炭疽病菌之族群調查，自臺灣地區之新竹縣、苗栗縣、嘉義縣與南投縣蒐集病株，這四個縣市的草莓栽種面積佔總栽種面積之 94%，其中以苗栗縣栽種面積最大，約佔總栽種面積之 90% (107 年農業統計資料)。分析 52 株炭疽病菌株，80% 以上於苗栗縣內不同地區分離出來。分離部位包含罹病株之冠部、葉片、走蔓、根部與果實等不同部位，經由 ITS 區域的序列比對結果顯示，有 45 株屬於 *Colletotrichum gloeosporioides* species complex，有 4 株屬於 *C. boninense* species complex，有 3 株屬於 *C. acutatum* species complex。結果顯示臺灣地區的草莓炭疽病菌至少有三種不同族群，其中又以 C. gloeosporioides species complex 的族群佔最大比例 (約 86%)，其分布區域亦包含了所有調查的縣市區域 (佔總栽種面積之 94%)。

為求更精確地鑑定不同族群內之炭疽病菌，所有分離純化的菌株皆以多基因並聯的方式進行類緣分析，分析的基因或區域包含 ITS、glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*)、chitin synthase (*CHS-1*)、actin (*ACT*)、β-tubulin 2 (*TUB2*)、calmodulin (*CAL*) 與 intergenic region of Apn2 and MAT1-2-1 (ApMAT)。序列經由 PCR 增幅後進行雙向定序，同一族群之序列經由 multiple sequence alignment 後，分別進行貝葉氏分析 (Bayesian inference)，

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最大似然法則分析 (Maximum likelihood) 與最大簡約法分析 (Maximum parsimony)，結果顯示 *C. gloeosporioides* species complex 的族群內包含兩種炭疽病菌，為 *C. siamense* 與 *C. fructicola*，*C. boninense* species complex 當中包含兩種炭疽病菌，為 *C. boninense* 與 *C. karstii*，*C. acutatum* species complex 包含一種炭疽病菌。在所有分離菌株當中，以 *C. siamense* (圖一) 出現的頻率最高，有約 75% 的出現頻率 (52 株當中佔 39 株) (Chung et al. 2019)。此外，於 *C. acutatum* species complex 當中分離的菌株，在演化樹分析上出現以獨立的分枝自成一個族群，且有著高度的可靠性，已命名為新種 C*olletotrichum miaoliense* sp. nov. (Chung et al. 2020)。

確立不同炭疽病菌族群後，於各族群當中各挑選至少 2 株菌作為代表性菌株，進行培養基上菌落生長速度及草莓葉上致病性強弱之比較，結果顯示 *C. siamense* 在不同溫度下 (18 ° C、22 ° C、25 ° C、28 ° C、30 ° C 與 32 ° C) 皆生長最為快速，病原性亦最強，推測臺灣草莓炭疽病菌雖然至少有五種，但 *C. siamense* 為田間最普遍的種類，亦為病原性最強的一種。

炭疽病檢測技術開發

由於炭疽病菌可以藉由壓器 (appressoria) 於植物組織表面潛伏感染，並且可以產生二次孢子 (secondary conidia)，因此潛伏感染時仍舊可以產生分生孢子，造成田間感染源的增加與散播 (Leandro et al., 2001)。經長期田間監測發現，炭疽病菌會潛伏於種苗，然後隨著種苗傳播至本田，因而偵測此病原菌潛伏感染情形，應有助於大幅降低其散播。炭疽病潛伏感染之偵測，可使用除草劑 (paraquat) 或冷凍法殺死植物組織後，加速其內潛藏的炭疽病菌之生長，並使分生孢子盤較易產生，藉此觀察是否有潛伏感染情形 (Mertely and Legard, 2004)。Dr. Ishikawa 則將外觀無病徵之草莓葉片以酒精表面消毒後，經 10-14 天可觀察潛伏感染之炭疽病的分生孢子堆產生情形 (Ishikawa, 2004)。分子生物學發展後，最常用來做為炭疽病菌種間鑑定及偵測潛伏感染情形之方法為聚合酶鏈鎖反應 (PCR)，然而當目標 DNA 量非常低時 (潛伏感染時期)，一般 PCR 不見得適用，此時需以高靈敏度之技術 (如 real-time PCR 等) 才能偵測得到。

為提升檢測速度，爰開發巢式聚合酶鏈鎖反應 (nested PCR) 引子對，此種方法是將第一次的 PCR 產物作為第二次 PCR 循環的模版，可以提高特異性及靈敏度。引子設計的目標區域設定在尋找 *C. gloeosporioides* complex 當中保守、

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但對其他 *Colletotrichum* spp. 不保守，且其上下游序列對大部分 *Colletotrichum* 為保守的區域。設計的 nested PCR 引子對首先進行專一性的分析，分別以 *C. siamense* ML133 的 DNA (為正對照組)，比較對於其他 *C. siamense* 分離株及不同種類炭疽病菌菌株，如 *C. fructicola* 與 *C. karstii* 的專一性，結果顯示可偵測到目前田間最主要之炭疽病菌種類 *C. siamense* 及 *C. fructicola*，經第一次 PCR 增幅出 490 bp 片段，第二次 PCR 則增幅出 150 bp 片段 (圖二)。將 *C. siamense* ML133 的 DNA 序列稀釋 (1 ng、100 pg、10 pg、1 pg、100 fg、10 fg per μL) 後進行測試，結果顯示 nested PCR 可以偵測低至 100 fg 之 *C. siamense* ML133 DNA，代表具有高靈敏度 (圖二)。

結　語

在發展草莓抗病育種技術與防治技術之前，必須先瞭解目前田間的草莓炭疽病是單一或多種病原菌所造成，才有可能針對主要病原菌研發相對應的整合性管理技術。目前所開發之 nested PCR 技術，可以檢測臺灣主要的草莓炭疽病菌 *C. siamense* 與 *C. fructicola*，但不會檢測出別種的炭疽病菌，且於土壤中常見的 *Fusarium* spp. 或木黴菌 (*Trichoderma* sp.) 亦不會被檢測出來，本檢測技術除了具有好的專一性，亦具有高度靈敏度，只要有 100 fg 的炭疽病菌 DNA (約 2 個病原菌細胞) 即可被偵測，適合檢測在草莓上潛伏感染的少量炭疽病菌。本檢測技術使用 nested PCR 反應，相較於一般 PCR，其靈敏度與專一性皆較高，到達與 real-time PCR 相當的靈敏度，且每次反應的成本較 real-time PCR 低，唯有其反應所需要的時間稍長，但相較於需要 10-14 天才能完成的酒精檢測法，整個檢測流程可在 1-2 天之內完成，已經符合快速檢測所需。國內目前正在積極推動草莓種苗三級繁殖制度，本檢測技術可以實際應用或支援種苗病害驗證作業 (鐘，2017)，未來將持續開發其他草莓病害檢測技術，以提高國內健康草莓苗的品質，減少病害發生與降低化學藥劑使用。

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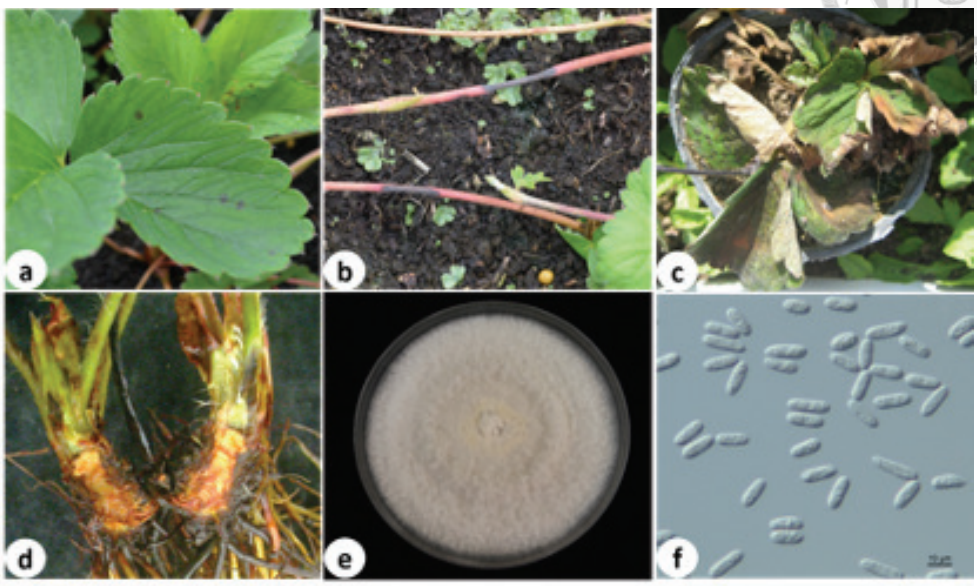

圖一、炭疽病菌在豐香草莓植株上各部位之病徵及菌落、分生孢子型態。a. 黑色壞疽葉斑。b. 走蔓上凹陷病斑。c. 炭疽病菌感染冠部造成草莓植株顯現出褪綠到枯萎症狀。d. 冠部的縱切面顯現褐色壞疽。e. 真菌菌落最初為白色，後來在上側逐漸轉為淺灰色。f. 分生孢子透明，長圓形到圓柱形，具圓形鈍角

Fig. 1. Anthracnose symptoms on strawberry and morphological characters of *Colletotrichum siamense*. (a) Black necrotic leaf spots. (b) Withering and girdling on a runner. (c) A strawberry plant with anthracnose crown rot showing chlorotic to blighted leaves and wilting symptoms. (d) Longitudinal section of an infected crown showing marbled reddish-brown necrosis. (e) The fungal colony was initially white, later became light gray on the upper side. (f) Conidia hyaline, oblong to cylindrical, with round obtuse ends. Scale bar = 10 μm

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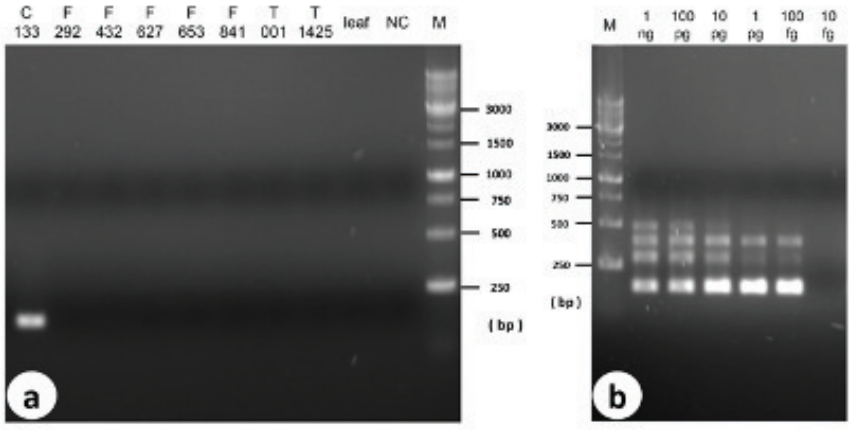

圖二、巢式聚合酶鏈鎖反應之專一性及靈敏度測試。a. DNA 樣本包含 *Colletotrichum siamense* ML133 (C133)、*Fusarium* spp. (F292、F432、F627、F653、F841)、*Trichoderma* sp. (T001、T1425)、草莓葉片 (leaf) 及負對照 (NC)。M：1 kb DNA 分子標記 (Faith Biotechnology)。b. 測試樣本為序列稀釋之 *Colletotrichum siamense* ML133 的 DNA : 1 ng/μL、100 pg/μL、10 pg/μL、1 pg/μL、100 fg/μL、10 fg/μL。M : 1 kb DNA 分子標記 (Faith Biotechnology)

Fig. 2. Specificity and sensitivity test of the nestd-PCR assay. (a) Samples included the DNA of *Colletotrichum siamense* ML133 (C133), *Fusarium* spp. (F292, F432, F627, F653、F841), *Trichoderma* sp. (T001, T1425), strawberry leaf (leaf), and negative control (NC). M: 1 kb DNA ladder (Faith Biotechnology). (b) Samples included serial dilutions of *C. siamense* ML133 DNA: 1 ng/μL, 100 pg/μL, 10 pg/μL, 1 pg/μL, 100 fg/μL, 10 fg/μL. M: 1 kb DNA ladder (Faith Biotechnology)

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Population analysis and development of a detection method for *Colletotrichum* species associated with strawberry anthracnose in Taiwan

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Abstract

In Taiwan, strawberry (*Fragaria* × *ananassa* Duch.) is a high-value crop with an average annual cultivated area of ~500 ha in the last 5 years. Anthracnose has become more destructive over the past decade in Taiwan. From 2010 to 2018, we surveyed anthracnose in major strawberry cultivation areas in Taiwan, and observed that more than 50% of diseased plants showed typical anthracnose crown rot (ACR) symptoms. Multi-gene analysis and morphological characterization of 52 isolates revealed that *C. siamense*, *C. fructicola* (*C. gloeosporioides* species complex), *C. karstii*, *C. boninense* (*C. boninense* species complex) and *Colletotrichum miaoliense* sp. nov of the *C. acutatum* species complex were associated with strawberry anthracnose in Taiwan. *C. siamense* was recognized as the most dominant, fast-growing and highly virulent causative agent of strawberry anthracnose in Taiwan. Our results would help develop a comprehensive control strategy to alleviate strawberry anthracnose in the future. Because *Colletotrichum* spp. can cause latent infections, use of healthy and pathogen-free strawberry seedlings will greatly reduce the occurrence of anthracnose rot in the field and the usage

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of fungicides. To produce healthy seedlings, it is important to diagnose anthracnose at the stage of latent infection. We conducted comparative genomics analysis of known *Colletotrichum* spp. genomes to search for ideal regions suitable for the design of specific primers. We developed a nested-PCR assay which can specifically detect *C. siamense* and *C. fructicola*, the predominant pathogens causing strawberry anthracnose in Taiwan, but not other pathogens and saprophytes associated with strawberry plants. It could detect as low as 100 fg genomic DNA of *C. siamense*, which corresponds to 2 cells of *C. siamense*, suggesting the high sensitivity of this new detection technique.

Key words: latent infection, healthy runner plants, nested-PCR

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Disease Note

Diseases Caused by Fungi and Fungus-Like Organisms

First Report of *Neopestalotiopsis rosae* Causing Leaf Blight and Crown Rot on Strawberry in Taiwan

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For the past 30 years, the most predominant strawberry cultivar in Taiwan has been 'Taoyuan No. 1', which produces fruit with rich flavor and aroma but is highly susceptible to anthracnose (Chung et al. 2019). Because epidemics of anthracnose became more destructive, farmers switched to anthracnose-tolerant cultivar 'Xiang-Shui' (~50 and ~80% of the cultivation area in 2018 and 2019, respectively). Since 2018, severe leaf blight and crown rot symptoms have been observed all year in Xiang-Shui in Miaoli, Nantou, Hsinchu, Taipei, Taoyuan, and Chiayi Counties. The disease became more prevalent and severe during 2019 to 2020 and caused up to 30% plant loss after transplanting. Symptoms appeared as brown necrotic lesions with black acervuli on leaves, slightly sunken dark-brown necrosis on stolons, and sunken reddish-brown necrosis on fruit. The diseased crown tissue showed marbled reddish-brown necrosis with a dark-brown margin, and plants with severe crown rot usually showed reddish-brown discoloration on leaves (the leaves initially turned reddish-brown between the veins and could become entirely scorched at later stages). To isolate the causal agent, small fragments of diseased leaves, crowns, stolons, and fruits were surface disinfested with 0.5% sodium hypochlorite for 30 s, rinsed with sterile water, and then placed on 1.5% water agar. Single hyphal tips extended from tissues were transferred to potato dextrose agar and cultured for 7 days at 25°C under a 12-h/12-h photoperiod. In total, 20 isolates were obtained from diseased leaves, crowns, stolons, and fruits. Colonies were white with cottony aerial mycelium, irregular margins, and black acervuli distributed in concentric rings. Conidia were fusiform to ellipsoid (five cells), with one basal appendage and three or four (usually three) apical appendages. From colony and conidial morphology, the causal agent was identified as *Neopestalotiopsis* sp. (Maharachchikumbura et al. 2014). The internal transcribed spacer (ITS) region, β-tubulin (*TUB*), and translation elongation factor 1-alpha (*TEF-1α*) of three isolates (ML1664 from diseased crown tissue collected in Hsinchu County; ML2147 and ML2411 from diseased leaves collected in Miaoli County) were sequenced (GenBank nos. MT469940 to MT469948). All three isolates clustered with the ex-type strain of *Neopestalotiopsis rosae* in the multilocus (ITS + *TUB* + *TEF-1α*) phylogenetic tree. To fulfill Koch's postulates, spore suspensions of ML1664 and ML2147 at 1×10^6 conidia/ml were used to spray inoculate Xiang-Shui seedlings at the three- to four-leaf stage until run-off (two trials, five seedlings per trial). Inoculated plants were put in a plastic bag (>90% relative humidity) at 25°C under a 12-h/12-h photoperiod. After 10 to 14 days, 80% of inoculated plants showed leaf or crown symptoms similar to those in the field. Control plants sprayed with sterile water showed no symptoms (four to five seedlings per trial). The fungi were reisolated from necrotic lesions with 100% frequency ($n \geq 3$ isolates per trial), and morphological characters and ITS sequences were identical to the original ones. This is the first report of *N. rosae* causing leaf blight and crown rot in strawberry in Taiwan. *N. rosae* and *N. clavispora* have been reported as new threats to strawberry in several other countries (Gilardi et al. 2019; Rebollar-Alviter et al. 2020). Clarification of the pathogen provides a basis for developing strategies to control the emerging disease. Further studies are needed to evaluate the resistance/susceptibility of major strawberry cultivars and the fungicide sensitivity of the pathogen.

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The author(s) declare no conflict of interest.

***e*-Xtra**

Keywords: *Neopestalotiopsis rosae*, strawberry, leaf blight

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Disease Note

Diseases Caused by Bacteria and Phytoplasmas

First Report of *Xanthomonas fragariae* Causing Angular Leaf Spot on Strawberry (*Fragaria* × *ananassa*) in Taiwan

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Angular leaf spot of strawberry, considered an A2 quarantine pest by the European and Mediterranean Plant Protection Organization (EPPO 2019), is an important bacterial disease in many regions. Since 2017, symptoms similar to angular leaf spot were observed in several strawberry cultivars including 'Taoyuan No. 1' and 'Xiang-Shui'. Early symptoms were angular, water-soaked lesions on the abaxial leaf surface, and later, reddish-brown irregular spots and coalesced lesions developed on the adaxial surface. In the humid conditions, sticky bacterial ooze exuding from lesions was observed. To isolate the causal agent, leaves showing water-soaked lesions were surface sterilized, cut into small pieces, and soaked in 5 ml of sterile water for at least 15 min. The supernatant from the cut-up pieces was serially diluted, followed by spreading on sucrose peptone agar (SPA) (Hayward 1960). After incubating at 20°C for 4 to 5 days, single colonies grown on SPA were transferred to a new SPA plate and cultured at 20°C until colonies appeared. The yellow, glossy, and mucoid colonies, which resembled the colony morphology of *Xanthomonas fragariae*, were selected as candidates for further confirmation. First, bacterial DNA of four candidate isolates, B001, B003, and B005 from Miaoli County and B004 from Taoyuan City, was PCR amplified with *X. fragariae*-specific primers: XF9/XF12 (Roberts et al. 1996) and 245A/B and 295A/B (Pooler et al. 1996). All four isolates could be detected by XF9/XF12 primer. Furthermore, isolates B003 and B004 could be detected by both 245A/B and 295A/B primers, whereas B001 and B005 could be detected by 295A/B only. Next, DNA gyrase subunit B (*gyrB*) was PCR amplified with the primers XgyrB1F/XgyrB1R (Young et al. 2008). The *gyrB* sequences of these four isolates were deposited in GenBank with accession numbers MT754942 to MT754945. The *gyrB* phylogenetic tree was constructed based on Bayesian inference analysis and maximum likelihood analysis. The *gyrB* sequences of the four isolates from Taiwan clustered in the clade containing the type strain of *X. fragariae* ICMP5715, indicating that they belong to *X. fragariae*. B001 and B005 formed a subgroup separated from B003 and B004, suggesting genetic differences between these isolates. To fulfill Koch's postulates, the abaxial surfaces of strawberry leaves were syringe infiltrated (Silva et al. 2017) or wound inoculated (Wang et al. 2017) with bacterial suspensions (final OD_{600} = 1.0 to 2.0) prepared from colonies of B001 and B003 washed from SPA plates. Inoculated plants were enclosed in a plastic bag (>90% relative humidity) at 25/20°C (day/night) under a 12-h/12-h photoperiod. After 7 to 14 days, water-soaked lesions similar to those observed in the field were developed on all inoculated leaves. The bacteria were successfully reisolated from lesions of inoculated leaves and confirmed by specific primers XF9/XF12, 245A/B, and 295A/B. We also found that the disease commonly occurs in the strawberry fields/nurseries with sprinkler irrigation during winter or early spring and was particularly serious in the windward side or near a riverside. To our knowledge, this is the first report of *X. fragariae* causing angular leaf spot on strawberry in Taiwan. Currently, the disease only occurs severely in certain regions, but establishment of effective management strategies will be needed to prevent spreading of this disease and potential economic loss in the future.

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***e*-Xtra**

Keywords: *Xanthomonas fragariae*, angular leaf spot, strawberry, Taiwan, *gyrB*

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PLOS ONE

RESEARCH ARTICLE

Development of a nested PCR assay for detecting *Colletotrichum siamense* and *Colletotrichum fructicola* on symptomless strawberry plants

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Abstract

Anthracnose is a major disease of strawberry that seriously impacts the strawberry industry. To prevent the spread of anthracnose through symptomless plants, it is important to detect pathogenic *Colletotrichum* spp. at the latent infection stage in the nursery. Previous PCR-based methods developed for the diagnosis or detection of *Colletotrichum acutatum* and *Colletotrichum gloeosporioides* have used primers targeting the internal transcribed spacer region of ribosomal DNA, β-tubulin gene, or mating type gene. In this study, to specifically detect *Colletotrichum siamense* and *Colletotrichum fructicola*, the most predominant and virulent *Colletotrichum* species causing strawberry anthracnose in Taiwan, we conducted a comparative genomics analysis of 29 *Colletotrichum* spp. and identified a non-conserved 1157-bp intergenic region suitable for designing specific primers for a nested PCR assay. *In silico* analysis and actual tests suggested that the new nested PCR assay could detect pathogenic *C. siamense* and *C. fructicola*, but not other strawberry pathogens (*Botrytis* sp., *Fusarium* spp., *Neopestalotiopsis rosae*, and *Phytophthora* sp.) or ubiquitous saprophytes (*Fusarium* spp. and *Trichoderma* spp.). The inner to outer primer ratio was optimized to 1:10 to eliminate unexpected bands and enhance the signal. The assay could detect as little as 1 pg of *C. siamense* genomic DNA, which corresponds to ~15 cells. Application of the new detection assay on 747 leaf samples collected from 18 strawberry nurseries in 2019 and 2020 showed that an average of 20% of strawberry mother plants in Taiwan were latently infected by *C. siamense* or *C. fructicola*. The newly developed assay is being applied to facilitate the production of healthy strawberry runner plants in Taiwan.

Introduction

Strawberry (*Fragaria* × *ananassa* Duch.) is an economically important small fruit crop that can grow in temperate, sub-tropical, and tropical regions around the world. The average

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Competing interests: The authors have declared that no competing interests exist.

strawberry cultivation area in Taiwan was about 514 ha from 2016 to 2020 [1], and Miaoli County is the predominant strawberry production region (~90% of the total cultivated area), where the major cultivars are 'Taoyuan No. 1,' and 'Xiang-Shui'. Farmers in Taiwan usually propagate strawberry runner plants from mother plants in the spring and summer. The high temperature and intermittent heavy rains during the monsoon and typhoon seasons create environmental conditions conducive to infectious disease and epidemics. In recent years, strawberry anthracnose has become a more serious problem, causing the death of about 20%–40% of plants [2]. A high percentage of severely diseased plants are removed during the nursery stage or within 1–2 months after transplanting, which eventually leads to considerable economic loss.

Colletotrichum is a fungal genus containing important plant pathogens causing anthracnose diseases of various economically valuable crops [3]. Several *Colletotrichum* species can infect strawberry, causing leaf spot, crown rot, stolon spot, petiole spot, and fruit rot [4]. Based on traditional morphological characteristics, the major pathogens of strawberry anthracnose are considered to be *C. fragariae*, *C. gloeosporioides*, and *C. acutatum* [5,6]. Recent advances in molecular phylogenetics revealed that *C. gloeosporioides* and *C. acutatum* are highly diverse species complexes and can be classified into several species [7,8]. *C. nymphaeae* in the *C. acutatum* species complex was identified as the major pathogen of strawberry anthracnose in the UK and USA [9,10]; *C. fructicola* (*C. gloeosporioides* species complex) in Japan (Chiba Prefecture) and China (Zhejiang province) [11,12]; and *C. siamense* (*C. gloeosporioides* species complex) in China (Hubei province) [13]. In Taiwan, a recent investigation of the diversity of infectious agents causing strawberry anthracnose in Miaoli, Hsinchu, Nantou, and Chiayi Counties from 2010 to 2018 revealed five *Colletotrichum* spp., i.e., *C. siamense* (75% of all isolates), *C. fructicola* (11%), *C. karstii* (6%), *C. miaoliense* (6%), and *C. boninense* (2%) [14].

Colletotrichum is a hemibiotrophic pathogen that can remain in a latent state in the host tissues. Its conidium germinates to form an appressorium on the plant surface, which is followed by penetration peg invasion of the epidermal cells and the production of primary hyphae (biotrophic phase). The time required from the adherence of the conidia to successful infection can be within 48 hours [15,16]. At this stage, no visible difference can be observed between healthy and infected tissues [17,18]. However, *Colletotrichum* is capable of producing secondary conidia [19,20], which makes the latently infected but asymptomatic mother plants an important inoculum source for runner plants in the nursery. Latently infected runner plants can also be an inoculum source in production fields [21–23]. After the lifestyle transition from biotrophy to necrotrophy, this polycyclic pathogen produces masses of conidia, spreads rapidly by rain splash, and causes epidemics under suitable conditions in the field [22].

The use of pathogen-free strawberry mother plants and their propagules is a critical step for disease control in the nursery and production fields and thus the reduction of losses caused by anthracnose [22]. Several methods for diagnosing latent infection or distinguishing *Colletotrichum* spp. infecting strawberry have been developed [21,23–33]. For instance, culture-based methods involve incubation of strawberry tissues after surface sterilization with ethanol or killing with paraquat or freezing [24,29–31]. These treatments promote the necrotrophic growth of the latently infected pathogens, resulting in the formation of yellow to orange conidiomata after 1–2 weeks of incubation. Culture-based methods are simple and convenient but a long time is required for sporulation; in addition, a great deal of experience is required to correctly differentiate *Colletotrichum* spp. from other fast-growing fungi with similar morphology [29,30]. Faster and more accurate PCR-based detection methods have also been developed, using specific primers targeting the internal transcribed spacer (ITS) region of ribosomal DNA [25,27,28,32], β-tubulin (*TUB2*) gene [21], or mating type gene *MAT1-2* [33]. During latent infection with *Colletotrichum* spp., the number of pathogen cells at the biotrophic phase is very

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low [34,35], and only methods with high sensitivity (e.g., nested PCR or qPCR) can be applied to detect such a low amount of target DNA.

PCR-based detection methods were previously designed to detect the anthracnose pathogens *C. acutatum* and *C. gloeosporioides* in strawberry [21,23,25,28,32,33,36]. However, *C. acutatum* and *C. gloeosporioides* are now considered a species complex based on recent evidence from multilocus molecular phylogenetic analyses [7,8], so the previous methods may not be able to distinguish current taxonomic species. This study aimed to develop a highly sensitive and specific method applicable for detecting the anthracnose pathogens on symptomless strawberry plants in Taiwan (the workflow of this study is shown in Fig 1). Due to the short period of time required for pathogenic *Colletotrichum* spp. to invade host tissues [15,16], the assay mainly targets the pathogens in the latent infection stage, although a small number of pathogens present on the host surface cannot be excluded. *C. siamense* and *C. fructicola*, the most predominant and virulent *Colletotrichum* species causing strawberry anthracnose in Taiwan [14], were targeted. Comparative genomics analysis of 29 available *Colletotrichum* spp. was conducted to search for a non-conserved region suitable for designing primers for a nested PCR assay (Fig 1A). *In silico* analysis and specificity tests were conducted to rule out detection of other pathogenic and saprophytic fungi frequently isolated from strawberries (Fig 1B), and the ratio of outer and inner primers used in the nested PCR were optimized to eliminate unexpected PCR products. To verify the new method and investigate the latent infection of strawberry plants by *Colletotrichum* spp. in Taiwan, a field survey was conducted on 747 asymptomatic mother plants in 18 strawberry nurseries (Fig 1C). As the production of strawberry runner plants is moving from propagation by small farmers toward professional propagation, it is expected that the highly specific and sensitive new method developed here will help reduce the disease incidence in mother plants, thereby increasing the rate of healthy runner plants in Taiwan.

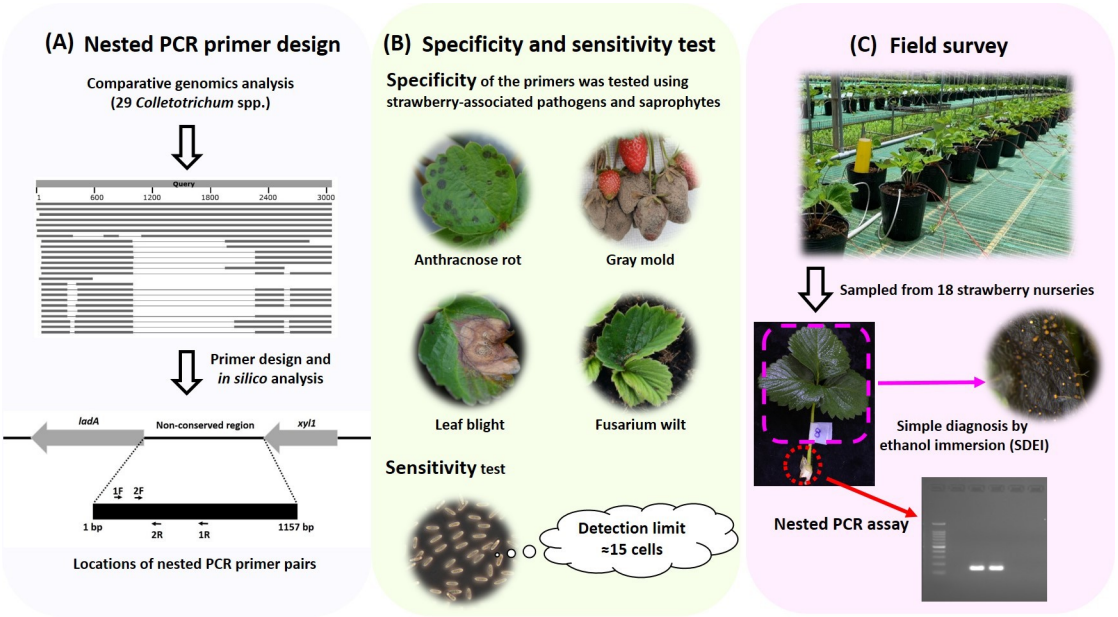

Fig 1. Workflow diagram for the nested PCR primer design, specificity and sensitivity tests, and field survey in this study. (A) Comparative genomics analysis and *in silico* analysis were conducted to identify non-conserved regions suitable for designing nested PCR primers. (B) Specificity and sensitivity tests of nested primers. Specificity was determined by testing the ability of primers to amplify strawberry-associated pathogens and saprophytes. The detection limit was ~15 cells of *C. siamense*. (C) Samples collected from 747 mother plants in 18 strawberry nurseries were assayed by the nested PCR and simple diagnosis by ethanol immersion (SDEI) methods.

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Materials and methods

Fungal isolation and cultivation

The isolates of *Botrytis* sp. and *Colletotrichum* spp. [14], *Fusarium* spp., *Phytophthora* sp., and *Neopestalotiopsis rosae* [37], and *Trichoderma* spp. [38] used in this study are listed in S1 Table. In addition to five species causing strawberry anthracnose (*C. siamense*, *C. fructicola*, *C. karstii*, *C. miaoliense*, and *C. boninense*) [14] and *N. rosae*, which causes strawberry leaf blight and crown rot [37], we isolated *Botrytis* sp. from strawberry fruit showing gray mold, *Fusarium* spp. from the root, crown, and nearby soil of diseased plants showing typical Fusarium wilt symptoms, and *Phytophthora* sp. from the root of a wilted strawberry plant. A *Fusarium* sp. and *Trichoderma* sp. isolated from the symptomless runner and petiole of a strawberry plant and a *Trichoderma asperellum* isolate collected from the rhizosphere soil of grape were also included. Fungal isolation from host tissues was conducted as described by Chung et al. (2020) [14]. Tissues approximately 3 x 3 mm in area were surface sterilized with 0.5%–1% sodium hypochlorite, rinsed with sterile deionized water three times, then placed onto 1.5% water agar at 25˚C. *Fusarium* isolation from soil was carried out by mixing 10 g of soil with 90 ml of 0.05% agar solution, then evenly spreading 200 μl of 10-fold serial dilutions on FoG1 medium (*Fusarium* colonies are purple on FoG1 medium) [39]. After 2 to 3 days of incubation, extended single hyphal tips from tissues were transferred to potato dextrose agar (PDA, BD Difco) and incubated for 5–7 days at 25˚C under a 12-h/12-h light/dark photoperiod. Fungal isolates were identified to the genus level by morphological characteristics and ITS sequences (as described below).

DNA extraction and sequence alignment

Genomic DNA was extracted from strawberry leaves or petioles using a plant genomic DNA extraction mini-prep system (VIOGENE) according to the procedures provided by the manufacturer. For extraction of fungal genomic DNA, the mycelium collected from a 7-day-old colony grown on PDA was frozen in liquid nitrogen and ground to a fine powder with a sterile mortar and pestle. The ITS was amplified with ITS1/ITS4 primers [40]. Amplicons were bidirectionally sequenced on an ABI 3730 DNA analyzer (Tri-I Biotech, Taiwan), and the sequences were used as queries in blast searches against the NCBI GenBank nr/nt database (blast.ncbi.nlm.nih.gov).

Target region selection and primer design

To identify ideal regions for primer design, we searched for non-conserved regions located in between two conserved regions in the genomes of *Colletotrichm* spp. The conserved regions were used to design primers to sequence the internal non-conserved regions, which are highly diverse and can be used to distinguish *Colletotrichum* species. Based on the initial results of blastn searches against 29 *Colletotrichum* genome sequences (S2 Table) using the ITS, chitin synthase (*CHS-1*), actin (*ACT*), *TUB2*, calmodulin (*CAL*), and intergenic region between the *Apn2* DNA lyase and *MAT1-2* (ApMAT) genes (sequences obtained from our previous study [2]) as queries, *C. gloeosporioides* strain 30206, *C. gloeosporioides* Cg-14, and *C. fructicola* Nara gc5 (which was designated *C. gloeosporioides* before 2018) were the closest strains to *C. siamense* ML133. Note that the primer design for this study was conducted in 2017, at a time when the genome sequence of *C. siamense* was not available (*C. siamense* ICMP18578 was released in 2019). Therefore, *C. gloeosporioides* 30206 was used as a query to search against the genomes of 26 *Colletotrichum* species (all strains in S2 Table except *C. gloeosporioides* Cg-14, *C. gloeosporioides* 30206, and *C. fructicola* Nara gc5). All genome sequences were downloaded

from the NCBI genome database [https://www.ncbi.nlm.nih.gov/genome/], and the genome blast was performed using BLAST Command Line Applications [41] following the user manual [https://www.ncbi.nlm.nih.gov/books/NBK279690/]. The blastn parameters were set to word size 28, e value $<10^{-5}$, and output format 5. The output file was parsed using Python [42]. The 500- to 2500-bp non-conserved regions between conserved hit regions (hereafter referred to as 'spacers') were selected. The 1000-bp upstream and 1000-bp downstream sequences of each selected spacer were blasted against the genome sequences of 29 *Colletotrichum* spp. (blastn word size 28, e value $<10^{-100}$). Spacers of 1000–1500 bp in length were selected from among those with upstream and downstream sequence hit numbers $\geq$ 50. Candidate spacers were checked manually and a region suitable for designing high-quality primers for a nested PCR assay was selected. The identified spacer region in *C. siamense* ML133 was sequenced by the primer pair 5'-TTGGCCTGCGCTTCAACGAC-3' (forward) and 5'-AACTCA CCCGCAAACACCAGT-3' (reverse). Primers for the first PCR (outer primers) and second PCR (inner primers) were designed based on the spacer sequences. Primers with high scores and that were compatible with each other were chosen using Oligo 7 software [43]. Furthermore, nested PCR primer candidates were blasted against *Fragariae* x *ananassa* (NCBI accession No. PRJDB1477) and other pathogen/microbial genomes, including *Fusarium* spp. (S3 Table), *Trichoderma* spp. (S4 Table), and strawberry pathogens *Botrytis cinerea*, *Phytophthora cactorum*, and *Xanthomonas fragariae*, to rule out possible non-target reactions *in silico*. The primers with lower hit numbers and lower e values were chosen.

Specificity and sensitivity of the nested PCR assay

Fifteen fungal isolates including pathogens and saprophytes isolated from strawberry or soil (S1 Table) and three strawberry cultivars (i.e., 'Taoyuan No. 1', 'Xiang-Shui,' and 'Miaoli No. 1') were used for evaluation of the specificity of the nested PCR assay. The primers targeting the ITS (ITS1/ITS4 [40]) and *ACTIN* gene (Actin-F/Actin-R [44]) were used to test the quality of fungal and strawberry DNA, respectively. Each PCR reaction was performed in a 50-μl mixture containing 2.5 U Taq polymerase (Prime Taq, GenetBio). For ITS and *ACTIN*, each reaction contained 1–20 ng DNA and 0.2 μM of each primer. For the nested PCR assay, the first PCR reaction contained 1–20 ng DNA and 0.02 μM of each outer primer (Col_nest-1F/Col_nest-1R), and the second PCR reaction contained 1 μl of the first PCR product and 0.2 μM of each inner primer (Col_nest-2F/Col_nest-2R) (primer sequences in Table 1). Different ratios of outer primers to inner primers (1:1, 1:2, 1:5, 1:10, 1:20, 1:50, and 1:100) were tested and 1:10 was found to be optimal. The conditions for the first PCR were an initial denaturation at 94˚C for 5 min, followed by 40 cycles of 94˚C denaturation for 30 sec, 55˚C annealing for 30 sec, and 72˚C extension for 30 sec with a final cycle of 72˚C for 5 min. The conditions for the second PCR were an initial denaturation at 94˚C for 1 min, followed by 30 cycles of 94˚C denaturation for 30 sec, 55˚C annealing for 30 sec, and 72˚C extension for 30 sec with a final cycle of 72˚C for 5 min. The PCR products were analyzed by electrophoresis on a 1.5% agarose gel in TAE buffer. Images were captured using a Fluorescent Gel Image System (FGIS-3, TopBio).

Table 1. Nested PCR primers used in this study.

Name	Sequence	Product size (bp)	Note
Col_nest-1F	5'- ACAAACGGTGATCCTTTCGTC -3'	490	Outer primers for the first PCR
Col_nest-1R	5'- GGTGCCCCTCAACACGAAC -3'		
Col_nest-2F	5'- CTCCCAACCGGATAATCTGC -3	151	Inner primers for the second PCR
Col_nest-2R	5'- ACCGACCGGAACATAGATCACA -3'		

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The expected sizes of the first and second PCR products were 490 bp and 151 bp, respectively. To test the detection limit, genomic DNA of *C. siamense* was 10-fold serially diluted from 1 ng/μl to 10 fg/μl. The first and second PCR reactions were performed as described above.

Detection of *Colletotricum* spp. on symptomless strawberry plants in nurseries

From 2019 to 2020, 747 asymptomatic leaf samples (747 mother plants) collected from 18 strawberry nurseries in Hsinchu City, Miaoli County, Taichung City, and Nantou County were tested using the nested PCR and simple diagnosis by ethanol immersion (SDEI) methods [30] (Table 2). In these nurseries, the mother plants were reproduced from field plants by the farmers themselves. Previous studies showed that latent infection with *C. acutatum* and *C. gloeosporioides* was more frequently detected in the older leaves and petioles [22,29]. In this study, the oldest leaf was removed from the crown of each tested plant. An approximately 1-cm segment of the basal petiole was used for the nested PCR assay. The remaining leaf and petiole were used for the SDEI assay. The SDEI assay was conducted following the procedures in Ishikawa (2004) with modification [30]. In brief, the collected leaf samples were washed with tap water, rinsed with deionized water, and blotted dry on tissue paper. The abaxial and adaxial surfaces of the leaves were sprayed thoroughly with 75% ethanol. At 30–60 sec after spraying, the leaves were washed with deionized water once, rinsed with sterile water, and blotted dry on sterile tissue paper. The leaves were put into a plastic bag with a wetted cotton pad to maintain high humidity (> 90%). Leaves were incubated for 7–14 days at 28–30˚C under a 12-h/12-h light/dark photoperiod.

Results

Identification of a highly diverse intergenic region for primer design

Through comparative genomics analysis of 29 *Colletotrichum* spp. isolates, 19 non-conserved regions (1000–1500 bp in length) located between conserved regions were identified. After manually checking the sequences, a non-coding region was selected for primer design. This region in *C. siamense* ML133 was 1157 bp in length (sequences uploaded to GenBank under accession number ON350970), which has 95.77% and 94.65% identity to the corresponding regions in *C. gloeosporioides* 30206 and *C. siamense* Cg363, respectively. An alignment of the sequences of different *Colletotrichum* spp. is shown in S1 Fig. In the genome of *C. siamense* Cg363, the region is located between the L-arabinitol 4-dehydrogenase (*ladA*) and NAD(P)H-dependent D-xylose reductase (*xyl1*) genes. Using this region as template, 79 pairs of primers were designed. After performing blast searches against the sequences of *Fragariae* x *ananassa*, strawberry pathogens, and saprophytes, two primer pairs suitable for nested PCR were selected. The sizes of the first and second PCR products were 490 bp and 151 bp, respectively.

Specificity and sensitivity of the nested PCR assay

Five *Colletotrichum* spp. causing strawberry anthracnose in Taiwan and a selected set of microorganisms commonly isolated from strawberry or soil were used for the specificity test. Among the five pathogenic *Colletotrichum* spp., *C. siamense* ML133 and *C. fructicola* ML348 but not *C. karstii* ML351, *C. boninense* ML521, or *C. miaoliense* ML1040 were detectable (Fig 2A). The first and second PCR resulted in specific bands of the expected sizes (490 bp and 151 bp, respectively). The pathogenic fungi *Neopestalotiopsis rosae* ML2147, *Fusarium* spp., *Botrytis* sp., and *Phytophthora* sp. and saprophyte fungi *Fusarium* spp. and *Trichoderma* spp. were not detectable (Fig 2B). No signal was detected from three strawberry cultivars, 'Taoyuan No.

Table 2. Field survey of *Colletotrichum* spp. on symptomless strawberry plants using the nested PCR and SDEI (simple diagnosis by ethanol immersion) assays.

Nursery site	Collection date	County/City	No. of leaf samples	Method	No. of positive samples	Detection rate (%)
1	2019/01/14	Miaoli County	50	Nested PCR	10	20
			50	SDEI	20	40
2	2019/03/08	Miaoli County	60	Nested PCR	9	15
			60	SDEI	16	27
3	2019/03/22	Hsinchu City	16	Nested PCR	2	13
			16	SDEI	5	31
4	2019/03/25	Taichung City	22	Nested PCR	6	27
			22	SDEI	14	64
5	2019/04/01	Miaoli County	50	Nested PCR	7	14
			50	SDEI	23	46
6	2019/04/09	Hsinchu City	6	Nested PCR	6	100
			6	SDEI	1	17
7	2019/05/01	Miaoli County	40	Nested PCR	1	3
			40	SDEI	2	5
8	2019/06/04	Miaoli County	41	Nested PCR	12	29
			41	SDEI	23	56
9	2019/06/21	Hsinchu City	7	Nested PCR	1	14
			7	SDEI	2	29
10	2019/07/04	Miaoli County	50	Nested PCR	19	38
			50	SDEI	11	22
11	2019/10/02	Nantou County	35	Nested PCR	1	3
			35	SDEI	6	17
12	2020/02/06	Miaoli County	50	Nested PCR	8	16
			50	SDEI	30	60
13	2020/02/13	Miaoli County	50	Nested PCR	2	4
			50	SDEI	21	42
14	2020/02/19	Miaoli County	50	Nested PCR	11	22
			50	SDEI	42	84
15	2020/03/04	Miaoli County	50	Nested PCR	0	0
			50	SDEI	14	28
16	2020/03/11	Miaoli County	50	Nested PCR	7	14
			50	SDEI	42	84
17	2020/04/08	Miaoli County	50	Nested PCR	11	22
			50	SDEI	45	90
18	2020/04/21	Miaoli County	70	Nested PCR	4	6
			70	SDEI	49	70
Total			747	Nested PCR	117	16
			747	SDEI	366	49
Average (per nursery)			41.5	Nested PCR	6.5	20
			41.5	SDEI	20.3	45

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1', 'Xiang-Shui', and 'Miaoli No. 1' (Fig 2C). PCR products of the expected sizes were observed from the controls (fungal ITS and strawberry *ACTIN*) (Fig 2).

In the sensitivity test, a bright and specific band was observed from the reactions using 1 ng, 100 pg, 10 pg, 1 pg, and 100 fg of the genomic DNA of *C. siamense* ML133. The product sometimes failed to be amplified when using 100 fg. The results showed that performing the first PCR with 40 cycles followed by the second PCR with 30 cycles can reliably detect as little

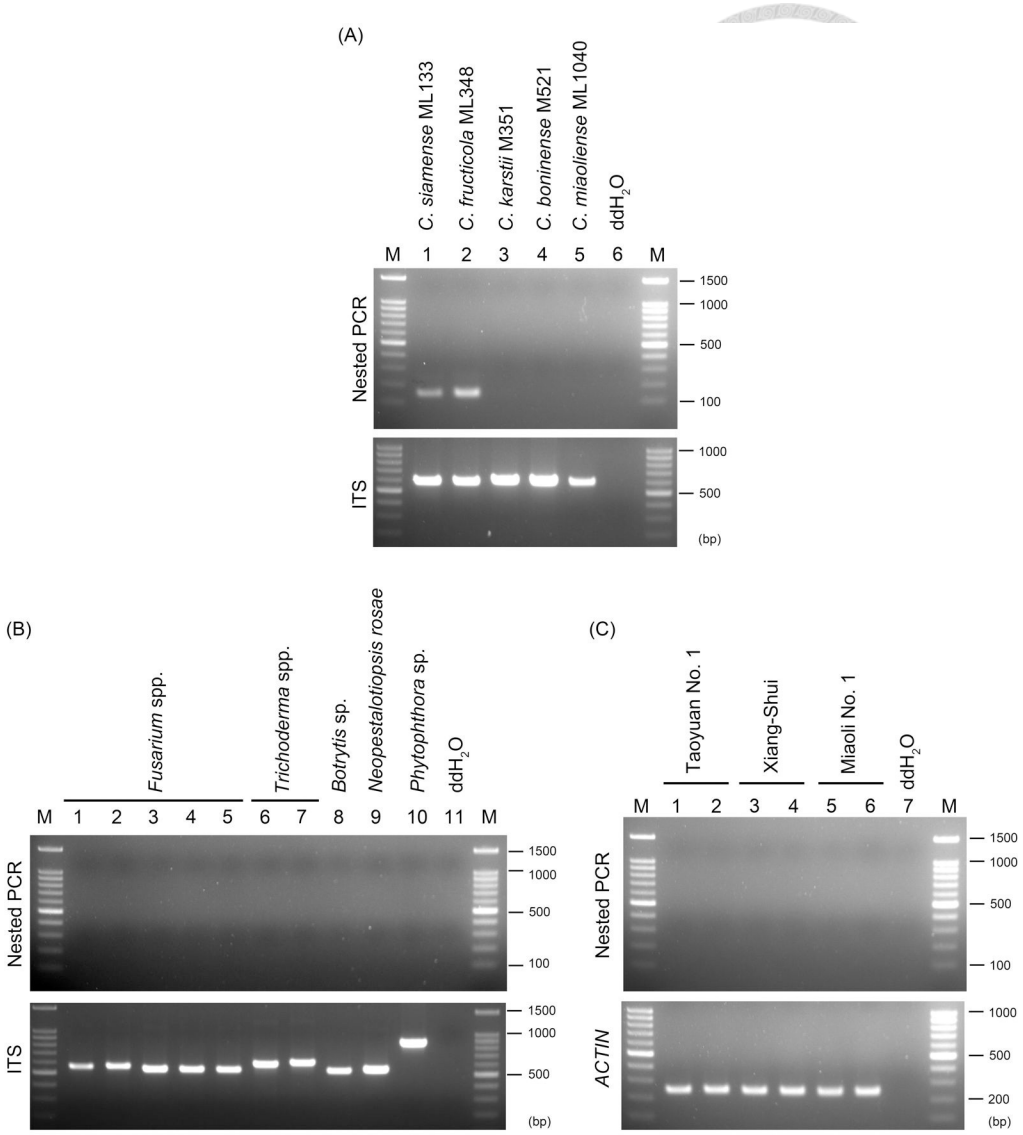

Fig 2. Specificity test of the nested PCR assay. (A) DNA of *Colletotrichum* spp. associated with strawberry anthracnose in Taiwan were used as template. *C. siamense* and *C. fructicola* are the most prevalent and virulent species, and *C. boninense*, *C. karstii*, and *C. miaoliense* are lowly pathogenic and present in a low percentage of strawberry plants. (B) DNA of pathogenic or saprophytic fungi isolated in the field were used as template. (C) DNA of different strawberry cultivars were used as template. The nested PCR assay was performed using primers Col_nest-1F/Col_nest-1R for the first PCR, and Col_nest-2F/Col_nest-2R for the second PCR. The quality of fungal and strawberry DNA was tested using primers targeting the ITS (ITS1/ITS4) and *ACTIN* (Actin-F/Actin-R), respectively. M, 100-bp DNA ladder (Faith Biotechnology Co., Ltd).

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as 1 pg genomic DNA (Fig 3), which corresponds to the DNA contents of ~15 cells of *C. siamense* (based on the genome size of *C. siamense* Cg363: ~62.9 Mb) [45].

Optimization of the nested PCR assay by changing outer and inner primer ratio

It was observed that the nested PCR often resulted in four bands of approximately 500 bp, 400 bp, 250 bp, and 150 bp. The sizes of these bands suggested that they may have come from amplification directed by different combinations of the outer primers and inner primers. To

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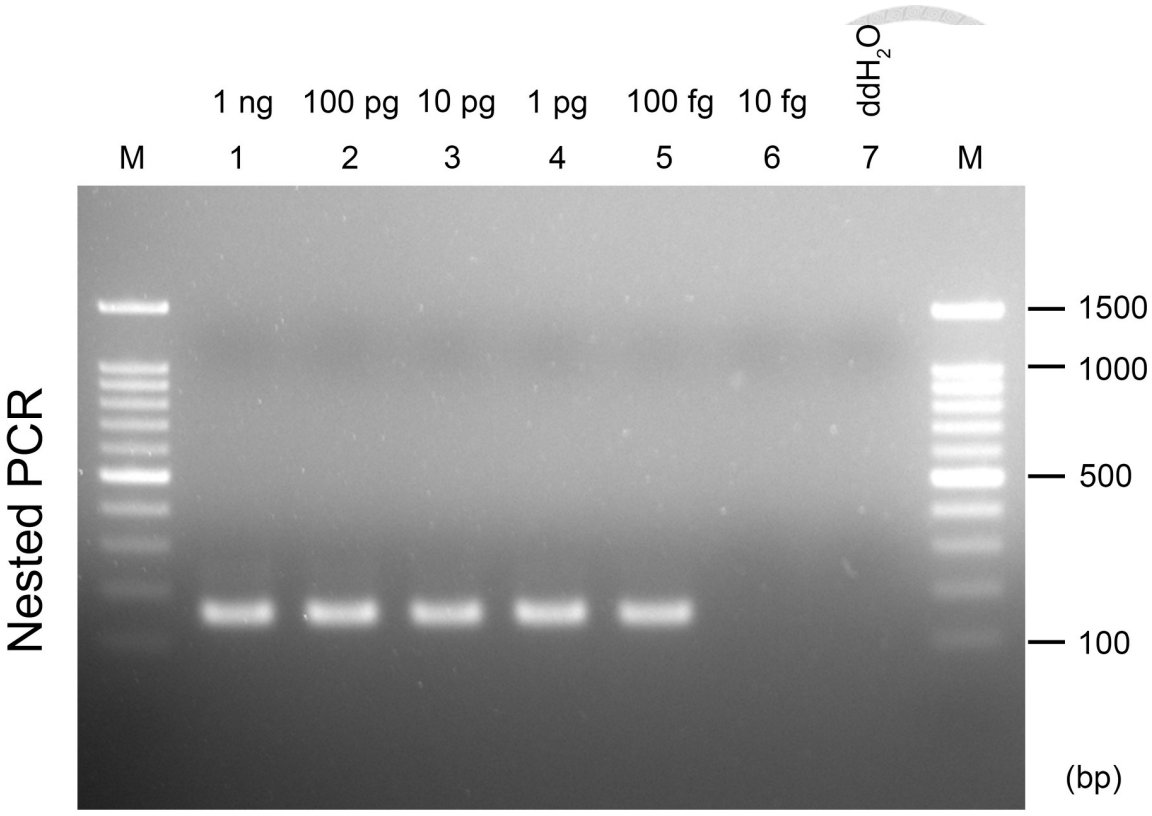

Fig 3. Sensitivity test of the nested PCR assay. Ten-fold dilutions (1 ng to 10 fg) of *C. siamense* ML133 genomic DNA were used as the template. The nested PCR assay was performed using primers Col_nest-1F/Col_nest-1R for the first PCR, and Col_nest-2F/Col_nest-2R for the second PCR. M, 100-bp ladder (Faith Biotechnology Co., Ltd).

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reduce the non-target signals, we tested different ratios of the outer and inner primers ranging from 1:1 to 1:100. When the ratio was 1:1 or 1:2, all four bands appeared. A single band of the expected size (151 bp) was observed for ratios 1:5, 1:10, and 1:20 (Fig 4). Notably, the signal was stronger with ratios of 1:5 and 1:10.

Field survey of *Colletotricum* spp. on symptomless strawberry plants in nurseries

From 2019 to 2020, 747 asymptomatic leaf samples collected from 18 strawberry nurseries were tested for strawberry anthracnose pathogens. The number of samples per nursery ranged from 6 to 70 (average 42 samples/nursery) (Table 2). Using our nested PCR assay, the detection rates ranged from 0% to 100% (average 20%); using the SDEI method [30], the detection rates ranged from 5% to 90% (average 45%) (Table 2). For the samples from 16 out of 18 nurseries, the detection rates from the nested PCR assay were lower than those from the SDEI method.

Discussion

Symptomless runner plants carrying the inoculum of *Colletotrichum* spp. is an important route for the spread of strawberry anthracnose from the nursery to the field [23,33]. In Taiwan, *C. siamense* and *C. fructicola* are the most prevalent and virulent strawberry anthracnose pathogens [14]. Several PCR-based methods (conventional PCR, nested-PCR, and quantitative

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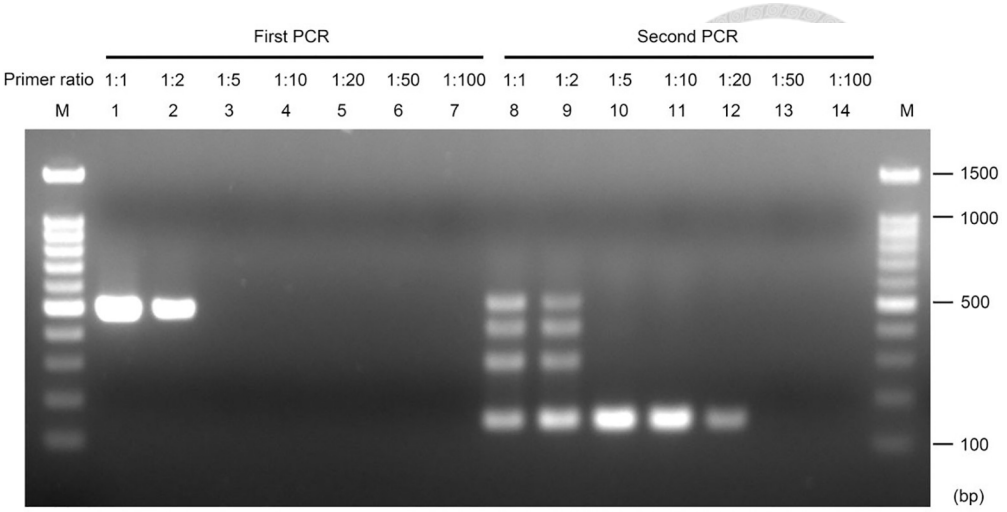

Fig 4. Optimization of the primer ratio for the nested PCR assay. Lanes 1–7, first PCR product; lanes 8–14, second PCR product. The nested PCR assay was performed using primers Col_nest-1F/Col_nest-1R for the first PCR, and Col_nest-2F/Col_nest-2R for the second PCR. Primer ratio represents the ratio of the concentrations of outer primers (first PCR) to inner primers (second PCR). The genomic DNA of *C. siamense* ML133 was used as the template. M, 100-bp ladder (Faith Biotechnology Co., Ltd).

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PCR) and culture-based methods (incubation of leaves treated with ethanol, herbicide, or freezing) have been developed for the diagnosis or detection of strawberry anthracnose [21,23–33]. However, a highly sensitive PCR-based detection method was previously not available for *C. siamense* and *C. fructicola*. In previous studies, PCR primers for detecting *Colletotrichum* spp. associated with strawberry were designed using the ITS, *TUB2*, or *MAT1-2* as the template [21,25,27,28,32,33]. However, among *Colletotrichum* spp., there is a high degree of sequence similarity between phylogenetic markers (ITS, *CHS-1*, *ACT*, *TUB2*, *CAL*, and ApMAT). The non-coding regions of *CHS-1*, *ACT*, *TUB2*, and *CAL* are more variable but too short (mostly $<$ 100 bp) for designing highly specific nested PCR primers. In this study, we conducted comparative genomic analysis and identified an intergenic region between *ladA* and *xyl1* that was ideal for distinguishing *C. siamense* and *C. fructicola* from the other 16 *Colletotrichun* spp. *In silico* analysis and actual tests suggested that our newly developed nested PCR assay could detect pathogenic *C. siamense* and *C. fructicola*, but not other strawberry pathogens (*Botrytis* sp., *Fusarium* spp., *Neopestalotiopsis rosae*, and *Phytophthora* sp.) or ubiquitous saprophytes (*Fusarium* spp. and *Trichoderma* spp.). Although *C. boninense*, *C. karstii*, and *C. miaoliense* (the other three *Colletotrichun* spp. causing strawberry anthracnose in Taiwan) were not detectable, the assay is expected to detect most cases of latent infection that can lead to serious disease. *C. boninense*, *C. karstii*, and *C. miaoliense* are present in Taiwan at low percentage (total 14%) and cause tiny lesions (0.07–0.35 cm in diameter) only on wounded leaves even under a conducive high temperature (30˚C) condition [14]. The assay can detect as low as 1 pg genomic DNA, which corresponds to ~15 cells of the pathogen. The ratio of the concentrations of nested primer pairs is critical for specificity [46,47], and the optimal outer and inner primer ratio for our nested PCR assay is 1:10. The high sensitivity and specificity of this assay allows the detection of trace amounts of pathogenic *C. siamense* and *C. fructicola*, without the problem of unexpected PCR products amplification.

Anthracnose spores are mainly disseminated by rain and overhead irrigation water. Older leaves at lower positions have more chances to be exposed to the pathogen inoculum; therefore, they are more likely to be infected than younger leaves at higher positions. In previous

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studies, old strawberry leaves/petioles were used as materials for detecting latent anthracnose infection [22,29]. Since older leaves are often removed by farmers for pest control purposes, they are good materials available all year round for detecting the source of pathogen inoculum.

Strawberry is propagated from stolons (runners) and transplanted in the form of runner plants. In our field survey conducted from 2019 to 2020, the nested PCR assay detected *C. siamense* and *C. fructicola* in an average of 20% of symptomless mother plants (Table 2). The percentage of plants latently infected or carrying the pathogen inoculum on surface was > 20% in 6 out of 18 nurseries. This reflects the severe epidemic of strawberry anthracnose in recent years [2,14] and indicates the importance of early detection and removal of latently infected mother plants before they are used for propagation. In 16 out of 18 nurseries, higher detection rates were observed using the culture-based SDEI method, perhaps because the nested PCR assay targets only two of five known pathogenic *Colletotrichum* spp. and only the basal petiole was assayed, whereas the SDEI method nonspecifically detects any viable *Colletotrichum* spp. that forms conidial masses on the whole leaf. In the remaining two nurseries (sites 6 and 10), higher detection rates were observed using the nested PCR assay than the SDEI method. This could be due to more frequent usage of fungicides or the farmers just sprayed fungicides before our sampling. When most *Colletotrichum* spp. were killed, the dead cells could only be detected by the nested PCR assay.

The use of overhead irrigation in strawberry nurseries or open field cultivation often increases the latent *Colletotrichum* spp. infection rate in strawberry runner plants, which leads to disease outbreaks in fruit-producing fields [48,49]. In some strawberry nurseries in Taiwan, the frequency of spraying fungicides can be as high as once every three days during the six-month nursery period. Frequent application of fungicides not only increases production costs but also causes the emergence of fungicide resistance in the pathogen population [22,48]. To prevent the spread of diseases and improve the health of runner plants produced by strawberry nurseries, the Council of Agriculture (COA) in Taiwan has established a voluntary pathogen-free certification system for strawberry propagation in 2018. According to the guidelines, anthracnose is one of the key diseases required to be tested and excluded from strawberry propagation. The nested PCR assay developed in this study has been applied for certification of 'pathogen-free' strawberry plants, from which healthy mother plants and runner plants can be supplied to farmers. The use of healthy plant materials combined with integrated control measures will contribute to the production of safe and high-quality strawberries, which is a win-win situation for both producers and consumers.

Supporting information

S1 Fig. Alignment of the spacer region sequences from different *Colletotrichum* spp. The locations of the nested PCR primers in the alignment region (indicated by arrows) are 381–401 bp (Col_nest-1F), 912–930 bp (Col_nest-1R), 510–529 bp (Col_nest-2F), and 645–680 bp (Col_nest-2R). Parts of the sequences of the L-arabinitol 4-dehydrogenase (*ladA*) and NAD(P)H-dependent D-xylose reductase (*xyl1*) genes are also shown.
(TIF)

S1 Table. Fungal isolates used in this study.
(DOCX)

S2 Table. List of *Colletotrichum* spp. genomes used for comparative genomics analysis in this study.
(XLSX)

S3 Table. List of *Trichoderma* spp. genomes used in this study.
(XLSX)

S4 Table. List of *Fusarium* spp. genomes used in this study.
(XLSX)

S1 Raw images.
(PDF)

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