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Identification and characterization of *Colletotrichum* species associated with mango anthracnose in Taiwan

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Abstract Mango is widely grown in Taiwan and anthracnose is one of the most important diseases of this crop. The aim of this study was to investigate *Colletotrichum* species associated with mango and the pathogenicity of these fungal species. From 2006 to 2017, mango tissue from 33 mango orchards were collected. Eighty-seven isolates associated with mango were analyzed preliminarily by comparing partial glyceraldehyde-3-phosphate dehydrogenase sequences. Four species belonging to *C. gloeosporioides* complex were preliminarily identified, namely *C. asianum* (68 isolates), *C. fructicola* (four isolates), *C. siamense* (eight isolates) and *C. tropicale* (two isolates). The other five isolates were identified as belonging to the *C. acutatum* complex. Ten isolates, belonging to different *Colletotrichum* species according to glyceraldehyde-3-phosphate dehydrogenase sequences prediction, were used for further morphology and multi-gene phylogenetic analysis. Five species were identified, namely *C. asianum*, *C. fructicola*, *C. siamense*, *C. tropicale* and *C. scovillei*. All five species showed pathogenicity on fruit, and *C. asianum* isolates C-1076 and C-1646 as well as *C. siamense* isolate C-526 caused larger lesions than the other isolates. On mango leaves, *C. asianum*, *C. fructicola*, *C. siamense* and *C. scovillei* isolates were pathogenic, while *C. tropicale* isolates, C-141 and C-303, failed to cause significant foliar lesions. In addition, *C. siamense* isolates C-526 and C-848 caused significantly larger lesions on leaves than other isolates. This study reports the identification and pathogenicity of *Colletotrichum* species related to mango anthracnose in Taiwan.

Keywords *Colletotrichum* spp. · Multi-gene phylogenetic analysis · Mango anthracnose · Pathogenicity test

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Introduction

Mango (*Mangifera indica*) is an important fruit crop in Taiwan. According to the Agricultural Statistics Yearbook 2018 (https://agrstat.coa.gov.tw/sdweb/public/book/Book.aspx), the total planted area in Taiwan in 2018 was 16,109 ha, and 146,672 metric tons of mango fruits were produced, with the value of over 243 million US dollars. The main mango-planting areas are Tainan, Pingtung, Kaohsiung, and Chiayi Counties.

The main cultivar planted in Taiwan, Irwin, is very sensitive to anthracnose, which is caused by *Colletotrichum* species. The disease attacks the leaves, flowers and fruits of Irwin. On young plant tissues, small red spots appear first, and gradually the spots expand and become larger black necrotic lesions. In addition, post-harvest outbreaks of anthracnose symptoms substantially shorten the shelf life of mature mango fruits (Arauz 2000).

In the past, morphological characteristics were relied upon to identify *Colletotrichum* species. However, since the shapes and sizes of spores are similar among *Colletotrichum* species and colony diversity is observed under different growth conditions, it is hard to distinguish *Colletotrichum* species solely by morphology. Therefore, multiple-gene phylogenetic analysis has been introduced to separate *Colletotrichum* species. For example, Weir et al. (2012) used a set of nuclear gene regions, actin (ACT), calmodulin (CAL), chitin synthase (CHS), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and the ribosomal internal transcribed spacer (ITS) to distinguish species within the *C. gloeosporioides* complex. On other research, ACT, CHS, GAPDH, histone H3, ITS and beta-tubulin (TUB) were used to analyze species within the *C. acutatum* complex (Damm et al. 2012).

In the past, mango anthracnose was considered to be caused by *C. gloeosporioides* and *C. acutatum* (Dodd et al. 1997; Freeman et al. 1998; Jayasinghe and Fernando 2009), and these species were also reported as the causal agents of mango anthracnose in Taiwan (Ann 1995; Weng and Chuang 1995). However, with the development of phylogenetic analysis, recent studies have demonstrated that mango anthracnose is caused by multiple *Colletotrichum* species. For instance, in northeastern Brazil, there are five species, *C. asianum*, *C. fructicola*, *C. tropicale*, *C. karstii* and *C. dianesei*, responsible for mango anthracnose (Lima et al. 2013). In Guangxi, China, *C. asianum*, *C. fructicola*, *C. siamense* and *C. scovillei* were reported to cause anthracnose (Mo et al. 2018; Qin et al. 2019). In Taiwan, *C. asianum*, *C. fructicola*, *C. tropicale* and *C. siamense* have also been indicated as pathogens of mango anthracnose (Lin 2018).

Anthracnose pathogens used in this study were collected from 33 orchards located in main mango producing areas in Taiwan from 2006 to 2017. There were two main objectives in this study. The first objective was to identify the fungal species associated with mango anthracnose in Taiwan by using multi-gene phylogenetic analysis methods, and the second was to evaluate the pathogenicity of different fungal species on leaves and fruits.

Materials and methods

Fungal isolation

Mango fruits, stems and pedicels from diseased and healthy plants were collected from 33 orchards in different areas in Taiwan in 2006–2017, Chiayi, Tainan, Kaohsiung and Pingtung, and *Colletotrichum* species were isolated from these plant tissues. For surface disinfection, fruits with anthracnose symptoms were rinsed with 75% ethanol and air-dried. Stems and pedicels were cut into 1-cm-long fragments, soaked for 30 s in 75% ethanol and 30 s in 0.6% NaOCl, and then rinsed twice with sterilized water. After disinfection, the pieces of stem and pedicels were plated onto acidified potato dextrose agar [APDA, 300 mL potato dextrose agar (PDA) with 750 μL 50% (*v*/v) lactic acid] and incubated for 7 d under 25 °C. The margins of colonies were transferred to PDA. After sporulation, for each isolate, an ooze of spores was mixed with a drop of sterilized water and spread on water agar using a wire loop. A germinating spore was cut from water agar to obtain a single colony.

DNA extraction, PCR and DNA sequencing

For DNA extraction, the mycelium of each isolate was collected from the surface of PDA and ground in 0.5 N NaOH with an electronic grinder. After centrifugation, the supernatant was mixed with 0.1 M Tris buffer (pH 8.0) in a ratio of 1:9 (*v*/v) and used as the DNA template for PCR (White et al. 1990).

PCR amplification of the ACT, CAL, CHS, GAPDH, ITS and TUB genes was carried out using the primer pairs ACT-512F and ACT-783R (Carbone and Kohn 1999), CL1C and CL2C (Weir et al. 2012), CHS-79F and CHS-345R (Carbone and Kohn 1999), GDF and GDR (Templeton et al. 1992), ITS-1F (Gardes and Bruns 1993) and ITS-4 (White et al. 1990), and T1 (O'Donnell and Cigelnik 1997) and Bt2b (Glass and Donaldson 1995), respectively. The PCR mixtures contained 11.9 μL of double-distilled sterilized water, 4 μL of 5× Phusion HF buffer, 0.4 μL of dNTPs (2.5 mM each dNTP), 1 μL of each primer (10 μM), 0.2 μL (2 U) of Phusion High-fidelity DNA polymerase (ThermoFisher, USA), and 1.5 μL of DNA template. The reactions were performed in a ThermoFisher SensoQuest Labcycler thermal cycler. The PCR program for all genes was 98 °C for 30 s, followed by 35 cycles of 98 °C for 10 s, 52 °C for 30 s, 72 °C for 30 s, and 72 °C for 10 min. The amplification products were sent to Tri-I Biotech Incorporation Company for purification and sequencing, and the sequences for each isolate in this study were deposited in GenBank (Table 1).

Table 1 A subsample of isolates of *Colletotrichum* spp., obtained from mango in Taiwan, that were used in this study, and their GenBank accession numbers

		Culture accession numbers[z]		Genbank accession number[y]					
Isolate	Location		Tissues	ACT	CAL	CHS-1	GAPDH	ITS	TUB
C. asianum									
C-1076	Jiadong, Pingtung	FU31254	young fruit[x]	MK462966	MK497049	MK347246	MK376934	MK318769	–
C-1646	Yujing, Tainan	FU31255	mature fruit	MK462967	MK497050	MK347247	MK376935	MK326570	–
C. fructicola									
C-517	Chiayi County, Chiayi	FU31256	mature fruit	MK358124	MK497051	MK347248	MK473907	MK326583	–
C-557	Chiayi County, Chiayi	FU31257	mature fruit	MK358125	MK497052	MK347249	MK473908	Mk326868	–
C. siamense									
C-526	Guantian, Tainan	FU31252	mature fruit	MK358126	MK387144	MK347250	MK473909	MK326903	–
C-848	Fangshan, Pingtung	FU31253	mature fruit	MK358127	MK387145	MK347251	MK473910	MK326904	–
C. tropicale									
C-141	Yujing, Tainan	FU31250	pedicel	MK358128	MK387146	MK520816	MK376936	MK329245	–
C-303	Jiali, Tainan	FU31251	mature fruit	MK358129	MK387147	MK520817	MK376937	MK327139	–
C. scovillei									
C-212	Jiaxian, Kaohsiung	FU31258	mature fruit	MK462968	–	–	MK473911	MK327142	MK462970
C-1428	Chiayi Agricultural Experiment Station	FU31259	young fruit	MK462969	–	–	MK473912	MK329246	MK462971

[z] Culture accession numbers were provided by Bioresource Collection and Research Center, Hsinchu, Taiwan

[y] *ACT* actin gene, *CAL* calmodulin gene, *CHS* chitin synthetase gene, *GAPDH* glyceraldehyde-3-phosphate dehydrogenase gene, *ITS* ribosomal internal transcribed spacer region, *TUB* β-tubulin gene

[x] Young fruit are green fruit with diameter about 1 cm; mature fruit indicates post-harvest mature fruit

Phylogenetic analysis

Bayesian inference was used to construct phylogenetic trees. The ACT, CAL, CHS, GAPDH and ITS nuclear gene regions were used in *C. gloeosporioides* species complex analysis, and ACT, ITS and TUB were used in *C. acutatum* species complex analysis. Sequences of *Colletotrichum*-type species from the GenBank database were included in the analysis (Table 2). Multiple sequence alignment of each gene was conducted using ClustalX v. 2.1 (Larkin et al. 2007), and these alignments were concatenated using SequenceMatrix v. 1.7.8 (Vaidya et al. 2011). jModeltest (Posada 2008) was used to choose the best-fit DNA substitution model under the Bayesian information criterion (BIC) (Table 3). Phylogenetic tree construction was conducted with MrBayes v. 3.2.6 (Ronquist et al. 2012). The analysis was run twice for 5×10^7 generations, and samples were taken from the posterior every 10,000 generations. The first 25% of generations were discarded as burn-in. Convergence of all parameters was checked using the Tracer program (Rambaut and Drummond 2007).

Morphology

For colony observation, isolates were grown on PDA (Merck, Germany) for 7 days at 25 °C under near-UV fluorescent tubes (12 h light/12 h dark). The lengths and widths of 50 conidia from each isolate grown on PDA were observed and measured. To measure the growth rate, five plates of each isolate were incubated at 25 °C in darkness, and the diameters of colonies were measured after 5 and 7 days of incubation. These values were used to calculate the growth rate.

Pathogenicity tests

Pathogenicity tests were conducted on detached young leaves and mature fruits of mango (cv. Irwin).

For the leaf inoculation assay, detached, asymptomatic young leaves were disinfected with 75% ethanol and air-dried. The inoculum was grown on water agar (WA, 2% agar) plates for 5 days in darkness. Two stab wounds were made on both sides of a leaf using a sterilized needle, and one hyphal disc 5 mm in diameter was cut from the WA plate and placed on each wound. Five leaves were inoculated for each isolate. WA discs without fungi were used as a negative control. Inoculated leaves were placed on flat shelves and incubated in sealed plastic boxes with water on the bottom at 25b°C for 5 days, and the diameters of necrotic areas were measured.

On fruits, isolates were incubated on PDA for 5 days at 25 °C, and 5-mm-diameter agar discs were cut from the margins of the colonies for inoculation. For surface disinfection, half-ripe fruits were first soaked in warm water (58 °C) for 2 min, and then soaked immediately in water at room temperature to avoid heat damage. After air-drying, fruits were sealed in paper boxes with 450 g of solid calcium carbide (CaC_2) for 2 days to ripen the fruits. Fruits were rinsed with 75% ethanol and air-dried before inoculation. A stab wound was made on each fruit with a sterilized needle, and a hyphal disc was placed on the wound. Ten fruits were inoculated for each isolate, and PDA discs were used as a negative control. The inoculated fruits were sealed in plastic bags with open petri dishes contained water for 2 days. The lesion diameter was measured at 7 days post-inoculation to assess the aggressiveness of different isolates. Differences in aggressiveness caused by *Colletotrichum* species were examined by one-way ANOVA, and means were compared using Fisher's LSD test. An F value with $P < 0.05$ was considered significant. For each isolate, the margin of the lesion was cut and incubated on APDA agar to complete Koch's postulate.

Results

Fungal isolation

A total of 682 isolates from 560 samples were obtained. Eighty-seven isolates were chosen arbitrarily for initial analysis based on partial sequences of the GAPDH gene. By performing BLASTn searches of these sequences against the GenBank database, the 87 isolates were preliminarily classified into five *Colletotrichum* species. Four species belonged to the *C. gloeosporioides* species complex: *C. asianum* (68 isolates), *C. fructicola* (four isolates), *C. siamense* (eight isolates), and *C. tropicale* (two isolates). In addition, there were five isolates belonging to the *C. acutatum* species complex.

Phylogenetic analysis

Ten isolates (two each of five different *Colletotrichum* species) based on GAPDH classification were chosen

Table 2 *Colletotrichum* spp. used as comparison in phylogenetic analysis, with the host, location, and GenBank accession numbers of gene sequences

			Genbank accession number[z]					
	host	country	ITS	GAPDH	CAL	ACT	CHS-1	TUB
C. gloeosporioides species complex								
C. aenigma ICMP 18608	*Persea americana*	Israel	JX010244	JX010044	JX009683	JX009443	JX009774	–
C. aeschynomenes ICMP 17673	*Aeschynomene virginica*	USA	JX010176	JX009930	JX009721	JX009483	JX009799	–
C. alatae CBS 304.67	*Dioscorea alata*	India	JX010190	JX009990	JX009738	JX009471	JX009837	–
C. alienum ICMP 12071	*Malus domestica*	New Zealand	JX010251	JX010028	JX009654	JX009572	JX009882	–
C. aotearoa ICMP 18537	*Coprosma* sp.	New Zealand	JX010205	JX010005	JX009611	JX009564	JX009853	–
C. asianum ICMP 18696	*Mangifera indica*	Australia	JX010192	JX009915	JX009723	JX009576	JX009753	–
C. asianum ICMP 18605	*Mangifera indica*	Thailand	JX010194	JX010021	JX009726	JX009465	JX009787	–
C. asianum CBS 130418	*Coffea arabica*	Thailand	FJ972612	JX010053	FJ917506	JX009584	JX009867	–
C. boninense CBS 123755	*Crinum asiaticum* var. *sinicum*	Japan	JX010292	JX009905	HM582004	JX009583	JX009827	–
C. clidemiae ICMP 18658	*Clidemia hirta*	USA, Hawaii	JX010265	JX009989	JX009645	JX009537	JX009877	–
C. cordylinicola ICMP 18579	*Cordyline fruticosa*	Thailand	JX010226	JX009975	HM470238	HM470235	JX009864	–
C. fructicola ICMP 12568	*Persea americana*	Australia	JX010166	JX009946	JX009680	JX009529	JX009762	–
C. fructicola ICMP 18613	*Limonium sinuatum*	Israel	JX010167	JX009998	JX009675	JX009491	JX009772	–
C. fructicola ICMP 18610	*Pyrus pyrifolia*	Japan	JX010174	JX010034	JX009681	JX009526	JX009788	–
C. fructicola ICMP 18581	*Coffea arabica*	Thailand	JX010165	JX010033	FJ917508	FJ907426	JX009866	–
C. fructicola (syn. *C. ignotum*) CBS 125397	*Tetragastris panamensis*	Panama	JX010173	JX010032	JX009674	JX009581	JX009874	–
C. fructicola (syn. *Glomerella cingulata var. minor*) CBS 238.49	*Ficus edulis*	Germany	JX010181	JX009923	JX009671	JX009495	JX009839	–
C. gloeosporioides ICMP 17821	*Citrus sinensis*	Italy	JX010152	JX010056	JX009731	JX009531	JX009818	–
C. gloeosporioides (syn. *Gloeosporium pedemontanum*) CBS 273.51	*Citrus limon*	Italy	JX010148	JX010054	JX009745	JX009558	JX009903	–
C. hippeastri CBS 241.78	*Hippeastrum* sp.	Netherlands	JX010293	JX009932	JX009740	JX009485	JX009838	–
C. horii ICMP 12942	*Diospyros kaki*	New Zealand	GQ329687	GQ329685	JX009603	JX009533	JX009748	–
C. horii ICMP 10492	*Diospyros kaki*	Japan	GQ329690	GQ329681	JX009604	JX009438	JX009752	–
C. kahawae subsp. *ciggaro* ICMP 18539	*Olea europaea*	Australia	JX010230	JX009966	JX009635	JX009523	JX009800	–
C. kahawae subsp. *ciggaro* (syn. *Glomerella cingulata* var. *migrans*) ICMP 17922	*Hypericum perforatum*	Germany	JX010238	JX010042	JX009636	JX009450	JX009840	–
	Vaccinium sp.	USA	JX010228	JX009950	JX009744	JX009536	JX009902	–

Table 2 (continued)

	host	country	Genbank accession number[z]					
			ITS	GAPDH	CAL	ACT	CHS-1	TUB
C. kahawae subsp. *ciggaro* (syn. *Glomerella rufomaculans* var. *vaccinii*) CBS 124.22								
C. kahawae subsp. Kahawae ICMP 17816	*Coffea arabica*	Kenya	JX010231	JX010012	JX009642	JX009452	JX009813	–
C. musae CBS 116870	*Musa* sp.	USA	JX010146	JX010050	JX009742	JX009433	JX009896	–
C. nupharicola CBS 470.96	*Nuphar lutea subsp. polysepala*	USA	JX010187	JX009972	JX009663	JX009437	JX009835	–
C. psidii CBS 145.29	*Psidium* sp.	Italy	JX010219	JX009967	JX009743	JX009515	JX009901	–
C. queenslandicum ICMP 1778	*Carica papaya*	Australia	JX010276	JX009934	JX009691	JX009447	JX009899	–
C. salsolae ICMP 19051	*Salsola tragus*	Hungary	JX010242	JX009916	JX009696	JX009562	JX009863	–
C. siamense ICMP 12565	*Persea americana*	Australia	JX010249	JX009937	JX009698	JX009571	JX009760	–
C. siamense ICMP 18121	*Dioscorea rotundata*	Nigeria	JX010245	JX009942	JX009715	JX009460	JX009845	–
C. siamense ICMP 18739	*Carica papaya*	South Africa	JX010161	JX009921	JX009716	JX009484	JX009794	–
C. siamense ICMP 18578	*Coffea arabica*	Thailand	JX010171	JX009924	FJ917505	FJ907423	JX009865	–
C. siamense (syn. *C. hymenocallidis*) CBS 125378	*Hymenocallis americana*	China	JX010278	JX010019	JX009709	GQ856775	GQ856730	–
C. siamense (syn. *C. jasmini-sambac*) CBS 130420	*Jasminum sambac*	Vietnam	HM131511	HM131497	JX009713	HM131507	JX009895	–
C. theobromicola CBS 124945	*Theobroma cacao*	Panama	JX010294	JX010006	JX009591	JX009444	JX009869	–
C. theobromicola (syn. *C. fragariae*) CBS 142.31	*Fragaria* × *ananassa*	USA	JX010286	JX010024	JX009592	JX009516	JX009830	–
C. theobromicola (syn. *C. gloeosporioides f. stylosanthis*) CBS 124251	*Stylosanthes viscosa*	Australiax	JX010289	JX009962	JX009597	JX009575	JX009821	–
C. ti ICMP 4832	*Cordyline* sp.	New Zealand	JX010269	JX009952	JX009649	JX009520	JX009898	–
C. tropicale ICMP 18672	*Litchi chinensis*	Japan	JX010275	JX010020	JX009722	JX009480	JX009826	–
C. tropicale CBS 124949	*Theobroma cacao*	Panama	JX010264	JX010007	JX009719	JX009489	JX009870	–
C. tropicale ICMP 18651	*Annona muricata*	Panama	JX010277	JX010014	JX009720	JX009570	JX009868	–
C. xanthorrhoeae CBS 127831	*Xanthorrhoea preissii*	Australia	JX010261	JX009927	JX009653	JX009478	JX009823	–
Glomerella cingulata "f.sp. *Camelliae*" ICMP 18542	*Camellia sasanqua*	USA	JX010223	JX009994	JX009628	JX009488	JX009857	–
C. acutatum species complex								
C. acerbum CBS 128530	*Malus domestica*	New Zealand	JQ948459	–	–	JQ949780	–	JQ950110
C. acutatum CBS 127545	*Aspalathus linearis*	South Africa	JQ948383	–	–	JQ949704	–	JQ950034
C. acutatum CBS 144.29	*Capsicum annuum*	Sri Lanka	JQ948401	–	–	JQ949722	–	JQ950052

Table 2 (continued)

	host	country	Genbank accession number[z]					
			ITS	GAPDH	CAL	ACT	CHS-1	TUB
C. acutatum CBS 112996	*Carica papaya*	Australia	JQ005776	–	–	JQ005839	–	JQ005860
C. acutatum CBS 369.73	*Lupinus angustifolius*	New Zealand	JQ948350	–	–	JQ949671	–	JQ950001
C. australe CBS 116478	*Trachycarpus fortunei*	South Africa	JQ948455	–	–	JQ949776	–	JQ950106
C. australe CBS 131325	*Hakea* sp.	Australia	JQ948456	–	–	JQ949777	–	JQ950107
C. brisbanense CBS 292.67	*Capsicum annuum*	Australia	JQ948291	–	–	JQ949612	–	JQ949942
C. chrysanthemi IMI 364540	*Chrysanthemum coronarium*	China	JQ948273	–	–	JQ949594	–	JQ949924
C. chrysanthemi CBS 126518	*Carthamus* sp.	Netherlands	JQ948271	–	–	JQ949592	–	JQ949922
C. chrysanthemi CBS 126519	*Chrysanthemum coronarium*	Netherlands	JQ948272	–	–	JQ949593	–	JQ949923
C. cosmi CBS 853.73	*Cosmos* sp.	Netherlands	JQ948274	–	–	JQ949595	–	JQ949925
C. costaricense CBS 330.75	*Coffea arabica*	Costa Rica	JQ948180	–	–	JQ949501	–	JQ949831
C. costaricense CBS 211.78	*Coffea* sp.	Costa Rica	JQ948181	–	–	JQ949502	–	JQ949832
C. cuscutae IMI 304802	*Cuscuta* sp.	Dominica	JQ948195	–	–	JQ949516	–	JQ949846
C. fioriniae CBS 125396	*Malus domestica*	USA	JQ948299	–	–	JQ949620	–	JQ949950
C. fioriniae CBS 129947	*Vitis vinifera*	Portugal	JQ948343	–	–	JQ949664	–	JQ949994
C. godetiae CBS 133.44	*Clarkia hybrida*, cv. Kelvon Glory	Denmark	JQ948402	–	–	JQ949723	–	JQ950053
C. godetiae CBS 129942	*Mahonia aquifolium*	Germany	JQ948428	–	–	JQ949749	–	JQ950079
C. guajavae IMI 350839	*Psidium guajava*	India	JQ948270	–	–	JQ949591	–	JQ949921
C. indonesiense CBS 127551	*Eucalyptus* sp.	Indonesia	JQ948288	–	–	JQ949609	–	JQ949939
C. johnstonii IMI 357027	*Citrus* sp.	New Zealand	JQ948443	–	–	JQ949764	–	JQ950094
C. johnstonii CBS 128532	*Solanum lycopersicum*	New Zealand	JQ948444	–	–	JQ949765	–	JQ950095
C. kinghornii CBS 198.35	*Phormium* sp.	UK	JQ948454	–	–	JQ949775	–	JQ950105
C. laticiphilum CBS 112989	*Hevea brasiliensis*	India	JQ948289	–	–	JQ949610	–	JQ949940
C. laticiphilum CBS 129827	*Hevea brasiliensis*	Colombia	JQ948290	–	–	JQ949611	–	JQ949941
C. limetticola CBS 114.14	*Citrus aurantifolia*	USA, Florida	JQ948193	–	–	JQ949514	–	JQ949844
C. lupini CBS 507.97	*Lupinus albus*	France	JQ948166	–	–	JQ949487	–	JQ949817
C. lupini CBS 109225	*Lupinus albus*	Ukraine	JQ948155	–	–	JQ949476	–	JQ949806

Table 2 (continued)

	host	country	Genbank accession number[z]					
			ITS	GAPDH	CAL	ACT	CHS-1	TUB
C. melonis CBS 159.84	*Cucumis melo*	Brazil	JQ948194	–	–	JQ949515	–	JQ949845
C. nymphaeae CBS 119294	*Leucaena* sp.	Mexico	JQ948205	–	–	JQ949526	–	JQ949856
C. nymphaeae CBS 515.78	*Nymphaea alba*	Netherlands	JQ948197	–	–	JQ949518	–	JQ949848
C. orchidophilum CBS 631.80	*Ascocenda* sp.	USA	JQ948152	–	–	JQ949473	–	JQ949803
C. orchidophilum CBS 632.80	*Dendrobium* sp.	USA	JQ948151	–	–	JQ949472	–	JQ949802
C. paxtonii CBS 502.97	*Musa nana*	"West Indies"	JQ948286	–	–	JQ949607	–	JQ949937
C. paxtonii IMI 165753	*Musa* sp.	Saint Lucia	JQ948285	–	–	JQ949606	–	JQ949936
C. phormii CBS 118201	*Phormium* sp.	New Zealand	JQ948449	–	–	JQ949770	–	JQ950100
C. phormii CBS 118194	*Phormium* sp.	Germany	JQ948446	–	–	JQ949767	–	JQ950097
C. pseudoacutatum CBS 436.77	*Pinus radiata*	Chile	JQ948480	–	–	JQ949801	–	JQ950131
C. pyricola CBS 128531	*Pyrus communis*	New Zealand	JQ948445	–	–	JQ949766	–	JQ950096
C. rhombiforme CBS 129953	*Olea europaea*	Portugal	JQ948457	–	–	JQ949778	–	JQ950108
C. rhombiforme CBS 131322	*Vaccinium macrocarpum*	USA	JQ948458	–	–	JQ949779	–	JQ950109
C. salicis CBS 129356	*Rhododendron* sp.	Latvia, Riga	JQ948470	–	–	JQ949791	–	JQ950121
C. salicis CBS 607.94	*Salix* sp.	Netherlands	JQ948460	–	–	JQ949781	–	JQ950111
C. scovillei CBS 126529	*Capsicum* sp.	Indonesia	JQ948267	–	–	JQ949588	–	JQ949918
C. scovillei CBS 126530	*Capsicum* sp.	Indonesia	JQ948268	–	–	JQ949589	–	JQ949919
C. scovillei CBS 120708	*Capsicum annuum*	Thailand	JQ948269	–	–	JQ949590	–	JQ949920
C. scovillei P-14	*Capsicum* sp.	China	MH306141			MH306142		MH306143
C. scovillei YN51–1	*Mangifera indica*	China	MH636512			MH622590		MH622722
C. simmondsii CBS 122122	*Carica papaya*	Australia	JQ948276	–	–	JQ949597	–	JQ949927
C. simmondsii CBS 111531	*Protea cynaroides*	USA	JQ948282	–	–	JQ949603	–	JQ949933
C. sloanei IMI 364297	*Theobroma cacao*	Malaysia	JQ948287	–	–	JQ949608	–	JQ949938
C. tamarilloi CBS 129814	*Solanum betaceum*	Colombia	JQ948184	–	–	JQ949505	–	JQ949835
C. tamarilloi CBS 129954	*Solanum betaceum*	Colombia	JQ948188	–	–	JQ949509	–	JQ949839
C. walleri CBS 125472	*Coffea* sp.	Vietnam	JQ948275	–	–	JQ949596	–	JQ949926

[z] *ACT* actin gene, *CAL* calmodulin gene, *CHS* chitin synthetase, *GAPDH* glyceraldehyde-3-phosphate dehydrogenase gene, *ITS* the ribosomal internal transcribed spacer gene, *TUB* β-tubulin gene

Table 3 Nucleotide substitution models used in phylogenetic analysis

Gene[z]	DNA substitution model	
	C. gloeosporioides species complex	*C. acutatum* species complex
ACT	TrNef+G	TrNef+G
CAL	TrNef+I	–
CHS	TrNef+G	–
GAPDH	HKY + I + G	–
ITS	TrNef+I	K80 + I
TUB	–	HKY + G

[z] *ACT* actin gene, *CAL* calmodulin gene, *CHS* chitin synthetase gene, *GAPDH* glyceraldehyde-3-phosphate dehydrogenase gene, *ITS* the ribosomal internal transcribed spacer region, *TUB* β-tubulin gene

for multiple-gene phylogenetic analysis. For the *C. gloeosporioides* species complex analysis, the aligned genes boundaries in the alignment were: ACT: 1–309, CAL: 310–1107, CHS: 1108–1406, GAPDH: 1407–1715, ITS: 1716–2349. For *C. acutatum* species complex analysis, the aligned genes boundaries were: ACT: 1–255, ITS: 256–822, TUB: 823–1604. The classification of species belonging to the *C. gloeosporioides* species complex based on the multi-gene phylogenetic tree shown in Fig. 1a was consistent with that based on GAPDH sequences. C-1076 and C-1646 belonged to *C. asianum*, and C-517 and C-557 belonged to *C. fructicola*. C-141 and C-303 were identified as *C. tropicale*, and C-526 and C-848 were identified as *C. siamense*. C-212 and C-1428 were grouped into the same clade as *C. scovillei* in the multi-gene phylogenetic tree (Fig. 1b).

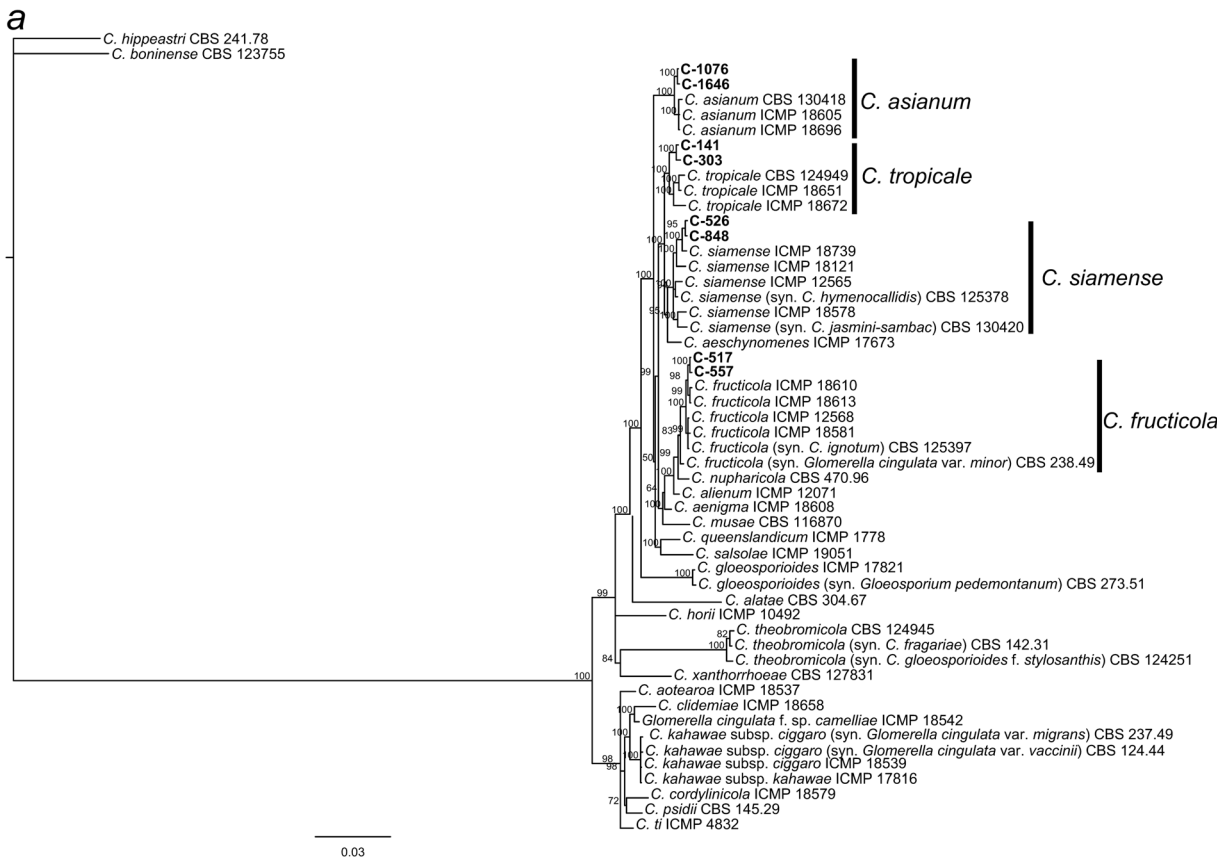


Fig. 1 Bayesian phylogenetic trees of *Colletotrichum* isolates from mango in Taiwan and from GenBank. **a** *C. goloeosporioides* species complex tree was built using concatenated sequences of partial ACT, CAL, CHS, GAPDH and ITS genes. *C. hippeastri* and *C. boninense* were used as outgroups. **b** *C. acutatum* species complex tree was built using concatenated sequences of partial ACT, ITS and TUB genes. Two *C. orchidophilum* strains were used as outgroups. The scale bars indicate the number of expected changes per site. Isolates from this study are emphasized in bold

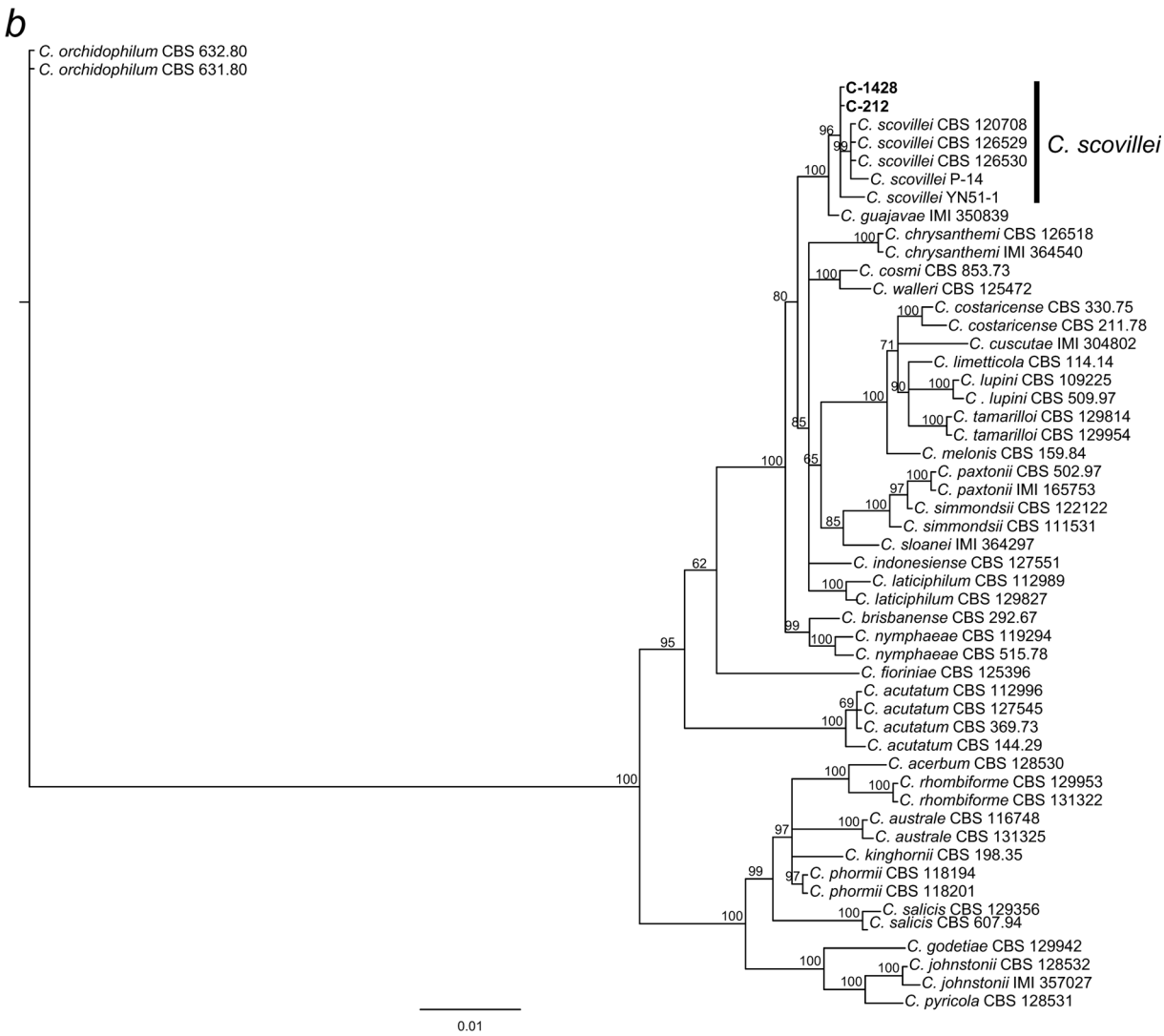
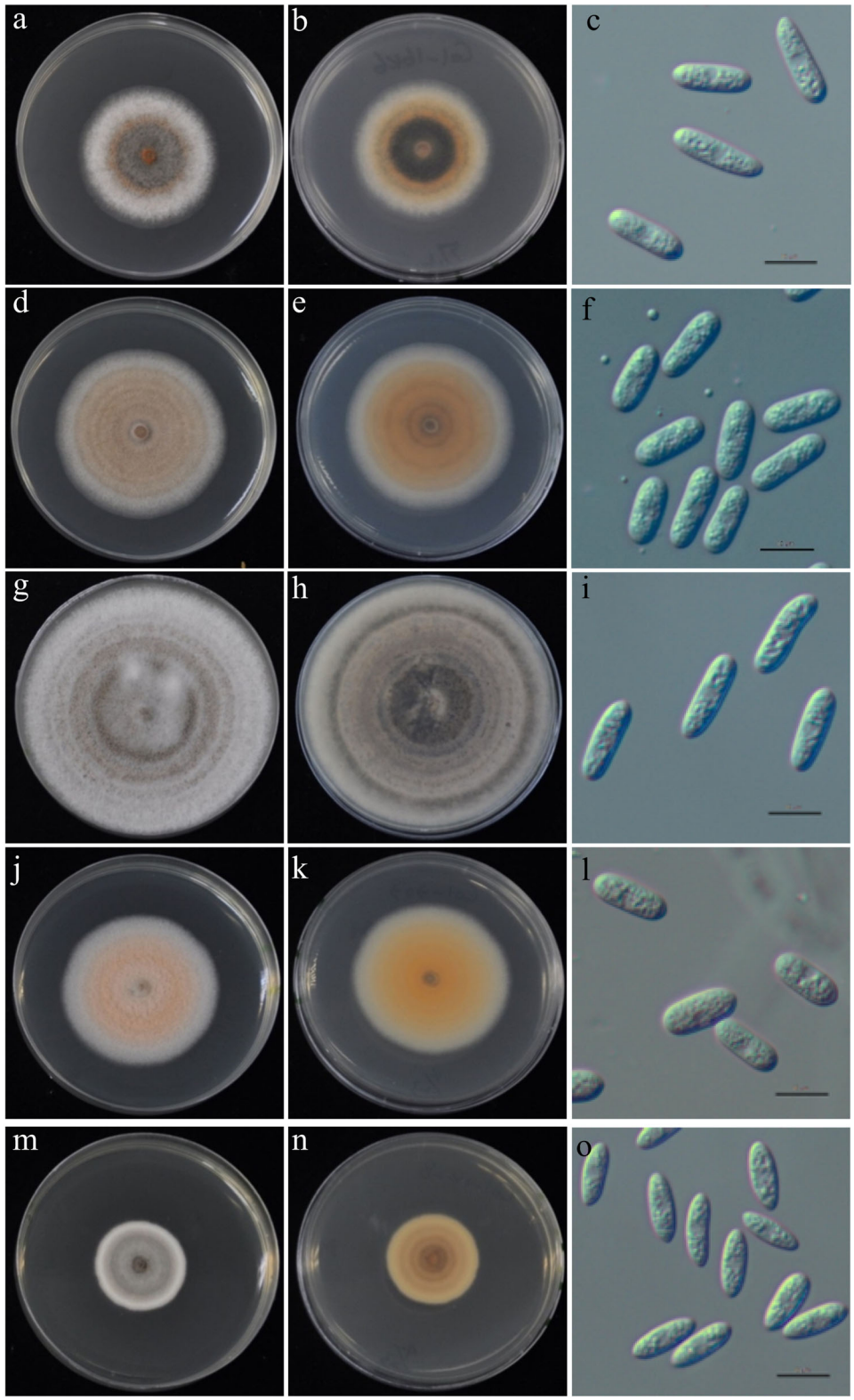


Fig. 1 (continued)

Morphology

Colonies of all isolates were white in color during the first two or three days of cultivation, after which some of them turned gray or salmon in color at the upper or lower surface. Colonies were mainly separated into four groups based on their appearance, but diversity in colony morphology was evident even within the same species (data not shown). Among the 10 isolates investigated, conidial sizes and shapes were similar among the four species belonging to *C. gloeosporioides* species complex (Fig. 2). Conidia of these species were aseptate, hyaline and cylindrical, and size ranged from 13.6 to 16.5 μm long × 5.3 to 6.0 μm wide. Conidia of *C. scovillei* isolates C-212 and C-1428 were hyaline, unicellular and cylindrical to slightly fusiform with both ends acute, and size ranged from 12.4 to 12.8 μm long × 4.6–4.8 μm wide.

Fig. 2 Morphological characteristics of colonies and conidia of mango *Colletotrichum* isolates on PDA at 7 days after incubation. Upper (**a**) and reverse (**b**) sides of PDA plate and conidia (**c**) of *C. asianum* isolate C-1646. Upper (**d**) and reverse (**e**) sides of PDA plate and conidia (**f**) of *C. fructicola* isolate C-517. Upper (**g**) and reverse (**h**) sides of PDA plate and conidia (**i**) of *C. siamense* isolate C-848. Upper (**j**) and reverse (**k**) sides of PDA plate and conidia (**l**) of *C. tropicale* isolate C-303. Upper (**m**) and reverse (**n**) sides of PDA plate and conidia (**o**) of *C. scovillei* isolate C-1428. Bars: 10 μm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Pathogenicity test

All five *Colletotrichum* species were pathogenic on wounded mango fruits with the level of aggressiveness varying among the 10 *Colletotrichum* isolates (Table 4). Typical symptoms were sunken and dark lesions expanding from the inoculation sites. The lesion diameters ranged from 4.9 mm to 21.5 mm. *Colletotrichum asianum* isolates C-1076 and C-1646 and *C. siamense* isolate C-526 caused significantly larger lesions on mango fruits than other isolates, and *C. tropicale* isolates C-141 and C-303 showed relatively weak ability to cause necrosis on fruits. No lesions were observed in the negative control.

On leaves, all *Colletotrichum* species were pathogenic except *C. tropicale*. Small, needle-like spots emerged on leaves near the fungal agar disc 5 days after inoculation with *C. tropicale*. Larger, round lesions appeared 6 days after inoculation, but *C. tropicale* was not isolated from the margins of the round lesions. Therefore, the pathogenicity of *C. tropicale* on mango leaves was uncertain. In contrast, the other four *Colletotrichum* species caused black lesions with clear edges on leaves 5 days post-inoculation, and pathogens were re-isolated from the edges of the lesions, completing Koch's postulates. Agar discs used as a negative control did not cause lesions on leaves.

Discussion

This is the latest large-scale survey of diversity of *Colletotrichum* pathogens associated with mango anthracnose in Taiwan. A recent study by Lin (2018) also surveyed diversity of *Colletotrichum* pathogens associated with mango anthracnose in Taiwan; however, only the abstract of this research is accessible by internet search engines. In this study, five species of *Colletotrichum* (*C. asianum*, *C. fructicola*, *C. scovillei*, *C. siamense* and *C. tropicale*) were found to be associated with mango anthracnose disease, and *C. asianum* was the most commonly isolated species. This result is different from that of an earlier investigation in Taiwan, in which only two species, *C. gloeosporioides* (about 82%) and *C. acutatum* (about 18%), were determined to be the causal agents of mango anthracnose in Taiwan (Weng and Chuang 1995). However, in this study, the 87 strains analyzed were

Table 4 Morphological and aggressiveness descriptions of *Colletotrichum* isolates from mango in this study

Isolate	Conidial size (μm)	Mycelial growth (mm/d)	Lesion diameter on mango leaves (mm)	Lesion diameter on mango fruits (mm)
C. asianum				
C-1076	14.6 ± 1.3 μm × 6.0 ± 0.4 μm	9.4	15.5 c[x]	25.1 a
C-1646	16.0 ± 1.1 μm × 5.3 ± 0.3 μm	9.7	22.9 ab	22.6 b
C. fructicola				
C-517	15.5 ± 0.9 μm × 5.5 ± 0.2 μm	9.9	–[y]	6.0 ef
C-557	–[z]	10.6	19.3 bc	16.2 c
C. siamense				
C-526	15.4 ± 0.8 μm × 5.4 ± 0.3 μm	11.9	23.3 ab	23.4 ab
C-848	16.5 ± 0.9 μm × 5.4 ± 0.2 μm	12.2	24.7 a	18.1 c
C. tropicale				
C-141	14.7 ± 0.8 μm × 5.4 ± 0.3 μm	7.6	–	7.9 e
C-303	13.6 ± 0.6 μm × 5.7 ± 0.2 μm	11.2	–	4.9 f
C. scovillei				
C-212	12.4 ± 1.3 μm × 4.6 ± 0.5 μm	8.0	11.0 d	6.6 ef
C-1428	12.8 ± 1.8 μm × 4.8 ± 0.6 μm	8.4	12.4 cd	10.4 d

[z] C-557 does not produce conidia on PDA plate

[y] C-517, C-141 and C-303 do not cause significant lesions on mango leaves

[x] Columns with different letters differ significantly according to Fisher's LSD test ($P \leq 0.05$)

chosen arbitrarily, which raised the possibility that some *Colletotrichum* species are not identified.

The species mentioned in this paper were also described from other countries or areas. In Brazil, of the five species identified as being pathogens of mango anthracnose (*C. asianum*, *C. fructicola*, *C. tropicale*, *C. karstii* and *C. diansei*) (Lima et al. 2013), three were also found to be associated with mango anthracnose in the current study. In Guangxi, China, *C. asianum*, *C. fructicola*, *C. siamense* and *C. scovillei* are responsible for mango anthracnose disease, and the aggressiveness differed significantly among isolates of the same species (Mo et al. 2018; Qin et al. 2019). *C. scovillei* was reported to cause mango disease only in China and this report currently, which suggests this mango-pathogenic *C. scovillei* might be endemic or restricted in some areas of Asia. In India, the pathogenicity of *C. asianum*, *C. fructicola* and *C. siamense* on mango was documented (Sharma et al. 2013). In addition to the countries mentioned above, *C. asianum*, the most frequently isolated species in this paper, was also identified as a cause of mango anthracnose in South Africa, Sri Lanka and Malaysia (Krishnapillai and Wilson Wijeratnam 2014; Latiffah et al. 2015; Sharma et al. 2015). *Colletotrichum tropicale*, on the contrary, is much less reported, which suggests that this species is not commonly found in mango crops or less aggressive.

Wound inoculation is a common method for evaluating pathogenicity of *Colletotrichum* spp. causing mango anthracnose (Lima et al. 2013; Mo et al. 2018; Sharma et al. 2013). In our study, a pretest of fungal disc inoculation with no wounding was conducted on leaves; *C. siamense* isolate C-848 caused lesions on five of six inoculation points 7 days post inoculation, and *C. asianum* isolate C-1076 caused lesions on all six inoculation points. However, results from wound inoculation were more consistent than from non-wounded inoculations. In nature, wounding on plant surface caused by strong winds or insects is common, and it facilitates the invasion of pathogens. Nevertheless, removing host surface defense may lead to overestimation of pathogen aggressiveness in the field. Further studies should be conducted to determine the aggressiveness of pathogens under non-wounded condition.

All species identified in this study have all been demonstrated to cause anthracnose on other crops. For example, *C. scovillei* was reported as the causal agent of pepper anthracnose in Brazil and China (Caires et al. 2014; Zhao et al. 2016). *C. asianum* and *C. fructicola* were also able to cause lesions on peppers (Phoulivong et al. 2012), and. *C. tropicale* was pathogenic on cassava in Brazil (Oliveira et al. 2019). Phoulivong et al. (2012) found that *C. siamense* isolated from coffee also caused lesions on wounded papaya and orange. Giblin et al. (2018) reported that *C. siamense* infected both mango and avocado without wounding. In Taiwan, *C. siamense* has only been reported to cause pepper spot disease on lychee (Ni et al. 2017) and crown rot disease on strawberry (Chung et al. 2019). However, in other countries, *C. siamense* also infects banana (Kumar et al. 2017), citrus (Cheng et al. 2013), dragon fruit (Meetum et al. 2015) and pear (Fu et al. 2019). All of the fruits mentioned above are important crops in Taiwan and are affected by anthracnose, suggesting that *C. siamense* might be a potential causal agent of anthracnose disease on these fruits in Taiwan. Phoulivong et al. (2012) also found that *C. asianum* and *C. fructicola* were able to cause cross infection by the same isolates among different hosts.

The sensitivity of *Colletotrichum* species on mango to fungicide treatment has been investigated in Taiwan, and resistance has emerged in some species and areas. A previous study based on restriction fragment length polymorphism analysis indicated that fungicide resistance against benzimidazoles occurred in *Colletotrichum* species isolated from mango in Tainan (Lou et al. 2010). A recent study demonstrated that most *Colletotrichum* species on mango show resistance to thiophanate-methyl except *C. tropicale* (Lin 2018). In addition, our investigation of lychee anthracnose also indicated that thiophanate-methyl resistance is present in *Colletotrichum* species collected from post-harvest lychee fruits in Tainan, and single nucleotide polymorphisms related to fungicide resistance in these strains were confirmed (unpublished data). These results and the cross-infection ability of *Colletotrichum* species suggests the possibility that the hazard of fungicide resistance may shift among crops, and this should be taken into consideration by growers when choosing a fungicide for one crop when resistance has been detected in *Colletotrichum* species on other crops.

In conclusion, this study characterized the identity and pathogenicity of the species causing mango anthracnose in Taiwan. These results are the basis of future studies such as diversity of fungicide sensitivity or host range among these anthracnose species. The information from this and future studies will help pathologists and producers enact plant protection management programs.

Compliance with ethical standards

Conflict of interest There are no potential conflicts of interest.

This research is not involving human participants and/or animals Therefore, there is no informed consent needed.

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