Identification and Characterization of *Colletotrichum* Species Associated with Mango Anthracnose in Guangxi, China

Jianyou Mo, Institute of Plant Protection, Guangxi Academy of Agricultural Sciences, Nanning, Guangxi, 530007, China, and Guangxi Key Laboratory of Biology for Crop Diseases and Insect Pests, Nanning, Guangxi, 530007, China; **Guang Zhao**, Department of Guangxi Forestry Pest Management, Nanning, Guangxi, 530028, China; **Qili Li**,[†] **Ghulam S. Solangi**, **Lihua Tang**, **Tangxun Guo**, and **Suiping Huang**, Institute of Plant Protection, Guangxi Academy of Agricultural Sciences, Nanning, Guangxi, 530007, China, and Guangxi Key Laboratory of Biology for Crop Diseases and Insect Pests, Nanning, Guangxi, 530007, China; and **Tom Hsiang**, School of Environmental Sciences, University of Guelph, ON, Canada

Abstract

Mango (*Mangifera indica*) is widely grown across southern China, especially in the provinces of Guangxi, Hainan, Yunnan, Sichuan, and Taiwan. Guangxi itself has over 86,667 ha of mango production. The purpose of this study was to identify *Colletotrichum* species associated with mango in different parts of Guangxi and examine their pathogenicity on leaves and fruits of mango in vitro. Diseased leaves were collected from 25 mango orchards in different areas of Guangxi province. Sixty-five isolates were obtained from mango leaves with anthracnose symptoms, and these were further characterized based on morphology and DNA sequencing. Twenty-nine isolates from different areas were selected for sequencing and analyses of the internal transcribed spacer region, glyceraldehyde-3-phosphate dehydrogenase, partial actin,

β -tubulin, and chitin synthase genomic regions. The most common fungal isolates were these three species: *Colletotrichum asianum*, *C. fructicola*, and *C. siamense*. *C. asianum* was the most common and widely distributed in Guangxi (51.7%), followed by *C. fructicola* (37.9%) and *C. siamense* (10.2%), both found in Tiandong, Tianyang, and Wuming counties. There was no evidence of geographical specialization of the different species. Pathogenicity assays showed that all isolates were pathogenic to mango leaves and fruit (cultivar Tainong). No relationship was found between origin of isolates and their virulence. This is the first description of *C. asianum*, *C. fructicola*, and *C. siamense* as causal agents of mango leaf anthracnose from Guangxi province, China.

Mango, *Mangifera indica* (L.), is considered one of the most important tropical or subtropical fruit crops (Nelson 2008). In China, mango is cultivated mainly in southern and southwestern provinces. The planted area of mango was almost 130,000 ha, producing almost 900,000 tons in China during 2012 (Li et al. 2013). Guangxi province is the largest producing area of mango in China, and the mango industry contributes significantly to the local economy of Guangxi (Li et al. 2013). Anthracnose disease of mango caused by several *Colletotrichum* species is considered the most important disease of mango in China (Hong et al. 2016). Mango anthracnose is mainly caused by *Colletotrichum gloeosporioides*, but sometimes *C. acutatum* has also been reported as the causal agent (Freeman et al. 1998; Jayasinghe and Fernando 2009; Phoulivong et al. 2010). Mango anthracnose appears as irregularly shaped, black necrotic spots on upper and lower sides of mango leaves. Symptoms can also be found on stalks and flowers. Although many parts of the plant may be affected, the major losses occur during ripening and postharvest, when black lesions appear on mango fruit surfaces (Arauz 2000; Freeman et al. 1998).

Identification and characterization of *Colletotrichum* species is usually based on morphological characteristics (Hyde et al. 2009; Photita et al. 2005), such as conidial morphological, production of perithecia (Freeman et al. 2000), and sensitivity to benomyl (Valero et al. 2010). However, morphological differences are not sufficient to separate *Colletotrichum* species because there are differences for the

same specimen when grown in the lab and under different growth conditions. Hence, molecular techniques are needed to separate species of *Colletotrichum* and to examine genetic variation within and between species and populations (Cai et al. 2009; Hyde et al. 2009; Phoulivong et al. 2010). Studies on the systematics of *Colletotrichum* species recently have placed species of this genus into nine major clades plus small clusters and isolated species, with clades probably representing species complexes (Cannon et al. 2012).

The internal transcribed spacer region (ITS) of ribosomal DNA has been frequently used to separate *Colletotrichum* species (Freeman et al. 2000) but not always satisfactorily (Cannon et al. 2012). Other markers, such as actin (ACT), β -tubulin (TUB2), chitin synthase 1 (CHS-1), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene have been used to resolve relationships among *Colletotrichum* species (Weir et al. 2012). Considering recent changes in classification of *Colletotrichum* species, and the lack of information on the identity and characteristics of isolates causing mango anthracnose in China, the objectives of this study were as follows: (i) identify and characterize species of *Colletotrichum* associated with mango anthracnose in Guangxi, China, based on morphology and DNA sequence comparisons; (ii) examine virulence of isolates from different parts of Guangxi province and whether there are geographical differences; and (iii) investigate relationships among isolates of *Colletotrichum* spp. from mango using sequences of ITS, ACT, TUB2, CHS-1, and GAPDH.

Materials and Methods

Fungal isolation. Mango leaves with symptoms of anthracnose were collected from production fields of six counties (Tiandong, Tianyang, Tianlin, Youjiang, Wuming, and Qinzhou) in Guangxi province, ranging over an area of 450 by 300 km. Small (3 × 3-mm) sections were cut from lesion margins and were surface sterilized in 75% ethanol for 15 s and 0.1% (v/v) mercuric chloride for 45 s, followed by rinses in sterile water. The sections were then plated onto potato dextrose agar (PDA) and incubated at 25°C. Fungal growth was examined daily up to 14 days. Isolates were subcultured onto fresh PDA and purified by single-spore culture. Isolates were stored on PDA slants at 4°C.

[†]Corresponding author: Qili Li; E-mail: 65615384@qq.com, liqili@gxaas.net

Funding: The research was supported by National Natural Science Foundation of China (Grant No. 31600029, 31560526), Guangxi Natural Science Foundation (Grant No. 2016GXNSFCB380004), Guangxi Key Laboratory of Biology for Crop Disease and Insect Pests (Grant No. 15-140-45-ST-2), and Foundation for Development of Science and Technology Guangxi Academy of Agricultural Sciences (2016JZ24, 2017JZ01).

Accepted for publication 29 December 2017.

Morphological characterization. Isolates were cultured on PDA plates at 25°C under continuous fluorescent light for 5 days. Plugs (6-mm diameter) from colony margins were placed in the center of each 90-mm-diameter plate with four replications. The colony color and culture diameters (two perpendicular directions) were recorded after 3, 7, and 10 days at 25°C. Colony diameters were used to calculate hyphal growth rate (mm/day). Isolates were incubated at 25°C for 21 days on PDA plates to examine conidial production and the shape and size of conidia. The length and width of 150 conidia per isolate were measured.

DNA isolation, polymerase chain reaction (PCR), and sequencing. Isolates were cultured on PDA in Petri plates overlaid with cellophane for 7 days at 25°C. Mycelia were harvested with

a flamed spatula, and genomic DNA of the isolates was extracted with the cetyltrimethyl ammonium bromide method (Taylor et al. 1993). All strains in this study were subjected to analysis of the rDNA-ITS, partial GAPDH, partial ACT, partial TUB2, and partial CHS-1 genomic regions (Table 1). PCR amplification of GAPDH was done using the primers GDF1 and GDR1 (Templeton et al. 1992) and that of CHS-1 with CHS79F and CHS354R (Carbone and Kohn 1999). The partial ACT, TUB2, and ITS regions were amplified with the primer pairs ACT512F and ACT783R (Carbone and Kohn 1999), T1 and Bt2b (Glass and Donaldson 1995), and ITS1 and ITS4 (White et al. 1990), respectively. All PCR reactions were conducted in 50-μl volumes containing 1× PCR buffer, 0.2 mM concentrations of each dNTP, 4 mM MgCl₂, 0.5 μM concentrations of each

Table 1. Primers used for PCR amplification and DNA sequencing

Gene ^z	Primer	Primer sequences	References
Actin	ACT-512F ACT-783R	ATGTGCAAGGCCGGTTTCGC TACGAGTCCTTCTGGCCCAT	Carbone and Kohn (1999)
GAPDH	GDF1 GDR1	GCCGTCAACGACCCCTTCATTGA GGGTGGAGTCGTACTTGAGCATGT	Guerber et al. (2003) Templeton et al. (1992)
CHS-1	CHS I-79F CHS I-354R	TGGGGCAAGGATGCTTGGAGAAG TGGAAGAACCATCTGTGAGAGTTG	Carbone and Kohn (1999)
β-Tubulin	T1 βt2b	AACATGCGTGAGATTGTAAGT ACCCTCAGTGTAGTGACCCCTGGC	O'Donnell et al. (2000) Glass and Donaldson (1995)
ITS	ITS1 ITS4	TCCGTAGGTGAACCTGCGG TCCTCCGCTTATTGATATGC	White et al. (1990)

^z GAPDH = glyceraldehyde-3-phosphate dehydrogenase; CHS-1 = chitin synthase; and ITS = rDNA internal transcribed spacer region.

Table 2. Sequences of *Colletotrichum* isolates used in the phylogenetic study

Species	Culture ^y	Host	Country	GenBank accession numbers ^z				
				ITS	GAPDH	ACT	CHS-1	TUB2
<i>C. asianum</i>	IMI 313839	<i>Mangifera indica</i>	Australia	JX010192	JX009915	JX009576	JX009753	JX010384
	ICMP 18580	<i>Coffea arabica</i>	Thailand	FJ972612	JX010053	JX009584	JX009867	JX010406
<i>C. fruticola</i>	ICMP 18613	<i>Limonium sinuatum</i>	Israel	JX010167	JX009998	JX009491	JX009772	JX010388
	ICMP 18727	<i>Fragaria ananassa</i>	U.S.A.	JX010179	JX010035	JX009565	JX009812	JX010394
<i>C. ignotum</i>	CBS 125397	<i>Tetragastris panamensis</i>	Panama	JX010173	JX010032	JX009581	JX009874	JX010409
	UASB-Cg-50	Pepper	India	JN248667	JN861219	JN861154	KC790583	JN861169
<i>C. siamense</i>	ICMP 12567	<i>Persea americana</i>	Australia	JX010250	JX009940	JX009541	JX009761	JX010387
	ICMP 17795	<i>Malus domestica</i>	U.S.A.	JX010162	JX010051	JX009506	JX009805	JX010393
	ICMP 18121	<i>Dioscorea rotundata</i>	Nigeria	JX010245	JX009942	JX009460	JX009845	JX010402
<i>C. gloeosporioides</i>	IMI 356878	<i>Citrus sinensis</i>	Italy	JX010152	JX010056	JX009531	JX009818	JX010445
<i>C. acutatum</i>	CBS 126521	<i>Anemone hybrida</i>	Netherlands	JQ948366	JQ948697	JQ949687	JQ949027	JQ950017
<i>C. aenigma</i>	ICMP 18686	<i>Pyrus pyrifolia</i>	Japan	JX010243	JX009913	JX009519	JX009789	JX010390
<i>C. aeschynomenes</i>	ICMP 17673*, ATCC 201874	<i>Aeschynomene virginica</i>	U.S.A.	JX010176	JX009930	JX009483	JX009799	JX010392
<i>C. alienum</i>	IMI 313842, ICMP 18691	<i>Persea americana</i>	Australia	JX010217	JX010018	JX009580	JX009754	JX010385
<i>C. nupharicola</i>	CBS 469.96, ICMP 17938	<i>Nuphar lutea</i> subsp. <i>polysepala</i>	U.S.A.	JX010189	JX009936	JX009486	JX009834	JX010397
<i>C. queenslandicum</i>	ICMP 1778*	<i>Carica papaya</i>	Australia	JX010276	JX009934	JX009447	JX009899	JX010414
<i>C. aotearoa</i>	ICMP 18532	<i>Vitex lucens</i>	New Zealand	JX010220	JX009906	JX009544	JX009764	JX010421
<i>C. musae</i>	CBS 116870*, ICMP 19119	<i>Musa</i> sp.	U.S.A.	JX010146	JX010050	JX009433	JX009896	HQ596280
<i>C. salsolae</i>	ICMP 19051*	<i>Salsola tragus</i>	Hungary	JX010242	JX009916	JX009562	JX009863	JX010403
<i>C. siamense</i> (syn. <i>C. hymenocallidis</i>)	CBS 125378(*), ICMP 18642	<i>Hymenocallis americana</i>	China	JX010278	JX010019	GQ856775	GQ856730	JX010410
<i>C. ti</i>	ICMP 5285	<i>Cordyline australis</i>	New Zealand	JX010267	JX009910	JX009553	JX009897	JX010441
<i>C. theobromicola</i>	ICMP 18566	<i>Olea europaea</i>	Australia	JX010282	JX009953	JX009496	JX009801	JX010376
<i>C. xanthorrhoeae</i>	BRIP 45094*, ICMP 17903, CBS 127831	<i>Xanthorrhoea preissii</i>	Australia	JX010261	JX009927	JX009478	JX009823	JX010448

^y IMI = culture collection of CAB International, Egham, UK; ICMP = International Collection of Microorganisms from Plant, Landcare Research, Auckland, New Zealand; CBS = CBS-KNAW, Fungal Biodiversity Centre, Utrecht, Netherlands; UASB = University of Agricultural Sciences, Bangalore, India; and BRIP = Plant Pathology Herbarium, Department of Employment, Economic, Development and Innovation, Queensland, Australia.

^z ITS = rDNA internal transcribed spacer region; ACT = actin gene; GAPDH = glyceraldehyde-3-phosphate dehydrogenase; CHS-1 = chitin synthase; TUB2 = β-tubulin; and * = ex-holotype or ex-epitype cultures.

primer, 0.5 units of Taq DNA polymerase (Takara), and 1 µl of template DNA (20 ng/µl). The PCR program for GAPDH, ACT, and TUB2 included a denaturation step at 94°C for 2 min, followed by 35 cycles at 94°C for 45 s, 60°C for 45 s, and 72°C for 1 min, and a final cycle at 72°C for 10 min. The PCR program for the ITS region included a 2-min denaturing step at 94°C followed by 34 cycles at 94°C for 1 min, 55°C for 30 s, and 72°C for 1 min, and a final cycle of 10 min at 72°C. The amplification products were sent to Shanghai Sangon Company for purification and sequencing. Sequences of mango *Colletotrichum* isolates obtained from Guangxi were submitted to GenBank.

Phylogenetic analyses. Sequences were edited and assembled using DNAMAN (version 5.2.2; Lynnon Biosoft) to produce consensus sequences that were compared by BLAST (Altschul et al. 1990) against the NCBI nonredundant database. Sequences from ex-type or ex-epitype isolates of *Colletotrichum* species from GenBank were selected for use in the phylogenetic analyses (Table 2). Sequence alignments of each gene and combined genes were made with ClustalX version 1.83. Phylogenetic trees were constructed with MEGA4 software version 4.0 using the maximum parsimony method.

Pathogenicity and virulence on mango tissues. Select isolates were used for pathogenicity and virulence tests on detached fruits and leaves of mango (cultivar Tainong) in controlled conditions. Isolates were incubated on PDA plates for 7 to 10 days at 25°C in the laboratory. Fresh young leaves and fresh harvested mango fruits without visible disease from Tiandong, Guangxi, were used for inoculation. The leaves and mango fruits were washed with running water, surface sterilized in 70% ethanol for 30 s and 1% NaClO for 1 min, and finally rinsed with sterile distilled water.

After air drying, detached young healthy leaves (12 to 15 cm) were placed into plastic containers (252 × 174 × 93 mm) lined with moist absorbent paper and, and six stab wounds were made in the mid-region forming a circle with a 5 mm diameter using a sterilized needle.

Hyphal plugs from actively growing margins of PDA cultures were placed on each wound spot on the leaves. The experiment was completely randomized with three replicates per isolate involving five leaves per replicate. Fifteen leaves were inoculated for each isolate, and in controls, leaves were treated with sterile agar plugs. The containers were partially sealed in plastic and incubated at 25°C in the dark in a growth chamber. Virulence was assessed by measuring lesion length at 7 days post inoculation (DPI) in two perpendicular directions on each leaf.

After washing and air drying, nearly ripened mango fruits (~200 g) were wounded in the protruding fleshy parts by stabbing with a flamed needle through the skin a depth of 3 mm, again forming a circle with a 5 mm diameter. Mycelial plugs of 6-mm diameter from PDA cultures were placed on wound sites. Control treatments used sterile agar plugs. Inoculated fruits were placed in plastic containers (252 × 174 × 93 mm) overlaid with moist absorbent paper to maintain humidity. The containers were placed in unsealed bags and incubated at 25°C in the dark. Fruits were checked for development of symptoms up to 10 days. Isolates were considered pathogenic when lesions expanded beyond the 5-mm diameter initial wound site. Virulence was evaluated by measuring lesion length at 10 DPI in two perpendicular directions. Significant differences in virulence caused were assessed by an analysis of variance, and when significant treatment effects were found, means were compared by LSD ($P = 0.05$) using Data Processing System software (DPS 3.0) for Windows (Zhejiang University, Hangzhou, China).

Results

Fungal isolation. From 2011 to 2014, leaves of mango with anthracnose symptoms were collected from 25 mango orchards in Guangxi province, China. At least one isolate from each field was chosen for further analysis. In total, 65 *Colletotrichum* isolates were obtained from different areas of Guangxi, and 29 single-spore

Table 3. Mango isolates of *Colletotrichum* spp. from Guangxi used in the study and their GenBank accession numbers for several genomic regions

Isolate	Species	Geographic area	GenBank accession numbers ^a				
			ACT	TUB2	GAPDH	CHS-1	ITS
NN19	<i>C. asianum</i>	Tuanluo, Nanning	MF039745	MF039774	MF040763	MF039803	MF039832
WM52	<i>C. asianum</i>	Lijian, Wuming	MF039746	MF039775	MF040764	MF039804	MF039833
QZ-3	<i>C. asianum</i>	Wuli, Lingshan	MF039747	MF039776	MF040765	MF039805	MF039834
TD6-7	<i>C. asianum</i>	Sitang, Tiandong	MF039748	MF039777	MF040766	MF039806	MF039835
NN133	<i>C. asianum</i>	Dingdang, Long'an	MF039749	MF039778	MF040767	MF039807	MF039836
LY12	<i>C. asianum</i>	Junting, Lingyun	MF039750	MF039779	MF040768	MF039808	MF039837
LA130	<i>C. asianum</i>	Dingdang, Long'an	MF039751	MF039780	MF040769	MF039809	MF039838
LA1	<i>C. asianum</i>	Tanliang, Long'an	MF039752	MF039781	MF040770	MF039810	MF039839
NN105	<i>C. asianum</i>	Jinlin, Nanning	MF039753	MF039782	MF040771	MF039811	MF039840
NN8	<i>C. asianum</i>	Guangxi Subtropical Crops Research Institute	MF039754	MF039783	MF040772	MF039812	MF039841
BS13	<i>C. asianum</i>	Youjiang, Baise	MF039755	MF039784	MF040773	MF039813	MF039842
NN7	<i>C. asianum</i>	Guangxi Academy of Agricultural Sciences	MF039756	MF039785	MF040774	MF039814	MF039843
TL110	<i>C. asianum</i>	Leli, Tianlin	MF039757	MF039786	MF040775	MF039815	MF039844
TL107	<i>C. asianum</i>	Leli, Tianlin	MF039757	MF039787	MF040776	MF039816	MF039845
NKY-1	<i>C. asianum</i>	Guangxi Academy of Agricultural Sciences	MF039758	MF039788	MF040777	MF039817	MF039846
WM01-2	<i>C. fruticicola</i>	Lijian, Wuming	MF039759	MF039789	MF040778	MF039818	MF039847
LZ120	<i>C. fruticicola</i>	Longzhou, Chongzuo	MF039760	MF039790	MF040779	MF039819	MF039848
NN10	<i>C. fruticicola</i>	Tunli, Nanning	MF039761	MF039791	MF040780	MF039820	MF039849
TD6-5	<i>C. fruticicola</i>	Sitang, Tiandong	MF039762	MF039792	MF040781	MF039821	MF039850
TD1	<i>C. fruticicola</i>	Pingma, Tiandong	MF039763	MF039793	MF040782	MF039822	MF039851
TL111	<i>C. fruticicola</i>	Leli, Tianlin	MF039764	MF039794	MF040783	MF039823	MF039852
NN20	<i>C. fruticicola</i>	Tanluo, Nanning	MF039765	MF039795	MF040784	MF039824	MF039853
TD8	<i>C. fruticicola</i>	Pingma, Tiandong	MF039766	MF039796	MF040785	MF039825	MF039854
WM03-1	<i>C. fruticicola</i>	Lijian, Wuming	MF039767	MF039797	MF040786	MF039826	MF039855
TD7	<i>C. fruticicola</i>	Sitang, Tiandong	MF039768	MF039798	MF040787	MF039827	MF039856
TD4	<i>C. fruticicola</i>	Pingma, Tiandong	MF039769	MF039799	MF040788	MF039828	MF039857
TD3	<i>C. siamense</i>	Silin, Tiandong	MF039770	MF039800	MF040789	MF039829	MF039858
TY9	<i>C. siamense</i>	Tianzhou, Tianyang	MF039771	MF039801	MF040790	MF039830	MF039859
WM03-2	<i>C. siamense</i>	Lijian, Wuming	MF039772	MF039802	MF040791	MF039831	MF039860

^a ACT = actin gene; TUB2 = β-tubulin; GAPDH = glyceraldehyde-3-phosphate dehydrogenase; CHS-1 = chitin synthase; and ITS = rDNA internal transcribed spacer region.

isolates were used for further study (Table 3) (one isolate from each orchard, but for some large orchards more than one isolate was chosen for further study). Among the 29 isolates, 15 were identified as *C. asianum*, 11 as *C. fructicola*, and 3 as *C. siamense* using morphological and molecular methods. Other species isolated such as species of *Alternaria*, *Phomopsis*, *Botryosphaeria*, or *Pestalotia* were not considered further in this study.

Phylogenetic analyses. In the present study, 29 isolates from different areas were selected for sequencing and analyses of ITS, GAPDH, ACT, TUB2, and CHS-1. BLASTn searches of generated DNA sequences from 15 isolates against GenBank showed high similarity (>99%) with the sequences of *C. asianum*, 11 isolates showed high similarity with the sequences of *C. fructicola*, and 3 isolates showed high similarity (99 or 100%) with the sequences of *C. siamense*.

Fig. 1. Phylogenetic tree using sequences of 29 *Colletotrichum* isolates from mango trees in Guangxi and from ex-type or ex-epitype isolates in GenBank using combined internal transcribed spacer, actin, glyceraldehyde-3-phosphate dehydrogenase, β -tubulin, and chitin synthase 1 genes. Bootstrap values > 50% (1,000 replication) are given at the nodes. *Colletotrichum acutatum* was used as the outgroup. Isolates from this study are emphasized in bold.

ACT, TUB2, CHS-1, GAPDH, and ITS sequences of *Colletotrichum* from mango trees in Guangxi province were submitted to GenBank (Table 3) and aligned with GenBank sequences of *Colletotrichum* species from different hosts and other countries (Table 2). The gene boundaries in the alignment were as follows: ACT (282 to 289 bp), TUB2 (699 to 701 bp), CHS-1 (299 bp), GAPDH (277 to 280 bp), and ITS (546 bp).

A phylogenetic tree of 29 *Colletotrichum* isolates from this study and sequences from ex-type or ex-epitype isolates from GenBank using combined ITS, ACT, GAPDH, TUB2, and CHS-1 genes with maximum parsimony analysis is shown in Figure 1. The combined data set produced three well-separated clades. Fifteen *Colletotrichum* isolates composed a highly supported clade (99% bootstrap support) with *C. asianum* isolates ICMP18580 from *Coffea arabica* in Thailand and IMI313839 from *Mangifera indica* in Australia. Eleven isolates composed a supported clade (69% bootstrap support) with

C. fruticola isolates ICMP18727, ICMP18613, and ICMP12568 and *C. ignotum* (= *C. fruticola*) isolates ICMP125397 and UASB-Cg-50. Three isolates composed a supported clade (51% bootstrap support), which closed with *C. siamense* isolate ICMP17795.

The diversity analyses from 29 isolates of *Colletotrichum* species showed the following distribution: 15 isolates (51.7%) belonging to *C. asianum*, 11 isolates (37.9%) to *C. fruticola*, and 3 isolates (10.2%) to *C. siamense*.

Morphological and cultural characterization. All tested isolates developed white colonies that turned to gray to black after 14 days on PDA (Fig. 2). Colony sectoring was observed in 8 of 29 isolates. There was considerable variation in mycelial growth rate among the 29 isolates of *Colletotrichum* (Table 2). The average mycelial growth rate for *C. asianum* was 6.7 to 10.7 mm/day, for *C. fruticola*, 7.3 to 12.4 mm/day, and for *C. siamense*, 9.4 to 11.1 mm/day. Conidia were all hyaline, guttulate, one-celled, and cylindrical. The

Fig. 2. Morphological characteristics of colonies and conidia of mango *Colletotrichum* isolates from Guangxi. Upper (A) and reverse (B) sides of *C. asianum* NN8 PDA plate at 14 days after inoculation; and conidia of *C. asianum* (C). Upper (D) and reverse (E) sides of *C. fruticola* isolate WM01-2 culture on PDA 14 days after inoculation; and conidia of *C. fruticola* (F). Upper (G) and reverse (H) sides of *C. siamense* isolate TY9 culture on PDA 14 days after inoculation; and conidia of *C. siamense* (I). Bars: 10 μ m.

average conidial sizes for isolates were *C. asianum*, 12.1 to 18.6 × 4.2 to 5.6 µm; *C. fruticola*, 14.1 to 20.2 × 3.7 to 4.3 µm; and *C. siamense*, 15.0 to 18.5 × 5.0 to 5.5 µm. The various *Colletotrichum* species reported here showed differences in conidial size and growth rates (Table 4).

Pathogenicity and virulence in tissues. All 29 tested isolates were pathogenic to mango leaves and fruits. Ripened fruits with anthracnose develop sunken, dark brown to black decay. The virulence of the 29 isolates of *Colletotrichum* from Guangxi province showed significant differences, with lesion diameters ranging from 7.0 to 30.0 cm on leaves and from 6 to 21 mm on fruits. The lesion diameter of *C. asianum* on leaves ranged from 9.2 to 28.2 mm and on fruits ranged from 6.2 to 21 mm. For *C. fruticola* it was 8.8 to 30.0 mm on leaves and 5.8 to 13.6 mm on fruits. For *C. siamense* it was 7.0 to 22.2 mm on leaves and 6.0 to 6.5 mm on fruits. The virulence of *C. fruticola* isolates TD1 and TD4 from the same field showed significant differences ($P \leq 0.05$) (Table 4).

Discussion

This is the first report characterizing *Colletotrichum* species associated with anthracnose of mango in Guangxi province, China, using morphological identification and phylogenetic analysis. The morphological features of the isolates agreed with the description of *C. gloeosporioides*, but cultural characteristics, which may be diverse owing to cultural conditions, and conidial morphology cannot distinguish between species within the *C. gloeosporioides* complex (Weir et al. 2012). Based on morphological identification, single gene sequence analysis, and the five-gene combined phylogenetic analysis,

the isolates obtained from mango in Guangxi included in this study belong to the *C. gloeosporioides* complex. *C. gloeosporioides* has previously been shown to be the causal agent of tropical fruit rots including mango, and the present study showed that all 29 isolates from mango leaves with anthracnose symptoms across a 450 by 300 km region belonged to the species complex *C. gloeosporioides*. In the contrast, Phoulivong et al. (2010) reported that *C. gloeosporioides* was not a common pathogen in the tropics and was not the cause of mango anthracnose in Thailand or Laos.

The species complex *C. gloeosporioides* has been separated into many different species (Weir et al. 2012). Three species were found to be associated with mango leaf anthracnose: *C. asianum*, *C. fruticola*, and *C. siamense*. These species have also been previously recorded from mango in Brazil (Phoulivong et al. 2010). Among the genomic regions tested, *C. asianum*, *C. fruticola*, and *C. siamense* could be separated by all except for ITS. This does not agree with the results of Weir et al. (2012), who found *C. asianum* can be separated from these other taxa using any of the genes tested, including ITS. ITS sequences do not reliably separate *C. asianum*, *C. siamense*, and *C. fruticola*, but *C. siamense* and *C. fruticola* can be distinguished using TUB2, and this agrees with the previous reports (Weir et al. 2012).

C. asianum was well separated using five-gene combined phylogenetic analysis in this study, and it was the predominant species associated with mango in Guangxi, South China, in agreement with Vieira et al. (2014), who found that *C. asianum* was the most common endophytic species from mango trees. *C. asianum* is a well-known pathogen of mangoes from India, Australia, Panama, and Thailand (Sharma et al. 2015).

Table 4. Morphological and virulence descriptions of *Colletotrichum* isolates from mango trees in Guangxi, China

Isolate	Conidial size (µm) ^x	Mycelial growth (mm/day) ^y	Lesion diameter on mango leaves (mm) ^z	Lesion diameter on mango fruit (mm) ^z
<i>C. asianum</i>				
NN19	18.6 ± 0.5 × 5.6 ± 0.3	8.4 bcdef	12.6 bcd	18.2 hi
WM52	17.8 ± 0.6 × 4.4 ± 0.3	7.9 bc	14.4 bcde	12.0 abcdefg
TL107	17.3 ± 1.0 × 6.0 ± 0.4	9.2 efghi	28.2 ij	11.5 abcdefg
TL110	16.4 ± 0.6 × 5.8 ± 0.3	9.5 fghij	14.0 bcde	9.7 abcdef
BS13	15.6 ± 0.5 × 6.5 ± 0.4	8.3 bcde	9.2 b	13.5 defgh
NN7	15.5 ± 0.4 × 4.7 ± 0.2	8.4 bcdef	15.4 bcdef	8.9 abcde
LA1	15.2 ± 0.6 × 5.7 ± 0.3	8.9 cdefg	24.8 hij	12.4 bcdefg
LY12	15.2 ± 0.5 × 4.9 ± 0.3	9.1 defgh	14.2 bcde	6.3 ab
QZ-3	14.2 ± 0.8 × 5.8 ± 0.3	10.7 klmn	26.2 hij	15.2 fgh
NN105	13.3 ± 0.5 × 4.6 ± 0.2	8.2 bcd	20.4 efgh	6.2 ab
LA130	13.1 ± 0.5 × 5.2 ± 0.4	8.3 bcde	20.2 efgh	9.5 abcdef
NN8	12.9 ± 0.4 × 4.8 ± 0.2	6.7 a	16.6 cdefg	16.6 gh
TD6-7	12.4 ± 0.6 × 5.3 ± 0.3	10.5 jklmn	24.0 hij	21.1 i
NN133	12.1 ± 0.5 × 4.2 ± 0.3	7.9 bc	14.6 bcde	12.6 cdefg
NKY-1	16.7 ± 0.3 × 4.6 ± 0.3	9.9 hijkl	30.0 j	15.1 fgh
<i>C. fruticola</i>				
TL111	20.2 ± 0.4 × 4.3 ± 0.3	10.7 jklmn	22.0 fghi	12.1 abc
TD6-5	16.9 ± 0.3 × 4.8 ± 0.3	7.3 ab	8.8 b	6.3 ab
TD4	15.4 ± 0.6 × 5.2 ± 0.4	10.3 ijklm	9.0 b	7.9 abcd
TD7	15.3 ± 0.5 × 4.9 ± 0.2	10.9 lmno	26.0 hij	10.9 abcdef
WM03-1	15.2 ± 0.3 × 4.0 ± 0.2	9.7 ghijk	25.0 hij	13.6 defgh
TD8	15.0 ± 0.5 × 5.3 ± 0.4	10.8 klmn	23.0 ghij	8.7 abcde
NN20	14.6 ± 0.7 × 4.6 ± 0.3	11.7 nop	30.0 j	7.9 abcd
TD1	14.1 ± 0.8 × 3.7 ± 0.4	11.9 op	22.2 fghi	7.0 abc
NN10	20.1 ± 0.5 × 4.2 ± 0.2	9.4 fghij	13.6 bcde	5.8 a
WM01-2	17.2 ± 0.5 × 5.4 ± 0.2	12.4 p	18.8 defgh	10.9 abcdefg
LZ120	14.9 ± 0.6 × 4.1 ± 0.2	9.5 ghijk	19.8 defgh	6.8 abc
<i>C. siamense</i>				
TY9	18.5 ± 0.3 × 5.0 ± 0.3	11.1 mno	22.2 fghi	6.5 abcdefg
TD3	15.4 ± 0.5 × 4.2 ± 0.1	10.4 ijklm	7.0 a	6.0 a
WM03-2	15.0 ± 0.5 × 5.5 ± 0.3	9.4 fghij	10.6 bc	14.4 efgh

^x The length and width of 150 conidia per isolate on PDA were measured after 21 days at 25°C.

^y The colony diameter (mm) was measured in two perpendicular directions. The colony diameter data were used to calculate the mycelial growth rate (mm/day).

^z The virulence of the isolates was evaluated by measuring lesion length at 7 and 10 days postinoculation in two perpendicular directions on leaves and fruits, respectively.

C. fruticola is diverse morphologically and geographically. The species has been found on coffee berries (Thailand), *Pyrus pyrifolia* (Japan), *Limonium* (Israel), *Malus* sp. and *Fragaria × ananassa* (U.S.A.), *Persea americana* (Australia), *Ficus* sp. (Germany), *Malus* sp. (Brazil), *Dioscorea* sp. (Nigeria), *Theobroma* sp. and *Tetragastris* sp. (Panama) (Weir et al. 2012), and *Vitis* sp. (China) (Peng et al. 2013). *C. fruticola* has been described in association with mango in Brazil and Korea (Joa et al. 2016); however, this is the first report of *C. fruticola* infecting mango in China. *C. ignotum* = *C. fruticola* (Weir et al. 2012), and our genetic analyses showed the isolates from these two species were in one clade but two subgroups.

C. siamense has been associated with mango anthracnose in Colombia (Pardo-De la Hoz et al. 2016) and on mango fruit (Qin et al. 2017) in Sanya, Hainan province, China, and also in postharvest of mango fruits from supermarkets in Changchun, northeast China (Liu et al. 2017), but this species is not common on mango in Guangxi province, and only three isolates were found to be *C. siamense*. In this study, this species was not well separated from the *C. gloeosporioides* complex with the combined five genes, and more genes may be needed in future study.

Pathogenicity tests using three species of *Colletotrichum* isolates showed that all species were pathogenic to mango leaves and fruits in wound-inoculation studies. Symptoms may vary considerably with factors such as variety and conditions of the fruit, humidity and temperature, and the inoculum concentration (Freeman et al. 1998). Hence, the observations from this study may not reflect the full virulence potential. Additional research should be conducted to determine the virulence potential of *Colletotrichum* species under field conditions with natural inoculum rather than just in vitro artificial inoculation.

The *Colletotrichum* isolates from mango examined in this study showed diversity based on sequence analysis and morphological features. Higher genetic diversity is often correlated with higher evolutionary potential and with greater ability to adapt to changing environmental conditions (McDonald and Linde 2002). In addition, sensitivity of *C. gloeosporioides* and *C. acutatum* species from mango to carbendazim and thiabendazole are significantly different from each other in China (Hu et al. 2005). Such information can be used by pathologists and breeders to develop strategies and cultivars toward more effective management of mango anthracnose disease. Future studies should focus on surveys of more isolates and tests on other features such as fungicide sensitivity.

Literature Cited

- Altschul, S. F., Gish, W., Miller, W., Myers, E. W., and Lipman, D. J. 1990. Basic local alignment search tool. *J. Mol. Biol.* 215:403-410.
- Arauz, L. F. 2000. Mango anthracnose: Economic impact and current options for integrated management. *Plant Dis.* 84:600-611.
- Cai, L., Hyde, K. D., Taylor, P. W. J., Weir, B., Waller, J. M., Abang, M. M., Zhang, J. Z., Yang, Y. L., Phoulivong, S., Liu, Z. Y., Prihastuti, H., Shivas, R. G., McKenzie, E. H. C., and Johnston, P. R. 2009. A polyphasic approach for studying *Colletotrichum*. *Fungal Divers.* 39:183-204.
- Cannon, P. F., Damm, U., Johnston, P. R., and Weir, B. S. 2012. *Colletotrichum*—Current status and future directions. *Stud. Mycol.* 73:181-213.
- Carbone, I., and Kohn, L. 1999. A method for designing primer sets for speciation studies in filamentous ascomycetes. *Mycologia* 91:553-556.
- Freeman, S., Katan, T., and Shabi, E. 1998. Characterization of *Colletotrichum* species responsible for anthracnose diseases of various fruits. *Plant Dis.* 82:596-605.
- Freeman, S., Minz, D., Jurkevitch, E., Maymon, M., and Shabi, E. 2000. Molecular analyses of *Colletotrichum* species from almond and other fruits. *Phytopathology* 90:608-614.
- Glass, N. L., and Donaldson, G. C. 1995. Development of primer sets designed for use with the PCR to amplify conserved genes from filamentous ascomycetes. *Appl. Environ. Microbiol.* 61:1323-1330.
- Guerber, J. C., Liu, B., Correll, J. C., and Johnston, P. R. 2003. Characterization of diversity in *Colletotrichum acutatum sensu lato* by sequence analysis of two gene introns, mtDNA and intron RFLPs, and mating compatibility. *Mycologia* 95:872-895.
- Hong, K., Gong, D., Zhang, L., Hu, H., Jia, Z., Gu, H., and Song, K. 2016. Transcriptome characterization and expression profiles of the related defense genes in postharvest mango fruit against *Colletotrichum gloeosporioides*. *Gene* 576:275-283.
- Hu, M., Li, M., Yang, F., and Zheng, F. 2005. Biological characteristics of 2 species of *Colletotrichum* causing mango postharvest anthracnose. *Southw. China J. Agric. Sci.* 18:306-310.
- Hyde, K. D., Cai, L., McKenzie, E. H. C., Yang, Y. L., Zhang, J. Z., and Prihastuti, H. 2009. *Colletotrichum*: A catalogue of confusion. *Fungal Divers.* 39:183-204.
- Jayasinghe, C. K., and Fernando, T. H. P. S. 2009. First report of *Colletotrichum acutatum* on *Mangifera indica* in Sri Lanka. *Ceylon J. Sci. Biol. Sci.* 38:31-34.
- Li, R. W., Huang, G. D., Su, M. H., Zhou, J. A., and Chen, Y. S. 2013. Status and developmental strategies of mango industry in China. *J. South. Agric.* 44:875-878.
- Liu, L., Shu, J., Zhang, L., Hu, R., Chen, C., Yang, L. N., Lu, B. H., Liu, Y. N., Yu, L., Wang, X., and Li, Y. 2017. First report of post-harvest anthracnose on mango (*Mangifera indica*) caused by *Colletotrichum siamense* in China. *Plant Dis.* 101:833.
- McDonald, B. A., and Linde, C. 2002. The population genetics of plant pathogens and breeding strategies for durable resistance. *Euphytica* 124:163-180.
- Nelson, S. C. 2008. Mango Anthracnose (*Colletotrichum gloeosporioides*). Publication PD-48. Cooperative Extension Service, College of Tropical Agriculture and Human Resources, University of Hawai'i at Manoa, U.S.A.
- O'Donnell, K., Nirenberg, H. I., Aoki, T., and Cigelnik, E. 2000. A multigene phylogeny of the *Gibberella fujikuroi* species complex: Detection of additional phylogenetically distinct species. *Mycoscience* 41:61-78.
- Pardo-de la Hoz, C. J., Calderón, C., Rincón, A. M., Cárdenas, M., Danies, G., López-kleine, L., Restrepo S., and Jiménez, P. 2016. Species from the *Colletotrichum acutatum*, *Colletotrichum boninense* and *Colletotrichum gloeosporioides* species complexes associated with tree tomato and mango crops in Colombia. *Plant Pathol.* 65:227-237.
- Peng, L. J., Sun, T., Yang, Y. L., Cai, L., Hyde, K. D., Bahkali, A. H., and Liu, Z. Y. 2013. *Colletotrichum* species on grape in Guizhou and Yunnan provinces, China. *Mycoscience* 54:29-41.
- Photita, W., Taylor, P. W., Ford, R., Hyde, K. D., and Lumyong, S. 2005. Morphological and molecular characterization of *Colletotrichum* species from herbaceous plants in Thailand. *Fungal Divers.* 18:117-133.
- Phoulivong, S., Cai, L., Chen, H., McKenzie, E. H., Abdelsalam, K., Chuakeatiro, E., and Hyde, K. D. 2010. *Colletotrichum gloeosporioides* is not a common pathogen on tropical fruits. *Fungal Divers.* 44:33-43.
- Qin, L. P., Huang, S. L., Lin, S. H., and Lin, C. H. 2017. First report of anthracnose of *Mangifera indica* caused by *Colletotrichum siamense* in Sanya city in China. *Plant Dis.* 101:1038.
- Sharma, G., Gryzenhout, M., Hyde, K. D., Pinnaka, A. K., and Shenoy, B. D. 2015. First report of *Colletotrichum asianum* causing mango anthracnose in South Africa. *Plant Dis.* 99:725.
- Taylor, B. H., Manhart, J. R., and Amasino, R. M. 1993. Isolation and characterization of plant DNAs. Pages 37-65 in: *Methods in Plant Molecular Biology and Biotechnology*. B. R. Glick and J. E. Thompson, eds. CRC Press, Boca Raton, FL.
- Templeton, M. D., Rikkerink, E. H., Solon, S. L., and Crowhurst, R. N. 1992. Cloning and molecular characterization of the glyceraldehyde-3-phosphate dehydrogenase-encoding gene and cDNA from the plant pathogenic fungus *Glomerella cingulata*. *Gene* 122:225-230.
- Valero, M., García-Martínez, S., Giner, M. J., Alonso, A., and Ruiz, J. J. 2010. Benomyl sensitivity assays and species-specific PCR reactions highlight association of two *Colletotrichum gloeosporioides* types and *C. acutatum* with rumpel disease on *Primofiori lemons*. *Eur. J. Plant Pathol.* 127:399-405.
- Vieira, W. A., Michereff, S. J., de Moraes, M. A., Jr., Hyde, K. D., and Câmara, M. P. 2014. Endophytic species of *Colletotrichum* associated with mango in northeastern Brazil. *Fungal Divers.* 67:181-202.
- Weir, B. S., Johnston, P. R., and Damm, U. 2012. The *Colletotrichum gloeosporioides* species complex. *Stud. Mycol.* 73:115-180.
- White, T. J., Bruns, T., Lee, S. J. W. T., and Taylor, J. W. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. Pages 315-322 in: *PCR Protocols: A Guide to Methods and Applications*. M. Innis, D. Gelfand, J. Sninsky, and T. White, eds. Academic Press, San Diego, CA.