Characterization, Phylogenetic Analyses, and Pathogenicity of *Colletotrichum* Species on *Morus alba* in Sichuan Province, China

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Abstract

Brown spot disease caused by *Colletotrichum* species was found on leaves of mulberry (*Morus alba* L.) in Dujiangyan, Sichuan Province, China. Fungal isolates from leaf lesions were identified as six *Colletotrichum* species based on morphological characteristics and DNA analysis of the combined sequences ITS, *GAPDH*, *ACT*, *CHS-1*, *TUB2*, and *GS*. These included *Colletotrichum fioriniae*, *C. fructicola*, *C. cliviae*, *C. karstii*, *C. kahawae* subsp. *ciggaro*, and *C. brevisporum*. Results showed that the most important causal

agent of mulberry anthracnose was *C. fioriniae*, causing typical brown necrotic spots or streaks, followed by *C. brevisporum*, *C. karstii*, and *C. kahawae* subsp. *ciggaro*, whereas the two other species (*C. fructicola* and *C. cliviae*) showed no pathogenicity to mulberry. This study is the first report of these species associated with mulberry in China.

Keywords: fungi, mulberry, brown spot disease, anthracnose

Mulberry (*Morus alba* L.) is a woody tree belonging to the family *Moraceae* and is distributed widely in China (Song et al. 2009). Mulberry is increasingly becoming an important economic crop worldwide and is mainly cultivated for its nutritional fruits, silkworm feeding, medicine, and beverage production. The fruit of mulberry is widely used as a nutritious food (Chang et al. 2011); the leaves, root bark, and twigs have been used in Chinese medicine for a long time to treat many illnesses, such as fever and other human diseases, and to lower blood pressure (Jia et al. 1999). Because leaves of *M. alba* contain 1-deoxynojirimycin as a characteristic constituent (Singab et al. 2005; Tsuduki et al. 2013), the leaves are currently used in teas for health support (Fan et al. 2016). Mulberry leaves are also used as fodder for animals in the winter and spring seasons (Cai et al. 2019), and research has shown that mulberry feed could enhance milk yield in dairy animals (Dillard and German 2000).

Mulberry anthracnose is a common and widespread leaf disease. Leaves infected with *Colletotrichum dematium* display brown necrotic spots or streaks (Yoshida et al. 1995) from early summer to autumn (Yoshida and Shirata 1998). Accordingly, this disease limits the productivity and quality of leaves, thus directly resulting in significant loss of leaves for both silkworm feeding (Yoshida et al. 2002) and tea production. Yoshida and Shirata (1999) showed that *C. dematium* can overwinter in infected or latently infected leaves, which could be the primary infection sources in the following year. Therefore, it has been a major problem to control this disease in mulberry cultivation (Yoshida et al. 2002).

However, there have been few studies on anthracnose of mulberry up to now. Anthracnose on the mulberry leaves caused by *C. morifolium* Hara and *C. morina* Hara was first reported by Hara (1925, 1954). Yoshida et al. (1995) isolated anthracnose fungi from diseased mulberry leaves in various areas of Japan and identified their species based on the taxonomy proposed by von Arx (1957); they showed that the most frequent species identified was *C. dematium*, which was found to be the most aggressive causal agent of the disease based on pathogenicity tests, followed by *C. gloeosporioides* and *C. acutatum*. *Colletotrichum* species also reported on mulberry were *C. morina* in Japan (Kobayashi 2007) and *C. moricola* in India (Mathur 1979). In China, anthracnose on mulberry was mainly owing to *C. morifolium* (Tai 1979; Tian 1981) and *C. morium* (Zhang et al. 2011), but *Colletotrichum* spp. associated with mulberry were not identified molecularly. Therefore, the purpose of this study was to accurately identify and determine *Colletotrichum* species causing mulberry anthracnose according to their morphology, DNA sequences, and pathogenicity, which would be crucial to control this disease effectively.

Materials and Methods

Sample collection and isolation. In 2016 and 2017, infected leaves of mulberry (*M. alba*) were collected from a mulberry orchard (about 15 ha in total, 30.81098° N, 103.69836° W) in Dujiangyan city, Sichuan Province, China. Three pieces (5 × 5 mm²) of leaf tissues at the border of lesions from each plant of 40 random plants were surface sterilized (70% ethanol for 60 s, 5% commercial NaClO for 3 min) and placed on potato dextrose agar (PDA) supplemented with 50 mg/liter of ampicillin sodium salt and incubated at 25°C. Fungal hyphae grown from the leaf tissues were picked up and transferred to fresh PDA within 2 to 8 days. Referring to the procedure described by Cai et al. (2009), single-spore isolates of each *Colletotrichum* isolate were obtained for the follow-up studies. All isolates were preliminarily identified by morphology and the internal transcribed spacer (ITS) and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) sequences. The numbers of fungi were recorded, and the percentage of each *Colletotrichum* species was calculated.

Morphological and cultural characterization. Mycelial plugs (6 mm) of the single-spore cultures were transferred from the edge of the growing colonies to fresh PDA plates. The plates were then placed in an incubator (GXZ, Ningbo Jiangnan Instrument Co.,

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Funding: This work was supported by the Study on the Etiology of the Early Apple Wilt Disease (2017LWJJ-004), USDA-NIFA Multistate Project W3157, and the New Jersey Agricultural Experiment Station.

The author(s) declare no conflict of interest.

Accepted for publication 17 April 2019.

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Zhejiang, China) at 5, 10, 15, 20, 25, 30, and 35°C in the dark. Referring to the method of Han et al. (2016), colony diameters (five replicates) were measured after 5 days, and daily growth rate = (colony diameter – 6)/2 mm. Colonies of all isolates except for group 4 produced pale orange conidia masses after 7 to 15 days; for group 4 isolates, they were first incubated at 25°C for 5 days, and then mycelia were wounded with a sterilized wire loop (PDA + W); orange conidia masses were produced on the edge of the wounds after 2 to 10 days. Appressoria were produced using a slide culture technique in which a 1-cm² square of a *Colletotrichum* culture on PDA was placed in an empty Petri dish, and a sterile cover slip was placed over the agar (Cai et al. 2009). After 7 days, the appressoria were observed on the underside of the cover slip. Microscopic preparations were made in sterile water and observed with a Nikon ECLIPSE *Ti* microscope (Nikon, Tokyo, Japan), with 30 measurements per each type of structure. Colony colors were rated according to the method of Rayner (1970). All relevant data were compared by Duncan's test ($P \leq 0.05$) using IBM SPSS Statistics 19.0.

DNA extraction, PCR amplification, and DNA sequencing. Mycelia were scraped from the fresh colony using a sterile medicine spoon. Genomic DNA was extracted by using a Fungal DNA Kit (Omega Bio-Tek, Doraville, CA). The concentration of DNA was manually adjusted at 100 ng/μl after measurement by a Nanodrop 2000 spectrophotometer (Thermo Scientific). The rDNA-ITS region, partial *GAPDH*, calmodulin (*CAL*), actin (*ACT*), chitin synthase 1 (*CHS-1*), histone (*HIS3*), and β-tubulin (*TUB2*) genes were amplified by ITS-1F/ITS-4, GDF1/GDR1, CAL 228F/CAL 737R,

ACT-512F/ACT-783R, CHS-354R/CHS-79F, CYLH3F/CYLH3R, and T1/Bt-2b, respectively (Damm et al. 2012b). The amplifications were performed in a T100TM Thermal Cycler (Bio-Rad, Hercules, CA) in a total volume of 20 μl containing 1 μl of genomic DNA, 1 μl of each forward/reverse primer (10 μM), 10 μl of 1-5 2× High-Fidelity Master Mix (MCLAB, South San Francisco, CA), and 7 μl of sterile water. DNA sequencing was performed by Tsingke Biological Technology Co. (Chengdu, China).

Phylogenetic analysis. The combined ITS, *GAPDH*, *ACT*, *CHS-1*, and *TUB2* sequences were aligned using Clustal X as implemented in MEGA version 6 and manually adjusted to allow maximum sequence similarity. The aligned file was analyzed using the criteria of both neighbor-joining and maximum likelihood in MEGA version 6 (Tamura et al. 2013), respectively. Trees were decorated in Micro-soft Office Word 2007.

Pathogenicity of *Colletotrichum* isolates. The single-spore strains of *Colletotrichum* were cultured at 25°C for 5 days, and then 4-mm PDA plugs were cut from the edge of fresh colonies for pathogenicity tests. Conidia were obtained by growing the isolates on PDA or PDA + W for 7 to 15 days as described above. The conidial suspension was adjusted to two concentrations of 1×10^6 and 1×10^9 conidia/ml in sterile water with 0.01% (vol/vol) Tween 80. Pathogenicity assays were performed on healthy and young leaves of detached mulberry twigs. Mulberry twigs were randomly divided into six groups, which were inoculated with six groups of isolates. For each isolate, 20 leaves on detached mulberry twigs were inoculated with and without wounding (Than et al. 2008). Nonwound

Fig. 1. Anthracnose on mulberry caused by *Colletotrichum* spp. under natural conditions: **a**, infected mulberry plants; **b**, symptoms under wet, humid conditions; and **c and d**, typical anthracnose symptoms.

inoculation was performed by placing a 15- μ l conidial suspension (1×10^6 or 1×10^9 conidia/ml) or 4-mm PDA plugs onto the middle of the right side of the leaves. According with the inoculation method of Scandiani et al. (2011), wound inoculation involved pin-pricking the middle of the leaves (two sites per leaf along the longitudinal axis) and then placing PDA plugs (4-mm diameter) with the mycelium in contact with the wound. Plants inoculated with sterile water

suspension containing Tween 80 or 4-mm PDA plugs without the fungus were used as controls. After inoculation, the twigs were placed in transparent plastic boxes to maintain high relative humidity and maintained at 25°C in an incubator. Disease incidence (infected leaves) was assessed 10 days after inoculation, and the diameters of lesions were measured by the crossing method (width and length of lesions) to evaluate the virulence. To fulfill Koch's postulates, all fungal isolates included in the pathogenicity tests were reisolated from infected leaves to confirm their identity by molecular and morphological approaches as described above. The data of the lesion diameters were compared by Tukey's test ($P \leq 0.05$) using IBM SPSS Statistics 19.0.

Results

Symptoms and separation rate of *Colletotrichum* species. Mulberry anthracnose was frequently observed in the mulberry orchard during 2016 and 2017. Symptoms on leaves developed on the middle and lower leaves of twigs from late June to the following January to February (when all the leaves dropped off). In the field, the leaves showed brown, necrotic, circular to irregular spots or streaks, and the orange masses of conidia were always visible on the abaxial leaf surface in the center of lesions (Fig. 1c and d). The symptoms initially appeared as small pale or red brown spots, which later became dark brown or tan, irregular or circular in shape, more or less sunken lesions. Gradually, the necrotic lesions enlarged, coalesced, and covered a large percentage of the leaf area, and eventually the leaves became yellow and then withered and died (Fig. 1a). Under wet, humid conditions, orange masses of conidia were always visible on both leaf surfaces in the center of lesions (Fig. 1b).

In total, 128 *Colletotrichum* isolates were obtained (Fig. 2a). Based on the high similarity of morphology as well as ITS and *GAPDH* sequences, *C. fruticicola* (group 2) was the most common species, representing 40% of all *Colletotrichum* isolates, followed by *C. fioriniae* (group 3), *C. karstii* (group 4), *C. cliviae* (group 5), *C. kahawae* (group 1), and *C. brevisporum* (group 6), which accounted for 36, 9, 7, 5, and 3%, respectively (Fig. 2b).

Fig. 2. Proportions of *Colletotrichum* species found on mulberry: **a**, percentage of isolates of each *Colletotrichum* species on mulberry; and **b**, number of *Colletotrichum* species recovered from mulberry.

Table 1. Morphological and cultural characteristics of *Colletotrichum* isolates from mulberry^z

Species (group)	Colony characters	Conidia			Appressoria		
		Length (μ m)	Width (μ m)	Shape	Length (μ m)	Width (μ m)	Characters
<i>C. kahawae</i> subsp. <i>ciggaro</i> (group 1)	Gray to gray-brown with numerous black spots in reverse	14.82 $\pm$ 3.55 b 7.5–18.9	4.53 $\pm$ 0.93 c 2.4–6.2	Cylindrical	9.55 $\pm$ 2.93 b 4.8–12.7	5.33 $\pm$ 1.81 b 3.2–8.8	Hyaline to light brown, globose, ovate to slightly irregular
<i>C. fruticicola</i> (group 2)	Gray to dark gray with visible conidial masses in the center; reverse greyish green with white halos	13.50 $\pm$ 1.91 c 7.9–17.8	4.66 $\pm$ 0.60 bc 3.5–6.3	Cylindrical	9.78 $\pm$ 2.13 b 7.2–13.8	6.12 $\pm$ 2.39 b 4.2–12.4	Brown to dark brown, ovoid, clavate, and slightly irregular to irregular
<i>C. fioriniae</i> (group 3)	White to light pink; reverse brownish pink to vinaceous with a few black spots	11.30 $\pm$ 1.18 d 7.8–13.8	3.84 $\pm$ 0.34 d 3.3–4.8	Fusiform	9.02 $\pm$ 1.71 bc 6.3–11.2	6.31 $\pm$ 1.54 b 5.1–9.8	Brown to dark brown, circular to clavate, and lightly irregular to irregular
<i>C. karstii</i> (group 4)	Grew to white with dark conidiomata scattered over the surface; reverse colorless to pale orange with some dark spots	14.18 $\pm$ 1.42 bc 10.8–17.2	6.14 $\pm$ 0.71 a 4.5–7.6	Cylindrical	7.23 $\pm$ 1.30 c 5.2–8.8	5.71 $\pm$ 1.45 b 3.8–8.4	Light brown to brown, circular to clavate, and lightly irregular to irregular
<i>C. cliviae</i> (group 5)	Gray to dark gray; reverse gray-yellow to gray-black with some black spots	12.02 $\pm$ 1.72 d 8.7–14.8	4.46 $\pm$ 0.47 c 3.6–5.2	Cylindrical	...	...	...
<i>C. brevisporum</i> (group 6)	Gray to dark gray; reverse gray-black with little black spots	18.64 $\pm$ 2.04 a 14.0–21.3	4.89 $\pm$ 0.41 b 3.9–5.6	Long cylindrical	14.36 $\pm$ 2.40 a 8.8–20.2	7.67 $\pm$ 1.04 a 6.4–10.8	Brown to dark brown with a light brown guttule in the middle, ovoid or slightly irregular

^z Columns with the same letter do not differ significantly according with Duncan's test ($P \leq 0.05$).

Morphological and cultural characterization. *Colletotrichum* isolates were classified into six morphological groups according to morphological and cultural characteristics (Table 1). Significant differences in colony morphology were observed among these six

groups of isolates identified when grown on PDA (Fig. 3). In total, three types of conidia were observed: cylindrical (observed in groups 1, 2, 4, 5), fusiform (group 3), and long cylindrical (group 6). Appresoria in groups 1 to 6 were brown and lightly irregular to irregular,

Fig. 3. Cultural traits of *Colletotrichum* species isolated from mulberry.

and the sizes of appressoria were not different ($P \leq 0.05$) among all the isolates except for group 6. Coupled with the below phylogenetic analyses, the specific descriptions are as follows: Group 1 isolates were olive to gray with a white edge, and after 10 days, gray to gray-brown with numerous black spots in reverse. Conidia were hyaline, cylindrical, aseptate, and measured 7.5 to 18.9×2.4 to $6.2 \mu\text{m}$ ($x = 14.82 \pm 3.55 \times 4.53 \pm 0.93$, $n = 30$). These characteristics were basically consistent with published descriptions of *C. kahawae* on *Coffea arabica* in Kenya (Waller et al. 1993) and *Eruca sativa* in Italy (Garibaldi et al. 2016). With the analysis of the glutamine synthetase (*GS*) gene, group 1 isolates were finally identified as *C. kahawae* subsp. *ciggaro*. The colonies of group 2 were gray to dark gray, and they produced pale orange conidia masses in the center and in reverse greyish green with white halos after 12 days. Conidia were hyaline, aseptate, smooth-walled with a large guttule, cylindrical with obtuse to rounded ends, and measured 7.9 to 17.8×3.5 to $6.3 \mu\text{m}$ ($x = 13.50 \pm 1.91 \times 4.66 \pm 0.60$, $n = 30$). Compared with *C. fruticola* described by Prihastuti et al. (2009), for which conidial masses were not visible in the colony, group 2 showed visible conidial masses after 10 days. This species is similar to reports for *C. fruticola* on *Citrus* (Huang et al. 2013) and *Annona* (Costa et al. 2017). Group 3 isolates were light pink, and the reverse was brownish pink to vinaceous with a few black spots. Conidia were 7.8 to 13.8×3.3 to $4.8 \mu\text{m}$ ($x = 11.30 \pm 1.18 \times 3.84 \pm 0.34$, $n = 30$), hyaline, aseptate, straight, and fusiform with acute ends. These characteristics were similar to the published descriptions of both *C. fioriniae* and *C. acutatum* that were reported by Damm et al. (2012a). Coupled with the analysis of multiple loci, group 3 isolates were identified as *C. fioriniae*. The characteristics of group 4 (Table 1) were highly similar to the descriptions of *C. karstii* (Damm et al. 2012b). Colonies produced by group 5 were gray to dark gray, and the reverse was gray-yellow to gray-black with some black spots. Conidia produced on PDA + W were hyaline, cylindrical, 8.7 to 14.8×3.6 to $5.2 \mu\text{m}$ ($x = 12.02 \pm$

$1.72 \times 4.46 \pm 0.47$, $n = 30$). Phylogenetic analysis showed that group 5 isolates were identified as *C. cliviae*. Group 6 isolates were first white, becoming gray to dark gray at the center with age, and in the reverse were gray-black with little black spots. Conidia measured 14.0 to 21.3×3.9 to $5.6 \mu\text{m}$ ($x = 18.64 \pm 2.04 \times 4.89 \pm 0.41$, $n = 30$) and were hyaline, aseptate with a large guttule at the center, and cylindrical with round ends. The sizes of conidia and appressoria were larger than the other five species (Table 1). These characters were consistent with the published descriptions of *C. brevisporum* on *Lycium chinense* (Paul et al. 2014) and *Capsicum annuum* (Liu et al. 2016a) in China.

Phylogenetic analyses. The sequences of single-spore strains and the reference sequences obtained from GenBank are shown in Table 2. The analysis of the individual *CAL*, *HIS3*, and *GS* sequences and combined ITS, *GAPDH*, *ACT*, *CHS-1*, and *TUB2* sequences of our six isolates compared with sequences of 19 *Colletotrichum* species reported on different hosts from GenBank (Table 2) allowed us to allocate the isolates into the molecular groups of known *Colletotrichum* species. Phylogenetic analyses were performed by both neighbor-joining and maximum likelihood methods, and only one tree constructed by the maximum likelihood method is shown in Figure 4. In the maximum likelihood tree (six representative isolates were used for further molecular identification), S37 (from group 5) and two *C. cliviae* isolates (CSSK4 and CSSS1) (Yang et al. 2009) formed a subclade with 99% bootstrap support; S38 (group 6) and the *C. brevisporum* isolate COUFAL7300 (Bezerra et al. 2016) formed a subclade with 100% bootstrap support; S29 (group 4) and two *C. karstii* isolates (CBS 118401 and CBS 130235) (Damm et al. 2012b) formed a subclade with 100% bootstrap support; S22 (group 3) and three *C. fioriniae* isolates (CBS 235.49, CBS 490.92, and CBS 129948) (Damm et al. 2012a) formed a subclade with 91% bootstrap support; and S8 (group 1) and the ex-type *C. kahawae* isolate ICMP 18539 (Weir et al. 2012) formed a subclade with 100% bootstrap support. Furthermore, an

Table 2. GenBank accession numbers of isolates included in this study

Species	Accession	Host	Locality	GenBank accessions ^a	
				ITS	GAPDH
<i>Colletotrichum acutatum</i>	CBS 129919, CPC 13876	<i>Hoodia</i> sp.	South Africa	JQ948370	JQ948701
	CBS 369.73, NRCC 10081	<i>Lupinus angustifolius</i>	New Zealand	JQ948350	JQ948681
<i>C. alienum</i>	ICMP 12071	<i>Malus domestica</i>	New Zealand	JX010251	JX010028
	ICMP 18621	<i>Persea americana</i>	New Zealand	JX010246	JX009959
<i>C. kahawae</i> subsp. <i>ciggaro</i>	S8	<i>Morus alba</i>	China	KY986888	KY986894
	ICMP 18539	<i>Olea europaea</i>	Australia	JX010230	JX009966
<i>C. fruticola</i>	CBS 237.49, ICMP 17922	<i>Hypericum perforatum</i>	Germany	JX010238	JX010042
	S21	<i>Morus alba</i>	China	KY986889	KY986895
	ICMP 18613	<i>Limonium sinuatum</i>	Israel	JX010167	JX009998
	ICMP 18581, CBS 130416	<i>Coffea arabica</i>	Thailand	JX010165	JX010033
<i>C. fioriniae</i>	S22	<i>Morus alba</i>	China	KY986890	KY986896
	CBS 235.49	<i>Malus</i> sp.	U.S.A.	JQ948325	JQ948655
	CBS 490.92, ATCC 60260	<i>Solanum lycopersicum</i>	New Zealand	JQ948326	JQ948656
	CBS 129948, RB128	<i>Tulipa</i> sp.	U.K.	JQ948344	JQ948674
<i>C. karstii</i>	S29	<i>Morus alba</i>	China	KY986891	KY986897
	CBS 118401	<i>Pachira</i> sp.	China	JQ005192	JQ005279
	CBS 130235	<i>Gossypium hirsutum</i>	Germany	JQ005190	JQ005277
	S37	<i>Morus alba</i>	China	KY986892	KY986898
<i>C. cliviae</i>	CSSK4	<i>Clivia miniata</i>	China	GQ485607	GQ856756
	CSSS1	<i>Clivia miniata</i>	China	GU109479	GU085867
<i>C. brevisporum</i>	S38	<i>Morus alba</i>	China	KY986893	KY986899
	COUFAL7300	<i>Sechium edule</i>	Brazil	KT285382	KT285381
<i>C. nupharicola</i>	CBS 469.96, ICMP 17938	<i>Nuphar lutea</i> subsp. <i>polysepala</i>	U.S.A.	JX010189	JX009936
	CBS 470.96, ICMP 18187	<i>Nuphar lutea</i> subsp. <i>polysepala</i>	U.S.A.	JX010187	JX009972
<i>Monilochaetes infuscans</i>	CBS 869.96	<i>Ipomoea batatas</i>	South Africa	JQ005780	JX546612

(Continued on next page)

^a ITS = internal transcribed spacers 1 and 2 together with 5.8S nrDNA; *GAPDH* = partial glyceraldehyde-3-phosphate dehydrogenase gene; *ACT* = partial actin gene; *CAL* = partial calmodulin gene; *CHS-1* = partial chitin synthase 1 gene; *HIS3* = histone3; *TUB2* = partial β -tubulin gene; and *GS* = glutamine synthetase. Sequences generated in this study are indicated in bold.

analysis of the *GS* gene sequences indicated that isolate S8 belonged to *C. kahawae* subsp. *ciggaro* (Weir et al. 2012).

Effect of temperature on mycelial growth. The optimum temperature for growth of all species was 25°C (Table 3). The six species were easily differentiated at 20 and 25°C. Group 1 had a significantly higher growth rate than the other five groups ($P \leq 0.05$) at temperatures less than or equal 25°C but was sensitive to higher temperatures (30°C), and no mycelial growth was observed at 35°C. Relatively, *C. brevisporum* (group 6) was tolerant to high temperatures (30, 35°C), and its average growth rate was still 14.0 mm/5 days at 35°C. Additionally, group 1 (*C. kahawae* subsp. *ciggaro*) was the most tolerant to low temperatures (5, 10°C), followed by *C. fioriniae* (group 3) and *C. karstii* (group 4), whereas no mycelial growth of group 2 (*C. fruticola*), group 5 (*C. cliviae*), and group 6 (*C. brevisporum*) was observed at 5°C.

Pathogenicity. Pathogenicity tests showed that *C. fioriniae* was the most aggressive species, *C. karstii*, *C. kahawae* subsp. *ciggaro*, and *C. brevisporum* were weak pathogens, and *C. fruticola* and *C. cliviae* could not infect detached mulberry leaves (Table 4). Ten days after wounded or nonwounded inoculation, distinct water-soaked, brown or dark necrotic spots or streaks developed (Fig. 5). No symptoms developed on the corresponding controls. The symptoms caused by *C. fioriniae* developed brown necrotic spots after 10 days, which were similar to those that occur under natural conditions in the field (Fig. 5a). Only in the inoculation series using mycelial plugs of group 1, *C. kahawae* subsp. *ciggaro* showed weak pathogenicity on the intact and punctured mulberry leaves, respectively (Fig. 5h and i). Obviously, under the high-concentration conidial suspension (1×10^9 conidia/ml) and wounded plants inoculated with PDA plugs conditions, *C. karstii* (group 4) produced lesions (Fig. 5d to g). When nonwounded plants were inoculated by group 6 (*C. brevisporum*) with 1×10^6 conidia/ml, few symptoms developed; however, when inoculated with 1×10^9 conidia/ml, up to 43% of the nonwounded

leaves developed dark necrotic lesions (Fig. 5c); wounded and nonwounded plants inoculated with noncolonized PDA plugs remained nonsymptomatic. The *Colletotrichum* species were reisolated from the symptomatic leaves, and their identification was confirmed by morphology and molecular assays as described above.

Discussion

In the past few decades, anthracnose on mulberry (*M. alba*) has been mainly attributed to *C. dematium* (Babu et al. 2008; Yoshida et al. 1995). So far, *Colletotrichum* species on mulberry have been only identified by using morphological characteristics (Tai 1979; Tian 1981; Yoshida et al. 1995; Zhang et al. 2011). However, many investigations have shown that it is difficult to accurately identify *Colletotrichum* species only by morphological and cultural features (Baroncelli et al. 2015; Cai et al. 2009; Cannon et al. 2000, 2012; Damm et al. 2009; Faedda et al. 2011). Therefore, phylogenetic analysis is indispensable for the identification of *Colletotrichum* species (Cai et al. 2009, 2011). This is the first time that *Colletotrichum* species on mulberry have been identified by both morphological data and sequence analysis of multiple loci. Accordingly, 128 isolates were accurately identified as *C. kahawae* subsp. *ciggaro* (six strains), *C. fruticola* (52 strains), *C. fioriniae* (46 strains), *C. karstii* (11 strains), *C. cliviae* (nine strains), and *C. brevisporum* (four strains). None of these species was previously reported on mulberry in China.

In recent years, *C. fioriniae* has been widely reported in China as a leaf spot pathogen in *Cinnamomum subavenium* (Sun et al. 2012), *Ficus virens* (Xue et al. 2017), and *Juglans regia* (Zhu et al. 2015); and as a fruit rot pathogen in *Capsicum annuum* and *Capsicum* spp. (Diao et al. 2017), *Citrus* spp. (Chen et al. 2017), and *Lycium barbarum* (Liu et al. 2016b). *C. kahawae* was recorded on chili peppers in China (Diao et al. 2017), and it was further identified as *C. kahawae* subsp. *ciggaro* by analyzing the *GS* gene in this study.

Table 2. (Continued from preceding page)

GenBank accessions ^a						References
<i>ACT</i>	<i>CAL</i>	<i>CHS-1</i>	<i>HIS3</i>	<i>TUB2</i>	<i>GS</i>	
JQ949691	...	JQ949031	JQ949361	JQ950021	...	Damm et al. (2012a)
JQ949671	...	JQ949011	JQ949341	JQ950001	...	Damm et al. (2012a)
JX009572	JX009654	JX009882	...	JX010411	JX010101	Weir et al. (2012)
JX009552	JX009657	JX009755	...	JX010386	JX010075	Weir et al. (2012)
KY986900	KY986906	KY986912	MF033876	MF033882	MK585939	This study
JX009523	JX009635	JX009800	...	JX010434	JX010132	Weir et al. (2012)
JX009450	JX009636	JX009840	...	JX010432	JX010120	Weir et al. (2012)
KY986901	KY986907	KY986913	MF033877	MF033883	MK585940	This study
JX009491	JX009675	JX009772	...	JX010388	JX010077	Weir et al. (2012)
FJ907426	FJ917508	JX009866	...	JX010405	JX010095	Weir et al. (2012)
KY986902	KY986908	KY986914	MF033878	MF033884	MK585941	This study
JQ949646	...	JQ948986	JQ949316	JQ949976	...	Damm et al. (2012a)
JQ949647	...	JQ948987	JQ949317	JQ949977	...	Damm et al. (2012a)
JQ949665	...	JQ949005	JQ949335	JQ949995	...	Damm et al. (2012a)
KY986903	KY986909	KY986915	MF033879	MF033885	MK585942	This study
JQ005540	JQ005713	JQ005366	JQ005453	JQ005626	...	Damm et al. (2012b)
JQ005538	JQ005711	JQ005364	JQ005451	JQ005624	...	Damm et al. (2012b)
KY986904	KY986910	KY986916	MF033880	MF033886	MK585943	This study
GQ856777	GQ849464	GQ856722	...	GQ849440	...	Yang et al. (2009)
GU085861	GU085863	GU085865	...	GU085869	...	Yang et al. (2009)
KY986905	KY986911	KY986917	MF033881	MF033887	...	This study
KT285378	KT285379	KT285380	...	KT285383	...	Bezerra et al. (2016)
JX009486	JX009661	JX009834	...	JX010397	JX010087	Weir et al. (2012)
JX009437	JX009663	JX009835	...	JX010398	JX010088	Weir et al. (2012)
JQ005843	...	JQ005801	JQ005822	JQ005864	...	O'Connell et al. (2012)

C. kahawae subsp. *ciggaro* has been widely reported in many countries, including Australia, Brazil, Germany, New Zealand, South Africa, the United States, Colombia, Italy, Portugal, and Spain (Afanador-Kafuri et al. 2014; Liu et al. 2013; Schena et al. 2014; Weir et al. 2012). *C. brevisporum* has been reported on *Citrus medica* (Jayawardena et al. 2016; Peng et al. 2012) and *Passiflora edulis* (Du et al. 2017) from China. *C. karstii* was recorded as an endophyte from *Bletilla ochracea* (Tao et al. 2013) and as a pathogen on *Citrus* spp. (Huang et al. 2013), *Hevea brasiliensis* (Cai et al. 2016), *Stylosanthes guianensis* (Jia et al. 2017), and *Nandina domestica* (Li et al. 2018) in China. *C. fructicola* has been reported as an endophyte of several plants in Central America (Rojas et al. 2010). In this research, *C. fructicola* was the most prevalent species, but it showed no pathogenicity to mulberry leaves. In addition, although *C. cliviae* was recently reported to cause anthracnose on *Glycine max* (Barbieri et al. 2017), *Capsicum* spp. (Saini et al. 2017), and *Zamioculcas zamiifolia* (Zhou and Li 2017), our studies showed that *C. cliviae* was not pathogenic to

mulberry leaves. This is the first report of two *Colletotrichum* species as leaf endophytes in mulberry.

Temperature is a primary factor that affects epidemics of plant disease (Bonde et al. 2012). The maximum growth temperature is also an important biological feature and widely used to characterize microorganisms (Damm et al. 2012a, 2012b; Prihastuti et al. 2009; Weir et al. 2012). In this study, temperature data showed that each species had a stable and distinguishable maximum temperature, which may be useful to distinguish *Colletotrichum* species on mulberry. On the other hand, these data may also provide useful information for the effective management of this disease in the future.

According to both the field and indoor inoculation symptoms, *C. fioriniae* forms typical lesions on mulberry leaves, but no clear lesions caused by *C. karstii*, *C. kahawae* subsp. *ciggaro*, and *C. brevisporum* were observed. *C. fioriniae* is the most important causal agent of mulberry leaf anthracnose, whereas *C. karstii*, *C. kahawae* subsp. *ciggaro*, and *C. brevisporum* showed a low pathogenicity to leaves.

Fig. 4. Phylogram generated from maximum likelihood analysis based on alignment of ITS, GAPDH, ACT, CHS-1, and TUB2. Bootstrap values (1,000 replicates) above 70% are shown at the nodes. Host and country of origin are listed next to the isolate numbers. Isolates obtained in this study are shown in bold. *Monilochaetes infuscans* (CBS 869.96) is used as an outgroup.

Table 3. Growth rate (mm/5 days) of *Colletotrichum* isolates from mulberry cultured on PDA at different temperatures^z

Species (group)	5°C	10°C	15°C	20°C	25°C	30°C	35°C
<i>C. kahawae</i> subsp. <i>ciggaro</i> (group 1)	1.6 ± 0.3 a	5.4 ± 0.7 a	16.0 ± 0.3 a	27.7 ± 0.6 a	35.8 ± 0.7 a	7.0 ± 0.6 e	0 e
<i>C. fructicola</i> (group 2)	0 c	2.4 ± 0.5 c	11.4 ± 0.7 c	24.1 ± 0.7 b	34.3 ± 0.5 b	20.1 ± 0.7 b	3.7 ± 0.3 b
<i>C. fioriniae</i> (group 3)	1.2 ± 0.6 b	2.5 ± 0.2 c	8.9 ± 0.5 d	15.6 ± 0.4 f	19.2 ± 0.3 f	6.2 ± 0.3 d	1.6 ± 0.2 d
<i>C. karstii</i> (group 4)	0.8 ± 0.3 b	2.5 ± 0.5 c	9.6 ± 0.2 d	18.3 ± 0.3 e	25.1 ± 0.5 e	14.1 ± 0.3 c	1.5 ± 0.3 d
<i>C. cliviae</i> (group 5)	0 c	2.7 ± 0.9 bc	13.0 ± 1.0 b	22.8 ± 0.4 c	32.9 ± 0.4 c	20.0 ± 0.4 b	3.1 ± 0.1 c
<i>C. brevisporum</i> (group 6)	0 c	3.6 ± 0.5 b	13.3 ± 0.3 b	21.8 ± 0.5 d	30.2 ± 0.4 d	29.7 ± 0.1 a	14.0 ± 0.2 a

^z Columns with the same letter do not differ significantly according with Duncan's test ($P \leq 0.05$).

Table 4. Pathogenicity of *Colletotrichum* isolates on leaves of detached twigs, 10 days after wound and nonwound inoculation^w

Species	Infected leaves (%)				Lesion diameter (mm)			
	Conidial suspension		PDA plugs (4 mm diameter)		Conidial suspension		PDA plugs (4 mm diameter)	
	$1 \times 10^6 \text{ ml}^{-1}$	$1 \times 10^9 \text{ ml}^{-1}$			$1 \times 10^6 \text{ ml}^{-1}$	$1 \times 10^9 \text{ ml}^{-1}$		
	Nonwounded ^x	Nonwounded ^x	Nonwounded ^y	Wounded ^z	Nonwounded ^x	Nonwounded ^x	Nonwounded ^y	Wounded ^z
Check	...	...	...	...	...	...	...	...
<i>C. kahawae</i> subsp. <i>cigarro</i>	...	...	18.3 ± 5.8	31.7 ± 7.6	...	...	9.5 ± 1.9 × 12.0 ± 2.0	10.6 ± 0.4 × 13.1 ± 0.2
<i>C. fruticola</i>	...	...	...	...	...	...	...	...
<i>C. fioriniae</i>	81.7 ± 7.6	100.0 ± 0.0	70.0 ± 10.0	91.7 ± 2.9	4.4 ± 0.5 × 5.4 ± 0.6	10.1 ± 1.7 × 13.2 ± 1.7	12.8 ± 1.0 × 15.0 ± 1.3	16.3 ± 1.6 × 19.5 ± 1.2
<i>C. karstii</i>	...	16.7 ± 2.9	...	28.3 ± 5.8	...	6.5 ± 0.6 × 7.8 ± 1.1	...	8.1 ± 1.3 × 10.0 ± 1.6
<i>C. cliviae</i>	...	...	...	...	...	...	...	...
<i>C. brevisporum</i>	3.3 ± 2.9	43.3 ± 10.4	...	...	1.3 ± 1.2 × 1.7 ± 1.5	5.4 ± 0.8 × 6.7 ± 0.7	...	...

^w Table shows incidence of disease by percent and lesion diameter following inoculation of wounded and unwounded mulberry leaves. Data represent the mean of three replications, each with 20 leaves on detached twigs. Check = mulberry twigs inoculated with sterile water suspension or PDA plugs without the pathogens (4 mm diameter) (as controls). Ellipsis (...) indicates no symptoms developed on detached leaves.

^x Unwounded, inoculated with 15 µl of conidial suspension.

^y Unwounded, inoculated with a 4-mm PDA plug.

^z Wounded by sterile pinprick, inoculated with a 4-mm PDA plug.

Fig. 5. Symptoms observed in pathogenicity tests on leaves of detached twigs 10 days after inoculation: **a**, symptoms induced with 10^6 conidial suspension of isolate S22; **b**, symptoms induced with a 4-mm PDA plug of isolate S22 without wounding; **c**, symptoms induced with 10^9 conidial suspension of isolate S38; **d and e**, symptoms induced with a 4-mm PDA plug of isolate S29 with wounding; **f and g**, symptoms induced with 10^9 conidial suspension of isolate S29; and **h and i**, symptoms induced with a 4-mm PDA plug of isolate S8 with wounding.

Additionally, *C. fruticicola* and *C. cliviae* were reported for the first time as endophytes on mulberry in China. *C. dematium*, which was once reported to be responsible for mulberry anthracnose in Japan (Yoshida and Shirata 1999), was not found in this study. With the changes of a number of factors such as species, inoculation methods, and conditions, symptoms of inoculation varied considerably (Freeman et al. 1998; Simmonds 1966). Therefore, in order to more accurately reflect the true virulence potential of these species, further research should be conducted to determine the pathogenicity according to natural infections rather than artificial inoculations (Niu et al. 2016; Prihastuti et al. 2009).

Since several decades ago, leaf spots caused by *Colletotrichum* species had been a common fungal disease on mulberry in several countries, including Japan (Hara 1925, 1954; Kobayashi 2007; Yoshida et al. 1995), India (Mathur 1979), and China (Tai 1979; Tian 1981; Zhang et al. 2011). In this study, we accurately identified six *Colletotrichum* species isolated from mulberry leaves based on the morphological and molecular characteristics; pathogenicity tests showed that *C. fiorinae* was the most aggressive species. This information is useful for implementing management strategies for mulberry anthracnose in China.

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