Characterization and Fungicide Sensitivity of *Colletotrichum* Species Causing Strawberry Anthracnose in Eastern China

Liqing Zhang,^{1,2} Lili Song,³ Xiangming Xu,⁴ Xiaohua Zou,¹ Ke Duan,^{1,3,†} and Qinghua Gao^{1,†}

¹ Shanghai Key Laboratory of Protected Horticultural Technology, Forestry and Fruit Tree Research Institute, Shanghai Academy of Agricultural Sciences, Shanghai 201403, China

² Shanghai Agricultural Seed & Seedling Co., Ltd.

³ College of Food Science, Shanghai Ocean University, Shanghai 201306, China

⁴ NIAB EMR, New Road, East Malling, Kent ME19 6BJ, U.K.

Abstract

Strawberry anthracnose caused by *Colletotrichum* spp. is one of the most serious diseases in the strawberry fields of China. In total, 196 isolates of *Colletotrichum* were obtained from leaves, stolons, and crowns of strawberry plants with anthracnose symptoms in eastern China and were characterized based on morphology, internal transcribed spacer (ITS), and β -tubulin (*TUB2*) gene sequences. All 196 isolates were identified as the *Colletotrichum gloeosporioides* species complex. In total, 62 strains were further identified at the species level by phylogenetic analyses of multilocus sequences of ITS, *glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*), *actin* (*ACT*), *Apn2-Mat1-2 intergenic spacer and partial mating type* (*ApMat*), *calmodulin* (*CAL*), and *TUB2*. Three species from the *C. gloeosporioides* species complex were identified: *Colletotrichum siamense*, *C. fruticola*, and *C. aenigma*. Isolates of *C. siamense* were tolerant to high temperatures, with a significantly larger colony diameter

than the other two species when grown above 36°C. The inoculation of strawberry plants confirmed the pathogenicity of all three species. *C. siamense* isolates resulted in the highest disease severity. The in vitro sensitivities of *C. siamense* and *C. fruticola* isolates to azoxystrobin and three demethylation-inhibitor (DMI) fungicides (difenoconazole, tebuconazole, and prochloraz) were determined. Both species were sensitive to DMI fungicides but not to azoxystrobin. *C. siamense* isolates were more sensitive to prochloraz, while *C. fruticola* isolates were more sensitive to difenoconazole and tebuconazole. The present study provides valuable information for the effective management of strawberry anthracnose.

Keywords: anthracnose, *Colletotrichum gloeosporioides*, fungicide sensitivity, strawberry

Strawberry (*Fragaria × ananassa*) is a popular and widely consumed fruit (Khoshnevisan et al. 2013, 2014). Strawberry production has expanded rapidly in recent years, and China has become one of the world's largest strawberry producers and exporters. In 2016, the global production of strawberry fruit was 9.2 million tons, with China accounting for 41% of the total (FAOSTAT 2016). With the increase in production and expansion of land allocated to strawberry growth, fungal diseases have become a major factor influencing strawberry yield and quality in China.

Anthracnose, caused by *Colletotrichum* spp., is a major strawberry disease in subtropical climates, in which hot and humid conditions favor disease development and can result in significant crop losses (Anciro et al. 2018). Strawberry anthracnose symptoms occur on all plant parts (Buddie et al. 1999). Lesions on stolons and petioles, as well as irregular leaf spots, are mainly found in plant nurseries (Freeman et al. 2001). In field crops, flower blight and fruit rot are common symptoms (Howard et al. 1992). Crown rot is characterized

by reddish-brown necrotic areas (Cai et al. 2009, 2011) and, in some cases, stunting and chlorosis have been associated with root necrosis (Freeman et al. 2001).

Colletotrichum is an important genera of plant-pathogenic fungi (Damm et al. 2012; Udayanga et al. 2013). The internal transcribed spacer (ITS) sequences alone, which have long been used to classify fungal species, cannot fully differentiate *Colletotrichum* spp. (Weir et al. 2012). A multilocus phylogeny coupled with morphological data are now usually used for the identification of *Colletotrichum* spp. (Cai et al. 2009, 2011). Three species have been reported as causal agents of strawberry anthracnose: *Colletotrichum acutatum*, *C. fragariae* (which is now known as *C. theobromicola*), and *C. gloeosporioides* (Denoyes-Rothan et al. 2003). Based on multilocus phylogeny and morphological data, *C. acutatum* and *C. gloeosporioides* have been defined as species complexes, containing 36 and 38 species, respectively (Damm et al. 2012; Marin-Felix et al. 2017; Weir et al. 2012).

C. gloeosporioides was described for the first time as a strawberry pathogen in the southeastern United States in 1930 (Brooks 1931). In the United States, epidemics of strawberry crown rot are mainly caused by *C. gloeosporioides* (Ureña-Padilla et al. 2002) but *C. gloeosporioides* is found only occasionally on strawberry in Europe (Freeman and Katan 1997; Van Hemelrijck et al. 2010). In Asia, *C. gloeosporioides* is the main cause of strawberry anthracnose. In Japan, Korea (Nam et al. 2013; Suzuki et al. 2010), and China (Jayawardena et al. 2016), *C. fruticola* is the predominant species.

Strawberry anthracnose has been mainly managed with fungicide applications. Since the 1990s, the most commonly used fungicides are benzimidazole fungicides such as carbendazim and thiophanate methyl (Han et al. 2009). However, since 2009 in China, benzimidazole-resistant *C. gloeosporioides* isolates have been frequently found in strawberry, leading to disease control failures (Han et al. 2009). At present, there are no other types of fungicides registered for controlling strawberry anthracnose in China (Xu et al. 2014). Demethylation-inhibitor (DMI) fungicides are alternatives to benzimidazole fungicides, and some of them (prochloraz

†Corresponding authors: Q. Gao; qhgao20338@sina.com; and K. Duan; kduan936@126.com

L. Zhang and L. Song contributed equally to this work.

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and tebuconazole) have been registered for use against grape anthracnose. The baseline sensitivity levels of *C. gloeosporioides* isolates to prochloraz and tebuconazole in strawberry and grape have been determined (Xu et al. 2014). Quinone outside inhibitor (QoI) fungicides are the primary choice for anthracnose control caused by *Colletotrichum* spp. in commercial strawberry fields in the United States (Brannen and Smith 2015).

Strawberry anthracnose was first reported in eastern China in 1990 (Hu 1990). Recent studies in some areas of China have identified the *Colletotrichum* spp. causing strawberry anthracnose to be *C. nymphaeae* (*C. acutatum* species complex) (Hubei Province and Beijing City), *C. fruticola* (Hubei, Liaoning, and Hainan Provinces and Beijing City), *C. gloeosporioides* (Hubei Province), *C. aenigma* (Hubei Province), and *C. changpingense* (*C. gloeosporioides* species complex) (Beijing City) (Han et al. 2016; Jayawardena et al. 2016). Strawberry is among the most widely grown fruit in eastern China, which has a moderate climate with plentiful rainfall (Wang et al. 2012). There have not been systematic studies of strawberry anthracnose in eastern China. The objectives of the present study were to (i) identify the *Colletotrichum* spp. associated with strawberry anthracnose in eastern China based on multilocus phylogeny and morphology, (ii) determine the pathogenicity of different *Colletotrichum* spp., and (iii) determine the sensitivity of *Colletotrichum* spp. to QoI (azoxystrobin) and DMI fungicides.

Materials and Methods

Fungal isolate collection. From 2016 to 2018, leaves, stolons, and crowns of strawberry plants with anthracnose symptoms were collected from six provinces of eastern China (Fig. 1). *Colletotrichum* spp. were isolated as described (Cao et al. 2019). Three pieces (5 by 5 mm) of tissue were taken from the margins between necrotic and apparently healthy tissues of each plant and surface sterilized in 70% ethanol for 30 s and then 1% NaClO₃ for 1 min. Finally, the samples were rinsed three times with sterilized distilled water and dried before being plated onto potato dextrose agar (PDA). PDA plates were incubated at 28°C under continuous darkness for 3 to 7 days. Single-spore cultures were obtained for each isolate in accordance with a previously published method (Cai et al. 2009), and then they were subcultured onto fresh PDA plates.

DNA extraction, PCR amplification, and DNA sequencing.

Isolates were grown on PDA at 28°C for 7 days. Mycelia of each isolate were scraped from the colony surface with a sterile 20-μl pipette tip. Total genomic DNA was extracted using the E.Z.N.A. Fungal DNA Mini Kit (Omega Bio-tek, Germany) according to the manufacturer's instructions.

As an initial analysis of genetic diversity, ITS and the β -tubulin (*TUB2*) gene were amplified from all of the isolates obtained in this study using the primers ITS1/ITS4 and T1/Bt2b, respectively (Glass and Donaldson 1995; O'Donnell and Cigelnik 1997; White et al. 1990).

A subset of 62 isolates, representing different geographic origins and genetic diversity (from initial ITS and *TUB2* analysis), was then selected for further investigation. The ITS and partial sequences of the *glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*), *actin* (*ACT*), *calmodulin* (*CAL*), *TUB2*, and the *Apn2-Mat1-2 intergenic spacer and partial mating type* (*Mat1-2*) gene (*ApMat*) were amplified and sequenced using the primer pairs ITS1/ITS4 (White et al. 1990), GDF1/GDR1 (Templeton et al. 1992), ACT512F/ACT783R (Carbone and Kohn 1999), CL1/CL2A (O'Donnell et al. 2000), T1/Bt2b (Glass and Donaldson 1995; O'Donnell and Cigelnik 1997), and AMF1/AMR1 (Silva et al. 2012), respectively.

The PCR products were examined by electrophoresis in 1.5% agarose gels stained with 4S Red Plus Nucleic Acid Stain (Sangon Biotech Co., Ltd., China) in a 1.0x Tris-acetate acid-EDTA buffer and photographed under UV light. The PCR amplifications were performed with a Mastercycler nexus (Eppendorf, Germany) in a 50-μl reaction volume. The PCR mixtures contained 1 μl of DNA template, 25 μl of TaKaRa Premix Taq (Ex Taq version; TaKaRa, Tokyo, Japan), and 1.5 μM each primer. The PCR amplification protocols were performed as described by Cao et al. (2019) but the denaturing temperatures were adjusted to 55°C for ITS and *CAL*, 58°C for *GAPDH*, 60°C for *ACT* and *TUB2*, and 61°C for *ApMat*. The PCR products were purified using the AxyPrep PCR Cleanup Kit (Axygen Biosciences, China) following the manufacturer's instructions. Sequencing the PCR amplicons was carried out by Sangon Biotech Co., Ltd.

Phylogenetic analyses. Multiple sequence alignments incorporating each gene were performed with BioEdit (Hall 1999) and ClustalX (Thompson et al. 2003). Alignments were manually adjusted to allow

Fig. 1. Map showing locations in eastern China where strawberry plants were sampled for *Colletotrichum* spp.

for the maximum alignment and to minimize gaps. The consensus sequences were deposited in GenBank (Supplementary Table S1). The partial DNA sequences of ITS, *GAPDH*, *ACT*, *ApMat*, *CAL*, and *TUB2* for 62 representative isolates of the *C. gloeosporioides* complex from different regions, 54 reference isolates of *C. gloeosporioides*, and the isolate MAFF305972 of *C. boninense* were used to establish a six-locus combined dataset (*ACT+ApMat+CAL+GAPDH+ITS+TUB2*) for the Bayesian inference analysis. The latter (MAFF305972) was used as an outgroup. The phylogenetic analysis was conducted using a Markov Chain Monte Carlo algorithm to generate trees with Bayesian posterior probabilities in MrBayes v.3.2.2 (Ronquist et al. 2012). MrModeltest v.2.3 (Nylander 2004) was used to carry out the statistical selection of best-fit models of nucleotide substitutions using the corrected Akaike information criteria. Specifically, the models selected were GTR+I for *ACT* and ITS, K80+G for *ApMat*, GTR+I+G for *CAL*, HKY for *GAPDH*, and SYM+G for *TUB2*; these models were incorporated into the analysis. The heating parameter was set to 0.2, and analyses were stopped once the average standard deviations of the split frequencies were below 0.01. Using these criteria, the first 25% of generations were discarded as burn-in. The generated 50% majority rule consensus tree was visualized using FigTree, version 1.4.2 (<http://tree.bio.ed.ac.uk/software/figtree/>). Clades were regarded as significantly supported if they had a posterior probability ≥ 0.95 (Ronquist et al. 2012).

Morphological analysis. Mycelial discs (9 mm in diameter) were taken from 7-day-old cultures, plated on PDA, and incubated at 28°C in the dark. The colony diameter (in millimeters) was measured in two perpendicular directions. The daily growth rate was calculated as the 7-day average of mean daily growth (millimeters per day), and colony characteristics were recorded. The experiment was designed as a randomized complete block. There were three replications of each isolate per temperature treatment. Characteristics of conidia, conidiophores, and appressoria were determined using methods described previously (Cai et al. 2009) and assessed using a digital microscope (DM2500; Leica, Germany). Appressoria induced on a synthetic nutrient-poor agar medium were measured using a slide culture technique (Cai et al. 2009).

Effects of temperature on mycelial growth. Based on the phylogenetic analyses, 13 *Colletotrichum* isolates obtained from different locations were evaluated to determine the effects of temperatures on radial colony growth in culture. The selected isolates consisted of five *C. siamense* isolates (GQHSD5, GQHHN12, GQHAH2, GQHZJ15, and GQH12), five *C. fructicola* isolates (GQHSD2, GQHZJ1, GQHAH6, GQHHN8, and GQH131), and three *C. aenigma* isolates (GQH124, GQH116, and GQHJS18). The protocols

performed were as described by Han et al. (2016). The plates were wrapped with parafilm and placed in an incubator (MJP-250; Shanghai Jing Hong Laboratory Instrument Co., Ltd., China) in the dark with the temperature set at one of the following values: 5, 10, 15, 20, 25, 28, 30, 32, 34, 36, or 38°C. After 5 days, the colony diameter (in millimeters) was measured and the daily growth rate was calculated. The experiment was designed as a randomized complete block. There were three replications of each isolate per temperature treatment.

Pathogenicity assay. Strawberry plants (cultivar Benihoppe) were used to confirm the pathogenicity of identified *Colletotrichum* spp. Three isolates of each identified species were chosen for pathogenicity tests: *C. siamense* (GQH12, GQHZJ15, and GQHAH8), *C. fructicola* (GQH26, GQHHN8, and GQHAH6), and *C. aenigma* (GQH124, GQHJS18, and GQH116). To further investigate the pathogenicity of the *C. gloeosporioides* species complex, 10 isolates of *C. siamense* (GQHSD5, GQH12, GQH39, GQH46, GQH137, GQHAH2, GQHAH8, GQHHN12, GQHZJ15, and GQHZJ19) and 10 isolates of *C. fructicola* (GQHSD2, GQH26, GQH64, GQH94, GQH131, GQH133, GQHJS19, GQHAH6, GQHHN8, and GQHZJ1) were chosen for pathogenicity tests. Conidial suspensions were prepared from 7-day-old cultures as previously described (Han et al. 2016). Stolon-derived healthy plants with more than 10 fully expanded compound leaves were inoculated by spraying a conidial suspension (10^6 conidia/ml with 0.01% [vol/vol] Tween 20 in sterile water) until runoff occurred (Freeman and Katan 1997). Mock inoculations of plants were carried out using just the Tween 20–water solution. Six plants, one per pot, for each isolate were inoculated, covered with plastic bags, and immediately placed in growth chambers at 90 to 100% relative humidity at 25°C in the dark for 48 h. The plastic bags were then removed and plants were maintained in growth chambers with a 12-h photoperiod at 25 and 20°C (day and night, respectively). Disease development was recorded 5 to 10 days after inoculation, as previously described (Han et al. 2016). The rating scale for leaves and petioles is listed in Table 1. An index of the disease was also calculated for each isolate, using Horiuchi and Hori's method: index of disease (%) = $[(n_0 \times 0 + n_1 \times 1 + n_3 \times 3 + n_5 \times 5 + n_7 \times 7 + n_9 \times 9) / 9N] \times 100$, where n represents the number of leaves or petioles in each class (0, 1, 3, 5, 7, and 9) and N represents the total number of leaves or petioles (Horiuchi and Hori 1980). These experiments were performed twice.

Fungicide sensitivity assays. In total, 15 isolates from *C. siamense* (GQHSD5, GQH17, GQH12, GQH39, GQH46, GQH137, GQHAH2, GQHAH8, GQHAH11, GQHHN11, GQHHN12, GQHHN13, GQHZJ15, GQHZJ19, and GQHZJ20) and 15 isolates from *C. fructicola* (GQHSD2, GQHSD7, GQH26, GQH64, GQH94, GQH131, GQH133, GQHAH6, GQHAH16, GQHHN8, GQHZJ1, GQHJS1, GQHJS3, GQHJS19, and GQHJS21) were selected based on geographical origin and species, and they were tested for their sensitivities. Each chemical was prepared separately in sterile distilled water and adjusted to a concentration of 10,000 µg/ml as the stock solution. The stock solutions were diluted to a series of concentrations and added to cooling, melted PDA (approximately 50°C) after autoclaving (Table 2). Mycelial plugs (5 mm in diameter) were removed from the margins of colonies of 7-day-old cultures actively growing on PDA and placed

Table 1. Rating scale for leaves and petioles inoculated by *Colletotrichum* spp.

Rating	Area of leaf surface (%)	Length of lesions (petioles) (mm)
0	Healthy leaves with no lesions	Healthy petioles with no lesions
1	0.1–5.0	0.1–2
3	5.1–15.0	2.1–5.0
5	15.1–30.0	5.1–9.0
7	30.1–50.0	9.1–15.0
9	50.1–100 or dead leaves	>15.0 or leaf wilting

Table 2. List of the fungicides used in this study^z

FRAC code	Chemical name	Manufacturer (in China)	Concentration (a.i. µg/ml)
11	Azoxystrobin	25SC; Syngenta Nantong Crop Protection Co. Ltd.	0, 100, 250, 500, 1,000, 2,000
3	Difenoconazole	10WDG; Syngenta Nantong Crop Protection Co. Ltd.	0, 0.01, 0.1, 2.5, 5, 10
3	Tebuconazole	43SC; Bayer Crop Sciences Co. Ltd.	0, 0.01, 0.05, 0.1, 10, 20
3	Prochloraz	45ME; Shenzhen Noposion Agrochemicals Co. Ltd.	0, 0.01, 0.05, 0.1, 0.5, 1

^z FRAC codes of fungicides according to the Fungicide Resistance Action Committee (FRAC) website (<https://www.frac.info/>); a.i. = active ingredient.

Fig. 2. Phylogenetic consensus tree based on Bayesian inference, illustrating the relationships within the *Colletotrichum gloeosporioides* species complex of isolates obtained from strawberry plants in eastern China. The tree was built with a Bayesian analysis of concatenated sequences of *actin* (ACT), *Apn2-Mat1-2 intergenic spacer and partial mating type* (ApMat), *calmodulin* (CAL), *glyceraldehyde-3-phosphate dehydrogenase* (GAPDH), internal transcribed spacer (ITS), and *β-tubulin* (TUB2). The Markov chain was run for 5,000,000 generations, each locus having a separate model of DNA evolution. *C. boninense* (MAFF305972) was used as the outgroup. Bayesian posterior probability values ≥ 0.95 are shown next to the nodes.

onto the center of plates containing fungicide. Plates were incubated at 28°C in the dark. The plates were incubated for 7 days at 28°C, and the diameter of each colony was measured in two perpendicular directions. There were three replicate plates, and the experiments were repeated twice.

The fungicide concentration at which fungal development is inhibited by 50% (EC_{50}) was used to measure fungicide sensitivity (Wong and Midland 2007; Wong and Wilcox 2002). The fungal relative growth (RG) at a given fungicide concentration was calculated as a percentage of fungal growth of the control plates. The EC_{50} value was estimated by the linear regression of the probit-transformed relative inhibition value ($1 - RG$) at the $\log_{10}$ transformed-fungicide concentration. The EC_{50} value for each isolate was calculated as the mean of the three experiments, and the correlation coefficients (r) among EC_{50} values of different fungicides were determined using the statistical algorithms of SAS software (version 9.4 for Windows; SAS Institute, Cary, NC, U.S.A.).

Data analyses. Daily growth rate data were subjected to an analysis of variance (ANOVA) followed by Duncan's multiple range test using SAS software. The minimum, optimum, and maximum growth temperature data were analyzed using the general linear model procedure in SAS. Species were treated as fixed effects and isolates were

Fig. 3. Culture characteristics and microscopic features of the *Colletotrichum gloeosporioides* species complex from strawberry anthracnose. **A**, Upper and reverse view of 1-week-old colonies on potato dextrose agar; **B**, conidiomata of the *C. gloeosporioides* species complex; **C**, micrographs of conidia of the *C. gloeosporioides* species complex; **D**, micrographs of mycelial appressoria of the *C. gloeosporioides* species complex; and **E**, micrographs of conidial appressoria of the *C. gloeosporioides* species complex conidia. Scale bars = 20 μ m.

treated as random effects. When F values were significant ($P < 0.05$), the least significant difference tests were used to compare the means of three species. Data from the pathogenicity assay were subjected to an ANOVA using SAS software to determine the significance levels of treatment differences. Means for different species from the pathogenicity test were separated using least significant difference tests ($P = 0.05$). Disease indices for *C. siamense* ($n = 10$) and *C. fructicola* ($n = 10$) isolates on strawberry plants were analyzed using Duncan's multiple range test. Mean logEC₅₀ values for *C. siamense* and *C. siamense* for the same fungicide were compared using two-tailed t tests with $\alpha = 0.05$. An ANOVA was performed on the logEC₅₀ values to compare the sensitivity of each species to the fungicides tested when the fungicide and isolate were treated as factors. To determine levels of cross-sensitivity between pairs of tested fungicides, coefficients of correlation using the logEC₅₀ values between any two fungicides were calculated for each species.

Results

Molecular identification and phylogenetic analyses. In total, 196 *Colletotrichum* strains were isolated from diseased strawberry plant samples (leaves, stolons, and crowns) from six provinces (Shanghai, Anhui, Henan, Shandong, Jiangsu, and Zhejiang) of eastern China (Fig. 1). Based on the phylogenetic analysis of the combined ITS and *TUB2* alignment and the colony morphological characteristics on PDA, all of these *Colletotrichum* isolates were initially identified as the *C. gloeosporioides* species complex. In total, 62 isolates were subsequently selected for further analyses on the basis of their origins, colony characteristics, and conidial morphologies (Supplementary Table S1).

Multilocus phylogenetic analysis. The six-locus phylogenetic analysis of the *C. gloeosporioides* species complex included 54 reference isolates and *C. boninense* (MAFF305972) as the outgroup (Fig. 2). The Bayesian inference was performed with the combined *ACT*, *ApMat*, *CAL*, *GAPDH*, *ITS*, and *TUB2* sequences, and comprised 2,916 characters after alignment. The boundaries of the loci used in the alignment were as follows: *ACT*, 1 to 226; *ApMat*, 227 to 1,011; *CAL*, 1,012 to 1,711; *GAPDH*, 1,712 to 1,946; *ITS*, 1,947 to 2,412; and *TUB2*, 2,413 to 2,916. The 62 tested isolates from the *C. gloeosporioides* species complex clustered in three clades: 29 isolates clustered with *C. siamense*, 30 isolates clustered with *C. fructicola*, and 3 isolates clustered with *C. aenigma* (Fig. 2).

Morphological and cultural characteristics. Distinct colony morphologies were observed for each species after 7 days on PDA. Isolates of *C. siamense* produced gray colonies with sparse, gray aerial mycelia. The reverse sides of the colonies were gray to black. Isolates of *C. fructicola* varied from gray to dark gray on PDA, with dense gray aerial mycelia and a few bright orange conidial masses near the inoculum point. Isolates of *C. aenigma* produced white colonies with dense gray to white aerial mycelia. The reverse sides of the colonies were pale orange toward the centers. Few differences in conidial morphology were observed among the three species. All three species had long elliptical conidia with obtuse to slightly rounded ends (Fig. 3). The growth rate of the *C. siamense* isolates was more rapid than those of *C. aenigma* and *C. fructicola* ($P = 0.0203$) (Table 3).

Effects of temperature on mycelial growth. The optimum temperature for all three species was 28°C. The tolerance levels to high temperatures were similar for *C. fructicola* and *C. aenigma*. For *C. fructicola* and *C. aenigma* isolates, no mycelial growth was observed at temperatures of 36°C and above. Isolates of *C. siamense* were tolerant to high temperatures, resulting in significantly larger colony diameters than the other two species when the temperature was >36°C ($P < 0.05$). Mycelial growth of *C. siamense* in vitro was observed at 38°C, with an average growth rate of 0.35 mm/day (Table 3; Fig. 4).

Pathogenicity tests. The pathogenicity levels of the *Colletotrichum* isolates were determined on both the leaves and petioles of strawberry plants. Necrotic spots were visible 3 days after inoculation. Disease symptoms were observed on petioles 7 days after inoculation. Disease severity caused by the three species was significantly different. Isolates of *C. siamense* showed the greatest disease severity ($P < 0.05$). The disease indices of the leaves and petioles were 91.3 and 83.7, respectively. *C. fructicola* species also showed strong pathogenicity on both the leaves and petioles of strawberry plants (Table 4). Plant mortalities caused by *C. siamense* and *C. fructicola* at 12 days postinoculation (dpi) were 60.0 and 51.7%, respectively. *C. aenigma*-infected leaves and petioles showed lower mean infection incidences (68.4 and 47.4%, respectively). For *C. siamense*, the disease development resulting from most isolates, except for GQH46 and GQHAH2, was similar. The disease index of GQH46 was significantly higher than those of the other isolates, especially at 4 and 6 dpi. In contrast, the disease index of GQHAH2 was significantly lower than those of the other tested isolates at almost all time points (Fig. 5A). For *C. fructicola*, compared with the other isolates, GQHS2 was the most virulent on strawberry leaves, especially at 8,

Fig. 4. Colony growth rate of the *Colletotrichum gloeosporioides* species complex from strawberry plants under different temperatures.

Table 3. Morphological characteristics of *Colletotrichum gloeosporioides* complex causing anthracnose of strawberry plants in eastern China

Species	Colony ^y	Conidial morphology ^x						Characteristics of appressoria	
		Length (μm)		Width (μm)		Shape	Growth ^z	Conidial	Mycelial
		Range	Mean	Range	Mean				
<i>C. siamense</i>	White to pale gray, dark gray	13.9–19.1	16.5 ± 0.5	4.7–6.5	5.4 ± 0.2	Long elliptical	6.84 ± 0.14 a	Brown to dark black, spindle to slightly irregular	Light brown to brown, spindle or irregular
<i>C. aenigma</i>	White to taupe	11.4–17.5	14.6 ± 0.7	4.7–5.9	5.3 ± 0.4	Long elliptical	6.59 ± 0.16 b	Brown to dark black, spindle to slightly irregular	Light brown to brown, spindle or irregular
<i>C. fructicola</i>	White to black/green	13.4–18.3	15.8 ± 0.4	4.2–5.5	4.9 ± 0.6	Long elliptical	6.43 ± 0.25 b	Brown to dark black, spindle to slightly irregular	Light brown to brown, spindle or irregular

^x Data are mean ± standard deviation; range is approximate.

^y Colony morphology.

^z Growth rate (mm day⁻¹). Means followed by the same letter were not significantly different at $P \leq 0.05$.

10, and 12 dpi. GQHZJ1 and GQH133 showed the lowest disease indices at almost all time points (Fig. 5B).

Sensitivity to fungicides. Isolates of *C. siamense* and *C. fructicola*, the main species causing strawberry anthracnose in this study, were chosen for the determination of sensitivities to fungicides using the mycelial growth method. For *C. siamense* isolates, the EC₅₀ value (mean $\pm$ standard deviation) was 0.056 ± 0.030 $\mu\text{g/ml}$ for

difenoconazole, 8.885 ± 0.53 $\mu\text{g/ml}$ for tebuconazole, 0.122 ± 0.070 $\mu\text{g/ml}$ for prochloraz, and >100 $\mu\text{g/ml}$ for azoxystrobin. For *C. fructicola* isolates, the EC₅₀ value was 0.036 ± 0.031 $\mu\text{g/ml}$ for difenoconazole, 6.305 ± 0.903 $\mu\text{g/ml}$ for tebuconazole, 0.140 ± 0.074 $\mu\text{g/ml}$ for prochloraz, and >100 $\mu\text{g/ml}$ for azoxystrobin (Table 5). The values indicated that both *C. siamense* and *C. fructicola* isolates were quite sensitive to prochloraz, difenoconazole, and tebuconazole but not to azoxystrobin. *C. siamense* isolates were more sensitive to prochloraz than *C. fructicola* isolates ($\alpha = 0.05$). There were no significant differences in the EC₅₀ values against difenoconazole and tebuconazole between the two species.

Strong correlations were obtained between difenoconazole and prochloraz for *C. fructicola* ($P < 0.05$) and between difenoconazole and tebuconazole for *C. siamense* ($P < 0.05$) (Table 6).

Discussion

This study aimed to characterize and compare the *Colletotrichum* spp. associated with anthracnose of strawberry in China using morphological characteristics and multilocus phylogenetic analyses. A subset of 62 isolates represented three distinct taxa: *C. siamense*

Table 4. Pathogenicity analyses of *Colletotrichum gloeosporioides* complex isolates from strawberry plants^y

Species	Disease index (12 days after inoculation)		
	Foliar	Petiole	Mortality (%) ^z
<i>C. siamense</i>	91.3 a	83.7 a	60.0 a
<i>C. fructicola</i>	84.9 a	70.0 ab	51.7 a
<i>C. aenigma</i>	68.4 b	47.4 b	30.0 b

^y Data are mean values. Mean values with the same letters were not statistically different ($P > 0.05$) according to the least significant difference test.

^z Percentages of plant mortality were evaluated at 12 days after inoculation.

Fig. 5. Disease index for **A**, *Colletotrichum siamense* ($n = 10$) and **B**, *C. fructicola* ($n = 10$) isolates on strawberry plants from eastern China. Mean scores of disease index. Mean values with the same letter do not differ significantly ($P = 0.05$) according to Duncan's multiple range test.

(29 isolates, 46.8%), *C. fructicola* (30 isolates, 48.4%), and *C. aenigma* (3 isolates, 4.8%). Their pathogenicity was confirmed in strawberry plants (leaves and petioles). The study also reported the fungicide sensitivities of *Colletotrichum* sp. complexes from strawberry in eastern China, which can optimize the selection of the most effective fungicides.

Both *C. siamense* and *C. fructicola* were originally described as causal agents of anthracnose on coffee berries in northern Thailand (Prihastuti et al. 2009). Different species in the *C. gloeosporioides* species complex are associated with strawberry anthracnose (Gan et al. 2017; Nam et al. 2013; Weir et al. 2012). Our results were in agreement with previous studies indicating that the *C. gloeosporioides* species complex is the most prevalent agent of strawberry anthracnose in China (Han et al. 2016; Jayawardena et al. 2016). Recently, Japanese strawberry cultivars have been the main cultivars grown in eastern China. *C. siamense*, *C. fructicola*, and *C. aenigma* are also the dominant fungal species causing strawberry anthracnose in Japan (Gan et al. 2017). We hypothesize that the Japanese strawberry cultivars are more susceptible to *C. siamense*, *C. fructicola*, and *C. aenigma*, resulting in the prevalence of strawberry anthracnose in both Japan and eastern China. *C. acutatum* and *C. theobromicola* (synonymous with *C. fragariae*, which is now in the *C. gloeosporioides* species complex) infect strawberry plants (Brooks 1931; Buddie et al. 1999) but no isolates of these species were identified in this study.

Temperature is a major factor affecting plant disease epidemics (Bonde et al. 2012; Wilson et al. 1990). Leandro et al. (2003) found that the optimum temperature ranges for *C. acutatum* conidial germination and appressorial development were 23.0 to 27.7 and 17.6 to 26.5°C, respectively (Leandro et al. 2003). The temperature required for the optimal disease development of anthracnose on lentils ranged from 20 to 24°C, with the greatest damage occurring at 28°C (Chongo and Bernier 2000). In recent years, *Colletotrichum* spp. have been increasingly prevalent in several cities of China that have hot and rainy summers. The effects of temperature on the growth rate of *Colletotrichum* spp. in vitro was evaluated in this study. Although there were no significant differences observed among the minimum and optimum growth temperatures of the three *Colletotrichum* spp. examined, the maximum growth temperatures were different (Fig. 4). *C. siamense* showed a stronger pathogenicity to strawberry plants and more tolerance to high temperatures than *C. fructicola* and *C. aenigma*, suggesting the potential threat of *C. siamense*, especially in areas with hot and rainy weather such as cities in eastern China.

C. siamense, *C. fructicola*, and *C. aenigma* were all pathogenic to leaves and petioles of strawberry plants. The species *C. fructicola* and *C. siamense* have broad host ranges and are recognized pathogens of chili, peach, pear, blueberry, and apple (De Silva et al. 2017; Ivic et al. 2013; Munir et al. 2016). The assessed pathogenicity levels of *C. siamense* and *C. fructicola* isolates were consistent with those of previous reports, indicating that *C. siamense* was more aggressive on strawberry than *C. fructicola*. *C. aenigma* isolates infect strawberry fruit and cause anthracnose disease symptoms (Baroncelli et al. 2015). Isolates of *C. fructicola* showed stronger pathogenicity

levels than *C. aenigma* on both leaves and petioles of strawberry in Wuhan, China (Han et al. 2016), and isolates of *C. aenigma*-infected leaves and petioles had lower mean infection incidences than *C. siamense* and *C. fructicola*. These data, together with our results, indicate that, in comparison with *C. siamense* and *C. fructicola*, *C. aenigma* was probably not the dominant pathogen causing strawberry anthracnose in China.

More importantly, 10 isolates each of *C. siamense* and *C. fructicola* were selected for further pathogenicity testing. Most *C. siamense* and *C. fructicola* isolates shared a similar disease development process yet some individual isolates caused significantly greater or lower disease indices than other isolates. In particular, the disease index of GQHSD2 from *C. fructicola* was greater than that of GQHAH2 from *C. siamense*, although *C. fructicola* resulted in a lower disease severity than *C. siamense* ($P < 0.05$). Previous studies selected only a few isolates of *Colletotrichum* spp. isolated from different hosts for pathogenicity testing (Niu et al. 2016; Sharma and Shenoy 2014), and the variation in pathogenicity among isolates of different *Colletotrichum* spp. has rarely been reported. Gan et al. (2017) classified 115 isolates collected from strawberry plants in Japan into four species of the *C. gloeosporioides* species complex. In addition, although *C. fructicola* was identified as the predominant virulent species in Japan, both virulent and less virulent isolates of *C. fructicola* and *C. siamense* were also identified. This result indicated that specific species were not strictly correlated with virulence. These findings, together with the results in our study, imply that pathogenicity testing is required to select a large number of isolates to ensure the accuracy of the results and avoid a bias.

The application of agrochemicals is still an essential strategy for controlling strawberry anthracnose in China. Since the 1990s, benzimidazoles, including carbendazim and thiophanate-methyl, have been the main fungicide group extensively used for the control of strawberry anthracnose in China. QoI fungicides are commonly used to control anthracnose in many countries, including China (Baggio et al. 2018; Forcelini et al. 2016; Hu et al. 2015; Ji et al. 2014; Kongtragoul et al. 2020; Li et al. 2005; Yokosawa et al. 2017). Azoxystrobin is a QoI fungicide that was registered for strawberry in China in 2015 (China Pesticide Information Network; <http://www.icama.org.cn/>). The recommended concentration for strawberry anthracnose was approximately 500 µg/ml. Furthermore, the resistance of the *C. gloeosporioides* species complex against QoI (azoxystrobin) fungicides has also been reported in various crops from many countries, such as *C. gloeosporioides* from strawberry in Japan (Inada et al. 2008), *C. siamense* from peach and blueberry in South Carolina in the United States (Hu et al. 2015), *C. gloeosporioides* from boxthorn and apple in Korea (Kim et al. 2016), and *C. fructicola* from apple in Japan (Yokosawa et al. 2017). Here, the *C. siamense* and *C. fructicola* isolates were all resistant to QoI (azoxystrobin) fungicides, indicating that these fungicides may not be suitable for the control of strawberry anthracnose, at least not in eastern China. The resistance could be addressed in the following ways: (i) *C. fructicola* has a teleomorph

Table 5. Summary of the sensitivity to four fungicides for *Colletotrichum siamense* and *C. fructicola* from strawberry plants in eastern China^w

FRAC ^x	Chemical	EC ₅₀ (µg/ml)					
		<i>C. siamense</i>			<i>C. fructicola</i>		
		N ^y	Range	Mean ^z	N ^y	Range	Mean ^z
11	Azoxystrobin	15	216.153–768.342	>100	15	100.968–745.629	>100
3	Difenoconazole	15	0.009–0.096	0.056 D ± 0.030	15	0.003–0.089	0.036 D ± 0.031
3	Tebuconazole	15	1.259–16.661	8.885 B ± 0.532	15	0.853–12.950	6.305 B ± 0.903
3	Prochloraz	15	0.011–0.358	0.122* C ± 0.070	15	0.017–0.541	0.140* C ± 0.074

^w Data were transformed using a log₁₀ transformation before statistical analysis. EC₅₀ = the fungicide concentration at which fungal development is inhibited by 50%.

^x Fungicide Resistance Action Committee (FRAC) code.

^y Number of isolates.

^z Means followed by an asterisk (*) are significantly different between different species based on the two-tailed *t* tests of transformed data with $\alpha = 0.05$. Means followed by the same letter are not significantly different between fungicides within each species, based on the least significant difference tests of transformed data with $\alpha = 0.05$.

and mandatory sexual recombination that increases the risk of fungicide resistance development (Yokosawa et al. 2017) and (ii) point mutations in the mitochondrial *cytochrome b* gene confer high levels of resistance to QoI fungicides (Ma and Michailides 2005). However, DMI fungicides are potential alternatives to benzimidazole fungicides for the management of strawberry anthracnose (Xu et al. 2014). In this study, the sensitivity levels of three DMI fungicides (difenoconazole, tebuconazole, and prochloraz) were determined. The sensitivities of *C. siamense* and *C. fruticola* to difenoconazole and prochloraz were consistently high. Members of the *C. gloeosporioides* species complex isolated from rubber trees in Hainan are more sensitive to prochloraz than to difenoconazole (Cao et al. 2017). Our results indicate that the *C. gloeosporioides* species complex isolated from strawberry plants was most sensitive to difenoconazole, less sensitive to prochloraz, and least sensitive to tebuconazole. This discrepancy indicates that variations in sensitivity to fungicides may be the result of different hosts and regions. The present study confirmed that the isolates of *C. siamense* were more sensitive to prochloraz than *C. fruticola*. The mean EC₅₀ value for prochloraz in all of the tested *C. siamense* isolates in the present study was 0.122 µg/ml. The isolates of *C. fruticola* were more sensitive to difenoconazole and tebuconazole than *C. siamense* isolates. In all the tested *C. fruticola* isolates in the present study, the mean EC₅₀ values for difenoconazole and tebuconazole were 0.036 µg/ml and 6.305 µg/ml, respectively. The sensitivities of these two species to difenoconazole, tebuconazole, and prochloraz were significantly different, revealing the importance of understanding their relative prevalence in a given region. Thus, more field studies are needed in the future.

In the present study, for *C. fruticola* only, there were correlations in the sensitivity levels between difenoconazole and prochloraz. For *C. siamense*, strong correlations were obtained between difenoconazole and tebuconazole. These may be related to DMI resistance. A positive cross-resistance was observed between tebuconazole and difenoconazole on the *C. gloeosporioides* species complex isolates from strawberry and grape plants. The DMI resistance resulted from multiple mechanisms such as mutations in the *CYP51* gene that lead to the reduced affinity of DMI for the sterol 14 α -demethylase enzyme encoded by the *CYP51* gene (Leroux and Walker 2011; Sang et al. 2015).

This study identified three *Colletotrichum* spp. (*C. siamense*, *C. fruticola*, and *C. aenigma*) from the *C. gloeosporioides* species complex affecting strawberry plants in six provinces of eastern China. *C. siamense* and *C. fruticola* were identified as the predominant virulent species. The fungicide sensitivity test showed that QoI fungicides (azoxystrobin) were not suitable for strawberry anthracnose control in eastern China. Instead, two DMI fungicides, difenoconazole and tebuconazole, could be used as alternatives. This provides insights into fungicide application strategies for the effective control of the disease. Further work is needed to analyze the influences of environmental variables and to conduct field trials to verify the effects of DMI fungicides. This may increase the ability to successfully manage strawberry anthracnose.

Table 6. Cross-sensitivity between azoxystrobin, difenoconazole, and tebuconazole inhibitor fungicides for isolates of *Colletotrichum siamense* and *C. fruticola* from strawberry plants in eastern China^z

Chemical name	Azoxystrobin	Difenoconazole	Tebuconazole	Prochloraz
Azoxystrobin	...	0.21	0.20	0.01
Difenoconazole	0.04	...	0.28	0.60*
Tebuconazole	0.38	0.67*	...	0.34
Prochloraz	0.11	0.24	0.31	...

^z The correlation coefficient (*r*) for the *C. fruticola* species complex (*n* = 15) is presented above the ellipses (...), whereas the *C. siamense* species complex (*n* = 15) is presented below the ellipses. The *r* was determined via a comparison of log 50% effective concentration values for each fungicide from the individual species complex; an asterisk (*) indicates that the *r* is significant at *P* = 0.05.

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