Genetic Diversity of *Colletotrichum* spp. Causing Strawberry Anthracnose in Zhejiang, China

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Abstract

Anthracnose is a serious fungal disease that primarily infects strawberry roots and stolons during development. Here, 91 isolates from different areas of Zhejiang province, China, were collected. Morphological characteristics were analyzed, and a phylogenetic analysis based on multiple genes (actin, internal transcribed spacer, calmodulin, glyceraldehyde-3-phosphate dehydrogenase, and chitin synthase) was performed. We found that all of the *Colletotrichum* species causing strawberry anthracnose belonged to the *Colletotrichum gloeosporioides* complex. Among them, we identified 48 isolates of *C. fructicola*, 21 isolates of *C. siamense*, 13 isolates of *C. gloeosporioides*, and 9 isolates of *C. aenigma*. *C. siamense* was distributed in the central and eastern regions of Zhejiang province (Hangzhou, Jinhua, Shaoxing,

Ningbo, and Taizhou). This is the first report of *C. siamense* causing strawberry anthracnose in Zhejiang province. *C. fructicola* was the most dominant species causing strawberry anthracnose in Zhejiang province. We identified the four species causing strawberry anthracnose in Zhejiang province, which will improve our understanding of the strawberry anthracnose epidemic and will benefit the development of future control measures.

Keywords: *Colletotrichum fructicola*, *Colletotrichum gloeosporioides* complex, *Colletotrichum siamense*, fruit, fungi, morphological characterization, pathogen diversity, phylogenetic analysis, small fruits, strawberry anthracnose

Strawberries (*Fragaria × ananassa*), which are native to South America, are widely cultivated in China (Henz et al. 1992). The area devoted to strawberry cultivation increased from 90,100 ha in 1985 to 330,000 ha in 2012; China produces 35.7% of all strawberries worldwide, and it is the largest producer of strawberries globally (Peng and Peng 2011). Zhejiang province is the one of the top eight strawberry-producing regions in China (Shen 2014).

Strawberry anthracnose is a serious fungal disease that can affect plants at any stage during their lifecycle (Henz et al. 1992). However, plants are most susceptible to strawberry anthracnose at the seedling stage and immediately after planting (Wang et al. 2008). Anthracnose primarily affects strawberry roots, petioles, and stolons, but flowers and fruit can also be infected, leading to severe outbreaks (Mertely and Legard 2004). Symptoms are irregular ulcer-like lesions, reddish brown stolons during the initial stages of stolon development, leaves and petioles with black spindle-shaped lesions, wilt, and dead roots (Debode et al. 2009; Freeman and Katan 1997). In Zhejiang, nearly 50% of all seedling deaths and >40% of all production losses in the nursery are attributed to this disease (Xie et al. 2010). During the entire plant growth cycle, strawberry was threatened by anthracnose disease. If environmental conditions are suitable, anthracnose decreased the strawberry yield by 80%. Transplants are believed to be the main source of primary inoculum, but complete epidemiology of *Colletotrichum* spp. on strawberry in China is unclear.

Colletotrichum spp. often cause anthracnose disease in herbaceous or woody plants in tropical and subtropical regions (Cannon et al. 2012). The genus *Colletotrichum* is one of the eight plant pathogens worldwide (Dean et al. 2012). Three *Colletotrichum* species or species complexes have been reported to cause strawberry anthracnose: the *Colletotrichum gloeosporioides* complex, *Colletotrichum fragariae*, and the *Colletotrichum acutatum* complex (Denoyes-Rothan et al. 2003). The *C. gloeosporioides* complex is found worldwide and has a wide range of hosts, whereas *C. acutatum* and *C. fragariae* both have narrow host ranges (MacKenzie et al. 2008). Although a few *C. acutatum* infections in Chinese strawberries have been reported, the primary species affecting strawberries in China are believed to be *C. gloeosporioides* and *C. fragariae* (Buddie et al. 1999; Han et al. 2016). The pathogens causing strawberry anthracnose in Hubei province, China, were divided into two groups: the *C. gloeosporioides* species complex (*Colletotrichum aenigma*, *Colletotrichum fructicola*, *C. gloeosporioides*, and *Colletotrichum murrayae*) and the *C. acutatum* complex (*C. acutatum* and *Colletotrichum nymphaeae*). In Florida, Ureña-Padilla et al. (2002) suggested that the pathogen-causing anthracnose on the strawberry root crowns and stolons was *C. gloeosporioides*, whereas the pathogens causing anthracnose on the strawberry fruit were members of the *C. acutatum* complex.

Based on morphological characters and analysis of internal transcribed spacer (ITS) ribosomal DNA, Xie et al. (2010) showed that strawberry anthracnose infections in Zhejiang and Shanghai were caused by *C. gloeosporioides*, *C. acutatum*, and *C. fragariae*. However, because this study only analyzed a single region and did not use multiple genes, the results may not be accurate. Indeed, multigene phylogenetic analyses in conjunction with morphological characters have been used to identify many important pathogens (Cai et al. 2009; Damm et al. 2012; Weir et al. 2012). The classification and delimitation of *Colletotrichum* species have been debated (Weir et al. 2012). Many *Colletotrichum* isolates have been successfully identified based on multigene phylogenies and morphological characters, indicating that this method is mature and can accurately identify pathogens (Damm et al. 2012). Morphological characters and multigene phylogenies have not been used to identify the *Colletotrichum* species associated with strawberry anthracnose in China or to determine the distributions of these species.

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The objectives of this study were (i) to use morphological and molecular characters to identify the species causing strawberry anthracnose in Zhejiang province and (ii) to determine the distributions and pathogenicity of *Colletotrichum* species throughout the region.

Materials and Methods

Source and collection of fungal isolates. Zhejiang province (27°02' to 31°11' N, 118°01' to 123°10' E) is located on the southeast coast of the Yangtze River in southeastern China (He et al. 2013), and it has a humid, subtropical monsoon climate. We collected 234 anthracnose samples from strawberry plants cultivated in Zhejiang province between 2013 and 2017 (Fig. 1). The samples included irregularly shaped lesions from the stem; black, spindle-shaped lesions from the leaves or petioles; reddish brown stolons; or the entire plant if roots were withered (Supplementary Fig. S1).

Pathogens were isolated from diseased petiole, leaf, stolon, or root tissues. The diseased plant tissues were first cut into 5 × 5-mm pieces using sterilized scissors. Excised tissue pieces were rinsed in 75% ethanol solution for 30 s, soaked in a 3% sodium hypochlorite solution (active chlorine: 6.8%) for 3 min, rinsed three times with sterile distilled water, and then, dried on sterile filter paper. After it was dried, each sample was placed on a plate containing potato dextrose agar (PDA) medium supplemented with a final concentration of 50 mg/ml of kanamycin sulfate and streptomycin sulfate to inhibit bacterial growth. The plate was incubated in an incubator at 25°C in darkness for 3 to 5 days. Hyphal growth was aseptically transferred to a new PDA plate (Cai et al. 2009; Phoulivong et al. 2010). Anthracnose isolates were identified and selected based on the morphological characteristics of each species. Isolates were maintained on PDA slants at 4°C.

DNA extraction and PCR. When about 67% of the PDA plate (90 mm) was covered by mycelia, the hyphae were scraped off with sterile slides. DNA was extracted from the hyphae using a fungal genomic DNA rapid extraction kit (B518229-0100; Sangon Biotech). DNA extracted from each isolate was stored at -20°C. The target genes amplified in this study were actin (ACT), calmodulin (CAL), chitin synthase (CHS-1), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and the ITS. The corresponding primers used for PCR amplification are shown in Table 1.

Fig. 1. Distribution of *Colletotrichum* spp. associated with strawberry anthracnose in Zhejiang province, China. For each region, the percentage occurrences of the four *Colletotrichum* species are given: Hangzhou ($n = 43$), Shaoxing ($n = 18$), Ningbo ($n = 10$), Taizhou ($n = 6$), Jinhua ($n = 4$), and Wenzhou ($n = 10$). *C. aenigma*, *Colletotrichum aenigma*; *C. fructicola*, *Colletotrichum fructicola*; *C. gloeosporioides*, *Colletotrichum gloeosporioides*; *C. siamense*, *Colletotrichum siamense*.

The PCRs were performed in an Applied Biosystems Veriti Thermal Cycler in a total volume of 50 μ l. The PCR mixtures contained 31.6 μ l of UV-sterilized ultrafiltered water, 5 μ l of 10× PCR buffer (with 20 mM MgCl₂), 5 μ l of dNTPs (each 20 μ M), 2 μ l of each primer (10 μ M), 2 μ l of albumin from bovine serum (BSA), 2 μ l of genomic DNA, and 0.4 μ l (1 U) of Roche FastStart Taq DNA Polymerase.

PCRs were performed in a thermal cycler (PTC-100TM Peltier Thermal Cycler). The parameter for GAPDH is as follows: denaturation at 94°C for 5 min followed by 30 cycles of 94°C for 30 s, 60°C for 30 s, and 72°C for 0.5 min and a final cycle at 72°C for 10 min. The parameter for ITS is as follows: 94°C for 5 min followed by 30 cycles of 94°C for 1 min, 52°C for 30 s, and 72°C for 40 s and a final step at 72°C for 10 min. The parameter for CAL is as follows: 94°C for 5 min followed by 30 cycles of 94°C for 1 min, 55°C for 45 s, and 72°C for 40 s and a final step at 72°C for 10 min. The parameter for the ACT and CHS-1 is as follows: denaturation at 94°C for 4 min followed by 30 cycles of 94°C for 30 s, 58°C for 30 s, and 72°C for 1 min and a final cycle of 10 min at 72°C (Weir et al. 2012).

PCR products were assessed using electrophoresis: 50 μ l of each amplicon was run on a 1% agarose gel stained with ethidium bromide in 1% Tris-acetate EDTA buffer for 30 min and then, photographed under UV light (Han et al. 2016). Gels were sent to Kingsley (Nanjing, China) for sequencing.

All sequences were grouped by gene and aligned using DNAMAN v. 7.0. Sequences with single-nucleotide polymorphisms were amplified and sequenced three times to ensure that the observed polymorphisms were not because of sequencing errors.

Molecular identification and phylogenetic analyses. Sequences homologous to the ACT, CAL, CHS-1, GAPDH, and ITS gene sequences from the isolates were downloaded from GenBank. *Colletotrichum boninense* (International Collection of Microorganisms from Plants [ICMP] 17904) was used as the outgroup (Hyde et al. 2014; Weir et al. 2012). All sequences were aligned using Clustal X 2.0.10 (Larkin et al. 2007). Gene sequences were compared and corrected using the “W” function in MEGA 5.0 (Tamura et al. 2011). Gene sequences were concatenated. Modeltest3.7.win, Win-paup4b10-console, and Mrmodeltest2 (Nylander 2004) as implemented in the MrMTgui were used to estimate the best model of nucleotide substitution (Vaidya et al. 2011). Bayesian inference (BI) phylogenies were constructed using Mr. Bayes v. 3.1.2 (Ronquist and Huelsenbeck 2003). Six simultaneous Markov chains were run for 1,000,000 generations each, and trees were sampled every 100th generation. The first 2,000 trees, representing the burn-in phase of the analyses, were discarded, and the remaining 8,000 trees were used to calculate posterior probabilities in the majority rule consensus tree. Phylogenetic trees were drawn using Treeview (Page 1996). The alignments and trees have been deposited in TreeBASE. The fungal isolates used in this study are listed in Supplementary Table S1.

Morphological and cultural characterization. Morphological and cultural characterizations were performed for all of the 91 isolates according to the methods described by Cai et al. (2009). Mycelia plugs (5.0 mm in diameter) from the edge of an actively growing 5-day-old colony of each tested isolate on PDA were transferred to PDA plates, and they were incubated at 28°C under a mixture of white and near-UV fluorescent light sources with a 12-h light/12-h dark cycle. The experiment was designed as a randomized complete block, with three replicates of each isolate. Colony color, conidial dimensions, and morphology were assessed using previous described methods (Cai et al. 2009; Peng et al. 2013; Weir et al. 2012). Conidial suspensions of each species were prepared in lactic acid from conidial masses grown on PDA. The shape and color of the conidia were observed, and the sizes of 50 conidia from each isolate were measured at 400× magnification under a light microscope (Carl Zeiss Microscopy GmbH).

Pathogenicity testing. Ninety-one isolates were cultured on PDA medium for 5 days at 25°C. Samples of these isolates were collected from the colony edge using a 5-mm punch. The mycelial surface of sample was placed in the center of an oat medium plate and cultured at 25°C for 5 days. The hyphae on the surface of the resulting colony were scraped off and grown at 25°C under a mixture of white and

near-UV fluorescent light sources with a 12-h light/12-h dark cycle for 10 to 15 days (de los Santos García de Paredes and Romero Muñoz 2002). After the production of a large number of conidial heaps, spore suspensions were collected in 1.5-ml centrifuge tubes using sterilized toothpicks. Spore suspensions were adjusted to 2.0×10^6 conidia per milliliter using a hemocytometer.

Six-week-old leaves of strawberry healthy plants (variety of Zhang Ji) that had not been treated with any chemicals were used for inoculations. Both wounded leaves, which were scratched but not pierced with a sterilized insect needle, and nonwounded leaves were adopted to assess the pathogenicity and evaluate the effect of wounds on the infection. Each leaf was inoculated by drop of 10- μ l spore suspensions, and the negative control was inoculated with sterile water. Additionally, nine plants were inoculated for each isolate. Then, plants were cultured in a greenhouse at $25 \pm 5^\circ\text{C}$ for 7 days under a light/dark cycle. Disease was assessed after 7 days by measuring the diameter of the disease lesion (Han et al. 2016). We calculated the disease incidence rate as the number diameter of the disease lesion (Han et al. 2016). Data from the pathogenicity test were subjected to one-way analysis of variance using the software IBM SPSS Statistics v22 to determine the significance of differences. Means for different species from the pathogenicity test were separated using the least significance difference test at $P = 0.05$ level.

Results

Isolates of *Colletotrichum* spp. from strawberries in Zhejiang. Between 2013 and 2017, 234 samples with typical anthracnose symptoms (Supplementary Fig. S1) were collected in Hangzhou, Ningbo, Taizhou, Shaoxing, and Wenzhou of Zhejiang province, China. From these samples, 91 isolates causing anthracnose were isolated. The distributions of the isolates across the region sampled are shown in Figure 1.

Molecular identification and phylogenetic analyses. The ACT, CAL, CHS-1, GAPDH, and ITS genes from all isolates were successfully amplified and sequenced. All isolates were identified as members of the *C. gloeosporioides* species complex. BI was performed on the haplotypes belonging to the *C. gloeosporioides* species complex based on the concatenated ACT, CHS-1, CAL, GAPDH, and ITS sequences. The concatenated alignment included 2,486 characters. The boundaries of the loci used in the alignment were as follows: ACT: 1 to 311; CAL: 312 to 1,189; CHS: 1,190 to 1,496; GAPDH: 1,497 to 1,804; and ITS: 1,805 to 2,486.

The tested 91 isolates fell into four clades. BI values indicated that 48 isolates clustered with *C. fructicola*, 21 isolates clustered with *Colletotrichum siamense*, 13 isolates clustered with *C. gloeosporioides*, and 9 isolates clustered with *C. aenigma* (Fig. 2). *C. fructicola* was most frequently isolated, identified in 53% of all samples, and BI values of 1.0 between 48 tested isolates. The BI values between tested isolates and *C. fructicola* ICMP 17921*, ICMP 18646*, and ICMP 18581* were 1.0, 0.92, and 0.92, respectively. *C. siamense* (23% of all samples) was distributed mainly in the central and eastern regions of Zhejiang province (Hangzhou, Jinhua, Shaoxing, Ningbo, and Taizhou). Nine isolates had BI values of 0.5 with *C. siamense*

ICMP 18642*, whereas the other four and eight isolates both had BI values of 0.53 with *C. siamense* ICMP 19118* and *C. siamense* ICMP 18578*, respectively. The third species, identified as *C. gloeosporioides*, accounted for 14% of all samples with BI values of 1, and it was primarily distributed in the central part of Zhejiang province (Hangzhou, Jinhua, Shaoxing, and Ningbo). The fourth species (10%), *C. aenigma*, which was found in the central part of Zhejiang province as, had BI values of 0.89 with *C. aenigma* ICMP 18608* (Figs. 1 and 2).

Morphological characters. The morphologies of the colonies of the four species grown on PDA media did not differ substantially. *C. fructicola* produced white colonies with irregular gray edges, and colonies were black on the back of PDA plates. The orange conidia were cylindrical with blunt or pointed ends, and attached cells were single or scattered. Most conidia were spherical, cylindrical, or spindle shaped, and very few were irregular. A small number of conidia produced two nearly circular attached cells (Fig. 3). The *C. siamense* colony was fluffy, with gray-white edges. The colony was neatly arranged, with yellow margins in the middle of the back of PDA plates. Conidia were straight, cylindrical, colorless, and smooth, with blunt ends. Spores attached to cells were brown, oval, or spindle shaped, with complete edges. A few irregularly shaped spores were observed (Fig. 4). The *C. gloeosporioides* colony was gray in the middle with white edges, and the back of the colony was black to gray with white edges. The conidial heaps were orange, cylindrical, and blunt at both ends or slightly pointed at one end (Fig. 5). Attached cells were single or scattered, and most were spherical or elliptical, with a few irregular cells. The aerial hyphae of *C. aenigma* were sparse, light gray to white, and flocculent. The colony was light white on the back, with a gray-green center. Conidia heaps were rare. The conidia were stick like, with one end obtusely rounded or with both ends slightly rounded. Most conidia had oil balls. Conidia were singly or loosely distributed, dark brown, and ovate or irregularly shaped. Some conidia were clover shaped (Fig. 6). Mycelial growth rates, attached cells, and conidia sizes in details are described in Table 2.

Pathogenicity assays. All isolates of each species showed stronger pathogenicity on wounded leaves than on nonwounded leaves (Table 2). For example, all isolates of *C. fructicola* had mean disease incidence (percentage) rates of 79.9 ± 0.7 and 22.2 ± 0.7 and lesion diameters of 20.5 and 17.0 mm on wounded and nonwounded leaves, respectively. Meanwhile, significant difference ($P = 0.05$) was observed in lesion diameter but not in disease incidence between four species on wounded leaves. On nonwounded leaves, significant difference ($P = 0.05$) was observed in both lesion diameter and disease incidence between species, and *C. fructicola* had higher pathogenicity.

Significant difference existed between and within species (Table 2). In Supplementary Figure S2, one isolate with the strongest pathogenic ability within each species was presented: *C. fructicola* (HJD-49), *C. siamense* (HJD4-7), *C. gloeosporioides* (GT-10), and *C. aenigma* (nbg-18). HJD-49 (*C. fructicola*) had strong pathogenicity both on wounded leaves and on nonwounded leaves. Among *C. siamense*, the first reported species on strawberry in Zhejiang, only a small portion of isolates, such as HJD4-7, caused similar disease on both

Table 1. Primers used in this study with sequences and sources

Gene	Primers	Direction	Length (bp)	Sequence	Reference
ACT ^z	ACT-512F	Forward	399	ATGTGCAAGGCCGGTTTCGC	Carbone and Kohn (1999)
	ACT-783R	Reverse		TACGAGTCCTTCTGGCCCAT	Carbone and Kohn (1999)
CAL	CL1C	Forward	773	GAATTC AAGGAGGCCTTCTC	Weir et al. (2012)
	CL2C	Reverse		CTTCTGCATCATGAGCTGGAC	Weir et al. (2012)
CHS-1	CHS-79F	Forward	779	TGGGCAAGGATGCTTGGAAGAAG	Carbone and Kohn (1999)
	CHS-354R	Reverse		TGGAAGAACCATCTGTGAGAGTTG	Carbone and Kohn (1999)
GAPDH	GDF	Forward	870	GCCGTCAACGACCCCTTCATTGA	Templeton et al. (1992)
	FDR	Reverse		GGGTGGAGTCGTACTTGAGCATGT	Templeton et al. (1992)
ITS	Its-1F	Forward	593	CTTGGTCATTTAGAGGAAGTAA	Gardes and Bruns (1993)
	Its-4	Reverse		TCCTCCGCTTATTGATATGC	White et al. (1990)

^z ACT, actin; CAL, calmodulin; CHS-1, chitin synthase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; ITS, internal transcribed spacer.

wounded leaves and nonwounded leaves, whereas most isolates, such as ZJE-3, had strong pathogenicity on wounded leaves but weak pathogenicity on nonwounded leaves (Supplementary Fig. S3).

Discussion

This study performed a phylogenetic analysis using a combination of five genes (GAPDH, CAL, CHS-1, ACT, and ITS) (Damm et al. 2012; Weir et al. 2012) and used this phylogeny as well as morphological characters to identify 91 different isolates from samples of strawberry anthracnose collected in six cities in Zhejiang province, China. The species causing strawberry anthracnose were identified as *C. fructicola*, *C. gloeosporioides*, *C. aenigma*, and *C. siamense*. All four of these species belong to the *C. gloeosporioides* species complex. The *C. gloeosporioides* complex, *C. fragariae*, and the *C. acutatum* complex have been previously reported to infect strawberries and cause strawberry anthracnose (Denoyes-Rothan et al. 2003). The *C. gloeosporioides* complex includes >30 species, including *C. gloeosporioides* (Cannon et al. 2008; Weir et al. 2012).

This is the first report of *C. siamense* infecting strawberry plants and causing strawberry anthracnose in Zhejiang. We found that *C. siamense* was the second most common species causing strawberry anthracnose in Zhejiang province. *C. siamense* is a synonym of *Colletotrichum jasmini-sambac* (Wikee et al. 2011) and *Colletotrichum hymenocallidis* (Yan et al. 2011). Although this species was originally described from coffee plantations in Thailand, it has a wide geographical distribution and host range (Prihastuti et al. 2009). Based on the variability in *C. siamense* morphological characters, it has been suggested that environment conditions might affect mycelial growth (Li 2012). Different types of *C. siamense* colonies were observed in this study. The hyphal growth rate was relatively slow under the culture conditions used here. The colonies were generally grayish white, and a conidia pile was easily produced on PDA media. In the inoculation study, pathogenicity differed even within species. The isolates of *C. siamense* have rarely been studied, but our results indicate that future studies of the pathogenesis of these isolates are needed.

Fig. 2. Phylogeny of 91 isolates in the *Colletotrichum gloeosporioides* species complex. The tree was constructed based on concatenated actin, internal transcribed spacer, calmodulin, glyceraldehyde-3-phosphate dehydrogenase, and chitin synthase gene sequence; the evolution of each gene was modeled separately. Values next to nodes are Bayesian posterior probability values based on 6,000,000 generations. *Colletotrichum hippeastri* was used as an outgroup. Ex-type cultures are emphasized in bold and include the taxonomic name as originally described. The scale bar indicates the number of expected changes per site. *C. aenigma*, *Colletotrichum aenigma*; *C. fructicola*, *Colletotrichum fructicola*; *C. gloeosporioides*, *Colletotrichum gloeosporioides*; *C. siamense*, *Colletotrichum siamense*.

C. fruticola was originally reported in Thailand as a pathogen of coffee berries. This isolate grows quickly in hot, humid conditions, and it has a strong sporulation ability (Prihastuti et al. 2009). The species *C. fruticola*, *C. gloeosporioides*, and *C. aenigma* can cause strawberry anthracnose (Han et al. 2016). Here, *C. fruticola* accounted for 52.7% of all samples and was observed in all regions sampled, suggesting that this is the dominant species causing strawberry anthracnose in Zhejiang province. It is believed that *C. fruticola* is more pathogenic than *C. gloeosporioides* or *C. aenigma* (Han et al. 2016). Consistent with this, our pathogenicity experiments showed that *C. fruticola* was more pathogenic than *C. gloeosporioides* and *C. aenigma*. A previous study reported that *C. murrayae* was the dominant species causing strawberry anthracnose in Hubei, China, but no isolates of this species were found in this study (Han et al. 2016).

C. gloeosporioides was previously thought to infect many types of fruit, particularly in the tropics (Afanador-Kafuri et al. 2003; Freeman et al. 1998; Sangeetha and Rawal 2007). However, studies have shown that this species is uncommon in the tropics and rarely causes anthracnose in tropical fruits (Phoulivong et al. 2010; Prihastuti et al. 2009; Rojas et al. 2010; Udayanga et al. 2013). In addition, not all isolates of *C. gloeosporioides* were pathogenic to strawberries in greenhouse tests (Mertely and Legard 2004). Here, *C. gloeosporioides* was present in 14.3% of all samples, and it was the third most dominant group.

However, we found that the disease index of this species was low, consistent with previous studies (Hu et al. 2014).

C. aenigma was first described by Weir et al. (2012) based on phylogenetic analysis and morphological descriptions. This species primarily infects *Pyrus pyrifolia* in Japan and *Persea americana* in Israel (Weir et al. 2012), but it has been reported on grapes and a wide range of hosts (Yan et al. 2011). It is less virulent to strawberries, and it does not resist high temperatures (Han et al. 2016). The distribution of *C. aenigma* on strawberries in Zhejiang province is relatively sparse (Yan et al. 2011). The description of *Colletotrichum annigma* might be useful for the classification of the *C. gloeosporioides* complex, and the characteristics of this species require further study.

Strawberry anthracnose was previously thought to be caused by a complex of *C. gloeosporioides* species, *C. acutatum* species, and *C. fragariae* (Damm et al. 2012; Weir et al. 2012). It is thought that the incidence of this disease relates to the host plant (Weir et al. 2012). Ureña-Padilla et al. (2002) suggested that the *C. gloeosporioides* complex caused anthracnose in the root crown, that *C. acutatum* caused anthracnose in the fruit, and that the *C. gloeosporioides* complex caused the infection in the canopy. The *C. gloeosporioides* species complex most commonly causes strawberry anthracnose in China, whereas the *C. acutatum* species complex occasionally causes anthracnose (Xie et al. 2010). Three *C. acutatum* isolates were

Fig. 3. *Colletotrichum fruticola*. **a and b**, Cultures of HJD-49 on potato dextrose agar; 7-d growth from single conidia from above and below. **c**, Conidia. **d to f**, Appressoria. Scale bar in **c** = 20 μ m in **c to f**.

Fig. 5. *Colletotrichum gloeosporioides*. **a and b**, Cultures of GT-10 on potato dextrose agar; 7-d growth from single conidia from above and below. **c**, Conidia. **d to f**, Appressoria. Scale bar in **c** = 20 μ m in **c to f**.

Fig. 4. *Colletotrichum siamense*. **a and b**, Cultures of HJD4-7 on potato dextrose agar; 7-d growth from single conidia from above and below. **c**, Conidia. **d to f**, Appressoria. Scale bar in **c** = 20 μ m in **c to f**.

Fig. 6. *Colletotrichum aenigma*. **a and b**, Cultures of nbg-18 on potato dextrose agar; 7-d growth from single conidia from above and below. **c**, Conidia. **d to f**, Appressoria. Scale bar in **c** = 20 μ m in **c to f**.

Table 2. Summary of morphological data of *Colletotrichum* isolates and pathogenicity of four group strains on stabbing and nonstabbing of strawberry leaves

Species or isolate no. ^u	Characteristics ^v	Conidia ^w		Appresoria ^w		Growth rate (mm/d) ^x	Wounded leaves ^y		Nonwounded leaves ^y	
		Length (µm)	Width (µm)	Length (µm)	Width (µm)		Disease incidence (%)	Lesion diameter ^z (mm)	Disease incidence (%)	Lesion diameter ^z (mm)
<i>Colletotrichum fructicola</i>	Off-white, with	15.3 ± 1.1 (11.3–17.2)	5.2 ± 0.5 (2.3–6.1)	8.8 ± 0.6 (8.1–10.5)	7.2 ± 0.4 (6.5–8.2)	7.4 ± 0.2	79.9 a (76.9–82.9)	20.5 a	22.2 a (19.2–25.2)	17.0 a
<i>Colletotrichum stamense</i>	Off-white, with	14.8 ± 0.5 (12.1–16.8)	4.2 ± 0.4 (2.5–7.3)	8.4 ± 0.9 (7.5–9.4)	7.6 ± 0.5 (6.7–8.8)	5.7 ± 0.4	88.9 a (84.8–93.0)	15.5 ab	6.3 b (2.7–9.9)	1.0 d
<i>Colletotrichum gloeosporioides</i>	Off-white, lacking	13.4 ± 0.7 (12.8–15.3)	4.3 ± 0.4 (2.1–6.5)	7.1 ± 0.4 (6.6–8.3)	5.4 ± 0.6 (5.1–6.4)	7.7 ± 0.3	89.7 a (82.9–96.6)	13.5 b	12.8 ab (1.9–27.5)	6.9 c
<i>Colletotrichum aenigma</i>	Off-white, lacking	14.0 ± 0.8 (12.5–16.6)	4.5 ± 0.3 (2.9–4.9)	9.4 ± 0.7 (8.2–9.6)	6.4 ± 1.0 (5.6–7.2)	7.2 ± 0.2	81.5 a (73.0–90.0)	14.5 b	14.8 ab (6.8–22.8)	11.7 b

^u All of the 91 isolates were assessed.

^v Colony characteristics: off-white, lacking, off-white mycelia lacking conidial masses; off-white, with, off-white mycelia with mass conidial masses.

^w Data are mean ± standard deviation or standard error, with ranges in parentheses.

^x Data are mean ± standard deviation or standard error.

^y Disease was assessed 7 days after inoculation with conidial suspension of *Colletotrichum* spp. by spray.

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

identified in Hubei, China, by Han et al. (2016). This study did not found any *C. acutatum* species that have previously been reported to cause strawberry plant infections (MacKenzie et al. 2008).

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