Distribution and Characteristics of *Colletotrichum* spp. Associated with Anthracnose of Strawberry in Hubei, China

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Abstract

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Anthracnose caused by *Colletotrichum* spp. is a serious disease of strawberry. The etiology of anthracnose of strawberry is complex, and several *Colletotrichum* spp. have been regarded as causal agents. In the present study, multilocus (actin, β -tubulin, calmodulin, glyceraldehyde-3-phosphate dehydrogenase, and chitin synthase) phylogenetic analysis revealed that 100 isolates of *Colletotrichum* associated with anthracnose of strawberry in central China belong to five species. In total, 97 isolates were identified belonging to the *Colletotrichum gloeosporioides* species complex, with *C. murrayae*, *C. gloeosporioides*, *C. fructicola*, and *C. aenigma* accounting for 81, 8, 4, and 4% of the total isolates, respectively. Three isolates belonging to the *C. acutatum* complex were identified as *C. nymphaeae*. On inoculated strawberry plants, isolates of *C. fructicola* and *C. murrayae* species showed strong pathogenicity to both leaves and petioles of strawberry, with plant mortality 30 days after

inoculation of 77.8 and 55.6%, respectively. *C. gloeosporioides*, *C. aenigma*, and *C. nymphaeae* showed strong pathogenicity to leaves but weak pathogenicity to petioles, with plant mortality 30 days after inoculation of 5.6, 16.7, and 11.1%, respectively. The five species were divided into four classes based on their maximum growth temperatures. Isolates of *C. murrayae* and *C. gloeosporioides* were more tolerant to high temperature ($>34^{\circ}\text{C}$) than isolates of other species, followed by *C. fructicola* and *C. aenigma*. Isolates of *C. nymphaeae*, which are only distributed in areas of higher altitude (1,100 m), were highly sensitive to higher temperature. These results indicate that pathogenicity and adaptation to temperature are important factors in the distribution of *Colletotrichum* spp. on strawberry plants. This research may increase our understanding of how *Colletotrichum* spp. emerge and spread to geographical regions with different latitudes or elevations.

Strawberry (*Fragaria* $\times$ *ananassa*) is a small fruit crop of global importance. The area used for strawberry cultivation in China has increased rapidly in recent years. Anthracnose can cause considerable yield losses under appropriate environmental conditions (Henz et al. 1992). Strawberry infected with *Colletotrichum* spp. may develop lesions on the petiole and crown, in serious cases resulting in the collapse and death of the plant. In nurseries, lesions form on stolons, resulting in the wilting and death of unrooted daughter plants (Freeman and Katan 1997). Infected transplants are capable of spreading the disease from the nursery to the field, where typical anthracnose symptoms are later manifested as irregular and black leaf spots, crown rot, flower blight, and fruit rot (Howard et al. 1992). Three species have been reported as causal agents of strawberry anthracnose: *Colletotrichum gloeosporioides*, *C. fragariae*, and *C. acutatum* (Denoyes-Rothan et al. 2003).

Anthracnose disease caused by *Colletotrichum* spp. affects a wide variety of woody and herbaceous plants, particularly tropical and subtropical crops and fruit trees (Cannon et al. 2012; Freeman et al. 1998; Hyde et al. 2009; Sutton 1992; Wikee et al. 2011). The genus was recently denoted the world's eighth most important group of plant-pathogenic fungi, based on perceived scientific and economic importance (Dean et al. 2012). However, the identification of *Colletotrichum* spp. has long been problematic. Single-gene phylogenetic analyses have not been successful in delineating species (Hyde et al. 2009). Ko Ko et al. (2011) noted the need to resurvey plant pathogens such as *Colletotrichum* spp. using molecular approaches because previous data based on morphology and internal transcribed spacer sequences are likely incorrect. Understanding of *Colletotrichum* spp. has been significantly improved with the use

of multilocus phylogenetic analysis on species complexes, as well as the epitypification of many important pathogenic species (Cai et al. 2009; Cannon et al. 2012; Damm et al. 2012a; Doyle et al. 2013; Su et al. 2011; Weir et al. 2012). An important contribution to the taxonomy of the *C. gloeosporioides* and *C. acutatum* species complex was made by Weir et al. (2012) and Damm et al. (2012a). Hyde et al. (2014) reviewed the species identification of members of *Colletotrichum* and revealed that the *C. gloeosporioides* complex consisted of 34 taxa. The *C. acutatum* complex currently comprises at least 31 taxa (Damm et al. 2012a).

Hitherto, little was known about the species and distribution of *Colletotrichum* associated with strawberry anthracnose in China. The first objective of this study was to identify and characterize the species associated with strawberry anthracnose in central China based on morphological, molecular, and pathogenic characteristics. The second objective was to analyze the distribution of *Colletotrichum* spp. based on a large number of isolates.

Material and Methods

Fungal isolate collections. Hubei Province is in the central region of the Yangtze River and has a warm, humid, subtropical climate (Zhang et al. 2009). From 2012 to 2014, plants exhibiting stunting, chlorosis, and root necrosis were collected from nurseries and production fields in central China. Thirteen locations were sampled from different geographical regions in central China. The altitudes of the sampling locations were determined by GPS positioning using an HTC desire 816D (HTC Co., Ltd., Shanghai, China). Most areas of Hubei Province are plains area with an elevation of no more than 100 m. The 10-day average temperatures from 2012 to 2013 in Wuhan and Lichuan were supplied by the meteorological service center of Hubei Province, China. The 10-day average temperature in Wuhan (with an elevation of less than 100 m) from July to September was 20.7 to 32.4°C in 2012 and 2013 (Fig. 1). Enshi is a mountainous area to the west of Hubei Province; the elevation of the sampling location in Enshi was approximately 1,100 m. The 10-day average temperature from July to September in Lichuan

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(the sampling area in Enshi) was 16.1 to 25.2°C in 2012 and 2013 (Fig. 1).

Fungi were isolated from diseased tissues such as petioles, stolons, leaf lesions, or diseased crowns of infected strawberry plants. The diseased plant tissues were surface disinfected in 70% ethanol for 10 s and 1% NaClO₃ for 3 min, then rinsed three times with sterile distilled water and, finally, dried with sterilized tissue paper. The samples were cut into 5-by-5-mm pieces and plated onto potato dextrose agar (PDA) acidified with lactic acid at a final concentration of 0.05% to inhibit bacterial growth. The isolation plates were incubated under continuous fluorescent light in an incubator at 28°C for 3 to 7 days. The growing edges of any fungal hyphae developing on the PDA plates were then transferred aseptically to new PDA plates. The initial identification was based on the colony morphology on PDA plates. Single-spore subcultures were obtained for *Colletotrichum* isolates, and isolates without conidia on the PDA plates were purified by transferring samples from the edge of each colony to new PDA plates three to five times. In total, 100 *Colletotrichum* isolates were obtained from typical anthracnose lesions from strawberry petioles, stolons, and leaves. Of these, 98 isolates were from Hubei Province and 2 isolates (Nj-2 and Nj-122) were obtained from Jiangsu Province in October. All isolates were stored at 10°C for about 1 to 2 weeks before DNA extraction.

Extraction of fungal DNA. To obtain mycelia from *Colletotrichum* spp., a 5-mm-diameter plug from the growing margin of a 5-day-old colony was inoculated on the center of an autoclaved cellophane membrane placed on PDA (pH 7.0) in a petri dish (90 mm in diameter) (Lou et al. 2015). The dish was incubated at 28°C for 4 days. The mycelia of each isolate in the dish were collected using an autoclaved spatula and stored at -20°C until use. The mycelia of each isolate were ground to a fine powder in liquid nitrogen with a mortar and pestle. Genomic DNA (gDNA) was extracted and purified as described elsewhere (Lee and Taylor 1990). DNA samples from each isolate were stored at -20°C until use.

Fig. 1. The 10-day average temperature from 2012 to 2013. Data were supplied by the Hubei meteorological service center.

Polymerase chain reaction and DNA sequencing. Partial sequences corresponding to actin (ACT), β -tubulin (TUB2), calmodulin (CAL), glyceraldehyde-3-phosphate dehydrogenase (GPDH), and chitin synthase (CHS-1) were amplified from gDNA from the indicated *Colletotrichum* isolates (Table 1) by polymerase chain reaction (PCR). ACT and TUB2 were amplified using the primer pairs ACT512F and ACT-R2, and T1 and Tub2-R2, respectively. CAL, GPDH, and CHS-1 were amplified using the primer pairs CL1C and CL2C, GDF2 and GDR3, and CHS-88F and CHS-834R, respectively (Table 1). Primers of Act-R2, TUB-R2, GDR3, and CHS-834R were designed downstream of the binding site of standard primers (Weir et al. 2012) at these loci. All new primers designed in this study from the conserved domain in the corresponding genes in *C. crassipes*, *C. graminicola*, and *C. gloeosporioides* provided more specific amplification for species in this study (data not presented). The primers were used for PCR and PCR product sequencing unidirectionally, because this was more convenient than sequencing from both ends.

The PCR amplification mixtures were set up in a 0.25-ml tube containing 60 μ l of reaction mixture comprising 1 μ l of each primer solution (50 μ M), 2 μ l of dNTP mix solution (2.5 mM), 1 μ l of gDNA solution (approximately 50 ng per reaction mixture), 1 μ l of Taq DNA polymerase (5 U per reaction mixture; TAKARA Biotechnology Co., Ltd., Dalian, China), 6 μ l of PCR buffer, and 49 μ l of PCR-grade water.

PCR was performed in a thermal cycler (PTC-100TM Peltier Thermal Cycler; Hercules, CA). The cycling parameters for GPDH consisted of a denaturation step at 94°C for 5 min; followed by 30 cycles of 94°C for 30 s, 65°C for 30 s, and 72°C for 1 min; and a final cycle at 72°C for 10 min. The cycling parameters for CAL were 94°C for 5 min; followed by 30 cycles of 94°C for 1 min, 55°C for 30 s, and 72°C for 2 min; and a final step at 72°C for 10 min. The cycling parameters for the ACT, TUB2, and CHS-1 regions consisted of a 5-min denaturing step at 94°C; followed by 30 cycles of 94°C for 30 s, 59°C for 30 s, and 72°C for 1 min; and a final cycle of 10 min at 72°C. A 5- μ l aliquot of each PCR was separated by electrophoresis in a 1.5% agarose gel in 1.0 $\times$ Tris-acetate EDTA buffer and photographed under UV light after staining with Gel Red for 30 min. PCR products containing the target band were purified using the AxyPrep PCR Cleanup Kit (Axygen Biological Technology Co., Ltd., Hangzhou, Zhejiang Province, China) according to the manufacturer's instructions, then individually sequenced on an ABI PRISM 377-96 automatic sequencer (Beijing AuGCT Biotechnol. Co., Ltd., Beijing) via the dideoxynucleotide termination method using a Big Dye Terminator v2.0 Sequencing Kit (Applied Biosystems Inc., Foster City, CA).

All sequences were divided into 25 groups by genes and species (based on the result of preliminary phylogenetic analyses) and were aligned with DNAMAN (v. 7.0). Sequences with single-nucleotide polymorphisms were amplified and sequenced a total of three times to ensure that polymorphisms were not due to sequencing errors.

Molecular identification and phylogenetic analyses. The partial DNA sequences of ACT, CAL, CHS-1, GPDH, and TUB2 for all isolates, except Lch-5, Lch-9-1, and Lch-13 (Table 2), representative

Table 1. Primers used in this study

Gene ^y	Primers	Direction	Length (bp)	Sequence	Reference
ACT	ACT-512F	Forward	399	ATGTGCAAGGCCGTTTCGC	Carbone and Kohn (1999)
	ACT-R2 ^z	Reverse	...	AGGTGTGGTGCCAAATCTTCTC	This study
TUB2	T1	Forward	870	AACATGCGTGAGATTGTAAGT	O'Donnell and Cigelnik (1997)
	TUB2-R2 ^z	Reverse	...	TGGGTGATCTGGAACCCCTG	This study
CAL	CL1C ^z	Forward	773	GAATTCAAGGAGGCCCTTCTC	Weir et al. (2012)
	CL2C ^z	Reverse	...	CTTCTGCATCATGAGCTGGAC	Weir et al. (2012)
GAPDH	GDF2	Forward	870	TCCCATCAAGGTCGGCATCA	This study
	GDR3 ^z	Reverse	...	ACCTTGCCGACAGCCTTGG	This study
CHS-1	CHS-88F	Forward	779	GATGCCTGGAAGAAGATTGTCGT	This study
	CHS-834R ^z	Reverse	...	GTCTCGCCAGTAGCGGACTTGAC	This study

^y Actin (ACT), β -tubulin (TUB2), calmodulin (CAL), glyceraldehyde-3-phosphate dehydrogenase (GPDH), and chitin synthase (CHS-1).

^z Used for polymerase chain reaction product sequencing.

isolates of recognized species within the *C. gloeosporioides* complex (Hyde et al. 2014; Weir et al. 2012), and isolate CBS123755 of *C. boninense* (Damm et al. 2012b), were used to establish a five-locus combined dataset (ACT + CAL + CHS-1 + GPDH + TUB2) in the first Bayesian inference (A) analysis. *C. boninense* (ICMP17958) was used as an outgroup. The sequence of CAL was missing for several representative isolates within the *C. acutatum* species complex. Therefore, partial DNA sequences of ACT, CHS-1, GADH, and TUB2 for isolates Lch-5, Lch-9-1, and Lch-13 (Table 2) and for 31 representative isolates (Damm et al. 2012a; Hyde et al. 2014) were

used to establish a four-locus combined dataset (ACT + CHS-1 + GPDH + TUB2) in the second Bayesian inference (B) analysis. *C. pseudoacutatum* was used as an outgroup in this analysis.

Multiple sequence alignments of each gene were constructed using ClustalW as implemented in MEGA v.5 (Tamura et al. 2011). The sequences generated for the genes in this study were manually truncated to align with the reference sequences of the type isolates for constructing phylogenetic trees. Bayesian inference was used to reconstruct the phylogenetic trees using MrBayes v. 3.2.1 (Ronquist et al. 2012). The best-fit models of nucleotide substitution for ACT

Table 2. Strains and sequence used in this study

Species	Isolates	Substrate	Locality	Date ^z	GenBank accession number				
					GPDH	CHS-1	CAL	ACT	TUB2
<i>Colletotrichum aenigma</i>	Lbwuh-4-2	Leaf	Wuhan, Hubei, China	2013.05	KM053192	KM053001	KJ420134	KM053098	KJ420040
<i>C. aenigma</i>	Wha-2	Leaf	Wuhan, Hubei, China	2012.08	KM053193	KM053002	KJ420135	KM053099	KJ420041
<i>C. aenigma</i>	Hzaui-1	Leaf	Wuhan, Hubei, China	2014.10	KP339285	KP339292	KP339280	KP339273	KP339266
<i>C. aenigma</i>	Hzaui-2-1	Leaf	Wuhan, Hubei, China	2014.10	KP339286	KP339293	KP339281	KP339274	KP339267
<i>C. fruticola</i>	Fch-3	Stolons	Xiangyang, Hubei, China	2012.08	KM053201	KM053010	KJ420143	KM053107	KJ420049
<i>C. fruticola</i>	Sha-4	Crown	Jingzhou, Hubei, China	2012.12	KM053202	KM053011	KJ420144	KM053108	KJ420050
<i>C. fruticola</i>	Sab-4-3	Crown	Jingzhou, Hubei, China	2013.06	KM053203	KM053012	KJ420145	KM053109	KJ420051
<i>C. fruticola</i>	Lch-39-2	Leaf	Enshi, Hubei, China	2013.09	KM053204	KM053013	KJ420146	KM053110	KJ420052
<i>C. gloeosporioides</i>	Lch-1911	Petiole	Enshi, Hubei, China	2013.07	KM053194	KM053003	KJ420136	KM053100	KJ420042
<i>C. gloeosporioides</i>	Jsh-6-1	Leaf	Wuhan, Hubei, China	2013.07	KM053195	KM053004	KJ420137	KM053101	KJ420043
<i>C. gloeosporioides</i>	Jsh-6-2	Leaf	Wuhan, Hubei, China	2013.07	KM053196	KM053005	KJ420138	KM053102	KJ420044
<i>C. gloeosporioides</i>	Jsh-7-1	Petiole	Wuhan, Hubei, China	2013.07	KM053197	KM053006	KJ420139	KM053103	KJ420045
<i>C. gloeosporioides</i>	Wha-4	Petiole	Wuhan, Hubei, China	2012.08	KM053198	KM053007	KJ420140	KM053104	KJ420046
<i>C. gloeosporioides</i>	Yd-2	Petiole	Yichang, Hubei, China	2012.07	KM053199	KM053008	KJ420141	KM053105	KJ420047
<i>C. gloeosporioides</i>	Xya-221	Stolons	Xiangyang, Hubei, China	2012.08	KM053200	KM053009	KJ420142	KM053106	KJ420048
<i>C. gloeosporioides</i>	Hzaui-5-2	Leaf	Wuhan, Hubei, China	2014.10	KP339287	KP339294	KP339282	KP339275	KP339268
<i>C. murrayae</i>	7hao	Petiole	Wuhan, Hubei, China	2012.08	KM053111	KM052920	KJ420053	KM053017	KJ419959
<i>C. murrayae</i>	DS-10-1	Petiole	Wuhan, Hubei, China	2013.08	KM053112	KM052921	KJ420054	KM053018	KJ419960
<i>C. murrayae</i>	Ds-10-4	Petiole	Wuhan, Hubei, China	2013.08	KM053113	KM052922	KJ420055	KM053019	KJ419961
<i>C. murrayae</i>	Ds-12-1	Petiole	Wuhan, Hubei, China	2013.08	KM053114	KM052923	KJ420056	KM053020	KJ419962
<i>C. murrayae</i>	Ezh-2	Petiole	Ezhou, Hubei, China	2012.08	KM053115	KM052924	KJ420057	KM053021	KJ419963
<i>C. murrayae</i>	Ezh-3	Crown	Ezhou, Hubei, China	2012.08	KM053116	KM052925	KJ420058	KM053022	KJ419964
<i>C. murrayae</i>	Fch-2	Stolons	Xiangyang, Hubei, China	2012.08	KM053117	KM052926	KJ420059	KM053023	KJ419965
<i>C. murrayae</i>	Fch-3-1	Stolons	Xiangyang, Hubei, China	2012.08	KM053118	KM052927	KJ420060	KM053024	KJ419966
<i>C. murrayae</i>	Gay-7-3	Crown	Jingzhou, Hubei, China	2013.06	KM053119	KM052928	KJ420061	KM053025	KJ419967
<i>C. murrayae</i>	Gay-7-4	Crown	Jingzhou, Hubei, China	2013.06	KM053120	KM052929	KJ420062	KM053026	KJ419968
<i>C. murrayae</i>	Gay-9-1	Crown	Jingzhou, Hubei, China	2013.06	KM053121	KM052930	KJ420063	KM053027	KJ419969
<i>C. murrayae</i>	Gsh-2	Stolons	Suizhou, Hubei, China	2012.08	KM053122	KM052931	KJ420064	KM053028	KJ419970
<i>C. murrayae</i>	Hga-6-2	Crown	Huanggang, Hubei, China	2012.08	KM053123	KM052932	KJ420065	KM053029	KJ419971
<i>C. murrayae</i>	Hy-2-2	Stolons	Xianing, Hubei, China	2013.07	KM053124	KM052933	KJ420066	KM053030	KJ419972
<i>C. murrayae</i>	Hy-2-5	Stolons	Xianing, Hubei, China	2013.07	KM053125	KM052934	KJ420067	KM053031	KJ419973
<i>C. murrayae</i>	Hy-8	Leaf	Xianing, Hubei, China	2013.07	KM053126	KM052935	KJ420068	KM053032	KJ419974
<i>C. murrayae</i>	Jy-3-2	Leaf	Xianing, Hubei, China	2013.07	KM053127	KM052936	KJ420069	KM053033	KJ419975
<i>C. murrayae</i>	MC-10-1	Stolons	Huanggang, Hubei, China	2013.08	KM053128	KM052937	KJ420070	KM053034	KJ419976
<i>C. murrayae</i>	MC-10-3	Stolons	Huanggang, Hubei, China	2013.08	KM053129	KM052938	KJ420071	KM053035	KJ419977
<i>C. murrayae</i>	Mc-13-1	Stolons	Huanggang, Hubei, China	2013.08	KM053130	KM052939	KJ420072	KM053036	KJ419978
<i>C. murrayae</i>	MC-14-1	Stolons	Huanggang, Hubei, China	2013.08	KM053131	KM052940	KJ420073	KM053037	KJ419979
<i>C. murrayae</i>	MC-14-2	Stolons	Huanggang, Hubei, China	2013.08	KM053132	KM052941	KJ420074	KM053038	KJ419980
<i>C. murrayae</i>	MC-15-2	Stolons	Huanggang, Hubei, China	2013.08	KM053133	KM052942	KJ420075	KM053039	KJ419981
<i>C. murrayae</i>	MC-17-1	Stolons	Huanggang, Hubei, China	2013.08	KM053134	KM052943	KJ420076	KM053040	KJ419982
<i>C. murrayae</i>	MC-19-3	Stolons	Huanggang, Hubei, China	2013.08	KM053135	KM052944	KJ420077	KM053041	KJ419983
<i>C. murrayae</i>	MC-20-2	Stolons	Huanggang, Hubei, China	2013.08	KM053136	KM052945	KJ420078	KM053042	KJ419984
<i>C. murrayae</i>	MC-20-3	Stolons	Huanggang, Hubei, China	2013.08	KM053137	KM052946	KJ420079	KM053043	KJ419985
<i>C. murrayae</i>	MC-2-1	Petiole	Huanggang, Hubei, China	2012.08	KM053138	KM052947	KJ420080	KM053044	KJ419986
<i>C. murrayae</i>	Mc-21-2	Stolons	Huanggang, Hubei, China	2013.08	KM053139	KM052948	KJ420081	KM053045	KJ419987
<i>C. murrayae</i>	MC-2-2	Stolons	Huanggang, Hubei, China	2012.08	KM053140	KM052949	KJ420082	KM053046	KJ419988
<i>C. murrayae</i>	MC-221	Stolons	Huanggang, Hubei, China	2013.08	KM053141	KM052950	KJ420083	KM053047	KJ419989
<i>C. murrayae</i>	MC-22-2	Stolons	Huanggang, Hubei, China	2013.09	KM053142	KM052951	KJ420084	KM053048	KJ419990
<i>C. murrayae</i>	MC-23-2	Stolons	Huanggang, Hubei, China	2013.09	KM053143	KM052952	KJ420085	KM053049	KJ419991
<i>C. murrayae</i>	MC-23-3	Stolons	Huanggang, Hubei, China	2013.09	KM053144	KM052953	KJ420086	KM053050	KJ419992
<i>C. murrayae</i>	Mc-23-4	Stolons	Huanggang, Hubei, China	2013.09	KM053145	KM052954	KJ420087	KM053051	KJ419993

(continued on next page)

^z Sample date: year and month (01 = January through 12 = December) collected.

(K80+G), CAL (GTR+G), CHS-1 (K80+G), GPDH (HKY+I), and TUB2 (GTR+I) were selected with MrModelTest v. 2.3 (Nylander 2004) using the Akaike Information Criteria. The multiple nucleotide sequence alignments of all genes were concatenated, and analysis was performed twice on the full data set for 5×10^7 generations. Samples were taken from the results every 1,000 generations. The convergence of all parameters was verified using the Tracer program (Rambaut and Drummond 2007). The first 25% of generations were discarded as burn-in. The concatenated alignment and trees were submitted to TreeBASE (www.treebase.org) with a submission number of 16878.

Isolates for biological characteristics. Based on the result of phylogenetic analyses, 21 representative *Colletotrichum* isolates obtained from different locations were selected for further characterization of colony and mycelial growth at different temperatures. The selected

isolates included five *C. murrayae* isolates (Yd-8, Ydu-15, Nj-2, Nj-122, and MC-17-1), four *C. fructicola* isolates (Sab-4-3, Sha-4, Lch-39-2, and Fch-3), five *C. gloeosporioides* isolates (Lch-1911, Xya-221, Yd-2, Jsh-6-1, and Wha-4), four *C. aenigma* isolates (Wha-2, Lbwuh-4-2, Hzaui-1, and Hzaui-2-1), and three *C. nymphaeae* isolates (Lch-5, Lch-9-1, and Lch-13).

Morphological and cultural characterization. Morphological and cultural characterizations were performed according to the methods described by Cai et al. (2009). Mycelial discs (5 mm in diameter) were taken from areas near the colony edge of 5-day-old cultures, transferred to PDA, and incubated at 28°C under mixed white and near-UV fluorescent light sources under a cycle of 12 h of light and 12 h of darkness. The experiment was designed as a randomized complete block with three replicates of each isolate. Colony color, conidial dimensions, and morphology were assessed (Cai et al.

Table 2. (continued from preceding page)

Species	Isolates	Substrate	Locality	Date ^a	GenBank accession number				
					GPDH	CHS-1	CAL	ACT	TUB2
<i>C. murrayae</i>	MC-24-1	Stolons	Huanggang, Hubei, China	2013.09	KM053146	KM052955	KJ420088	KM053052	KJ419994
<i>C. murrayae</i>	Mc-3-1	Petiole	Huanggang, Hubei, China	2012.08	KM053147	KM052956	KJ420089	KM053053	KJ419995
<i>C. murrayae</i>	Mc-3-2	Petiole	Huanggang, Hubei, China	2012.08	KM053148	KM052957	KJ420090	KM053054	KJ419996
<i>C. murrayae</i>	MC-4-1	Petiole	Huanggang, Hubei, China	2012.08	KM053149	KM052958	KJ420091	KM053055	KJ419997
<i>C. murrayae</i>	MC-5-1	Petiole	Huanggang, Hubei, China	2012.08	KM053150	KM052959	KJ420092	KM053056	KJ419998
<i>C. murrayae</i>	MC-5-3	Petiole	Huanggang, Hubei, China	2012.08	KM053151	KM052960	KJ420093	KM053057	KJ419999
<i>C. murrayae</i>	MC-6-2	Stolons	Huanggang, Hubei, China	2012.08	KM053152	KM052961	KJ420094	KM053058	KJ420000
<i>C. murrayae</i>	MC-9-3	Stolons	Huanggang, Hubei, China	2012.08	KM053153	KM052962	KJ420095	KM053059	KJ420001
<i>C. murrayae</i>	Nj-122	Petiole	Nanjing, Jiangsu, China	2013.09	KM053154	KM052963	KJ420096	KM053060	KJ420002
<i>C. murrayae</i>	Nj-2	Petiole	Nanjing, Jiangsu, China	2013.09	KM053155	KM052964	KJ420097	KM053061	KJ420003
<i>C. murrayae</i>	Sab-552	Stolons	Jingzhou, Hubei, China	2013.06	KM053156	KM052965	KJ420098	KM053062	KJ420004
<i>C. murrayae</i>	Sab-6-1	Stolons	Jingzhou, Hubei, China	2013.06	KM053157	KM052966	KJ420099	KM053063	KJ420005
<i>C. murrayae</i>	Sha-1	Crown	Jingzhou, Hubei, China	2012.12	KM053158	KM052967	KJ420100	KM053064	KJ420006
<i>C. murrayae</i>	Sha-2	Crown	Jingzhou, Hubei, China	2012.12	KM053159	KM052968	KJ420101	KM053065	KJ420007
<i>C. murrayae</i>	Sha-3	Crown	Jingzhou, Hubei, China	2012.12	KM053160	KM052969	KJ420102	KM053066	KJ420008
<i>C. murrayae</i>	Sha-5	Crown	Jingzhou, Hubei, China	2013.03	KM053161	KM052970	KJ420103	KM053067	KJ420009
<i>C. murrayae</i>	Sha-6	Crown	Jingzhou, Hubei, China	2013.03	KM053162	KM052971	KJ420104	KM053068	KJ420010
<i>C. murrayae</i>	Sha-7-1	Crown	Jingzhou, Hubei, China	2013.03	KM053163	KM052972	KJ420105	KM053069	KJ420011
<i>C. murrayae</i>	Sha-7-2	Crown	Jingzhou, Hubei, China	2013.03	KM053164	KM052973	KJ420106	KM053070	KJ420012
<i>C. murrayae</i>	Wha-7	Petiole	Wuhan, Hubei, China	2012.08	KM053165	KM052974	KJ420107	KM053071	KJ420013
<i>C. murrayae</i>	Wha-8	Petiole	Wuhan, Hubei, China	2012.08	KM053166	KM052975	KJ420108	KM053072	KJ420014
<i>C. murrayae</i>	Wuh-13	Crown	Wuhan, Hubei, China	2013.04	KM053167	KM052976	KJ420109	KM053073	KJ420015
<i>C. murrayae</i>	Wuh-13-1	Crown	Wuhan, Hubei, China	2013.04	KM053168	KM052977	KJ420110	KM053074	KJ420016
<i>C. murrayae</i>	Wuh-13-2	Crown	Wuhan, Hubei, China	2013.04	KM053169	KM052978	KJ420111	KM053075	KJ420017
<i>C. murrayae</i>	Xt-1	Petiole	Xiantao, Hubei, China	2012.08	KM053170	KM052979	KJ420112	KM053076	KJ420018
<i>C. murrayae</i>	Xt-18-1	Crown	Xiantao, Hubei, China	2013.06	KM053171	KM052980	KJ420113	KM053077	KJ420019
<i>C. murrayae</i>	Ych-1-2	Crown	Xiaogan, Hubei, China	2012.08	KM053172	KM052981	KJ420114	KM053078	KJ420020
<i>C. murrayae</i>	Yd-3	Leaf	Yichang, Hubei, China	2012.07	KM053173	KM052982	KJ420115	KM053079	KJ420021
<i>C. murrayae</i>	Yd-8	Petiole	Yichang, Hubei, China	2012.07	KM053174	KM052983	KJ420116	KM053080	KJ420022
<i>C. murrayae</i>	Ydu-11	Petiole	Yichang, Hubei, China	2013.08	KM053175	KM052984	KJ420117	KM053081	KJ420023
<i>C. murrayae</i>	Ydu-12-1	Petiole	Yichang, Hubei, China	2013.08	KM053176	KM052985	KJ420118	KM053082	KJ420024
<i>C. murrayae</i>	Ydu-12-2	Petiole	Yichang, Hubei, China	2013.08	KM053177	KM052986	KJ420119	KM053083	KJ420025
<i>C. murrayae</i>	Ydu-14-1	Petiole	Yichang, Hubei, China	2013.08	KM053178	KM052987	KJ420120	KM053084	KJ420026
<i>C. murrayae</i>	Ydu-15	Petiole	Yichang, Hubei, China	2013.08	KM053179	KM052988	KJ420121	KM053085	KJ420027
<i>C. murrayae</i>	Zhd-11	Petiole	Wuhan, Hubei, China	2013.10	KM053180	KM052989	KJ420122	KM053086	KJ420028
<i>C. murrayae</i>	Zhd-13-1	Crown	Wuhan, Hubei, China	2013.10	KM053181	KM052990	KJ420123	KM053087	KJ420029
<i>C. murrayae</i>	Zhd-14-1	Crown	Wuhan, Hubei, China	2013.10	KM053182	KM052991	KJ420124	KM053088	KJ420030
<i>C. murrayae</i>	Zhd-4	Crown	Wuhan, Hubei, China	2012.12	KM053183	KM052992	KJ420125	KM053089	KJ420031
<i>C. murrayae</i>	Zhd-4-2	Crown	Wuhan, Hubei, China	2012.12	KM053184	KM052993	KJ420126	KM053090	KJ420032
<i>C. murrayae</i>	Zhd-6	Crown	Wuhan, Hubei, China	2012.12	KM053185	KM052994	KJ420127	KM053091	KJ420033
<i>C. murrayae</i>	Zhd-8-1	Petiole	Wuhan, Hubei, China	2012.12	KM053186	KM052995	KJ420128	KM053092	KJ420034
<i>C. murrayae</i>	Zhd-8-2	Petiole	Wuhan, Hubei, China	2012.12	KM053187	KM052996	KJ420129	KM053093	KJ420035
<i>C. murrayae</i>	Zj-1-2	Leaf	Xianning, Hubei, China	2013.07	KM053188	KM052997	KJ420130	KM053094	KJ420036
<i>C. murrayae</i>	Zj-3-2	Leaf	Xianning, Hubei, China	2013.07	KM053189	KM052998	KJ420131	KM053095	KJ420037
<i>C. murrayae</i>	Zj-6-1	Stolons	Xianning, Hubei, China	2013.07	KM053190	KM052999	KJ420132	KM053096	KJ420038
<i>C. murrayae</i>	Zj-9-2	Petiole	Xianning, Hubei, China	2013.07	KM053191	KM053000	KJ420133	KM053097	KJ420039
<i>C. nymphaeae</i>	Lch-5	Stolons	Enshi, Hubei, China	2013.07	KP339288	KP339295	...	KP339276	KP339269
<i>C. nymphaeae</i>	Lch-9-1	Leaf	Enshi, Hubei, China	2013.07	KP339289	KP339296	...	KP339277	KP339270
<i>C. nymphaeae</i>	Lch-13	Leaf	Enshi, Hubei, China	2014.08	KP339290	KP339297	...	KP339278	KP339271

2009; Peng et al. 2012; Weir et al. 2012). Conidial suspensions of each species were prepared in lactic acid from conidial masses on PDA. The shape and color of the conidia were observed, and the size of 50 conidia per isolate was measured using a microscope (BM2000; Nanjing Jiangnan Novel Optics Co., Ltd., Nanjing, China).

Effect of temperature on mycelial growth. In total, 21 isolates of the five species were evaluated to determine the effect of temperature on radial colony growth in culture. All isolates were maintained on PDA supplemented with lactic acid at 28°C for 5 days. Plugs of agar with a diameter of 5 mm were cut from the leading edge of colonies and placed in the center of petri plates containing PDA. The plates

were then wrapped with parafilm and placed in an incubator (BPH-9272; Shanghai Yiheng Scientific Instrument Co., Ltd., Shanghai, China) at 1 of 11 temperatures (6, 10, 14, 18, 22, 26, 28, 30, 32, 34, 36, 38, 40, and 42°C [$\pm 0.2^\circ\text{C}$]) in the dark. After 4 days, colony diameters were measured and used to calculate the mycelial growth rate (in millimeters per day). The experiment was designed as a randomized complete block, with three replicates of each isolate for each temperature treatment. The experiment was repeated once.

Pathogenicity assays. Not all the *Colletotrichum* isolates could sporulate on PDA plus 0.2% yeast extract (PDAY); therefore, one isolate of each species that could sporulate on PDAY was selected

Fig. 2. Bayesian inference phylogenetic tree of 132 isolates of the *Colletotrichum gloeosporioides* species complex. The tree was built using concatenated sequences of the *ACT*, *TUB2*, *CAL*, *GPDH*, and *CHS-1* genes, each with a separate model of DNA evolution. Values next to the nodes of the tree are Bayesian posterior probability values based on 5,000,000 generations. *C. boninense* 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.

for pathogenicity assays. *C. murrayae* isolate Nj-2, *C. gloeosporioides* isolate Jsh-7-1, *C. fruticola* isolate Lch-39-2, *C. aenigma* isolate Lbwuh-4-2, and *C. nymphaeae* isolate Lch-5 were utilized for pathogenicity tests. The five isolates were maintained on PDA supplemented with lactic acid at 28°C for 5 days. Plugs of agar with a diameter of 5 mm were cut from the leading edge of colonies and placed in the center of petri plates containing PDAY. The fungal isolates were grown at 28°C under mixed white and near-UV fluorescent light sources under a cycle of 12 h of light and 12 h of darkness (de los Santos Garcia de Paredes and Romero Muñoz 2002), and sporulated profusely after 7 to 10 days. Plates were flooded with sterile distilled water, the spore suspension was passed through cheesecloth to remove dislodged mycelia (Mangandi et al. 2015), and the suspension was adjusted to 2×10^6 conidia/ml.

Healthy 6-week-old strawberry plants ('Benihopp') devoid of any exposure to fungicides were used for plant inoculation experiments. Plants were inoculated by spraying conidial suspensions (2×10^6 conidia/ml) of the five isolates (Nj-2, Jsh-7-1, Lch-39-2, Lbwuh-4-2, and Lch-5) until runoff (Freeman and Katan 1997). Negative control plants received sterile water without conidia. Nine plants were inoculated with each isolate separately and maintained in a moisture chamber at 25 to 35°C for 48 h, then transferred to a greenhouse at 25 to 35°C for 30 days under natural daylight conditions.

The pathogenicity to strawberry of different species were evaluated by calculating the disease index (DI) according to the symptoms severity (SS) of leaves and petioles, respectively. The SS class of leaves, which was calculated based on a percentage of infected leaf

surface caused by *Colletotrichum* spp., was described on a six-point scale, as follows: 0 = healthy leaves with no lesions or 1 = 0.1 to 5.0, 3 = 5.1 to 15.0, 5 = 15.1 to 30.0, 7 = 30.1 to 50.0, and 9 = 50.1 to 100% infected leaf surface or dead leaves. The SS class of petioles, calculated based on the length of lesions caused by *Colletotrichum* spp. on petioles, was described on a six-point scale, as follows: 0 = healthy petiole with no lesions or lesions of 1 = 0.1 to 2, 3 = 2.1 to 5.0, 5 = 5.1 to 9.0, 7 = 9.1 to 15.0, and 9 = >15.0 mm in length or leaves wilting (Wang et al. 2008). All leaves and petioles on each plant were evaluated individually. The number of leaves or petioles in each SS class was recorded 20 days after inoculation (dai) and plant mortality was recorded 30 dai. These experiments were conducted twice.

SS were converted to DI that was calculated using the formula of Horiuchi and Hori (1980): $DI = [(n_0 \times 0 + n_1 \times 1 + n_3 \times 3 + n_5 \times 5 + n_7 \times 7 + n_9 \times 9)/9N] \times 100$, where n is the number of leaves or petioles in this SS class; the subscript numbers 0, 1, 3, 5, 7, and 9 are the SS class; and N is the total number of leaves or petioles.

Data analysis. Data from the pathogenicity test were subjected to analysis of variance using the Statistical Analysis Systems (SAS, version 8.0; SAS Institute, Cary, NC) in order to determine the significance of treatment differences. Means for different species from the pathogenicity test were separated using the least significance difference (LSD) test at $P = 0.05$ level. The minimum, optimum, and maximum growth temperature data were analyzed using the general linear model procedure (PROC GLM) in SAS. Species were considered fixed effects and isolates were considered random effects. When

Fig. 3. Bayesian inference phylogenetic tree of 34 isolates of the *Colletotrichum acutatum* species complex. The tree was built using concatenated sequences of the *ACT*, *TUB2*, *GPDH*, and *CHS-1* genes, each with a separate model of DNA evolution. Values next to the nodes of the tree are Bayesian posterior probability values based on 500,000 generations. *C. pseudoacutatum* 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.

F values were significant ($P < 0.05$), species means were compared using the LSD test.

Results

Molecular identification and phylogenetic analyses. ACT, CAL, CHS-1, GPDH, and TUB2 regions of all isolates were successfully amplified and sequenced. Among the 100 isolates, 97 isolates were identified belonging to the *C. gloeosporioides* species complex and 3 isolates were identified belonging to the *C. acutatum* species complex.

The Bayesian inference (A) with the haplotypes belonging to the *C. gloeosporioides* species complex was performed with the combined TUB2, ACT, CAL, GPDH, and CHS-1 sequences and comprised 2,352 characters after alignment. The boundaries of the loci used in the alignment were as follows: TUB2, 1 to 733; ACT, 734 to 1,028; CAL, 1,029 to 1,776; GPDH, 1,777 to 2,081; and CHS-1, 2,082 to 2,352. The Bayesian inference (B) with the haplotypes belonging to the *C. acutatum* species complex was performed with the combined TUB2, ACT, GPDH, and CHS-1 sequences and comprised 1,265 characters after alignment. The boundaries of the loci used in the alignment were as follows: TUB2, 1 to 495; ACT, 496 to 745; GPDH, 746 to 1,015; CHS-1, 1,016 to 1,265.

The isolates from strawberry belonged to five clades. In Bayesian inference (A), 81 isolates clustered with *C. murrayae* Li J. Peng & K. D. Hyde; 8 isolates clustered with *C. gloeosporioides* (Penz.) Penz. & Sacc.; 4 isolates clustered with *C. fructicola* Prihastuti, L. Cai & K. D. Hyde; and 4 isolates clustered with *C. aenigma* B. S. Weir & P. R. Johnst. (Fig. 2). In Bayesian inference (B), three isolates clustered with *C. nymphaeae* (Pass.) Aa (Fig. 3). *C. murrayae* was the most frequently isolated species, comprising 81% of all isolates, followed by *C. gloeosporioides* at 7%. Isolates of *C. murrayae* were widely distributed in Hubei Province. *C. gloeosporioides* was primarily distributed in Wuhan, Xiangyang, Yichang, and Enshi. *C. fructicola* was mainly distributed in Xiangyang, Jingzhou, and Enshi. All four isolates of *C. aenigma* were isolated from Wuhan. *C. nymphaeae* was distributed in Enshi (Fig. 4). The altitudes of the sampling locations were no higher than 100 m, with the exception of Enshi (with an altitude of 1,100 m).

Morphological and cultural characterization. Few differences of colony morphology were observed among the five species identified

when grown on PDA. All selected isolates developed white colonies that became gray after 10 days. Isolates of *C. nymphaeae* and *C. gloeosporioides* produced numerous conidia on PDA, while isolates of the other three species produced fewer conidia (Fig. 5). The conidia produced by *C. nymphaeae* were sharp at both ends (Fig. 6). This morphology was different from that of the other four species in this study (Table 3).

Effect of temperature on mycelial growth. The optimum temperature of all five *Colletotrichum* spp. in the current study was 28°C but the tolerance to high temperature (34°C) among species was different (Fig. 7). The five species were divided into four groups based on their maximum growth temperatures (Table 4). Isolates of *C. nymphaeae*, which were isolated only in Enshi, were sensitive to high temperatures. The average growth rates of the three isolates were 0.11 mm/day at 34°C, and no mycelial growth in vitro was observed at temperatures above 34°C (Table 4).

Isolates of *C. fructicola* and *C. aenigma* were similar in their tolerances to high temperatures. These two species were moderately sensitive to high temperature and grew very slowly at 36°C, with colony diameters smaller than those of isolates of *C. murrayae* and *C. gloeosporioides* but larger than those of isolates of *C. nymphaeae*. No mycelial growth in vitro of the *C. fructicola* and *C. aenigma* isolates was observed at temperatures above 36°C (Fig. 7). Isolates of *C. gloeosporioides* and *C. murrayae* were tolerant to high temperatures, with a significantly larger colony diameter than the other four species when the ambient temperature was above 36°C. Mycelial growth in vitro was observed even at 40°C, with average growth rates of 0.11 and 0.08 mm/day, respectively (Fig. 7).

Pathogenicity assays. All isolates of *Colletotrichum* tested were pathogenic to nonwounded strawberry leaves and petioles. Symptoms first appeared on leaves. Infected leaves developed sunken, prominent, dark-brown to black decay, and necrotic lesions, and typical orange conidial masses were often observed on leaves 7 to 10 dai. Symptoms of anthracnose were not observed on petioles at 10 dai. At 20 dai, symptoms appeared on both leaves and petioles of strawberry (Fig. 5). Disease severity caused by the five species showed significant differences. Isolates of *C. fructicola* and *C. murrayae* species showed strong pathogenicity on both leaves and petioles of strawberry (Table 5). Plant mortalities caused by *C. fructicola* and

Fig. 4. Distribution of *Colletotrichum* spp. associated with anthracnose of strawberry in Hubei Province, China.

C. murrayae at 30 dai were 77.8 and 55.6% respectively (Table 5). *C. gloeosporioides*, *C. aenigma*, and *C. nymphaeae* showed strong pathogenicity to leaves but weak to petioles. Plant mortalities caused by these species at 30 dai were 5.6, 16.7, and 11.1%, respectively (Table 5), as compared with 0% for the water control plants.

Discussion

This study represents the first attempt to characterize *Colletotrichum* spp. associated with anthracnose of strawberry in China using a polyphasic approach. GPDH, CAL, CHS-1, ACT, and TUB2 loci were employed in the phylogenetic analysis. Phylogenetic analysis showed that the 100 *Colletotrichum* isolates from strawberry plants belonged to five species. This study provides the first report of *C. gloeosporioides*, *C. aenigma*, *C. murrayae*, *C. fructicola*, and *C. nymphaeae* from strawberry in China, while *C. fructicola* and *C. nymphaeae* have been recorded from strawberry previously (Damm et al. 2012a; Weir et al. 2012). Together with the 9 species in the *C. gloeosporioides* and *C. acutatum* species complexes (Damm et al. 2012a; Weir et al. 2012), 12 *Colletotrichum* spp. are now known to be associated with strawberry hosts. *C. gloeosporioides*, which was previously recognized as a pathogen of strawberry, is actually a species complex. *C. gloeosporioides* was typified by Cannon

et al. (2008) and a recent study indicated this species was associated with the genera *Citrus*, *Ficus*, *Mangifera*, *Pueraria*, and *Vitis* (Weir et al. 2012). These results are in agreement with previous reports that *C. gloeosporioides* species complex is the most prevalent agent of strawberry anthracnose in China, while *C. acutatum* species complex only occasionally causes anthracnose (Xie et al. 2010). *C. theobromicola* (synonymous with *C. fragariae*) has been reported to infect strawberry plants (Brooks 1931; Xie et al. 2010) but no isolates of this species were identified in this study.

Important progress on taxonomy of the *C. gloeosporioides* and *C. acutatum* species complex have been made by Weir et al. (2012) and Damm et al. (2012a) but the biological characteristics such as temperature characteristics, host range, and pathogenicity of different *Colletotrichum* spp. is unclear. Many pathogens are sensitive to temperature, rainfall, and humidity (Harvell et al. 2002; Wilson et al. 1990). Temperature is a major factor affecting epidemics of plant disease; it can affect spore germination and infection, latent period, sporulation, spore survival, and host resistance to many pathogens (Bonde et al. 2012; Wilson et al. 1990). Temperature highs and their frequency can be factors in the delay or absence of plant disease (Bonde et al. 2012, 2013). On the other hand, the adaptation of pathogens to temperature is an important factor for disease emergence

Fig. 5. Culture characteristics and pathogenicity of five *Colletotrichum* spp. from strawberry. **A**, *Colletotrichum murrayae*; **B**, *C. fructicola*; **C**, *C. gloeosporioides*; **D**, *C. aenigma*; and **E**, *C. nymphaeae*. Cultures on potato dextrose agar, growth at 18°C for 10 days from mycelial discs (5 mm in diameter) from **1**, aerial and **2**, reverse view; **3**, symptoms caused by *Colletotrichum* spp. on strawberry 30 days after spray inoculation with conidia.

and spread. For example, Botrytis fruit rot of strawberry caused by *Botrytis cinerea* occurs mainly at low temperatures in winter and spring (Elad et al. 2007); however, *Colletotrichum* spp. require relatively hot temperatures and mainly cause anthracnose of strawberry in summer (Henz et al. 1992; Xie et al. 2010). The minimum, optimum, and maximum growth temperatures are important biological characteristics and widely used to characterize microorganisms (Damm et al. 2012a,b; Prihastuti et al. 2009; Weir et al. 2012). In this study, no significant differences were observed in the minimum and optimum growth temperatures of the five *Colletotrichum* spp. but the maximum growth temperatures were different (Table 4). The five *Colletotrichum* spp. were grouped into four classes based on their maximum growth temperatures.

C. murrayae was originally reported from *Citrus* leaves in China (Peng et al. 2012). *C. murrayae* is tolerant to high temperature and showed strong pathogenicity to strawberry plants. *C. murrayae* was the most frequently isolated species, distributed in nearly all sampling areas, and seems to be the most economically important species in strawberry nurseries in Hubei, China. *C. gloeosporioides* was tolerant to high temperature but was weakly pathogenic to strawberry plants. *C. gloeosporioides* was the second most prevalent species, with 8% of all isolates (Fig. 4). *C. fruticola* was originally reported causing coffee berry disease in Thailand (Prihastuti et al. 2009). Although *C. fruticola* showed stronger pathogenicity to strawberry plants than other four species in this study, it showed less tolerance to high temperature than *C. murrayae* and *C. gloeosporioides*, and occurred only sporadically.

C. aenigma was first described by Weir et al. (2012) and causes anthracnose of *Pyrus pyrifolia* in Japan and *Persea americana* in Israel (Weir et al. 2012). *C. nymphaeae* was originally described in detail in morphological and pathological terms by van der Aa (1978). A description in morphological and multilocus phylogenetic analysis of this species was provided by Damm et al. (2012a). *C. aenigma* and *C. nymphaeae* showed weak pathogenicity to strawberry plants and were less tolerant to high temperature than *C. murrayae* and *C. gloeosporioides*. *C. aenigma* and *C. nymphaeae* occurred very sporadically in Hubei, China (Fig. 4).

According to current data, the results revealed that each species possesses a stable and distinguishable maximum growth temperature, which may be used for distinguishing *Colletotrichum* spp., particularly morphologically similar species. The frequencies and distribution of these five species are related to differences in high temperature tolerance and pathogenicity. This research may increase our understanding of how *Colletotrichum* spp. emerge and spread to geographical regions with different latitudes or elevations. Due to climate change, pathogens are exposed to increasingly warmer environments, and the impact of anthropogenic climate change on terrestrial organisms is often predicted to increase with latitude and elevation (Colwell et al. 2008; Deutsch et al. 2008; Harvell et al. 2002). This research is also important for predicting changes that may occur due to global warming conditions. Further studies should be conducted on the host range, distribution, and pathogenicity of *Colletotrichum* spp. at different temperatures.

Fig. 6. Conidia of five *Colletotrichum* spp. from strawberry. **A**, *Colletotrichum murrayae*; **B**, *C. fruticola*; **C**, *C. gloeosporioides*; **D**, *C. aenigma*; and **E**, *C. nymphaeae*. Conidial suspensions of each species were prepared in lactic acid from conidial masses on potato dextrose agar.

Table 3. Summary of morphological data of *Colletotrichum* isolates

Species	Characteristics ^y	Conidia ^x		
		Length (μm)	Width (μm)	Shape ^z
<i>Colletotrichum murrayae</i>	White, lacking	14.3 ± 0.9 (10.4–17.0)	3.8 ± 0.8 (2.8–6.6)	Rounded
<i>C. gloeosporioides</i>	White, with	14.4 ± 1.5 (11.3–18.0)	5.1 ± 1.0 (2.8–6.6)	Rounded
<i>C. fruticola</i>	White, lacking	14.0 ± 0.8 (11.3–15.1)	4.8 ± 0.8 (2.8–6.6)	Rounded
<i>C. aenigma</i>	White, lacking	14.4 ± 0.9 (12.2–16.0)	4.1 ± 0.3 (3.8–4.7)	Rounded
<i>C. nymphaeae</i>	White, with	11.2 ± 0.8 (9.4–13.2)	3.9 ± 0.5 (2.8–4.7)	Pointed

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

^y Colony characteristics: White, lacking = white mycelia lacked conidial masses and White, with = white to gray mycelia with mass conidial masses.

^z Shape at ends.

Fig. 7. Colony growth rate of *Colletotrichum* spp. from strawberry under different temperatures. Isolates of *Colletotrichum murrayae* (five isolates), *C. gloeosporioides* (five isolates), *C. fruticola* (four isolates), *C. aenigma* (four isolates), and *C. nymphaeae* (three isolates) were cultured on potato dextrose agar in the dark at different temperatures for 4 days.

Table 4. Minimum, optimum, and maximum growth temperatures of *Colletotrichum* spp.

Species	Temperature (°C) ^a		
	Minimum	Optimum	Maximum
<i>Colletotrichum murrayae</i>	10.0 a	28.0 a	38.8 b
<i>C. gloeosporioides</i>	10.0 a	28.0 a	40.0 a
<i>C. fruticola</i>	10.0 a	28.4 a	36.0 c
<i>C. aenigma</i>	10.0 a	28.0 a	36.0 c
<i>C. nymphaeae</i>	10.0 a	28.0 a	34.0 d

^a Mean values with the same letters are not significantly different ($P \leq 0.05$). Isolates of *C. murrayae* (five isolates), *C. gloeosporioides* (five isolates), *C. fruticola* (four isolates), *C. aenigma* (four isolates), and *C. nymphaeae* (three isolates) were cultured on potato dextrose agar in the dark at different temperature for 4 days.

Table 5. Pathogenicity of *Colletotrichum* isolates on strawberry^x

Species	Disease index ^y		Mortality (%) ^z
	Foliar	Petiole	
<i>Colletotrichum murrayae</i>	70.0 ab	38.6 ab	55.6 b
<i>C. gloeosporioides</i>	57.7 ab	4.7 c	5.6 d
<i>C. fruticola</i>	78.5 a	42.4 a	77.8 a
<i>C. aenigma</i>	46.7 b	8.6 bc	16.7 c
<i>C. nymphaeae</i>	53.1 b	3.5 c	11.1 cd
LSD	24.8	32.0	9.0

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

^y Disease index was evaluated at 20 days after inoculation.

^z Percentage of plant mortality were evaluated at 30 days after inoculation. Mean percent mortality of strawberry with conidial suspension of *Colletotrichum* spp. by spray methods.

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