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ORIGINAL PAPER

Walnut anthracnose caused by *Colletotrichum siamense* in China

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Abstract The genus *Colletotrichum* includes a number of important plant pathogens, which cause anthracnose diseases on a broad range of hosts in the world. In recent years, walnut has been severely damaged by anthracnose disease in China with significant yield losses. Thus, it is necessary to verify the etiology of anthracnose on walnut using both morphological and molecular approaches. In 2014, walnut fruits with anthracnose symptoms were collected from five walnut orchards in Shandong Province, China, and 24 isolates were isolated. Among them, six similar single-spore isolates obtained were used for pathogenicity testing of walnut anthracnose. Acervuli were brown, circular and the average size was 50.4–101.8 μm. Conidiophores were hyaline, septate, not branched or branching at the base, conidiogenous cells were enteroblastic, phialidic, hyaline, cylindrical, ampulliform. Conidia were single celled, smooth-walled with a large guttule, colorless, fusiform to cylindrical, and had obtuse to slightly rounded ends. The size of conidia was (11.6-)13–15(−16.2) × (4.1-)4.6–5.6(−6) μm. Appressoria were brown, ovoid to ellipsoid or slightly irregularly to irregularly shaped, and the average size was 6.8–9 × 5.1–6.5 μm. Pathogenicity of the isolates to fruits and leaves were compared, and genes from all six isolates were sequenced. The isolates were identified as *C. siamense* based on four-gene phylogenetic analyses (ribosomal DNA-ITS, GAPDH, ACT and CHS-1) and morphological as well as cultural characters. This is the first report of *C. siamense* as a causal agent of anthracnose of walnut in China.

Keywords Coelomycete · *Juglans* · Multi-gene phylogeny · Sequence · Species complex

Walnut (*Juglans regia* L.) is a valuable woody nut and oil tree planted all over the world. Because of various nutrients in the nuts, walnut is hailed as a super food in the twenty-first century (Smith 2013). Walnut wood is used for high-end furniture and as an industrial material (Voulgaridis and Vassiliou 2004; Vassilioul and Aidinidis 2007). Walnut plantations can bring significant economic, social, and ecological benefits (Nie et al. 2016). Hence, in recent years, the walnut plantations have increased rapidly in China (Pan and Zhou 2012). Based on FAO statistics, the annual growth rates in walnut production area, production and yield were 13.5%, 21.3%, and 6.9%, respectively, from 2006 to 2013. The total production area was 425, 000 ha and the production was 1700, 000 tons in China (http://faostat3.fao.org/browse/Q/QC/E). Due to early-fruiting walnut varieties ('Xiangling', a major variety), tree height control pruning, dense planting, and rainy summers, walnut production has often been limited by walnut anthracnose and the yield losses observed were up to 50% (Wang et al. 2016).

The genus *Colletotrichum* Corda is one of the most important and destructive plant pathogens worldwide. Many crops of cereals, fruits, vegetables and ornamentals were affected by *Colletotrichum* spp. (Cannon et al. 2012; Damm et al. 2010; Honger et al. 2016) and the yield and quality were seriously reduced. From the perspective of scientific and economic

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significance, *Colletotrichum* was considered as the eighth most important group of phytopathogenic fungi worldwide (Dean et al. 2012). Several species of *Colletotrichum* have been reported on *Juglans regia*: *C. acutatum* from Australia (Simmonds 1966); *C. gloeosporioides* sense lato (in two cases reported as *Glomerella cingulata*) from China (reported as *C. glucocorticoids* = *Gloeosporium rufomaculans* (Berk.) Thüm.) (Chen 2003), Korea (Cho and Shin 2004), Japan (Kobayashi 2007), New Zealand (Gadgil et al. 2005; Pennycook 1989), South Africa (Crous et al. 2000; Gorter 1977); *C. fioriniae* (Zhu et al. 2015) from China; and *Colletotrichum* sp. from Mexico (Alvarez 1976).

It is generally accepted that *C. gloeosporioides* is the pathogen of walnut anthracnose in China (Qu et al. 2011; Wang et al. 2016), but it is not clear whether other species of *Colletotrichum* can cause walnut anthracnose. *C. fioriniae* was shown to cause walnut leaf spot disease in China and the symptoms were similar to anthracnose (Zhu et al. 2015). It is necessary to determine whether other species of *Colletotrichum* are responsible for walnut anthracnose. In this paper, the isolates causing walnut anthracnose in China were characterized based on multilocus phylogenetic analysis and morphological characteristics.

Materials and methods

Plant materials, pathogen isolation and purification In late July of 2014, 62 walnut fruits ('Xiangling') with anthracnose symptoms from five orchards in Shandong province, China were collected. Orchard 1 is located in Laodong village, Zhangfang town, Shanghe county, Jinan city (37°19′46.8″ N, 121°05′46.6″ E); Orchard 2: Laodou village, Zhangfang town, Shanghe county, Jinan city (37°19′26.6″ N, 121°05′ 58.7″ E); Orchard 3: Xiaoliu village, Zhangfang town, Shanghe county, Jinan city (37°20′21.2″ N, 121°03′30.1″ E); Orchard 4: North Gaoer village, Zhonggong town, Licheng district, Jinan city (36°25′22.1″ N, 117°02′9.4″ E); Orchard 5: South Guozi village, Yazi town, Rushan city (37°01′48.6″ N, 121°12′58.2″ E). The walnut anthracnose disease was severe in the five orchards.

To isolate the pathogenic fungus, the walnut fruits were surface sterilized with 70% ethanol. Then, the margins of the lesions on fruits were cut into 3 to 4 mm^2 segments, sterilized with 1% sodium hypochlorite for 60 s, 70% ethanol for 60 s, and rinsed three times in sterile distilled water. Finally, the segments were placed on potato dextrose agar (PDA, 1.5%, Difco-BD Diagnostics, Sparks, MD, USA), 5 segments per plate. The plates were incubated at 28 °C for 7 days. Hyphae were transferred to PDA plates for sporulation. Pure cultures were obtained by monosporic isolation using serial dilution (Choi et al. 1999; Than et al. 2008b) and stored at 4 °C on PDA slants. The isolates were deposited into the China Forestry Culture Collection Center for preservation (Table 1). Monosporic cultures were transferred to new PDA plates for extracting DNA, observing morpho-cultural characters and conducting pathogenicity tests.

Morphological analysis Colony characters on PDA were observed after 5 days at 28 °C. Colony diameter was measured daily for 5 days and the growth rate was calculated as mean daily growth (mm/day). The experiments were carried out three times and each treatment had four replicates. The color of colonies, conidia and appressoria, conidial masses and zonation were also documented. Conidial size was measured after 10 days based on previously described methods (Weir et al. 2012). Appressoria were induced to develop on the surface of hydrophobic glass plates in vitro. A drop of sterile water was used to prepare a wet mount; acervuli were gently crushed and a few drops of cedar oil were added to reduce desiccation. Acervuli, conidia, appressoria, conidiophores and conidiogenous cells were observed under a compound microscope (Nikon Eclipse 50i, Japan), photographed using a high resolution QImaging camera system (QImaging, Canada), and measured using cellSens standard software, respectively.

DNA extraction, amplification, sequencing, and phylogenetic analyses Fungal genomic DNA was extracted using a previously described method (Freeman et al. 1996). Mycelia were ground to a fine powder in liquid nitrogen, then dissolved in cetyltrimethylammonium bromide (CTAB) buffer [2% *w/v* CTAB, 1.42 M NaCl, 20 mM EDTA, 100 mM Tris HCl pH 8.0, 0.2% (*w/v*) beta-mercaptoethanol], and incubated at 65 °C for 30 min. Following a phenol/chloroform extraction, the genomic DNA was precipitated by isopropanol before dissolving in Tris-EDTA (TE) buffer (10 mM Tris-HCl, 1 mM EDTA pH 8.0). The extracted DNA was subjected to PCR amplification of 4 genes including the ribosomal internal transcribed spacer (ITS), actin (ACT), glyceraldehyde-3phosphate dehydrogenase (GAPDH) and chitin synthase (CHS-1). Four genes were amplified: ribosomal DNA region using universal primers ITS1 and ITS4 for ITS (White et al. 1990; Gardes and Bruns 1993); the gene coding for GAPDH using primers GDF-1 and GDR1 (Guerber et al. 2003); CHS-1 gene using primers CHS-79F and CHSI-354R (Carbone and Kohn 1999) and Actin gene using primers ACT-512F and ACT-783R (Carbone and Kohn 1999).

The PCR procedure was carried out as follows: 12.5 μL Taq DNA polymerase mix, 1 μL of each primer, 2 μL genomic DNA, 8.5 μL ddH_2O, and the total volume was 25 μL. The reaction consisted of 36 cycles including initial denaturizing (pre-denaturating) at 95 °C for 5 min, 30 s denaturing at 94 °C. The optimum annealing temperature for each gene was; ACT: 59 °C, ITS regions: 58 °C, CHS-1 and GAPDH: 56 °C for 30 s. The extension temperature was 72 °C for 1.5 min. Final extension for 7 min at 72 °C was

Table 1 Strains of *Colletotrichum* studied in this paper with details about host/substrate and location, and GenBank accessions of the sequences generated

Species	Cultrue accession #	Host	Country	GenBank accessions			
				ITS	GAPDH	ACT	CHS-1
C. alatae	**CBS 304.67**	***Dioscorea alata***	**India**	**JX010190**	**JX009990**	**JX009471**	**JX009837**
	ICMP18122	*Dioscorea alata*	Nigeria	JX010191	JX010011	JX009470	JX009846
C. aotearoa	**ICMP 18537**	***Coprosma* sp.**	**New Zealand**	**JX010205**	**JX010005**	**JX009564**	**JX009853**
	ICMP 18530	*Vitex lucens*	New Zealand	JX010268	JX009911	JX009521	JX009884
C. boninense	**MAFF 305972**	***Crinum asiaticum* var.*sinicum***	**Japan**	**JX010292**	**JX009905**	**JX009583**	**JX009827**
C. fructicola	**ICMP18581**	***Coffea arabica***	**Thailand**	**JX010165**	**JX010033**	**FJ907426**	**JX009866**
	ICMP 18645	*Theobroma cacao*	Panama	JX010172	JX009992	JX009543	JX009873
	CBS 125397(T)	*Tetragastris panamensis*	Panama	JX010173	JX010032	JX009581	JX009874
C. gloeosporioides	**IMI 356878**	***Citrus sinensis***	**Italy**	**JX010152**	**JX010056**	**JX009531**	**JX009818**
	CBS 273.51(T)	*Citrus limon*	Italy	JX010148	JX010054	JX009558	JX009903
	ICMP 18678	*Pueraria lobata*	USA	JX010150	JX010013	JX009502	JX009790
C. horii	**NBRC7478**	***Diospyros kaki***	**Japan**	**GQ329690**	**GQ329681**	**JX009438**	**JX009752**
	ICMP12942	*Diospyros kaki*	New Zealand	GQ329687	GQ329685	JX009533	JX009748
C. kahawae subp. *Ciggaro*	**ICMP18539**	***Olea europaea***	**Australia**	**JX010230**	**JX009966**	**JX009523**	**JX009800**
	CBS237.49(T)	*Hypericum perforatum*	Germany	JX010238	JX010042	JX009450	JX009840
C. kahawae subp. *Kahawae*	**ICMP17816**	***Coffea arabica***	**Kenya**	**JX010231**	**JX010012**	**JX009452**	**JX009813**
C. nupharicola	**CBS470.96**	***Nuphar lutea* subp. *Polysepala***	**USA**	**JX010187**	**JX009972**	**JX009437**	**JX009835**
	ICMP17940	*Nymphaea ordorata*	USA	JX010188	JX010031	JX009582	JX009836
	ICMP 17938	*Nuphar lutea* subsp. *polysepala*	USA	JX010189	JX009936	JX009486	JX009834
C. psidii	**CBS 145.29**	***Psidium* sp.**	**Italy**	**JX010219**	**JX009967**	**JX009515**	**JX009901**
C. queenslandicum	**ICMP 1778**	***Carica papaya***	**Australia**	**JX010276**	**JX009934**	**JX009447**	**JX009899**
	ICMP 1780	*Carica* sp.	Australia	JX010186	JX010010	JX009504	JX009900
C. salsolae	**ICMP 19051**	***Salsola tragus***	**Hungary**	**JX010242**	**JX009916**	**JX009562**	**JX009863**
	ICMP 18693	*Glycine max* (inoculated)	Hungary	JX010241	JX009917	JX009559	JX009791
C.siamense	**ICMP18578**	***Coffea arabica***	**Thailand**	**JX010171**	**JX009924**	**FJ907423**	**JX009865**
	ICMP 18574	*Pistacia vera*	Australia	JX010270	JX010002	JX009535	JX009798
	ICMP 12565	*Persea americana*	Australia	JX010249	JX009937	JX009571	JX009760
	ICMP 18118	*Commelina* sp.	Nigeria	JX010163	JX009941	JX009505	JX009843
	ICMP 17795	*Malus domestica*	USA	JX010162	JX010051	JX009506	JX009805
	SH-8* = CFCC 51552	*Juglans regia*	China	KX913951	KX913957	KX913948	KX913954
	SH-9* = CFCC 51940	*Juglans regia*	China	KY242352	KY242347	KY242336	KY242342
	SH-10* = CFCC 51941	*Juglans regia*	China	KY242351	KY242346	KY242338	KY242343
	SH-11* = CFCC 51942	*Juglans regia*	China	KY242353	KY242348	KY242339	KY242341
	WH-19* = CFCC 51943	*Juglans regia*	China	KY242355	KY242350	KY242340	KY242344
	GE-20* = CFCC 51944	*Juglans regia*	China	KY242354	KY242349	KY242337	KY242345
C. theobromicola	**CBS 124945**	***Theobroma cacao***	**Panama**	**JX010294**	**JX010006**	**JX009444**	**JX009869**
	CBS 142.31(T)	*Fragaria* × *ananassa*	USA	JX010286	JX010024	JX009516	JX009830
	MUCL 42294(T)	*Stylosanthes viscosa*	Australia	JX010289	JX009962	JX009575	JX009821

Table 1 (continued)

Species	Cultrue accession #	Host	Country	GenBank accessions			
				ITS	GAPDH	ACT	CHS-1
C. ti	**ICMP 4832**	***Cordyline* sp.**	**New Zealand**	**JX010269**	**JX009952**	**JX009520**	**JX009898**
	ICMP 5285	*Cordyline australis*	New Zealand	JX010267	JX009910	JX009553	JX009897
C. xanthorrhoeae	**BRIP 45094**	***Xanthorrhoea preissii***	**Australia**	**JX010261**	**JX009927**	**JX009478**	**JX009823**

Ex-type strains or authentic cultures are in bold. (T) = ex-type or authentic culture of synonymised taxon

* Strains collected in the present study

performed after the cycles ended. After PCR, all the PCR products were sent to Shanghai Personal Biotechnology Co., Ltd. for DNA sequencing. All sequences have been deposited to GenBank (Table 1).

The ITS, GAPDH, ACT and CHS-1 sequences were compared with sequences in Q-Bank (www.q-bank.eu) using Blast. The sequences of 35 *Colletotrichum* isolates were obtained from GenBank for phylogenetic analyses (Table 1), *C. boninense* (MAFF 305972, isolated from *Crinum asiaticum* var. *sinicum*) was used as an outgroup for comparison. Each gene sequence for isolates was aligned using Clustal W option in MEGA 7.0. Alignments were manually regulated to keep maximum alignment and maximum sequence similarity. Gaps were regarded as missing data. Four genes/region were combined according to'ITS-GAPDH-ACT-CHS-1'. Phylogenetic analysis was performed with the multiple sequence alignment of four genes using MEGA 7.0 software (Kumar et al. 2016).

A multilocus phylogenetic tree was generated using the maximum parsimony method or the maximum likelihood method by combining datasets and the tree was drawn to scale, with branch lengths measured in the number of substitutions per site. Percentage of bootstrap support for each node (calculated with 1, 000 replicates) (Felsenstein 1985), the consistency index (CI), retention index (RI), composite index (CI), and the highest log likelihood were used to evaluate the relative stability of the branches.

Pathogenicity and virulence in leaves and fruits The pathogenicity and virulence tests were conducted on both healthy fruits ('Xiangling', 1-mo old) and fully expanded leaves at the experimental station of the Research Center for the Prevention and Control of Invasive Forest Pests of Shandong Province. Six isolates: SH-8, SH-9, SH-10, SH-11, GE-20 and WH-19 were used. The hyphae of the isolates (5-day old) were cut into 5-mm diam plugs with a cork borer. Eighty-four healthy walnut leaves and 140 fruits were washed with running tap water for 60 s, surface disinfected with the 70% ethanol for 30 s, washed three times with sterile distilled water and wounded with a 0.5-mm diam red-hot needle. The leaf was inoculated with two hyphal plugs and the fruit with one hyphal plug. A blank agar plug was placed on pin-wounded leaves and fruits as control. Six isolates were used to confirm pathogenicity, and each isolate was inoculated to 20 fruits and 12 leaves, respectively; an additional 20 fruits and 12 leaves were treated with blank agar plugs as controls. The experiment was repeated three times. All the inoculated leaves and fruits were placed into sterile tissue culture bottles containing two layers of wet paper towels to maintain humidity and held at 28 °C under a 12 h light/dark cycle. The fungus was re-isolated from the lesions according to the aforementioned methods. In order to complete Koch's postulates, characteristics of colony, acervuli, conidia, appressoria, conidiophores and conidiogenous cells and daily growth rate were compared with those inoculated isolates. The lesion areas were measured at the 10th day after inoculation to evaluate the virulence. The virulence of the isolates was determined by one-way ANOVA and means were compared by the LSD test at the 1% significance level using DPS software 7.05.

Results

Description of the symptoms in the field The anthracnose disease on walnut leaves and fruits was observed in the field. In the early stages, there were some sub-circular or irregular shaped spots that occurred on the fruits. These spots were water soaked, sunken, turned brown to black gradually, enlarged and often amalgamated into large necrotic areas. Acervuli developed on these lesions in concentric rings. The older spots at the center became blackish and oozed gelatinous pink conidial masses. Finally, the fruits did not develop normally and many dropped prematurely (Fig. 1). The spots on leaves were sub-circular or irregular shaped, water soaked, brown or black and expanded along the vein, and formed long strip lesions. Later, the central lesions became necrotic and perforated, leading to early leaf abscission. Severe walnut anthracnose disease occurred in late July of 2014 and the disease indices were 57.6 and 47.8 in orchard 1 and orchard 5, respectively.

Fig. 1 Disease symptom on fruits of walnuts in field

Isolation and identification of the pathogen of walnut anthracnose Twenty-four isolates were obtained from the diseased fruits, and six isolates were used in this paper. All the diseased fruits and leaves developed anthracnose symptoms within 7 days (Fig. 2a–m), while the non-inoculated controls did not show any symptoms within 7 days (Fig. 2g, o). Lesions caused by infections on inoculated fruits and leaves were sub-circular or irregular shaped, water soaked, sunken, and turned brown to black gradually. The disease spot enlarged horizontally to a sub-circular or irregular shape and expanded vertically into the immature endocarp (nut shell) (Fig. 2n). The black lesions enlarged and often amalgamated into large necrotic areas. The older spots at the center became blackish and oozed gelatinous pink conidial masses. These symptoms were similar to the disease observed in the field. The fungus was re-isolated from the lesions according to the aforementioned methods. The morphology, including colony, acervuli, conidia, appressoria, conidiophores and conidiogenous cells of these re-isolates were similar to the inoculated isolates, and the same fungus was re-isolated from the lesions. Thus, the results confirmed that the isolates caused walnut anthracnose.

Morphological characteristics Colonies on PDA were initially white and became pale brown at the center with age, and produced strong black pigmentation near the center on the reverse. Aerial mycelium was cottony, dense, white. Acervuli developed visible orange to dark orange conidial masses at the inoculum point (Figs. 3a–f and 4m). Acervuli were present in aged cultures, brown, circular to subcircular and the average size was 50.4–101.8 μm ($\bar{x}$ = 76.1 ± 25.7, n = 55) (Fig. 4h). Setae were absent. Conidiophores were hyaline, septate, not branched or branching at the base. Conidiogenous cells were enteroblastic, phialidic, hyaline, cylindrical, and ampulliform (Fig. 4j–k). Conidia were single celled, smooth with a large guttula, colorless, fusiform to cylindrical, and had obtuse to slightly rounded ends. The size was (11.6-)13–15(−16.2) × (4.1-)4.6–5.6(−6) μm ($\bar{x}$ = 14.0 ± 1 × 5.1 ± 0.5, n = 45) (Fig. 4g). Conidia germinated and developed appressoria on hydrophobic surfaces in vitro. Most conidia formed one appressorium. Germ tubes in

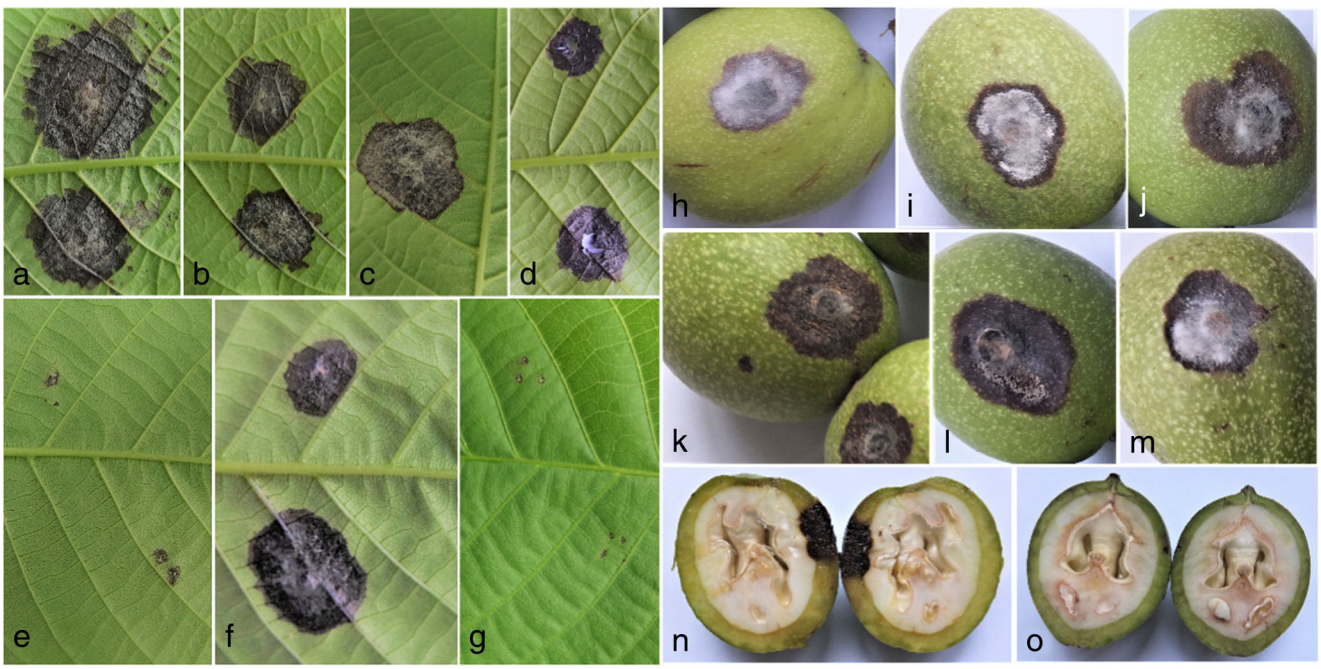

Fig. 2 Pathogenicity test; (**a**) – (**f**) necrotic lesion on walnut leaves inoculated with 6 isolates (SH-8, SH-9, SH-10, SH-11, WH-19, GE-20); (**g**) control with blank agar plug (**h**) – (**m**) necrotic lesions on walnut fruits inoculated with 6 isolates (SH-8, SH-9, SH-10, SH-11, WH-19, GE-20); (**n**) vertical section of walnut fruit inoculated with isolate SH-8; (**o**) vertical section of walnut fruit inoculated with blank agar plug

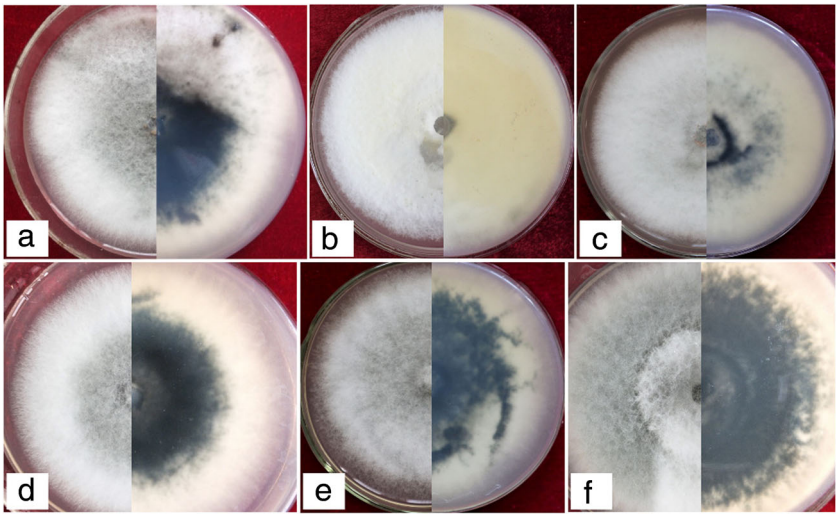

Fig. 3 Cultural characters of *C. siamense* on PDA, right: top view of culture; left: reverse view of culture. (**a**) – (**f**): isolates SH-8, SH-9, SH-10, SH-11, WH-19 and GE-20

different lengths grew from the conidial ends and produced pleurogenous or acrogenous, unicelluar appressoria. Appressoria were pale brown to brown, ovoid to ellipsoid or slightly irregularly to irregularly shaped and the average size was 6.8–9 × 5.1–6.5 μm ($\bar{x}$ = 7.9 ± 1.1 × 5.8 ± 0.7, n = 45) (Fig. 4a–f). Consequently, the isolates were determined to be *C. siamense* according to morphological characters (Prihastuti et al. 2009).

The growth rate of the colony in culture is an important distinctive feature. On PDA, the daily growth rates of isolate SH-8, SH-9, SH-10, SH-11, GE-20 and WH-19 were 11.0, 11.8, 19.8, 14.8, 13.9, 18.1 mm/d, respectively, and

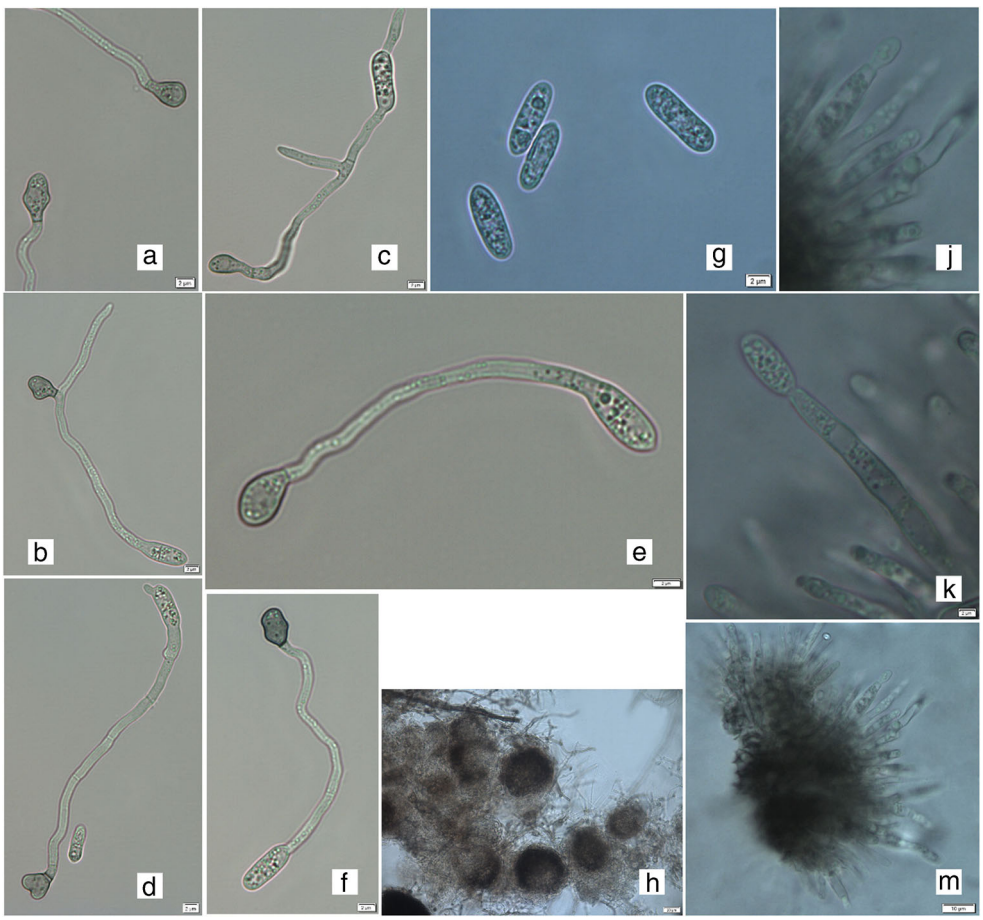

Fig. 4 *C. siamense* (**a**) – (**f**) appressoria; (**g**) conidia; (**j**) – (**k**) conidiophores and conidiogenous cells, Bars = 2 μm; (**h**) acervuli, Bars = 20 μm; (**m**) acervulus, conidiophores, and conidiogenous cells, Bars = 10 μm

significantly different. The daily growth was SH-10, WH-19 > SH-11 > GE-20 > SH-9 > SH-8 in a descending order.

Phylogenetic analysis DNA of 6 isolates was amplified and determined for the ITS, GAPDH, ACT and CHS-1 genes, respectively. The strains/taxa of *Colletotrichum* studied are listed in Table 1.

The ITS, GAPDH, ACT and CHS-1 sequences were compared with sequences in Q-Bank using Blast, respectively. The similarity of ITS sequences with the corresponding sequence of *C. siamense* ex-holotype culture **ICMP 18578** (JX010171) and *C. siamense* isolate ICMP18574 (JX010270) and were 99.1% and 100%, respectively. The GAPDH sequence showed 99.5% and 100% similarity with *C. siamense* ex-holotype culture **ICMP 18578** (JX009924) and *C. siamense* isolate ICMP18574 (JX010002). The similarity of the ACT sequence with *C. siamense* ex-holotype culture **ICMP 18578** (FJ907423) and *C. siamense* isolate ICMP18574 (JX009535) were all above 99%. The CHS-1 sequence showed 100% similarity with *C. siamense* isolate ICMP18574 (JX009798). The four sets of sequence data did not show major conflicts in phylogenetic trees, and the genes were combined. The concatenated sequences of four housekeeping genes (1, 048 bp) included ITS, GAPDH, ACT and CHS-1. The aligned sequence was above 99% homologous to *C. siamense* ex-holotype culture **ICMP 18578** and *C. siamense* isolate ICMP18574. In the maximum parsimony phylogenetic tree, the tree length is 391, the consistency index is 0.67, the retention index is 0.86, and the composite index is 0.65 (0.57) for all sites and parsimony-informative sites (in parentheses). The isolates studied are monophyletic with *C. siamense* with 80% bootstrap support (Fig. 5). In the maximum likelihood phylogenetic tree, the tree with the highest log likelihood is −3592.39. Isolates were in the same cluster with *C. siamense* with 94% bootstrap support (Fig. 6).

The morphological characteristics as well as phylogenetic analysis using sequences of four genes indicated that isolates are *C. siamense*.

Pathogenicity and virulence in leaves and fruits All six isolates were pathogenic to walnut leaves and fruits. The diameters of lesions on fruits were from 1.4 ± 0.4 cm to 1.8 ± 0.3 cm within 10 days (Fig. 7) and there were no significant differences ($P < 0.01$) among the six isolates on fruits. Based on the lesions on leaves, however, isolate SH-8 was the most virulent, and the lesions were 1.6 ± 0.4 cm. There was a significant difference in virulence among the other isolates ($P < 0.01$). The isolate SH-9 and SH-11 were the pathogenic strains with a highest virulence, and the lesions were 1.1 ± 0.6 cm, and 1.0 ± 0. 3 cm, respectively. Isolates SH-10 and GE-20 were in the same group, and the lengths were from 0.5 ± 0.4 cm to 0.7 ± 0.2 cm. There were no significant differences in lesion sizes ($P < 0.01$) between SH-10 and GH-20. Isolate WH-19 was the weakest in pathogenicity to walnut leaves, as lesion size was 0.2 ± 0.1 cm (Fig. 7).

Discussion

The genus *Colletotrichum* was observed by Tode in 1790, and then legitimately described by Corda (1831). Up to the present, significant progress has been made on the taxonomy of the *Colletotrichum* species (Weir et al. 2012; Damm et al. 2012a, b; Cannon et al. 2012), but the controversies in taxonomic relationships within the *Colletotrichum* are still worth further study. *Colletotrichum* has abundant genetic diversity among populations, and retains wide variation on morphological characters, cultural properties, virulence and genetic backgrounds (Zakaria et al. 2015; Santos et al. 2015; Mota et al. 2016). There are few nuances of the characters among some similar species, and more than one species can affect the same plant species. On the basis of morphology, host range and ITS data, most species of *Colletotrichum* were identified. To distinguish the species from each other, morphology and multi-regions/genes phylogenies have to be applied, and many *Colletotrichum* taxa have been successfully delineated (Yang et al. 2009; Weir et al. 2012; Than et al. 2008a). In this work, the isolates obtained from diseased walnut fruits were identified as *C. siamense* according to morphologic characters, cultural properties and phylogenetic analysis using four genes. The pathogenicity tests further confirmed that *C. siamense* was the pathogen responsible for walnut anthracnose.

C. siamense was initially described as a pathogen related to anthracnose of coffee berries in Thailand (Prihastuti et al. 2009). In addition, *C. siamense* has been found to cause anthracnose on *Carica papaya* L. in South Africa, *Dioscorea rotundata* Poir. in Nigeria, *Vitis vinifera* L., *Malus domestica* B. (Weir et al. 2012), *Amygdalus persica* L., *Vaccinium* spp. (Hu et al. 2015b) in the USA., *Bauhinia forficata* subsp. *pruinosa* in Argentina (Larran et al. 2015), *Fragaria* × *ananassa* Duch., *Mangifera indica* L. in Brazil (Vieira et al. 2014; Capobiango et al. 2016), *Annona muricata* L. in Colombia (Álvarez et al. 2014), *Ficus racemosa* L. (Weir et al. 2012; Hu et al. 2015a), *Zizyphus mauritiana* Lam., *Lilium* L., *Hosta* (Lam.) Aschers (Prihastuti et al. 2009; Phoulivong et al. 2012) in Thailand. *Artocarpus heterophyllus* Lam., *Eriobotrya japonica* Thumb., *Ficus carica* L., *Mentha* sp., *Persea americana* Mill., *Piper nigrum* L., *Pistacia vera* L., *Rosmarinus officinalis* L., *Theobroma cacao* L. in Australia (James et al. 2014), *Citrus reticulata* Blanco cv. Shiyue Ju (Cheng et al. 2013), *Camellia oleifera* Abel. (Li et al. 2015), *Corchorus capsularis* L. (Niu et al. 2016), *Cinnamomum kotoense* Kanehira & Sasaki (Zhou et al. 2016), *Hymenocallis americana* Roem., *Orchidaceae*, *Averrhoa carambola* L. (Yang et al. 2009, 2013, 2014), *Populus tomentosa* Carr (Li et al. 2012), *Sarcandra glabra*

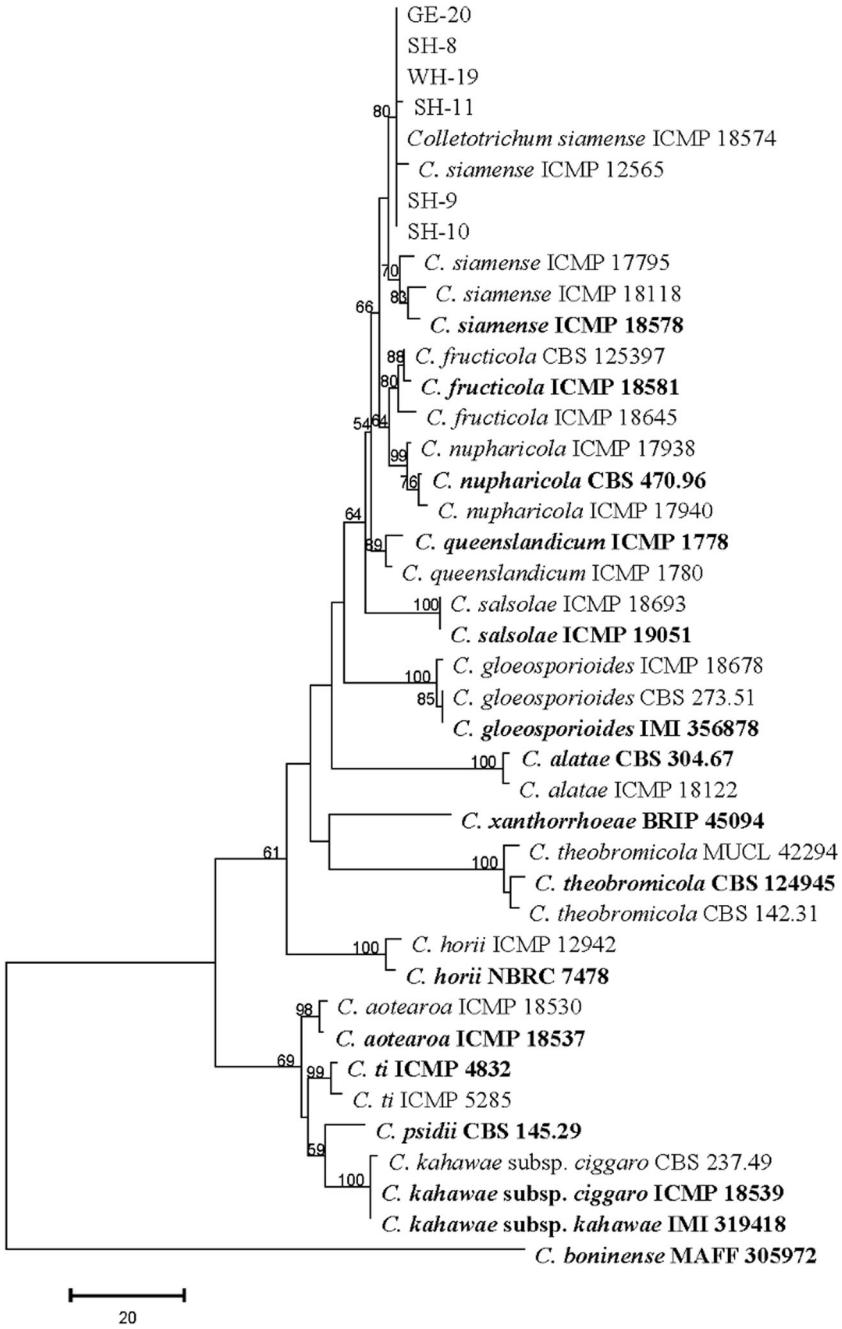

Fig. 5 Phylogenetic tree of isolates of walnut anthracnose with allied taxa calculated with sequence data of concatenated ITS, GPADH, ACT and CHS-1 using Maximum Parsimony method (1000 bootstrap replicates; bootstrap values indicated at nodes). *C. boninense* MAFF 305972 represents the out group. The scale bar indicates the number of expected changes per site

(Thunb.) Nakai (Ye et al. 2016) in China, *Jasminium sambac* L. (Wikee et al. 2011) in Vietnam, *Cocos nucifera* L., *Saraca* L., *Dieffenbachia*, *Cassia*, *Psidium*, *Bauhinia*, *Allium cepa* (Chowdappa et al. 2015), *Azadirachta india* Neem., *Punica* in India (Sharma et al. 2015), *Mandevilla* in Japan (Watanabe et al. 2016).

In conclusion, *C. siamense* is biologically and geographically diverse with a wide host range. In this paper, *C. siamense* is reported for the first time as a pathogen causing walnut anthracnose. Previously, Qu et al. (2011) obtained 17 isolates from walnut orchards in Shandong province, and all isolates were capable of causing walnut anthracnose. Conidia were single-celled with a large guttula, colorless, cylindrical, and the size was 10.4–15.0 × 4.6–6.4 μm. Appressoria were subcircular, trilobed, irregularly shaped, light brown or dark brown, and the average size was 10.2 × 7.3 μm (Qu et al. 2011). All isolates were identified as *C. gloeosporioides* on the basis of the morphological characters and ITS data. The result may be inconclusive due to the fact that *C. gloeosporioides* is a species complex containing 22 species plus one subspecies, which needs a multi-gene approach to accurately delineate species. Wang et al. (2016) obtained an

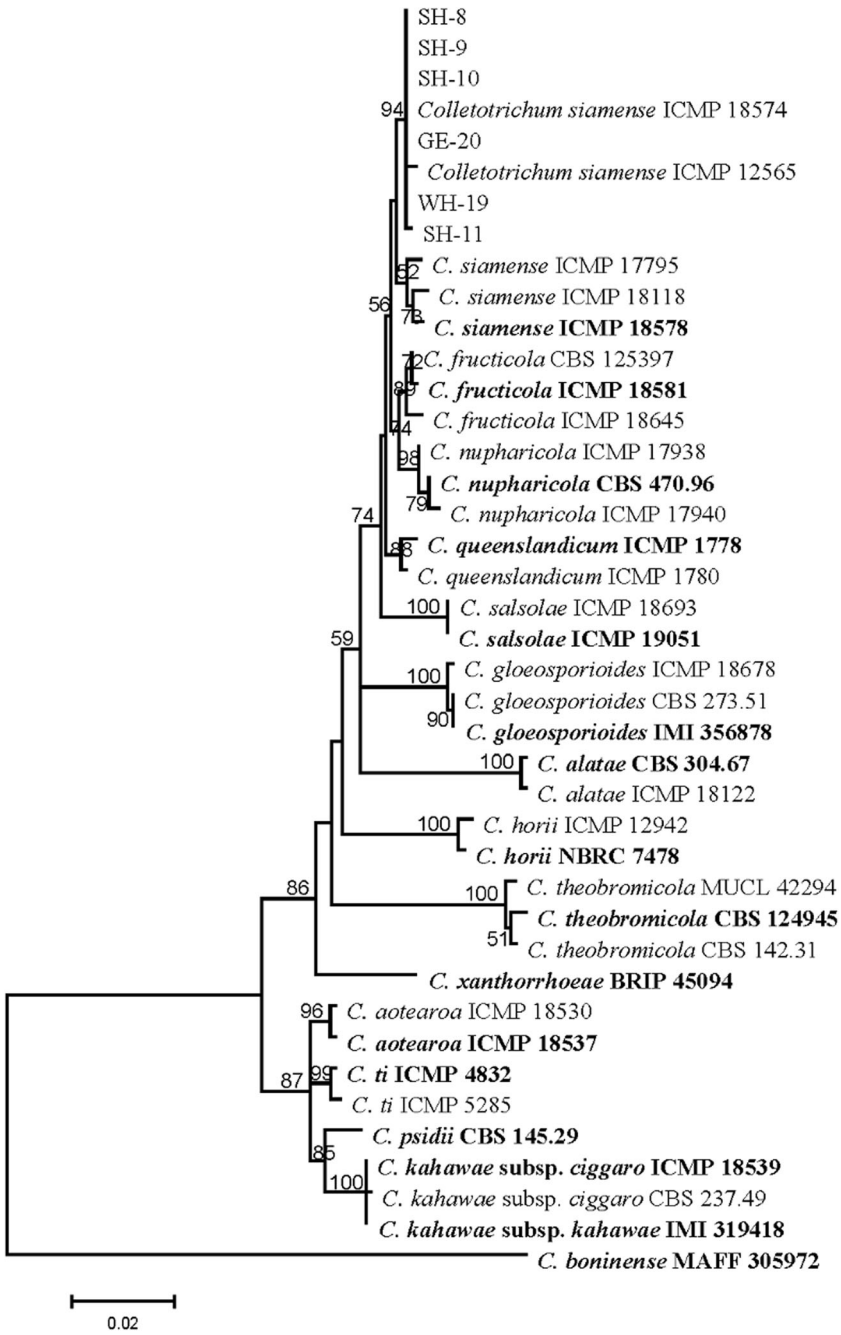

Fig. 6 Phylogenetic tree of isolates of walnut anthracnose with allied taxa calculated with sequence data of concatenated ITS, GPADH, ACT and CHS-1 using maximum likelihood method (1000 bootstrap replicates; bootstrap values indicated at nodes, the highest log likelihood = −3592.38). *C. boninense* MAFF 305972 represents the out group. The scale bar indicates the number of expected changes per site

isolate TS-09 from walnut fruits with anthracnose symptoms in Taian city. Based on the morphological characteristics and phylogenetic analysis using sequences of 4 genes (ACT, ITS, GAPDH and beta-tub2), isolate TS-09 was classified as *C. gloeosporioides*. Colonies of TS-09 on PDA were initially gray white and became dark gray at the center with age. Aerial mycelium was cottony. Conidia were single celled, colorless, cylindrical, and the size was 10.2 × 3.4 μm. Appressoria were pale brown to brown, ovoid. In comparison with the 6 isolates of *C. siamense* from the current study, morphological characteristics and cultural characters of isolate TS-09 were slightly different. Conidial shape and size are important distinctive features. Conidial shape of the 6 isolates of *C. siamense* was similar to that of the isolate TS-09, while the conidial size of *C. siamense* was greater than that of isolate TS-09. The color of the colonies on PDA was slightly different. The colonies of the *C. siamense* isolates were initially white and turned into pale brown at the center with age, while the colonies of isolate TS-09 were pale gray initially and turned into dark gray at the center with age.

In China, *C. siamense*, *C. gloeosporioides* and *C. fioriniae* (Zhu et al. 2015) could all cause walnut anthracnose. Further

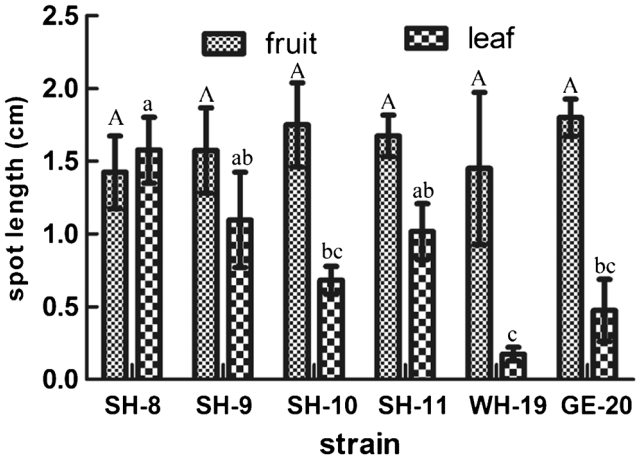

Fig. 7 Mean fruits and leaves spot length (cm) caused by *C. siamense* isolates associated with walnut anthracnose. Bars above columns are the standard errors. Capital letters and lowercase letters were used for the spot length on fruits and leaves, respectively. Columns with same letter means no significant difference according to LSD test ($P < 0.01$)

studies will be conducted to determine whether other species of *Colletotrichum* are also responsible for walnut anthracnose.

Pathogenicity testing using six isolates of *C. siamense* showed that all isolates were highly pathogenic to walnut fruits, and there was no significant difference in virulence. While the virulence to walnut leaves was significantly different. The isolate SH-8 was the most virulent in all isolates, while isolate WH-19 could hardly infect walnut leaves. The isolates' virulence to walnut fruits was not consistent with that to leaves. This result is in agreement with the conclusion of Zhao et al. (2013). Additional research should be conducted to confirm virulence based on natural infections.

C. siamense has recently been proposed as a new species from the *C. gloeosporioides* species complex (Prihastuti et al. 2009), and its biology, ecology, epidemiology, and population genetic structure are poorly understood. The epidemiology and control of walnut anthracnose disease caused by *C. siamense* will be conducted in the future.

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