Biological and Molecular Characterization of Five *Phomopsis* Species Associated with Pear Shoot Canker in China

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Abstract

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In recent years, a widespread canker disease that infects the branches of pear trees has been observed in many provinces in China; it kills the branches and results in high losses in fruit production. Symptomatic branches were collected for etiological isolation from 11 varieties of three pear species and from *Malus pumila*. Samples were collected from six provinces in China. In total, 143 *Phomopsis* isolates were obtained from 181 samples and these were identified as belonging to five species: *Phomopsis fukushii* ($n = 69$ isolates), *Diaporthe eres* ($n = 31$), *P. amygdali* ($n = 22$), *P. longicolla* ($n = 13$), and *D. neotheicola* ($n = 8$). Pathogenicity tests showed that only the first three species induced lesions on non-wounded branches of *Pyrus pyrifolia* var. Cuiguan. All the fungal species induced branch cankers following wound inoculations, and tests with additional pear varieties showed significantly higher virulence levels for the first three species than the latter two. A host range evaluation suggested

that the five species could infect most fruit trees belonging to the *Rosaceae* family as well as some non-Rosaceous species. Virulence varied depending on the species of both host and pathogen. Isolates of *Phomopsis amygdali* had significantly higher virulence in all the tested *Rosaceae* plants. Correlations among the host, pathogen, and sampling regions were noted, and the morphology, growth rate, and sporulation of these species in varied media were also characterized. This study presents the first attempt to perform a broad survey and characterization of the *Phomopsis* spp. associated with the pear shoot cankers in China. This study shows that *D. eres* and *P. amygdali* are just as responsible for the pear shoot canker diseases as *P. fukushii*, and it expands the host and geographic ranges of the five species. This report provides useful information for understanding and improving management strategies for controlling this economically important disease.

In recent years, a canker disease that causes brown canker tissue around buds on the shoots, twigs, or large branches of pear trees has been widely observed in many provinces in China (e.g., Jiangxi, Jiangsu, Shandong, Hubei, Fujian, and Yunnan). Disease incidence can be as high as 20% in pear trees of the species *Pyrus pyrifolia* and *P. communis* and 5 to 10% in other pear species (Huang et al. 2014). This disease always kills the infected shoots or branches and the attached blossom and leaf buds, resulting in a blight of numerous branches and a large loss of fruit production. Furthermore, the disease also results in decay of the xylem tissues of the infected branches, allowing the branches to be easily broken by the wind, and it has even killed entire pear trees. It has become a serious problem that limits the growth of pear trees in many pear-cultivating areas in China.

Several fungal pathogens can reportedly infect pear shoots or branches, inducing canker, dieback, or the death of the infected organs; for example, fungi belonging to the genera *Valsa* (Sawamura et al. 1990), *Phomopsis* (Smit et al. 1996), and *Botryosphaeria* (Zhai et al. 2014). Furthermore, more than one species belonging to the same genus can induce the same disease. It has been difficult to make a definite diagnosis by depending only on the symptoms observed in the field or with a limited amount of samples. Until the present study, the identification of the canker pathogen was unconfirmed, although the disease was primarily known as the “pear shoot canker” induced by *Phomopsis* spp. because the observed symptoms were similar to

previous descriptions for *P. pyrifolia* in Japan (Nasu et al. 1987; Tanaka and Endo 1930).

China is an important pear-growing country and leads other countries in pear cultivation; therefore, the identification and prevention of this disease has economic significance. China is also a likely center of origin for the pear. It is one of two genetic diversity subcenters for this fruit (Rubtsov 1944; Vavilov 1951; Wu et al. 2013; Zeven and Zhukovsky 1975), and numerous varieties and many species are cultivated. Therefore, studying the pathogens and their diverse ways of infecting pear trees of different varieties and species in China should provide important information for understanding the pear shoot canker. A broad survey for and characterization of *Phomopsis* spp. that infect pear trees is still lacking in China. Whether the disease is induced by the *Phomopsis* fungus remains unknown and, if so, it is unclear which species is responsible for the disease in different host varieties or species in different provinces. Given the significance of identifying the disease and characterizing the responsible pathogens, we collected numerous samples from most provinces where the disease occurs in China and isolated and identified the pathogens responsible for the disease. This study provides useful information for understanding this economically important disease.

Materials and Methods

Field symptom survey, sample collection, and pathogen isolation.

Field symptom surveys were carried out in 17 orchards with random screening of 141 trees in a total in six provinces (Jiangxi, Jiangsu, Shandong, Hubei, Fujian, and Yunnan) in China from May 2013 to May 2014. Samples with canker symptoms were collected from the branches of *P. pyrifolia* (vars. Cuiyu, Cuiguan, Chuxialv, Huanghua, Minfu, and Qingxiang), *P. bretschneideri* (vars. Suli and Zaobaisu), *P. communis* (vars. Packham, J6, and J23), and *Malus pumila* (var. Fushi) in the surveyed orchards.

Infected tissues (4 to 5 mm²) were excised from the xylem or phloem under the canker lesions neighboring the asymptomatic regions after they were surface sterilized with 0.1% HgCl₂ for 1 min and 75% ethanol for 1 min, then rinsed twice with sterilized

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water and air dried. The excised tissues were placed on potato dextrose agar (PDA; 20% diced potato, 2% glucose, and 1.5% agar) petri plates and incubated at 25°C in the dark for 3 to 5 days until fungal colony formation. Each isolate was further purified by culturing a single conidium. In brief, an isolate was cultured on PDA at 25°C with a photoperiod of 14 h to induce sporulation. The conidia produced were rinsed with sterilized water and diluted to a low concentration. The conidial dilutions were spread on PDA to generate discrete colonies, and six colonies of each isolate were selected and stored in 25% glycerol at -70°C for later use.

DNA extraction, amplification, sequencing, and phylogenetic analysis. Fungal genomic DNA was extracted according to a previous description (Freeman et al. 1996). In brief, mycelia were ground into a fine powder in liquid nitrogen, dissolved in cetyltrimethylammonium bromide (CTAB) buffer (2.0% [wt/vol] CTAB, 1,420.0 mM NaCl, 20.0 mM EDTA, 100.0 mM Tris HCl [pH 8.0], and 0.2% [wt/vol] β -mercaptoethanol), incubated at 65°C for 30 min, combined with an equivalent volume of phenol/chloroform, and centrifuged at 12,000 rpm for 10 min to remove the precipitated segments. The genomic DNA was dissolved in Tris-EDTA buffer (10.0 mM Tris-HCl and 1.0 mM EDTA, pH 8.0) after being precipitated by isopropanol. The extracted DNA was subjected to a polymerase chain reaction (PCR) amplification of three regions, including the ribosomal internal transcribed spacer (ITS; including a partial 18S ribosomal RNA [rRNA] gene, ITS1, 5.8S rRNA gene, ITS2, and partial 28S rRNA gene), translational elongation factor subunit 1- α gene (EF1 α), and actin (ACT) by using the primer pairs ITS1/ITS4, ACT547F/ACT768R (Baumgartner et al. 2013), and EF1-728F/EF1-986R (Carbone and Kohn 1999). The PCR conditions for all the genomic regions were 2 min at 95°C; followed by 35 cycles of 95°C for 60 s, 55°C for 60 s, 72°C for 90 s; and then 10 min at 72°C in a PCR Thermal Cycler (Model PTC-100; MJ Research). The PCR products were then gel purified with an agarose gel DNA purification kit (Wuhan More Biotechnology Corporation) and commercially sequenced by Nanjing Jinsirui Biotechnology Co., Ltd.

The sequences were subjected to a homologous sequence search in the database of the National Center for Biotechnology Information by using the basic local alignment search tool (BLASTn) to provide an approximate identification of the isolates. Sequences for the available ex-type cultures were obtained from GenBank. Multiple sequence alignments were performed with DNAMAN (version 5.2.2; Lynnon Biosoft), and a phylogenetic tree was constructed by the neighbor-joining method using the 1,000 bootstrap replicates in MEGA 5.2 (Tamura et al. 2011).

Colony morphologies, conidial morphologies, and growth rate analysis. To characterize the morphology, a purified isolate was established from a 7-day-old mycelial plug (4 mm in diameter) placed in the center of 9-cm petri dishes containing PDA. Cultures were incubated at 25°C with a photoperiod of 14 h until pycnidium formation. The colony appearance was recorded daily. The released conidia were diluted to a low concentration and observed under an optical microscope (Nikon Eclipse E600 FN; Nikon); 50 conidia were measured and then photographed with the SPOT program (version 4.6; Diagnostic Instruments, Inc.). These measurements were made in triplicate on three separate plates of each isolate.

Growth rate was quantified by similarly establishing each isolate on PDA. Cultures were incubated in triplicate at 25°C in the dark. The colony diameters were recorded daily until 7 days had passed, and their average diameters were statistically analyzed. The growth rate of each isolate was calculated by dividing the average colony diameter measured at 6 days after inoculation by 6.

Sporulation properties and germination tests. Barleycorn and pome juice decoction media were prepared according to a previous report (Zhao et al. 2012), with some modifications. In brief, 70.0 g of barleycorn in its shell was soaked in water for 1 h, moved to a conical flask, mixed with 20.0 ml of 6.0% honey and 20.0 ml of 1.0% peptone, and sterilized at 121°C for 40 min; for the decoction, 300.0 g of mature pear fruit was mashed, the juice was squeezed through a multilayer gauze filter into a container, 300.0 ml of distilled water and 30.0 g agar were added, and the mixture was sterilized at 121°C for 20 min. Corn flour and Czapek

medium were prepared according to another report (Fang 1998). In brief, 300.0 g of corn flour and 17.0 g of agar were diluted with distilled water into 1,000.0 ml and then sterilized at 121°C for 20 min; for the Czapek medium, 2.0 g of NaNO₃, 1.0 g of K₂HPO₄, 0.5 g of KCl, 0.5 g of MgSO₄ · 7H₂O, 0.01 g of FeSO₄, and 30.0 g of sucrose were quantified, brought up to a 1,000.0-ml volume with distilled water, and sterilized at 121°C for 20 min.

A sporulation test was performed using colonized plugs to inoculate the centers of three petri dishes containing the medium described above. Plates were incubated at 25°C for a photoperiod of 14 h per day. Sporulation and conidium release were considered according to the formulation of pycnidia and the appearance of spore tendrils on the medium, respectively.

Conidial germination was evaluated by dropping 20.0 μ l of spore suspension diluted in potato-glucose (PD) solution (20.0% diced potato and 2.0% glucose) on a glass slide, which was moved to a petri dish over several water-soaked filter papers to maintain high humidity, cultured at 25°C in the dark, and observed each hour postincubation under an optical microscope (Nikon Eclipse E600 FN; Nikon). Five microscope views with $\times$ 200 magnification, containing approximately 70 to 80 conidia in the each, were randomly observed for each slide until all the conidia observed germinated.

Pathogenicity analysis, host range analysis, and virulence assessment. A spore suspension was prepared by inoculating plates of barleycorn medium with mycelial plugs as described above, growing the cultures for 8 days at 25°C with a 14-h photoperiod, then rinsing the plates with PD solution to release spores and further diluting the spore suspension with PD solution to a final concentration of 10⁶ conidia/ml.

Intact branch inoculations were performed on 1-year-old branches of 3-year-old potted pear trees (*P. pyrifolia* var. Cuiguan) that were cultivated outdoors in Wuhan, Hubei province, China, from mid-March to April 2013 during the inoculation tests. Trees almost terminated their dormant period at the time of inoculation. The average daily lowest temperature was 13°C and the highest temperature was 21°C, with an average monthly rainfall of 137.0 mm after inoculation. Given an unwounded tree, a 20.0- μ l aliquot of spore suspension (10⁶ conidia/ml) was coated (using a pipette) onto a bud and its neighboring regions after they were disinfested by swabbing the branches with cotton balls containing 75% ethanol. PD solutions containing no conidia were used in parallel as controls. For wounded trees, intact branches that were 7.0 to 11.0 mm in diameter were disinfested with 75% ethanol, and a hole was punched between two of the closer buds with a puncher (5 mm in diameter) on each shoot. A colonized PDA plug (5 mm in diameter) was excised from the colony margins after being cultured on PDA at 25°C for 3 days, inoculated in the hole of each shoot, and wrapped with plastic film strips to maintain high humidity until 10 days postinoculation (dpi). Noncolonized PDA plugs were used in parallel as controls. The symptoms were monitored and recorded every 10 days until the experiment was complete. Each bud from one branch of an individual tree was used for each inoculation treatment, and two independent experiments were performed.

Inoculation of detached mature pear fruit (*P. bretschneideri* var. Huangguan) was performed by placing mycelium plugs on the skin over wounds (approximately 1.0 mm in depth) that had been prepared by puncturing the fruit three times with a needle. The mycelia plugs were covered with sterilized moist cotton balls and the inoculated fruit were incubated at 25°C in plastic containers covered with a plastic film. Three fruit were used for each inoculation treatment, and the symptoms were recorded daily.

Inoculation of detached branches was performed using 1-year-old branches (10.0 cm in length, 6.5 to 10.0 mm in diameter) with mycelia plugs in punched holes that were then covered with sterilized moist cotton balls and incubated under the same conditions as described above. Six branches were used for each inoculation treatment and the symptoms were recorded at 10 dpi.

Statistical analysis. Data were analyzed with SPSS Statistics 21.0 (WinWrap Basic; <http://www.winwrap.com>) by one-way analysis of variance, and means were compared using Tukey's test at

a significance level of $P = 0.05$. Homogeneity of variance was tested before analysis.

Results

Field symptom survey. The typical symptoms of the disease were always observed around the buds on shoots, twigs, or large branches, starting with small, sunken lesions that gradually expanded into oval or long cankers in March. The lesions were dark or reddish brown in appearance, with warped epidermal layers in some locations (Fig. 1A). As the lesions spread into the surrounding shoots or branches or merged together, the infected shoots or branches together with the attached leaves and buds were killed, and dieback in the areas above the lesions became visible by April to June (Fig. 1B). Under droughty circumstances or seasons, the lesions turned into dark-brown, stiff, shrunken depressions after dehydration, with cracks separating the diseased from the healthy tissues and the epidermal layers sloughing off the dried bark, leaving lesions with a reddish brown color and a withered, dried-up appearance (Fig. 1C). In one serious situation, most of the branches and even an entire pear tree were killed (Fig. 1D). In late summer, numerous small black protuberant spots (pycnidia) formed along the lenticels on the diseased bark (Fig. 1E), and the pycnidia released slim, round, curly white spore tendrils after rainfall in late summer (Fig. 1F).

Isolation and molecular phylogenetic analysis. Using symptomatic samples collected from six provinces in China, we recovered 181 fungal isolates from three pear species (11 varieties) and from apple trees. Of these, 143 isolates exhibited the colony morphologies of *Phomopsis* spp. The other 38 isolates were identified as species of *Pestalotiopsis* (8 isolates), *Botryosphaeria* (9 isolates), *Alternaria* (6 isolates), and *Valsa* (13 isolates) based on their morphology and ITS sequences (data not shown), and those isolates were not included in further studies. All the *Phomopsis* isolates initially appeared as small, sparse white colonies but colony morphology diverged after 7 days of incubation on PDA medium plates at 25°C in the dark.

For species identification, 100 isolates that included the full range of colony morphologies were subjected to PCR amplification of their ITS sequences. The target DNA bands (approximately 507 to 547 bp in length) were sequenced and aligned, indicating similarities ranging from 74.08 to 100%. BLASTn searches of the ITS sequences showed that these colonies shared considerably high similarities (more than 75.0%, $E = 4e^{-54}$) with *Phomopsis* spp., including *Phomopsis fukushii*, *Diaporthe eres* (anamorph *P. oblonga* (Desm.) Traverso), *P. amygdali*, *P. longicolla*, and *D. neotheicola* (anamorph: *P. theicola* Curzi). Phylogenetic analysis based on the ITS sequences with those of the *Phomopsis* spp. revealed that the 100 isolates clustered together with five fungal species (Fig. 2). In all, 22 representative isolates belonging to the five species (9, 4, 3, 3, and 2 isolates, respectively) were further selected from the 100 isolates according to their colony morphologies, and their actin and EF1- α gene regions were sequenced. The 22 isolates were further subjected to phylogenetic analysis based on the concatenated sequences, including EF1- α , actin, and ITS. Similar clades statuses were obtained for the respective isolates of the five species compared with the phylogenetic analysis based on the ITS sequences (Supplementary Fig. S1), confirming the species identification.

Relationships among *Phomopsis* spp., hosts, and sampling regions. *P. fukushii* isolates were frequently isolated from *Pyrus communis* and *M. pumila* (both of which were collected from Shandong); less frequently from *P. pyrifolia* collected from Jiangxi, Hubei, Fujian, and Jiangsu; and not at all from *P. bretschneideri* collected in Yunnan province (Table 1). *D. eres* was isolated from all host species collected from all the provinces except Jiangsu, where only two samples were collected. It was one of only two species recovered from *P. bretschneideri* collected in Yunnan province (Table 1). *Phomopsis amygdali* and *P. longicolla* were only isolated from *Pyrus pyrifolia* collected from Jiangxi and Fujian, except *Phomopsis longicolla* was also collected from the additional province of Hubei (Table 1). *D. neotheicola*

Fig. 1. Typical canker symptoms on branches of *Pyrus pyrifolia* var. Cuiguan in the field. **A**, Newly developed reddish-brown canker lesion around a bud; **B**, dieback symptoms resulting from lesion expansion around the branches; **C**, withered symptom of an infected branch; **D**, severe branch dieback or wilt of an infected tree; **E**, pycnidia formed on the lesions; **F**, white spore tendrils released from pycnidia.

was isolated most frequently from *Pyrus bretschneideri* that was collected in Yunan province (five of eight isolates), occasionally from *P. pyrifolia* from Jiangxi and Fujian, but not from other host species or provinces (Table 1).

Colony morphologies, conidial morphologies, and growth rate analysis. Characterization of the colony morphologies for the five *Phomopsis* spp. after 10 days of incubation on PDA

Fig. 2. Maximum-likelihood phylogenetic analysis of isolates obtained during this study along with *Phomopsis* (*Diaporthe*) spp. isolates registered in the GenBank database on the basis of their internal transcribed spacer (ITS) sequences. A heuristic search was performed with a bootstrap value of 1,000 replications; only the bootstrap values of more than 60% are shown, and the branch lengths are proportional to the genetic distances. The isolates obtained in this study are indicated by a triangle (Δ). Distance scale = 0.05. The GenBank accession numbers for the ITS sequences of the reference isolates are listed in the bracket as follows: MAFF625034 (JQ807469), DP0590 (JQ807464), DP0438 (JQ807461) and DP0460 (JQ807463) of *Phomopsis fukushii*; DF5017 (JQ807449) and AR4373 (JQ807442) of *Diaporthe eres*; FAU1052 (AF102998) of *P. amygdali*; SSLP-1 (HQ333500) of *P. longicolla*; DP0188 (JQ807454) of *D. neotheicola*; STE-U2660 (AF230751) of *P. viticola*; and 5.5.4r1(2) (KC143171) of *D. ambigua*. *Botryosphaeria dothidea* (ZH2211, KC960944) is added as an outgroup.

revealed a clear divergence in the number of aerial hyphal rings, mycelial density and pigmentation, and distribution and quantity of pycnidia.

Phomopsis fukushii (69 isolates) formed snow-white colonies with dense mycelia and contained no less than two wide concentric rings of aerial hyphae in each colony. Spherical or oblate black pycnidia formed over the concentric rings and they were uncoated. The isolates were divided into two subgroups depending on the amount of aerial hyphal rings, with subgroup 1a (21 isolates) producing more than three hyphal rings with pycnidia uniformly distributed along the hyphal rings, whereas subgroup 1b (48 isolates) produced two aerial hyphal rings with the inner rings being wider than the outer ones. Thirteen isolates produced purple or light-gray pigments that were visible when plates were viewed upside-down (Fig. 3A).

D. eres (31 isolates) formed white colonies with thin mycelia and contained no less than two concentric aerial hyphal rings in each colony, with spherical or oblate black pycnidia uniformly distributed around the concentric rings. The isolates were divided into two subgroups depending on the manner of pycnidia formation. Isolates in subgroup 1Ia (23 isolates) formed uncoated pycnidia over the hyphal rings whereas subgroup 1Ib (8 isolates) formed pycnidia coated by mycelia along the hyphal rings (Fig. 3A). Four isolates also produced purple or light-gray pigments on the undersides of the colonies (Fig. 3A).

P. amygdali (22 isolates) formed gray to black colonies with thin mycelia and contained inconspicuous aerial hyphal rings in each colony, with spherical or oblate black pycnidia scattered over the colony. Notable black pigments formed on the undersides of the colonies after 10 days of incubation but the mycelia on the upper colony surface remained white.

P. longicolla (13 isolates) formed white or oyster-colored colonies with thin, dense, and floccose mycelia. They produced no pigment on the undersides of the colonies, and their pycnidia were spherical or oblate, black, clearly smaller than those of other species, and were randomly scattered over the colonies (Fig. 3A).

D. neotheicola (8 isolates) formed a snow-white colony with sparse mycelia with abnormal or irregular margins, produced no pigment on the undersides of the colonies, and their pycnidia were spherical or oblate, black, coated with mycelia, and scattered over the colonies.

In all, 2 to 5 isolates (16 in total; Table 2) were chosen from each species according to their colony appearance as representatives for the characterization of their conidium morphology. After being cultured on PDA medium for 20 to 30 days, all the isolates could produce α-conidia but only one to three isolates of each species also produced β-conidia (Table 2).

The α-conidia of all the isolates were one-celled, hyaline, fusiform, aseptate, and frequently biguttulate (Fig. 3B), with lengths ranging from 4.97 to 11.01 μm and widths from 1.51 to 4.28 μm. The sizes were significantly different among different isolates ($F=11.139$ and $P < 0.001$ for the lengths and $F = 53.128$ and $P < 0.001$ for the widths) (Table 2). The β-conidia of all the isolates were one-celled, aseptate, hyaline, and filiform with straight or curved ends (Fig. 3B), with lengths ranging from 17.30 to 48.98 μm and widths from 0.99 to 2.69 μm. The sizes were significantly different among different isolates ($F = 133.179$ and

Table 1. Host, sampling region and numbers of *Phomopsis* (*Diaporthe*) isolates obtained in the study

Sampling province	Host	N ^z	Proportion of isolates from each species				
			<i>Phomopsis fukushii</i>	<i>Diaporthe eres</i>	<i>P. amygdali</i>	<i>P. longicolla</i>	<i>D. neotheicola</i>
Jiangxi	<i>Pyrus pyrifolia</i>	42	40.6	15.6	31.3	6.3	6.3
Hubei	<i>P. pyrifolia</i>	14	35.7	35.7	0.0	28.6	0.0
Fujian	<i>P. pyrifolia</i>	45	35.6	20.0	26.7	15.6	2.2
Shandong	<i>P. communis</i>	15	73.3	26.7	0.0	0.0	0.0
Jiangsu	<i>P. pyrifolia</i>	1	100.0	0.0	0.0	0.0	0.0
Yunnan	<i>P. bretschneideri</i>	11	0.0	54.5	0.0	0.0	45.5
Shandong	<i>Malus pumila</i>	15	86.7	13.3	0.0	0.0	0.0
Total	...	143	371.9	165.9	57.9	50.4	53.9

^z Number of *Phomopsis* (*Diaporthe*) isolates derived from the province.

$P < 0.001$ for the lengths and $F = 17.998$ and $P < 0.001$ for the widths) (Table 2).

Two representative isolates of each species were chosen to assess the growth rates (Fig. 4). The results showed that both isolates of each species had identical or similar growth rates, and the average growth

rates were 12.5, 10.8, 13.3, 15.0, and 5.7 to 6.1 mm per day for the *P. fukushii*, *D. eres*, *P. amygdali*, *P. longicolla*, and *D. neotheicola* isolates, respectively. The growth rate was significantly different among some of the species ($F = 89.440$, $P < 0.001$), of which *D. neotheicola* had the lowest growth rate, *D. eres* had a medium growth rate, and

Fig. 3. Morphologies of the representative isolates belonging to five *Phomopsis* (*Diaporthe*) spp. obtained in this study. **A**, Colony appearance of representative isolates which were cultured on potato dextrose agar (PDA), with images taken after 18 days from above and **B**, at the bottom, α - and β -conidia, scale bar = 10 μ m; **C**, above-colony appearance of JXWZ28 in *Phomopsis fukushii* cultured on PDA, pome decoct juice, Czapek, corn flour, and barleycorn medium at 13 days postinoculation.

Table 2. Morphological characteristics of the representative isolates of *Phomopsis* (*Diaporthe*) obtained in this study

Species, isolate	Conidia size ranges ^x				Means ± SD of conidia size ^y		Color ^z	Type
	α-Conidia		β-Conidia					
	Length	Width	Length	Width	α-Conidia	β-Conidia		
<i>Phomopsis fukushii</i>								
JXCY12	5.61–9.71 a	1.97–4.28 c	17.30–29.88 a	0.99–2.23 a	7.85 ± 0.32 × 2.89 ± 0.50	23.40 ± 2.99 × 1.46 ± 0.32	W	W
JXWZ25	5.93–8.99 a	1.98–2.23 a	...	...	7.73 ± 0.45 × 2.23 ± 0.31	...	W	W
JXWZ28	5.58–10.09 a	1.87–3.39 bc	23.37–43.85 b	1.04–2.69 ab	7.88 ± 0.5 × 2.45 ± 0.44	29.05 ± 2.98 × 2.75 ± 0.49	W-P	G
SDFS71	5.35–9.85 ab	2.03–3.15 a	30.84–48.98 e	1.58–2.18 cd	7.63 ± 0.5 × 2.35 ± 0.41	40.98 ± 4.65 × 1.87 ± 0.16	W	W
SDFS77	5.32–9.91 ab	2.08–3.39 c	...	...	7.61 ± 0.90 × 2.93 ± 0.42	...	W-P	G
<i>Diaporthe eres</i>								
JXCXL13	5.07–10.08 a	1.87–3.05 d	26.13–44.34 cd	1.32–2.64 d	7.70 ± 1.10 × 3.37 ± 0.34	33.20 ± 3.93 × 1.98 ± 0.38	G	G
HBJ2319	6.28–9.30 ab	1.87–3.97 c	...	...	7.61 ± 0.70 × 2.96 ± 0.47	...	W	W
YNDSS41	5.88–10.51 a	1.51–3.28 a	24.79–36.98 c	1.34–2.33 bc	7.73 ± 1.33 × 2.31 ± 0.42	31.38 ± 3.36 × 1.79 ± 0.24	G	G
FJCY110	4.97–8.18 d	1.51–3.14 a	20–35.61 b	1.03–2.08 a	6.43 ± 0.78 × 2.26 ± 0.36	27.07 ± 3.53 × 1.59 ± 0.27	W-G	W
<i>P. amygdail</i>								
JXCY15	5.36–9.38 ab	2.03–3.21 bc	...	...	7.65 ± 0.81 × 2.65 ± 0.39	...	G	G
FJCY118	6.47–9.43 a	2.03–2.98 ab	...	...	7.85 ± 0.82 × 2.55 ± 0.28	...	G	G
JXYT181	5.33–10.36 a	1.83–3.31 d	27.11–44.33 d	1.35–2.68 d	7.78 ± 1.03 × 3.39 ± 0.38	35.01 ± 3.96 × 1.99 ± 0.39	G	G
<i>P. longicolla</i>								
FJMF120	5.25–11.01 bc	1.83–3.2 b	29.03–40.31 d	1.34–2.33 bc	7.64 ± 1.01 × 2.38 ± 0.33	34.72 ± 2.64 × 1.79 ± 0.24	W	W
HBCG59	5.25–8.99 ab	2.05–3.36 ab	...	...	7.07 ± 0.76 × 2.62 ± 0.33	...	W	W
<i>D. neotheicola</i>								
FJHH134	5.61–8.10 ab	1.87–2.67 a	21.34–28.03 a	1.43–1.69 bc	6.74 ± 0.62 × 2.22 ± 0.17	25.01 ± 2.63 × 1.79 ± 0.28	W	W
YNZBS34	6.41–8.94 cd	1.93–2.59 a	...	...	7.62 ± 0.60 × 2.21 ± 0.17	...	W	W

^x Numbers indicate minimum and maximum sizes for length and width, respectively, of 50 conidia recorded from each selected *Phomopsis* spp. from China. Significance at $P = 0.05$ level.

^y Numbers indicate mean conidium sizes of the each isolate calculated by the statistical analysis. Data were analyzed with SPSS Statistics 21.0 (WinWrap Basic; <http://www.winwrap.com>) by one-way analysis of variance, and means were compared using Tukey's test at a significance level of $P = 0.05$. SD = standard deviation.

^z Colony color showed on potato dextrose agar plates cultured for 10 days, and types determined according to Kanematsu et al. (2000). W, G, and P = white, gray, and purple colony, respectively; W-G = white colony with gray pigment; and W-P = white colony with purple pigment.

P. longicolla had the highest growth rate, and the other two species (*P. fukushii* and *P. amygdali*) were positioned between *D. eres* and *P. longicolla* (Fig. 4).

Sporulation properties and germination tests. To prepare sufficient conidia to study the fungal pathogenicity and characterize the conidial sporulation properties, the five species were assessed by culturing one representative isolate (Supplementary Fig. S2) on five media: pome juice decoction, Czapek, corn flour, barleycorn, and PDA. The results showed a significant divergence in the time of pycnidial formation and conidial release among the five species and the five media tested. Most of the species formed pycnidia at earlier times (8 to 9 days) on the barleycorn medium, later times (20 to 30 days) on the pome juice medium, and somewhere in between on the other media (12 to 21 days) (Supplementary Fig. S2A). The pycnidia formation time was significantly different among the species on each medium, and *P. fukushii*, *D. eres*, and *P. amygdali* formed pycnidia earlier than *P. longicolla* and *D. neotheicola* on the PDA ($F = 5.177$, $P = 0.016$), pome juice decoction ($F = 11.613$, $P = 0.001$), and corn flour media ($F = 13.406$, $P = 0.001$); *D. eres* and *D. neotheicola* formed pycnidia later than the other species on Czapek medium ($F = 109.5$, $P < 0.001$), and all the species had a similar formation time on the barleycorn medium ($F = 2.250$, $P = 0.136$). The conidial release time was also significantly affected by the growing medium and the species. No conidia were released on the pome juice medium for any of the species, or on Czapek medium for *D. eres*, *P. longicolla*, and *D. neotheicola*. *P. fukushii*, *D. eres*, and *P. amygdali* released conidia earlier than *P. longicolla* and *D. neotheicola* on the barleycorn medium. The release time was similar on PDA and corn flour for these species, except that *D. neotheicola* had a later release time (Supplementary Fig. S2B). It is worth noting that large numbers of white spore tendrils were released from the pycnidia on barleycorn medium at 10 to 13 dpi for all of the species (Fig. 3C).

The *P. fukushii* isolate was further evaluated to determine how the five different media affected colony appearance and spore production. The number of concentric hyphal rings varied depending on the medium, with three, two, zero, seven, and zero rings produced on PDA, pome juice decoction, Czapek, corn flour, and barleycorn, respectively. Purple pigment was produced in pome juice decoction medium and not in other media. The pycnidia were produced without being coated in mycelia in PDA, Czapek, and barleycorn medium but they were half-coated in mycelia in corn flour medium and pome juice decoction (Fig. 3C). The numbers of pycnidia also varied depending on the medium type. Approximately 50, 3, 100, 90, and 1,430 pycnidia were produced per plate for PDA, pome juice decoction, Czapek, corn flour, and barleycorn medium, respectively. Other species also exhibited similar effects of media on pycnidial production (data not shown). The barleycorn

medium was chosen to prepare the conidium suspension for further pathogenicity analysis.

Sixteen isolates representative of the species (Table 2) were subjected to a germination test in PD culture solution. The α -conidia easily germinated in PD culture solution, with a germination incidence of 100% after 4 h of incubation, but the β -conidia did not germinate under this condition, even after the culture time was prolonged for 10 days.

Pathogenicity analysis, host range analysis, and virulence assessment. *Pathogenicity analysis on intact branches of Pyrus pyrifolia var. Cuiguan.* Fourteen representative isolates (Fig. 5) were selected for pathogenicity analysis after observing that they could form α -conidia in similar quantities over similar culture periods, whereas no β -conidia were ever observed in the spores released in this period. Pathogenicity analysis on intact branches of *Pyrus pyrifolia* var. Cuiguan showed that, under unwounded conditions, all the isolates belonging to *Phomopsis fukushii*, *D. eres*, and *P. amygdali* (except isolate FJHH135) started to induce small dark-brown canker lesions around the buds that were visible at 40 dpi. The small lesions quickly expanded into large brown or reddish lesions at high temperatures and under humid weather conditions (in May to June), and the lesions became depressed and cracked at the margins by 60 dpi (Fig. 5A). The induced symptoms matched the ones observed in the field. The lesion lengths varied significantly among the different isolates ($F = 7.945$, $P < 0.001$). None of the isolates of either *P. longicolla* or *D. neotheicola* induced any lesions, nor were lesions formed on the controls (Fig. 5A and B). After wounding, mycelium plug inoculations of attached branches from Cuiguan resulted in the quick emergence of the same reddish symptoms that were induced by all the isolates of *P. fukushii*, *D. eres*, *P. amygdali*, and *P. longicolla* at 15 dpi, whereas neither of the two isolates of *D. neotheicola* induced lesions (Supplementary Fig. S3A). No lesions developed on control branches inoculated with buffer. The lesion lengths varied significantly among the different isolates ($F = 9.796$, $P < 0.001$). In late summer to early autumn, numerous small pycnidia formed along with the lenticels in the canker lesions. White spore tendrils were forced out of the lenticels after it rained later in the autumn, and they were similar to the ones observed in the field (Fig. 1E and F). All branches showing canker symptoms elicited by the inoculations were subjected to fungal isolation, and the results showed that the reisolated colonies matched the inoculated ones in their morphologies and ITS sequences (data not shown).

Pathogenicity analyses on the branches of other pear varieties. Twenty isolates (Supplementary Fig. S3B) from the five species were used for pathogenicity analyses conducted with mycelial inoculation of wounded, detached branches of *Pyrus pyrifolia* var. Hohsui under indoor conditions. The results showed that, except for one isolate

Fig. 4. Growth rates of two representative isolates of five *Phomopsis* (*Diaporthe*) spp. The isolates and the species involved are shown at the bottom of the colonies. Data were analyzed with SPSS Statistics 21.0 (WinWrap Basic; <http://www.winwrap.com>) by one-way analysis of variance, and means were compared using Tukey's test at a significance level of $P = 0.05$. Letters over the bars indicate the significant difference at $P = 0.05$.

(YNZBS34) of *D. neotheicola* that produced no lesions, all the isolates started to induce black lesions at 2 dpi. The lesions turned reddish and significant differences were evident among different isolates and species by 15 dpi ($F = 14.307$, $P < 0.001$). Most isolates from

Fig. 5. Symptoms and lesion lengths induced by inoculation of unwounded attached pear branches (*Pyrus pyrifolia* var. Cuiguan) with spore suspensions (10^6 /ml) from five *Phomopsis* (*Diaporthe*) spp. **A**, Representative symptoms as photographed at 60 days postinoculation (dpi). **B**, Mean lesions lengths from three replicates of branches measured at 60 dpi. Statistical analysis was performed with SPSS Statistics 21.0 (WinWrap Basic; <http://www.winwrap.com>) by one-way analysis of variance, and means were compared using Tukey's test at a significance level of $P = 0.05$. Letters over the bars indicate the significant difference at the $P = 0.05$ level.

Phomopsis fukushii, *D. eres*, and *P. amygdali* induced significantly longer lesions (12.0 to 48.2 mm) than those produced by *D. neotheicola* (0 to 9.5 mm) or *P. longicolla* (10.2 to 15.8 mm). Similar virulence variation was obtained for the isolates (10 isolates of the 20 representative isolates) that were analyzed on *Pyrus communis* vars. Kndo and Pactor Jules Guyot on *P. pyrifolia* var. Hohsui (Supplementary Fig. S3C). No lesions were induced in the control branches inoculated with PDA plugs.

Host range analysis and virulence assessment. Two isolates from each *Phomopsis* spp. (Fig. 6) were chosen to analyze their host ranges in other rosaceous fruit species that are commonly grown in China, including apple (*M. pumila* Mill. var. Fushi), crabapple (*M. spectabilis* (Ait.) Borkh), hawthorn (*Crataegus pinnatifida* Bunge), cherry (*Cerasus pseudocerasus* (Lindl.) G. Don), and peach (*Prunus persica* (L) Batsch). Two nonrosaceous fruit species, kiwifruit (*Actinidia chinensis* Planch.) and citrus (*Citrus reticulata* Blanco), were also tested. The results showed that all the fungal species induced obvious lesions and decayed the xylem on the inoculated branches of apple, peach, crabapple, and cherry fruit, except that *D. neotheicola* isolate YNZB34 did not induce lesions in crabapple, and isolate FJHH134 induced no lesions on any of the inoculated branches (Fig. 6). None of the isolates induced lesions on hawthorn or citrus. Lesion lengths varied depending on both fungal species and plant species. In apple and crabapple branches ($F = 80.569$ and 394.432 , respectively, $P < 0.001$), both *Phomopsis amygdali* isolates induced significantly longer lesions than the other species, with gradually shorter lesion lengths for *P. fukushii*, *D. eres*, *P. longicolla*, and *D. neotheicola*. In cherry, all the species induced longer lesions than in other plant branches ($F = 64.948$ to 234.459 , $P < 0.001$), except the *D. neotheicola* isolate YNZB34, which still induced short lesions similar to the lesion lengths on other plants ($F = 1.414$, $P = 0.301$). In peach and kiwifruit branches ($F = 56.582$ and 87.265 , respectively, $P < 0.001$), most of the isolated species induced similarly sized but significantly longer lesions than those induced by the *D. neotheicola* isolate (Fig. 6).

Discussion

This study presents the first attempt to characterize the *Phomopsis* spp. associated with pear branch canker disease (i.e., pear shoot canker) in China; it provides solid evidence that *P. fukushii*, *D. eres*, and *P. amygdali* were responsible for the pear shoot canker disease in single or mixed infections and that *P. longicolla* and *D. neotheicola* only posed a virulence risk to wounded plants.

In this study, 181 samples were collected from 11 varieties of three pear species from the six primary pear-producing provinces. To our knowledge, this is the first survey of its kind of this disease. Given

Fig. 6. Lesion lengths induced by inoculation of three replicates of detached nonwounded branches of seven fruit species using mycelial plugs from representative isolates of five *Phomopsis* (*Diaporthe*) spp., as measured at 10 days postinoculation. Statistical analysis was performed with SPSS Statistics 21.0 (WinWrap Basic; <http://www.winwrap.com>) by one-way analysis of variance, and means were compared using Tukey's test at a significance level of $P = 0.05$. Letters over the bars indicate the significant difference at the $P = 0.05$ level.

that the pear and apple trees were grown in mixed plantings or at least in close proximity in many regions, some symptomatic apple samples close to pear orchards were also collected for fungal isolation, and 13 *P. fukushii* and 2 *D. eres* isolates were obtained from apple. The morphology, pathogenicity, and host range analysis of the representative isolates from apple exhibited no obvious difference from those from pear, and Koch's postulate was also followed with one isolate (SDFS71), showing that the isolate was also able to induce pear branch canker (Fig. 5A and B). These results suggest that the *P. fukushii* and *D. eres* isolates that infected apple trees had no clear biological divergence from those infecting pear trees, and diseased apple branches could also act as a source of infection that resulted in the pear branch canker observed in the field.

Molecular sequence analyses combined with morphology, host affiliation, and cultural and pathological data have been adopted to identify *Phomopsis* spp. (Aa et al. 1990; Mostert et al. 2001; Rehner and Uecker 1994; Santos and Phillips 2009; Santos et al. 2011; Shenoy et al. 2007; van Niekerk et al. 2005; van Rensburg et al. 2006). In fact, the current confirmation of the taxonomic status of the species in this genus relies heavily on molecular techniques (Castlebury 2005; Castlebury et al. 2003; Crous and Groenewald 2005; Santos et al. 2010; Udayanga et al. 2012). In this study, the species was identified by performing a phylogenetic analysis of ITS, which was confirmed by further phylogenetic analysis based on multilocus regions (ITS, actin, and EF1- α). Among these regions, rDNA ITS and partial sequences of the EF1- α have commonly been used for molecular identification (Santos et al. 2010, 2011; Sun et al. 2012; Udayanga et al. 2012; van Niekerk et al. 2005; van Rensburg et al. 2006), and the multiloci sequence analysis has been used in species identifications of *Phomopsis* isolates from grapevines (Baumgartner et al. 2013).

Analysis of our isolates revealed that *P. fukushii* was the most common species, that *D. eres* and *P. amygdali* were also associated with the disease, and that *P. longicolla* and *D. neotheicola* were far less common. An analysis of the species with their host and sampling regions showed a correlation between the species and the host or region (Table 1), suggesting that the specific species might be related to the disease that occurred on different host varieties in different regions. Among these species, *D. eres* could be responsible for the branch canker of *Pyrus bretschneideri* in Yunnan, both *Phomopsis fukushii* and *D. eres* in *Pyrus communis* and *M. pumila* from Shandong and *P. pyrifolia* in most provinces, as well as *Phomopsis amygdali* in *Pyrus pyrifolia* from Jiangxi and Fujian together with *Phomopsis fukushii* and *D. eres*.

Colony morphologies differed among the species in the current study, with obvious divergence in number and appearance of mycelial rings, pigment production, and the distribution, size, and quantity of pycnidia (Fig. 3C), although some *P. fukushii* and *D. eres* isolates exhibited similar colony morphologies. These two species were also poorly resolved in previous studies of host specificity (Kanematsu et al. 2000; Udayanga et al. 2011). Most of the features remained stable in subcultures and colony pigments were used only to allocate groups into white or gray types (W or G type) within a species in a previous study (Kanematsu et al. 2000). According to the pigments of the isolates obtained in this study, all the isolates of *P. longicolla* and *D. neotheicola* were W types, whereas all the *P. amygdali* isolates were G types, except that *P. fukushii* and *D. eres* contained both types of isolates (13 and 4 isolates, respectively; Table 2). *P. amygdali* had been shown to be cross-fertile with the G-type isolates in another study (Kanematsu et al. 2000). Consistent with both of the previous reports, we also found that the G-type isolates had distinctly higher virulence than the W-type for most isolates of the five species, especially for isolates of *P. amygdali*, *P. longicolla*, and *D. neotheicola* (Fig. 5; Table 2).

All the species we studied produced two types of conidia (α - and β -conidia), with similar shapes for each type (Table 2). Our results differ from a previous study, wherein the G-type isolates produced no β -conidia (Kanematsu et al. 2000), but are consistent with a recent report that *P. amygdali* isolates from *Pieris japonica* readily produced β -conidia (Bienapfl and Balci 2013). Compared with the α -conidia sizes from the *Phomopsis fukushii* that was isolated from Japanese pear and apple in Japan (6.1 to 6.9 by 2.1 to 2.7 μ m), the

isolates obtained in this study had larger α -conidia (7.61 to 7.88 by 2.23 to 3.37) (Kanematsu et al. 2000). The α - and β -conidia differed in size among different isolates but the difference was not related to the species types (Table 2). Therefore, as in previous reports, the micromorphology was not very useful for species definition (Baumgartner et al. 2013; Kanematsu et al. 2000). However, an evaluation of the growth rate revealed a significant difference among and within species, with isolates of *D. neotheicola* having the lowest growth rate (5.7 to 6.1 mm per day), which was similar to the slow-growing species on PDA (5.2 $\pm$ 0.2 mm/day) in a recent report (Udayanga et al. 2014).

Pathogenicity tests using conidial suspensions to inoculate intact branches of *Pyrus pyrifolia* var. Cuiguan and the subsequent reisolation of the same species from the diseased branches verified that three species (*Phomopsis fukushii*, *D. eres*, and *P. amygdali*) are responsible for pear branch canker disease. This conclusion was also supported by the observation that the three species were isolated at markedly higher frequencies (122 isolates) than either of the other two *Phomopsis* spp. (21 isolates) or than the other fungal genera (36 isolates) that were found in a broader range of pear species and regions (Table 1). The *Phomopsis* spp. induced canker symptoms after a conidia suspension was inoculated on the buds, which is consistent with the conclusion that the disease was invoked by fungal invasion via the buds (Melanson et al. 2002; Udayanga et al. 2011). Only α -conidia germinated whereas β -conidia did not, as reported (Uecker 1988), suggesting that infections are initiated by α -conidia alone rather than by β -conidia. When the plants were wounded, *P. longicolla* also induced canker lesions in the intact branches just as the first three species did, suggesting that the species could be an opportunistic pathogen associated with the disease. However, both *D. neotheicola* isolates induced no lesions when a wound was present, suggesting that the species might be nonpathogenic to this pear variety. A pathogenicity tests on pear fruit from *Pyrus bretschneideri* var. Huangguan showed that all five species can damage wounded pear fruit (data not shown). *Phomopsis fukushii* had previously been reported as the cause of a fruit rot of Chinese white pear (*Pyrus ussuriensis* Maximowicz var. *sinensis* Kikuchi) and Japanese pear (*P. serotina* Rehder var. *cuta* Rehder) (Nasu et al. 1987). *Phomopsis* spp. had been identified as the pathogen responsible for pear shoot canker in Japan, and it inhibits trunk growth and causes dead twigs and chronic canker or fruit rot of deciduous fruit trees (Tanaka and Endo 1930). We concluded that the pear branch canker disease in China is identical to the one described in Japan.

The host range evaluation suggested that all five *Phomopsis* spp. can attack most fruit trees belonging to the *Rosaceae* family as well as some nonrosaceous plants; however, virulence varied depending on the species of both the pathogen and the host. This result suggests that the fungus species derived from pear or apple has the potential to cause disease in other stone and pome fruit trees, and vice versa, as it did by cross-infection with three *Phomopsis* isolates from peach, plum, and Asian pear (Uddin and Stevenson 1998). It is notable that the *Phomopsis amygdali* isolates had dramatically higher virulence than other species in all the tested *Rosaceae* plants. All fungal species were highly pathogenic in cherry but had similar and lower virulence risks in peach and kiwifruit. *D. neotheicola* posed little or no virulence risk in all the tested plant branches (Fig. 6).

P. fukushii was identified first as the pathogen responsible for the pear shoot canker in Japan (Tanaka and Endo 1930) and then on more *Pyrus* and *Malus* spp. (Farr and Rossman 2015; Nasu et al. 1987; Tanaka and Endo 1930) and grapevine from North America showing wood canker (Baumgartner et al. 2013). *D. eres* was shown to infect a broad range of plants encompassing approximately 300 species and causes cankers, fruit rots, and leaf spot diseases (Udayanga et al. 2011), whereas pear trees have reportedly not been damaged by this species (Farr and Rossman 2015). *P. amygdali* was only reported as responsible for the shoot canker of peach and almond (Bienapfl and Balci 2013; Tuset and Portilla 1989). *P. longicolla* was originally described from soybean in association with soybean seed decay and isolated from *Abutilon theophrasti*, *Arachis hypogaea*, *Chamaesyce nutans*, *Ipomoea lacunosa*, and *Xanthium strumarium* (Li et al. 2010a,b; Udayanga et al. 2011). *D. neotheicola* was associated with

fruit trees, including *Prunus* and *Pyrus* spp., and *Vitis vinifera*, and there was no information about its pathogenicity. To our knowledge, this is the first clarification that *D. eres* and *P. amygdali* are responsible for the pear shoot canker disease, and our results also expand the host and geographic ranges of the five species. This study provides useful information to understand this pathogen and improve management strategies for controlling this economically important disease.

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