Alternaria malicola sp. nov., a New Pathogen Causing Fruit Spot on Apple in China

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Abstract

Alternaria spp. are pathogens of several diseases that pose significant threats to apple production. Several putative *Alternaria* sp. isolates were obtained from lesions of a disease commonly referred to as black dot on apple fruit in Shaanxi Province, China. Pathogenicity tests using mycelial plugs and conidial suspensions indicated that this isolate could cause leaf blotch, as well as moldy core and black dot on fruit. On the basis of sequence analysis of glyceraldehyde-3-phosphate dehydrogenase (GAPDH), RNA polymerase second largest subunit, and translation

elongation factor 1- α , an isolate clustered with the *Alternaria* sect. *Ulocladioides*. By combining GAPDH, major allergen Alt1, mating type protein 1-2-1, and the AGA1 gene sequence analysis and morphological description, the isolates were identified as a new species named *Alternaria malicola*. Our finding expands the documented diversity of apple pathogens within the genus *Alternaria* and clarifies the taxonomy of the pathogen assemblage that may be associated with three apple diseases.

The genus *Alternaria* consists of a diverse group of pathogens, saprotrophs, and endophytes. They have a global distribution and can be found on and in a large variety of crop plants, ornamentals, fruit trees, and other hosts. Saprophytic species cause putrefaction and sometimes produce mycotoxins in foods and other biomaterials (Bottalico and Logrieco 1992; Ren et al. 1998). Plant-pathogenic *Alternaria* spp. can reduce crop yield and pose serious threats to agricultural production (Strandberg 1992), causing a wide range of symptoms, including leaf spot, blossom rot, and fruit rot and blight (Andersen et al. 2005).

Several *Alternaria* spp. cause *Alternaria* leaf blotch, moldy core, and black dot diseases on apple, threatening apple production in many areas of the world (Filajdic and Sutton 1991). In China, *Alternaria* leaf blotch of apple was first reported in the 1970s, and the disease is currently considered to be among the four most serious diseases in all major production areas of that country (Hu et al. 2006; Li et al. 2011). The causal agent of *Alternaria* leaf blotch of apple was originally identified as *Alternaria mali* Roberts in the United States (Roberts 1924). Sawamura (1962) indicated that the leaf blotch pathogen was *Alternaria alternata* f. sp. *mali* on the basis of the role of its host-specific AM toxin. Moldy core is an economically important apple disease worldwide that reduces apple fruit quality and quantity. The genus *Alternaria* was considered to be associated with moldy core of apple (Combrink et al. 1985; Serdani et al. 1998; Spotts 1990). *A. alternata*, *A. arborescens*, *A. tenuissima*, and *A. infectoria* were later identified, and *A. tenuissima* was pinpointed as the main pathogen that caused moldy core in China (Gao et al. 2013).

Black dot (also known as black spot) on apple was first noted in China during the 1990s, after widespread introduction of the practice of bagging apple fruit during the period of fruit development. The disease is harmful to the quality of the fruit. Black to brown spots (1 to 5 mm in diameter) appear in the calyx dimple of the fruit; the lesion is sunken, and tissue in the center of the lesion dries and

may crack (Wang et al. 2014). The fungi *Cylindrosporium pomi* C. Brooks, *Phoma pomi* Pass. (Xu et al. 2000), *Trichothecium roseum* (Pers.) Link, *Alternaria* spp. (Hao et al. 2004), and *Acremonium strictum* W. Gams were considered to be causal agents of black dot in China (Guo et al. 2005).

The objective of this study was to identify the species of *Alternaria* isolates obtained from black dot symptoms on bagged apple fruit, based on morphological characteristics and a phylogenetic analysis of the glyceraldehyde-3-phosphate dehydrogenase (GAPDH), RNA polymerase second largest subunit (RPB2), translation elongation factor 1- α (TEF1), major allergen Alt1 (Alt1), mating type protein (MAT1-2-1), and the AGA1 (AGA) gene.

Materials and Methods

Isolates. Twelve apple fruit (*Malus domestica* ‘Fuji’) exhibiting symptoms of disease were collected in 2015 from commercial orchards located in Baishui and Qianxian Counties of Shaanxi Province. Two putative *Alternaria* isolates (QXHD_10 and BSBD_86) used in this study were obtained from the margins of black dot lesions on the pericarp and cultured on potato dextrose agar (PDA) slants at 25°C in darkness for a week. Purified colonies were preserved in glycerol (15%) at –80°C in the Fungal Herbarium of Northwest A&F University, Yangling, Shaanxi Province, China. For comparative purposes, 10 species (*Alternaria alternariae*, *A. obovoidea*, *Ulocladium allii-tuberosi*, *U. chartarum*, *U. consorsiale*, *U. dauci*, *U. multifforme*, *U. oudemansii*, *U. terricola*, and *U. zantedeschiae*) (Table 1) were obtained from the Herbarium of Shandong Agricultural University, Department of Plant Pathology.

A representative isolate, QXHD_10, was deposited in the China General Microbiological Culture Collection Center (CGMCC) (Beijing) and dried cultures were deposited at the Herbarium Mycologicum Academiae Sinicae (HMAS) (Beijing). New species names and descriptions were deposited in MycoBank (www.Mycobank.org) (Crous et al. 2004). The isolate of *Alternaria* and GenBank numbers used in the study are shown in Table 2.

Pathogenicity tests on apple. Mycelial plug method. Isolate QXHD_10 was used for pathogenicity tests on pericarps of cultivars Golden Delicious, Red Delicious, Gala, and Fuji. Five fruit of each cultivar were used for each inoculation method. Mature asymptomatic fruit harvested from a commercial orchard located near Yangling were cleaned with pure water and wiped gently with cotton wool dipped in 75% ethanol for 30 to 40 s, then air dried before inoculation. After the culture was incubated for a week in darkness at 25°C, 0.5-cm-diameter plugs containing both hyphae and conidia were excised from the actively growing margin of

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the colony and placed on the calyx dimple of the fruit (two plugs per fruit), with the culture side facing the fruit. Sterile water and agar plugs without the fungus were used as controls. Inoculated and control fruit were sealed in individual plastic bags with wet cotton wool (soaked with sterile water) to provide continuous high relative humidity (>90%) and incubated at 25°C in darkness.

Conidial suspension method. To produce abundant conidia, single-conidium isolates were grown on potato-carrot agar (PCA; 20 g of potato, 20 g of carrot, 20 g of agar, and 1 liter of distilled water) for 2 weeks under cool-white fluorescent light with a regime of alternating 8 h of light and 16 h of darkness at 20 to 23°C (Simmons 1998). Suspensions were then prepared by flooding culture dishes with sterile distilled water and disrupting the spores using a sterile scalpel (Harteveld et al. 2014), then filtering with Miracloth (polyester synthetic fiber filter cloth, aperture = 22 to 25 µm; Calbiochem, Merck Co. Inc., Darmstadt, Germany) to remove hyphae and transferring conidial suspensions to three to four 1.5-ml plastic centrifuge tubes. The suspensions were then refrigerated until use, which was within 2 h of preparation. Conidial suspensions (1 ml per fruit) were brushed on pericarps of apple fruit with a sterile calligraphy brush. All fruit were cleaned with pure water and wiped gently with cotton wool dipped in 75% ethanol for 30 to 40 s to remove possible adhering microbes before inoculation.

Cultivars of apple used for conidial inoculation were Golden Delicious, Red Delicious, Gala, and Fuji. Inoculations were performed on wounded and nonwounded leaves, carpels, and pericarps. Sterilized insect mounting pins (size 2; length = 40 mm and width = 0.38 mm; Beijing Zhecheng Technology Co. Ltd., Beijing) were used to make 8 to 10 wounds (epidermal punctures) located arbitrarily on fruit; 100-µl conidial suspensions were applied to each wound. Five individual fruit were inoculated by each method. Symptom development was photographed 10 days after incubation at 25°C in darkness. Three replicates were prepared for per apple cultivar.

Leaves (fifth to sixth leaves, counting proximally from the shoot tip) and carpels attached to the excised fruit of Golden Delicious, Red Delicious, Gala, and Fuji apple were also inoculated. Conidial suspensions (100 µl each) were placed on carpels or on each abaxial

side of the excised leaves. Five samples of each tissue type per apple cultivar were inoculated by each method. The inoculated fruit were wrapped with plastic cling wrap, whereas inoculated leaves were placed in a sealed plastic bag with wet cotton wool to provide continuous high relative humidity (>90%); both were incubated at 25°C in darkness. Symptom development was assessed 5 days after inoculation for carpels and 10 days after inoculation for leaves.

DNA extraction, polymerase chain reaction, and sequencing.

The protocol of Pryor and Gilbertson (2000) was used to extract genomic DNA from mycelium of single-conidium-origin colonies grown on PDA plates at 25°C in darkness for 7 days. Six genes were amplified: GAPDH, RPB2, TEF1, Alta1, MAT1-2-1, and AGA. Primers used for amplification and sequencing were *gpd1* and *gpd2* for GAPDH (Berbee et al. 1999), *fRPB2-5F2* (Sung et al. 2007) and *fRPB2-7cR* (Liu et al. 1999) for RPB2, *EF1-728F* and *EF1-986R* for TEF1 (Carbone and Kohn 1999), *Alta1-F* and *Alta1-R* for Alta1 (Hong et al. 2005), *MAT1-2F* and *MAT1-2R* for MAT1-2 (Geng et al. 2014), and *AlphaboxF* and *AlphaboxR* for AGA (Geng et al. 2014). The amplification reaction mixtures of GAPDH, RPB2, TEF1, and Alta1 gene, consisting of 1× polymerase chain reaction (PCR) buffer, 2 mM MgCl₂, 0.2 mM each dNTP, 0.4 µM each primer, 1 µl of template DNA (2 µl for RPB2 and TEF1), and 1 U of Taq polymerase (2 U for RPB2), were made up to a total volume of 25 µl with sterile water. The PCR mixture for MAT1-2-1 and AGA gene amplifications contained 5 µl of 10× ThermoPol reaction buffer (200 mM Tris-HCl [pH 8.3], 100 mM KCl, 100 mM [NH₄]₂SO₄, 20 mM MgSO₄, and 1% Triton X-100), 5 µl of 10 mM MgSO₄, 20 ng of template genomic DNA, 0.4 µM each primer, 4 µl of 2.5 mM dNTP, and 0.5 U of AmpliTaq polymerase; total volume was adjusted to 50 µl with deionized water (Geng et al. 2014). The PCR were performed on a Bio-Rad PCR System PTC-200TM. Conditions for PCR amplification consisted of an initial denaturation step of 5 min at 96°C followed by 30 cycles of 1 min at 96°C, 1 min at 56°C, and 45 s at 72°C for GAPDH; 5 min at 94°C followed by 40 cycles of 30 s at 94°C, 30 s at 59°C, and 45 s at 72°C for TEF1; 35 cycles of 1 min at 94°C, 30 s at 55°C, and 30 s at 72°C for Alta1 and AGA; 1 min at 94°C, 30 s at 58°C, and 1 min at 72°C for MAT1-2; followed by a final elongation

Table 1. Distinguishing characteristics of this new species and similar well known species of *Ulocladium* under growth conditions^a

Species	Shape	Size (µm)	Conidia		Conidial wall	Conidiophores (µm) ^b
			Transverse septa	Longitudinal or oblique septa		
<i>Alternaria malicola</i> CGMCC3.18704	Spheroidal or ellipsoid to long-ellipsoid with a slightly cuspidal top	16–35 × 8–16	1–5	1–3	Verrucose protuberances or densely tuberculate	10–60 × 3.0–4.5
<i>A. alternariae</i> CBS 116328	Obovoid to broadly obovoid	18–35 × 10–20	1–5	1–3	Smooth or slightly verrucose	16–70 × 2.5–4.0
<i>A. obovoidea</i> CBS 101229	Broadly obovoid with a conspicuously pointed base	20–28 × 12.5–17	3–4	1–4	Punctulate to verrucose	45–100 × 3.5–6.0
<i>A. terricola</i> CBS 202.67	Obovoid to broadly ellipsoid	13.5–22.5 × 10–18.5	1–2	0–1	Smooth or slightly verrucose	30–100 × 4.0–5.0
<i>Ulocladium allii-tuberosi</i> CBS 124112	Long-ellipsoid	15–45.5 × 8.5–15	1–3	0–3	Smooth	85–130 × 4.5–5.5
<i>U. chartarum</i> CBS 200.67	Ellipsoid or obovoid	20–35.5 × 10–20	1–4	0–2	Smooth or verrucose	40–65 × 3.0–5.0
<i>U. consortiale</i> CBS 201.67	Obovoid or long-ellipsoid	20.5–41 × 12–15	1–5	1–4	Smooth or slightly verrucose	19–74 × 3.0–4.5
<i>U. dauci</i> CBS 102062	Obovoid or spheroidal	20.5–24.5 × 11–14.5	1–2	1–2	Densely tuberculate to pustulate	50–65 × 4.0–5.0
<i>U. multiforme</i> CBS102060	Obovoid or spheroidal	20–25 × 15–18	1–2	1–2	Densely tuberculate to pustulate	55–75 × 4.5–5.0
<i>U. oudemansii</i> CBS 137.81	Obovoid to broadly ellipsoid	25–30 × 11–14	3–5	0–2	Smooth or verrucose	110–200 × 5.0–7.5
<i>U. zantedeschiae</i> CBS 124113	Ellipsoid or obovoid	15.5–32.5 × 7.5–14	1–5	1–5	Smooth or verrucose	36–71 × 3.0–5.0

^a New species in this study are printed in bold.

^b Dimensions of conidiophores (µm, length × width at base).

Table 2. *Alternaria* isolates used in the study with the corresponding GenBank accession numbers of gene sequences^a

Species name	Strain number	GenBank accession numbers ^b					
		RPB2	TEF1	GAPDH	Alta1	MAT1-2-1	AGA
<i>Alternaria alternantherae</i>	CBS 124392	KC584374	KC584633	KC584096	...	...	...
<i>A. alternata</i>	CBS 916.96	KC584375	KC584634	AY278808	AY563301	AB009452	AB239917
<i>A. argyranthemii</i>	CBS 116530	KC584378	KC584637	KC584098	...	...	...
<i>A. armoraciae</i>	CBS 118702	KC584379	KC584638	KC584099	...	...	...
<i>A. brassicicola</i>	CBS 118699	KC584383	KC584642	KC584103	...	...	...
<i>A. capsici-annui</i>	CBS 504.74	KC584385	KC584644	KC584105	...	...	...
<i>A. cheiranthi</i>	CBS 109384	KC584387	KC584646	KC584107	...	...	...
<i>A. cinerariae</i>	CBS 116495	KC584389	KC584648	KC584109	...	...	...
<i>A. dianthicola</i>	CBS 116491	KC584394	KC584653	KC584113	...	...	...
<i>A. ellipsoidea</i>	CBS 119674	KC584396	KC584655	KC584115	...	...	...
<i>A. eryngii</i>	CBS 121339	KC584397	KC584656	AY562416	...	...	...
<i>A. ethzedia</i>	CBS 197.86	KC584398	KC584657	AY278795	...	...	...
<i>A. gaisen</i>	CBS 632.93	KC584399	KC584658	KC584116	...	...	...
<i>A. gypsophilae</i>	CBS 107.41	KC584401	KC584660	KC584118	...	...	...
<i>A. infectoria</i>	CBS 210.86	KC584404	KC584662	AY278793	...	...	...
<i>A. japonica</i>	CBS 118390	KC584405	KC584663	KC584121	...	...	...
<i>A. longipes</i>	CBS 540.94	KC584409	KC584667	AY278811	...	...	...
<i>A. mimicula</i>	CBS 118696	KC584411	KC584669	AY562415	...	...	...
<i>A. molestata</i>	CBS 548.81	KC584412	KC584670	KC584125	...	...	...
<i>A. panax</i>	CBS 482.81	KC584417	KC584675	KC584128	...	...	...
<i>A. porri</i>	CBS 116698	KC584421	KC584679	KC584132	...	...	...
<i>A. radicina</i>	CBS 245.67	KC584423	KC584681	KC584133	...	...	...
<i>A. selini</i>	CBS 109382	KC584426	KC584684	AY278800	...	...	...
<i>A. solani</i>	CBS 116651	KC584430	KC584688	KC584139	...	...	...
<i>A. sonchi</i>	CBS 119675	KC584433	KC584691	KC584142	...	...	...
<i>A. tenuissima</i>	CBS 918.96	KC584435	KC584693	AY278809	...	...	...
<i>A. thalictrigena</i>	CBS 121712	KC584436	KC584694	KC584144	...	...	...
<i>A. triglochiticola</i>	CBS 119676	KC584437	KC584695	KC584145	...	...	...
<i>A. vaccariicola</i>	CBS 118714	KC584439	KC584697	KC584147	...	...	...
<i>A. penicillata</i>	CBS 116608	KC584440	KC584698	FJ357299	...	...	...
<i>A. cetera</i>	CBS 121340	KC584441	KC584699	AY562398	...	...	...
<i>A. embellisia</i>	CBS 339.71	KC584449	KC584708	KC584155	...	...	...
<i>A. conoidea</i>	CBS 132.89	KC584452	KC584711	FJ348227	...	...	...
<i>A. eureka</i>	CBS 193.86	KC584456	KC584715	JN383471	...	...	...
<i>A. lolii</i>	CBS 115266	KC584460	KC584719	JN383473	...	...	...
<i>A. botryospora</i>	CBS 478.90	KC584461	KC584720	AY278831	...	...	...
<i>A. phragmospora</i>	CBS 274.70	KC584462	KC584721	JN383474	...	...	...
<i>A. scirpicola</i>	CBS 481.90	KC584469	KC584728	KC584163	...	...	...
<i>A. alternariae</i>	CBS 126989	KC584470	KC584730	AY278815	...	...	...
<i>Stemphylium herbarum</i>	CBS 191.86	KC584471	KC584731	AF443884	...	...	...
<i>A. leucanthemi</i>	CBS 422.65	KC584473	KC584733	KC584165	...	...	...
<i>A. alternariae</i>	CBS 116328	...	...	AY278815	AY563316	HQ906726	HQ906746
<i>A. aspera</i>	CBS 115269	KC584474	KC584734	KC584166	...	...	...
<i>A. atra</i>	CBS 195.67	KC584475	KC584735	KC584167	AY563318	HQ906727	HQ906747
<i>Ulocladium allii-tuberosi</i>	CBS 124112	...	...	KF533907	KF533896	KF533884	HQ906745
<i>U. arborescens</i>	CBS 115269	...	...	KF533910	KF533899	KF533887	...
<i>A. bornmuelleri</i>	DAOM 231361	KC584491	KC584751	FJ357305	...	...	...
<i>A. botrytis</i>	CBS 197.67	KC584476	KC584736	KC584168	...	...	...
<i>Alternaria</i> sp.	CBS 198.67	KC584477	KC584737	KC584169	AY563317	KF533888	HQ906748
<i>A. brassicae-pekinensis</i>	CBS 121493	KC584478	KC584738	KC584170	KF533892	HQ906728	HQ906749
<i>A. cantlous</i>	CBS 123007	KC584479	KC584739	KC584171	EU684146	KF533881	HQ906750
<i>U. capsicumae</i>	CBS 120006	KC584480	KC584740	AY762950	EU684149	HQ906740	HQ906751
<i>U. castaneae</i>	CBS 124390	...	...	KF533902	KF533891	HQ906729	HQ906752
<i>U. chartarum</i>	CBS 200.67	...	...	AY278819	AY563319	HQ906741	HQ906753
<i>U. consortiale</i>	CBS 201.67	...	...	FJ266494	FJ266509	HQ906730	HQ906754
<i>A. consortialis</i>	CBS 104.31	KC584482	KC584742	KC584173	...	...	...
<i>A. cucurbitae</i>	CBS 483.81	KC584483	KC584743	AY562418	AY563315	HQ906731	HQ906755
<i>U. dauci</i>	CBS 102062	...	...	FJ266495	FJ266510	HQ906732	HQ906756
<i>U. microsporium</i>	CBS 124391	...	...	KF533901	KF533890	KF533880	HQ906758
<i>A. multiformi</i>	CBS 102060	KC584484	KC584744	KC584174	...	...	...
<i>U. oblong-obovoideum</i>	CBS 126317	...	...	KF533904	KF533893	HQ906734	HQ906759
<i>A. obovoidea</i>	CBS 101229	KC584485	KC584745	FJ266498	AY563315	HQ906731	HQ906755
<i>U. oudemansii</i>	CBS 137.81	...	...	EU684152	EU684148	HQ906742	HQ906761
<i>U. septosporum</i>	CBS 109.38	...	...	FJ266500	FJ266515	HQ906743	HQ906764
<i>A. heterospora</i>	CBS 123376	KC584488	KC584748	KC584176	EU855805	KF533885	HQ906765
<i>U. sorghi</i>	CBS 126315	...	...	KF533909	KF533898	KF533886	HQ906766
<i>A. subcucurbitae</i>	CBS 121491	KC584489	KC584749	EU855803	EU855807	HQ906736	HQ906767
<i>A. terricola</i>	CBS 202.67	KC584490	KC584750	KC584177	FJ266516	HQ906744	HQ906768
<i>U. zantedeschiae</i>	CBS 124113	...	...	KF533900	KF533889	HQ906737	HQ906769
<i>A. malicola</i> QXHD_10	CGMCC3.18704	MF426957	MF426959	MF426953	MF426951	MF426955	MF426949
<i>A. malicola</i> BSBD_86	...	MF426956	MF426958	MF426952	MF426950	MF426954	MF426948

^a New species and sequences generated in this study are printed in bold.^b RPB2 = RNA polymerase II second largest subunit (RPB2) gene, TEF1 = translation elongation factor 1- α gene, GAPDH = glyceraldehyde-3-phosphate dehydrogenase gene, Alta1 = major allergen alta1 gene, MAT1-2-1 = mating type 1-2-1 gene, and AGA = G protein α subunit AGA1 gene.

step of 7 min at 72°C. The partial RPB2 gene was obtained by using a touchdown PCR protocol of 5 cycles of 45 s at 94°C, 45 s at 60°C, and 2 min at 72°C; followed by 5 cycles with a 58°C annealing temperature and 30 cycles with a 54°C annealing temperature. Purifying and automated sequencing of the PCR products was performed at Organism Technology, Shanghai, China with forward and reverse primers.

Sequence alignment and phylogenetic analysis. All sequences generated in this study, as well as other sequences of *Alternaria* spp. and the outgroup sequences obtained from the National Center for Biotechnology Information's GenBank sequence database (Table 2), were imported into BioEdit v.7.0.5.2 (Hall 1999) for comparison and analysis. *Stemphylium herbarum* isolate CBS 191.86 and *A. alternata* isolate CBS 916.96 were used to root the three-locus GAPDH-RPB2-TEF1 and GAPDH-Alt1-MAT (1-2-1)-AGA phylogenetic trees. Preliminary alignments of the multiple sequences were conducted using MUSCLE (Edgar 2004), with manual adjustment using BioEdit for visual improvement where necessary.

We performed Bayesian and maximum-likelihood (ML) analyses of both the individual data partitions and the combined aligned dataset. Bayesian analyses were performed with MrBayes v. 3.2.1 (Ronquist and Huelsenbeck 2003). The Markov-Chain Monte Carlo analysis used four chains and started from a random tree topology. The sample frequency was set at 100 and the temperature value of the heated chain was 0.1. The temperature value was lowered to 0.05 when the average standard deviation of split frequencies did not fall below 0.01 after 5 million generations (RPB2 and Pleosporineae

phylogeny). Burn-in was set to 25% after which the likelihood values were stationary. ML analyses including 1,000 bootstrap replicates were run using RAxML v. 7.2.6 (Stamatakis 2006). The best nucleotide substitution model of different gene regions was selected by using MrModelTest v.2.3 (Nylander 2004).

Sequences of an isolate of *S. herbarum* (CBS 191.86) were used as the outgroup in the *Alternaria* phylogeny and sequences of *A. alternata* (CBS 916.96) were used in the *Ulocladium* phylogeny. The resulting trees were printed with TreeView v. 1.6.6 (Page 1996) and alignments and representative trees were deposited in TreeBASE (<https://www.treebase.org>).

Morphology of isolates. Hyphal tips of isolate QXHD_10 from mycelial growth that developed on PDA slants were transferred to PCA in plastic Petri dishes (Simmons and Roberts 1993) in vitro and descriptions of colony morphology were made after incubation for 7 days under cool-white fluorescent light with a regime of alternating 8 h of light and 16 h of darkness at 20 to 23°C (Simmons 1998). In order to measure and observe fungal structures, a sellotape technique was used for making slide preparations (Crous et al. 2009) with Shear's medium (3 g of potassium acetate, 60 ml of glycerin, 90 ml of 95% ethanol, and 150 ml of water) as mounting fluid. At least 30 measurements ($\times 1,000$ magnification) were made by Nikon NIS-Elements D 3.0 imaging software. The length/width ratio (L/W) of ascospores was measured according to the method of Verkley (1999) and the average was calculated.

To visualize how conidia, conidial hila, and conidiogenous scars were produced, scanning electron micrographs were taken

Fig. 1. Symptoms on pericarps of Golden Delicious, Red Delicious, and Gala inoculated with *Alternaria malicola* isolate QXHD_10. **A** to **D**, Golden Delicious; **E** to **H**, Red Delicious; **I** to **L**, Gala; and **M** to **P**, Fuji. **A**, **C**, **E**, **G**, **I**, **K**, **M**, and **O** were inoculated without wounding; **B**, **D**, **F**, **H**, **J**, **L**, **N**, and **P** were wound inoculated. **A**, **B**, **E**, **F**, **I**, **J**, **M**, and **N** were inoculated by hyphae; **C**, **D**, **G**, **H**, **K**, **L**, **O**, and **P** were inoculated by conidium suspensions. All symptoms were photographed 10 days after inoculation. Scale bars: **G**, **H**, **O**, and **P** = 1.0 cm; others = 1.5 cm.

of isolate QXHD_10. After cultures were maintained on PCA for a week in darkness at 25°C, 5-mm-diameter fragments of PCA with attached hyphae and conidia were fixed in 4% glutaraldehyde for 6 h and 1% osmium tetroxide in 0.1 M cacodylate buffer (pH 6.8) for 2 h, followed by a series of ethanol rinses, after which the hyphae and conidia were dehydrated in a critical-point dryer, sputter coated with gold, and examined under a scanning electron microscope (Joel JSM 6360LV) at accelerating voltages of 15 and 25 kV.

Results

Pathogenicity tests. Ten days after inoculation with conidial suspensions, sunken and water-soaked spots appeared on both wounded and nonwound pericarps (Fig. 1). The lesions were larger (diameter = 0.5 to 3.5 cm) and more ridged in the wound-inoculation assays than in nonwound-inoculation assays (diameter = 0.2 to 1.5 cm) on Golden Delicious, Red Delicious, and Gala. The symptoms of both wound-inoculation assays (diameter = 0.5 to 2.0 cm) and nonwound-inoculation assays (diameter = 0.2 to 0.8 cm) on Fuji were slighter than those on the other three cultures.

Isolate QXHD_10 caused characteristic moldy core symptoms on apple when inoculated with either hyphae or conidial suspensions. The moldy core symptoms on carpels were observed 5 days after inoculation. Lesions on Fuji were smaller than those on Golden Delicious, Red Delicious, or Gala (Fig. 2).

Ten days after leaves were inoculated with mycelial plugs, slight chlorosis (diameter = 0.6 to 1.5 cm) appeared around sites of inoculation

with mycelial plugs on Golden Delicious, Red Delicious, and Gala in the detached-leaf assay, whereas no lesions were observed after inoculation by conidial suspensions. On Fuji, however, no symptoms were observed when inoculated with either mycelial plugs or conidial suspensions (Fig. 3).

Phylogeny. The combined gene alignment of GAPDH, RPB2, and TEF1 that included 1,848 characters (including gaps) was used in the phylogenetic analysis of isolates BSBD_86 and QXHD_10. Sequences of 59 isolates listed in Table 2 were obtained from GenBank, including the outgroup *S. herbarum* (CBS 191.86), were used to construct a phylogeny of closely related *Alternaria* genera.

For Bayesian inference, this dataset included 1,848 characters (including gaps). The HKY+I+G model was selected for GAPDH and the GTR+I+G model for RPB2 and TEF1. The Bayesian analysis lasted 405,000 generations and the consensus trees and posterior probabilities (PP) were calculated from the 812 trees left after discarding 406 trees (the first 25% of generations). The Bayesian analysis produced phylogenetic trees with the same topology as the RAXML tree; therefore, only the Bayesian tree is shown (Fig. 4), with RAXML bootstrap support values (MLBS) plotted on the tree. The phylogenetic trees (Fig. 4) delimited 24 clades. Isolates BSBD_86 and QXHD_10 in this study were clustered into sect. *Ulocladioides*.

The combined gene alignment of the GAPDH-Alta1-MAT(1-2-1)-AGA dataset consisted of 24 isolates selected based on species of *Ulocladium* that were similar in morphology, as well as *A. alternata* (CBS 916.96) as the outgroup. This dataset included 3,367 characters (including gaps). The GTR+I+G model was selected for the Bayesian

Fig. 2. Symptoms on fruit carpels of Golden Delicious, Red Delicious, and Gala inoculated with *Alternaria malicola* isolate QXHD_10. **A** to **D**, Golden Delicious; **E** to **H**, Red Delicious; **I** to **L**, Gala; and **M** to **P**, Fuji. **A**, **C**, **E**, **G**, **I**, **K**, **M**, and **O** were inoculated without wounding; **B**, **D**, **F**, **H**, **J**, **L**, **N**, and **P** were wound inoculated. **A**, **B**, **E**, **F**, **I**, **J**, **M**, and **N** were inoculated by hyphae; **C**, **D**, **G**, **H**, **K**, **L**, **O**, and **P** were inoculated by conidium suspensions. All symptoms were photographed 5 days after inoculation. Scale bars = 1.0 cm.

analysis. This analysis lasted 480,000 generations and the consensus trees and PP were calculated from the 962 trees remaining after discarding 481 trees (the first 25% of generations) for burn-in. The Bayesian analyses produced phylogenetic trees with the same topology as the RAxML tree; therefore, only the Bayesian tree is presented (Fig. 5), with ML bootstrap values plotted on the tree. The Bayesian PP > 0.95 and MLBS > 50% were plotted against the tree (Fig. 5). Isolates BSBD_86 and QXHD_10 in this study were categorized from other species with high bootstrap support (1.0/90%).

Taxonomy. *Alternaria malicola* G. Y. Sun & Jiling Dang, sp. nov.

Colonies on PCA: Hyphae thin- to slightly thin-walled, hyaline to pale grayish, smooth, vegetatively branched, septate, ramose, aerial, 2.5 to 4 μm wide. Conidiophores produced laterally or terminally on the hyphae, erect or ascending, simple or branched, geniculate, pale brown, smooth, 1 to 4 septa, 10 to 60 $\times$ 3 to 4.5 μm , arising mostly at right angles from undifferentiated hyphae, conspicuous scars after conidia have fallen off (Figs. 6C to F and 7A and B). Conidia numerous, acrogenous or acropleurogenous, sympodial proliferations, solitary, noncatenulate. Neonatal conidia subhyaline or pale grayish, ellipsoidal or subacute, thick-walled, 1 to 3 septa, with few or no protuberance (Figs. 6C and E and 7C). Mature conidia brown to dark chocolate-brown, spheroidal or ellipsoid to long-ellipsoid, nonbeaked or with slightly cuspidal top, verrucose protuberances or densely tuberculate or irregular shaped protuberances, that were more common on mature conidia, with 1 to 5 transverse septa and 1 to 3 longitudinal septa or oblique septa, 16 to 35 $\times$ 8 to 16 μm , med. 25.5 $\times$ 12 μm , L/W = 2 to 2.2, med. 2.1 (Figs. 6C to F and 7E and F).

Colonies on PDA brown to dark brown, rapidly covering the Petri dish within 7 days. Concentric zonation of growth pronounced, and sporulation abundant. Mycelium subhyaline to dilute yellowish brown.

Holotype: CHINA. Shaanxi: Qianxian. From fruit of apple (*Malus $\times$ domestica*) 'Fuji', Oct 2015, J. L. Dang, (Holotypus HMAS 247403, ex-type culture CGMCC3.18704 = QXHD_10).

Specimen examined: China, Shaanxi Province, Baishui County. From fruit of apple (*Malus $\times$ domestica*) 'Fuji', Oct 2015, J. L. Dang, culture BSBD_86.

Notes: Distinguishing characteristics of this new species and other similar well-known species of *Ulocladium* are shown in Table 1. In conidium morphology, this new fungus is distinct from *U. capsici* (Xue and Zhang 2007), *U. leve* (Liu and Zhang 2008), *U. consortiale* (Simmons 1967), *U. oblong-obovoideum* (Zhang and Zhang 2002), *U. sorghi* (Zhang and Zhang 2006), *U. castaneae* (Zhang and Zhang 2002), *U. populi* (Shipunov et al. 2009), *A. cantlous* (Wang et al. 2010), *A. subcucurbitae* (Wang et al. 2008), and *U. microsporum* (Moubasher and Abdei-Hafez 1977) in having coarse or verrucose protuberances, whereas the other species possess conidial surfaces that are rarely or only slightly verrucose. The conidia of *U. lignicola* (Nagaraju et al. 2009) have 3 to 6 transverse septa with small tubercles and those of *U. gpararwalii* (Nagaraju et al. 2009) have 0 to 3 transverse septa, whereas our new species has 1 to 5 transverse septa. Conidial ornamentation similar to that of our new species occurs in *U. populi* (Shipunov et al. 2009); however, the latter conidia have more septa (3 to 6 transverse septa and 1 to 4 longisepta) than our new species. The conidia of *U. arborescens* (Simmons 2004) may remain solitary or more commonly proliferate in branching chains, and

Fig. 3. Symptoms on leaves of Golden Delicious, Red Delicious, and Gala inoculated by *Alternaria malicola* isolate QXHD_10. **A** to **D**, Golden Delicious; **E** to **H**, Red Delicious; **I** to **L**, Gala; and **M** to **P**, Fuji. **A**, **C**, **E**, **G**, **I**, **K**, **M**, and **O** were inoculated without wounding; **B**, **D**, **F**, **H**, **J**, **L**, **N**, and **P** were wound inoculated. **A**, **B**, **E**, **F**, **I**, **J**, **M**, and **N** were inoculated by hyphae; **C**, **D**, **G**, **H**, **K**, **L**, **O**, and **P** were inoculated by conidium suspensions. All symptoms were photographed 10 days after inoculation. Scale bars = 1.0 cm.

Fig. 4. Bayesian 50% majority rule consensus tree based on the glyceraldehyde-3-phosphate dehydrogenase, RNA polymerase second largest subunit, and translation elongation factor 1- α sequences of 59 strains representing the *Alternaria* complex. The Bayesian posterior probabilities (PP) and RAxML bootstrap support values (ML) are given at the nodes (PP/ML). The tree was rooted to *Stemphylium herbarum* (CBS 191.86). Monotypic lineages are indicated by black dots. New sequences (BSBD_86 and QXHD_10) generated in this study are printed in bold.

Fig. 5. Bayesian 50% majority rule consensus tree based on the glyceraldehyde-3-phosphate dehydrogenase, allergen Alta1, mating type protein 1-2-1, and the AGA1 gene sequences of strains representing *Ulocladium*. The Bayesian posterior probabilities (PP) and RAxML bootstrap support values (ML) are given at the nodes (PP/ML). The tree was rooted to *Alternaria alternata* (CBS 916.96). New sequences (BSBD_86 and QXHD_10) generated in this study are printed in bold.

secondary spore production is by means of well-defined secondary conidiophores; both are distinct from our new species, which proliferates without branching chains. Additional distinctions of the new species from other species of *Ulocladium* are shown in Table 1.

Discussion

The genus *Alternaria* was originally described by Nees von Esenbeck (1816), based on *A. tenuis* as the type species. In the past,

classification and identification of *Alternaria* spp. were dominated by description of morphological characteristics of conidia and conidiation patterns (Simmons 1967). Molecular phylogeny was used to supplement morphology-based identification (Kusaba and Tsuge 1995; Peever et al. 2004). Sequence analysis of the GAPDH, RPB2, and TEF1 gene regions were used most widely to analyze phylogenetic diversity among the *Alternaria* spp. groups. Currently, the genus *Alternaria* is considered to be a complex comprising 24 internal clades and

Fig. 6. Microscopic morphology of *Alternaria malicola* isolate QXHD_10. **A**, Colonies on apple in close-up view; **B**, colony on potato dextrose agar after 1 week; and **C** to **F**, sympodial conidiophores and conidia on potato carrot agar. Scale bars: **A** = 2 mm, **B** = 5 mm, and **C** to **F** = 10 μm.

Fig. 7. Scanning electron micrographs of *Alternaria malicola* isolate QXHD_10 showing conidiophores and scars, conidial ornamentation. **A** and **B**, Conidiophores and scars; **C**, neonatal conidia; and **D** to **F**, mature conidia and ornamentation. Scale bars: **A**, **B**, and **C** = 5 μm and **D**, **E**, and **F** = 10 μm.

six monotypic lineages. The 24 internal clades were treated as sections, which is a continuation of a recent proposal for the taxonomic treatment of lineages in *Alternaria* (Woudenberg et al. 2013). *Alternaria* sect. *Alternaria* included 11 phylogenetic species and one species complex based on genome and transcriptome comparisons and molecular phylogenies. In total, 35 morphospecies, which cannot be distinguished based on multigene phylogeny, are synonymized under *A. alternata* (Woudenberg et al. 2015).

The genus *Ulocladium* was first created by Preuss (1851), with *U. botrytis* designated as the type species. In the past, *Ulocladium* was equal with *Alternaria* and *Stemphylium* in taxonomic position, and shared several characteristics of the juvenile and mature shapes of conidia, as well as modes of proliferation of conidiophores and conidia (Simmons 1967). In combination with a review of literature and morphology, the species within the *Alternaria* clade are all recognized here as *Alternaria* sensu stricto, placing the genus *Ulocladium* in synonymy with *Alternaria*. Suggestions to synonymize *Ulocladium* with *Alternaria* have been made several times (Pryor and Gilbertson 2000), and the genus *Ulocladium* has been assigned to the *Alternaria* complex (Woudenberg et al. 2013). Species of *Ulocladium* were clustered into three sister clades of this complex, now called sect. *Ulocladium*, sect. *Ulocladioides*, and sect. *Pseudoulocladium* (Woudenberg et al. 2013). Our isolates QXHD_10 and BSBD_86 were clustered into sect. *Ulocladioides*.

The taxonomic classification of species of *Ulocladium* is based primarily on morphological characteristics, including conidial shape, size range, septation, ornamentation, catenulation, and presence or absence of a beak (apical extension) (Simmons 1967). However, morphological characteristics of species of *Ulocladium* can vary when grown under different cultural conditions, and have caused difficulty in establishing new taxa, even when comparing the sporulation elements under standardized conditions (Simmons 1998). Therefore, it became important to use molecular genetic analysis for further examination of the relationships among *Ulocladium* spp. The nuclear internal transcribe spacer, small subunit rRNA gene (mt SSU), GAPDH, and *Alta1* gene sequences have been used extensively in the resolution of phylogenetic relationships among *Ulocladium* spp. (Hong et al. 2005; Lawrence et al. 2012, 2013; Pryor and Gilbertson 2000). Later, using the MAT genes to identify and characterize the species of *Ulocladium* was suggested (Geng et al. 2014). MAT genes appear to evolve faster than some other regions in the genome, and high interspecific variation and low dissimilarity within species make the MAT genes more appropriate than other regions of the genome for species delimitation (Turgeon 1998; Voigt et al. 2005).

Species of *Ulocladium* colonize a variety of habitats, including soil, air, and plants (Preuss 1851; Saccardo 1886; Simmons 1967, 1998). A few species of *Ulocladium* isolated from plant leaves are associated with leaf lesions. *U. cucurbitae* incites a widespread leaf spot disease of cucurbits (Simmons 1998). *U. chartarum* is the causal agent of a leaf necrosis on *Quercus pubescens* (Vannini and Vettraino 2000). *U. capsicum* was discovered on leaves of *Capsicum annuum* L., Wang described species *U. subcucurbitae* and *U. brassicae* from diseased leaves of *Chenopodium glaucum* and *Brassica pekinensis* (Wang et al. 2008) and *U. cantlous* from *Cucumis* sp. (Wang et al. 2010). *U. obovoideum* was discovered on lemon (Simmons 1990) and *Citrus sinensis* (Zhang and Zhang 2002). However, no species has been described from apple.

Combining results of phylogeny, morphology, and pathogenicity assessments on apple in our study indicated that isolate QXHD_10 is a newly described pathogen of black dot of apple. This species can cause symptoms on leaves and carpels of apple in vitro as well. However, just two isolates were obtained and the symptoms have not been found in the field. We speculate that the species is a potential or secondary pathogen associated with all three diseases: black dot, *Alternaria* leaf blotch, and moldy core. The results of the present study further broaden understanding of the diversity of fungi in the *Alternaria* complex, and are likely to be useful to plant pathologists and fruit breeders in improving management of these diseases. However,

further studies are required to characterize the association of *A. mali-cola* with leaf lesions and moldy core of apple and its pathogenic potential.

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Literature Cited

- Andersen, B., Hansen, M. E., and Smedsgaard, J. 2005. Automated and unbiased image analyses as tools in phenotypic classification of small-spored *Alternaria* spp. *Phytopathology* 95:1021-1029.
- Berbee, M. L., Pirseyedi, M., and Hubbard, S. 1999. *Cochliobolus* phylogenetics and the origin of known, highly virulent pathogens, inferred from ITS and glyceraldehyde-3-phosphate dehydrogenase gene sequences. *Mycology* 91: 964-977.
- Bottalico, A., and Logrieco, A. 1992. *Alternaria* plant diseases in Mediterranean countries and associated mycotoxins. Pages 209-232 in: *Alternaria: Biology, Plant Diseases and Metabolites*. J. Chelkowski and A. Visconti, eds. Elsevier, Amsterdam.
- Carbone, I., and Kohn, L. M. 1999. A method for designing primer sets for speciation studies in filamentous ascomycetes. *Mycology* 91:553-556.
- Combrink J. C., Kotzé, J. M., Wehner, F. C., and Grobbelaar, C. J. 1985. Fungi associated with core rot of staking apples in South Africa. *Phytophylactica* 17:81-83.
- Crous, P. W., Gams, W., Stalpers, J. A., Robert, V., and Stegehuis, G. 2004. MycoBank: An online initiative to launch mycology into the 21st century. *Stud. Mycol.* 50:19-22.
- Crous, P. W., Verkley, G. J. M., Groenewald, J. Z., and Samson, R. A. 2009. *Fungal Biodiversity*. CBS Laboratory Manual Series 1. CBS-KNAW Fungal Biodiversity Centre, Utrecht, The Netherlands.
- Edgar, R. C. 2004. MUSCLE: Multiple sequence alignment with high accuracy and high throughput. *Nucleic Acids Res.* 32:1792-1797.
- Filajdic, N., and Sutton, T. B. 1991. Identification and distribution of *Alternaria mali* on apples in North Carolina and susceptibility of different varieties of apples to *Alternaria* blotch. *Plant Dis.* 75:1045-1048.
- Gao, L. L., Zhang, Q., Sun, X. Y., Jiang, L., Zhang, R., Sun, G. Y., Zha, Y. L., and Biggs, A. R. 2013. Etiology of moldy core, core browning, and core rot of Fuji apple in China. *Plant Dis.* 97:510-516.
- Geng, Y., Li, Z., Xia, L. Y., Wang, Q., Hu, X. M., and Zhang, X. G. 2014. Characterization and phylogenetic analysis of the mating-type loci in the asexual ascomycete genus *Ulocladium*. *Mycologia* 106:649-665.
- Guo, Y. Z., Sun, G. Y., Gao, B. W., Li, C. Y., Zhang, P. L., and Lei, X. L. 2005. Studies on the identification of pathogen and the biological characteristics of black-dot disease of bagged apple. *Acta Agric. Boreali-Occident. Sin.* 14: 18-21.
- Hall T. A. 1999. BioEdit: A user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symp. Ser.* 41:95-98.
- Hao, X. A., Wu, Y. F., Zhou, X. M., Yang, Y., Wang, X. M., and Liu, S. B. 2004. Preliminary studies on the identification and occurrence of black-dot disease on bagged apple fruit in Shaanxi. *Acta Agric. Boreali-Occident. Sin.* 13:54-57.
- Hartevel, D. O. C., Akinsanmi, O. A., and Drenth, A. 2014. Pathogenic variation of *Alternaria* species associated with leaf blotch and fruit spot of apple in Australia. *Eur. J. Plant Pathol.* 139:789-799.
- Hong, S. G., Cramer, R. A., Lawrence, C. B., and Pryor, B. M. 2005. *Alta1* allergen homologs from *Alternaria* and related taxa: Analysis of phylogenetic content and secondary structure. *Fungal Genet. Biol.* 42:119-129.
- Hu, T. L., Wang, S. T., Song, P., Zhang, F. Q., Zhen, W. C., and Cao, K. Q. 2006. Primary study on the crucial weather conditions of mass infection of *Alternaria mali* on apple. *J. Hebei Agric. Univ.* 29:63-66.
- Kusaba, M., and Tsuge, T. 1995. Phylogeny of *Alternaria* fungi known to produce host-specific toxins on the basis of variation in internal transcribed spacers of ribosomal DNA. *Curr. Genet.* 28:491-498.
- Lawrence, D. P., Gannibal, P. B., Peever, T. L., and Pryor, B. M. 2013. The sections of *Alternaria*: Formalizing species-groups concepts. *Mycology* 105: 530-546.
- Lawrence, D. P., Park, M. S., and Pryor, B. M. 2012. *Nimbya* and *Embellisia* revisited, with nov. comb for *Alternaria celosiae* and *A. perpunctulata*. *Mycol. Prog.* 11:799-815.
- Li, Y., Zhang, L. Y., Zhang, Z., Cong, P. H., and Cheng, Z. M. 2011. A simple sequence repeat marker linked to the susceptibility of apple to *Alternaria* blotch caused by *Alternaria alternata* apple pathotype. *J. Am. Soc. Hortic. Sci.* 136:109-115.
- Liu, H. M., and Zhang, T. Y. 2008. A new species and two new records of *Ulocladium* from China. *Mycosystema* 27:1-4.
- Liu, Y. J., Whelen, S., and Hall, B. D. 1999. Phylogenetic relationships among ascomycetes: Evidence from an RNA polymerase II subunit. *Mol. Biol. Evol.* 16:1799-1808.
- Moubasher, A. H., and Abdei-Hafez, S. I. 1977. *Ulocladium microsporum* sp. nov. *Trans. Br. Mycol. Soc.* 69:164-166.

- Nagaraju, D., Kunwar, I. K., Manoharachary, C., and Agarwal, D. K. 2009. *Ulocladium gpagarwalii* and *U. lignicola* two new sp. nov. from Andhra Pradesh. Indian Phytopathol. 62:237-239.
- Nees von Esenbeck C. G. D. 1816. Das system der pilze und schwämme. In der Stahelschen buchhandlung, 1816–1817, Würzburg, Germany.
- Nylander, J. A. A. 2004. MrModeltest V2. Program distributed by the author. Evolutionary Biology Centre, Uppsala University.
- Page, R. D. M. 1996. TreeView: An application to display phylogenetic trees on personal computers. Comput. Appl. Biosci. 12:357-358.
- Peever, T. L., Su, G., and Carpenter-Boggs, L. 2004. Molecular systematics of citrus-associated *Alternaria* species. Mycology 96:119-134.
- Preuss, C. G. T. 1851. Übersicht untersuchter pilze, besonders aus der Umgegend von Hoyerswerda. Linnaea 24:99-153.
- Pryor, B. M., and Gilbertson, R. L. 2000. Molecular phylogenetic relationships amongst *Alternaria* species and related fungi based upon analysis of nuclear ITS and mt SSU rDNA sequences. Mycol. Res. 104:1312-1321.
- Ren, P., Ahearn, D. G., and Crow, S. A. 1998. Mycotoxins of *Alternaria alternata* produced on ceiling tiles. J. Ind. Microbiol. Biotechnol. 20:53-54.
- Roberts, J. W. 1924. Morphological characters of *Alternaria mali* Roberts. J. Agric. Res. 27:699-712.
- Ronquist, F., and Huelsenbeck, J. P. 2003. MrBayes 3: Bayesian phylogenetic inference under mixed models. Bioinformatics 19:1572-1574.
- Saccardo, P. A. 1886. Sylloge Fungorum Omnium hucusque cognitorum, Vol. 4. Nabu Press, Padua, Italy.
- Sawamura, K. 1962. Studies on spotted disease of apple 1. Causal agent of *Alternaria blotch*. Bull. Tohoku Natl. Agric. Exp. Stn. 23:163-175.
- Serdani, M., Crous, P. W., Holz, G., and Petrini, O. 1998. Endophytic fungi associated with core rot of apples in South Africa, with specific reference to *Alternaria* species. Sydowia 50:257-271.
- Shipunov, A., Raghavendra, A. K., Ganley, R. J., and Newcombe, G. 2009. *Ulocladium populi* E.G. Simmons, G. Newcombe & A. Shipunov, sp. nov. Fungal Planet 34:1-2.
- Simmons, E. G. 1967. Typification of *Alternaria*, *Stemphylium*, and *Ulocladium*. Mycology 59:67-92.
- Simmons, E. G. 1990. *Alternaria* themes and variations. Mycotaxon 37:79-119.
- Simmons, E. G. 1998. Multiplex conidium morphology in species of the *Ulocladium atrum* group. Can. J. Bot. 76:1533-1539.
- Simmons, E. G. 2004. Novel dematiaceous hyphomycetes. Stud. Mycol. 50:109-118.
- Simmons, E. G., and Roberts, R. G. 1993. *Alternaria* themes and variations. Mycotaxon 48:109-140.
- Spotts, R. A. 1990. Moldy core and core rot. Pages 29-30 in: Compendium of Apple and Pear Diseases. A. L. Jones and H. S. Aldwinckle, eds. American Phytopathological Society, St. Paul, MN.
- Stamatakis, A. 2006. RAxML-VI-HPC: Maximum likelihood-based phylogenetic analyses with thousands of taxa and mixed models, v. 7.03. Bioinformatics 22: 2688-2690.
- Strandberg, J. O. 1992. *Alternaria* species that attack vegetable crops: Biology and options for disease management. Pages 175-208 in: *Alternaria: Biology, Plant Diseases and Metabolites*. J. Chelkowski and A. Visconti, eds. Elsevier, Amsterdam.
- Sung, G. H., Sung, J. M., Hywel-Jones, N. L., and Spatafora, J. W. 2007. A multi-gene phylogeny of *Clavicipitaceae* (Ascomycota, Fungi): Identification of localized incongruence using a combinational bootstrap approach. Mol. Phylogenet. Evol. 44:1204-1223.
- Turgeon, B. G. 1998. Application of mating type gene technology to problems in fungal biology. Annu. Rev. Phytopathol. 36:115-137.
- Vannini, A., and Vettraino, A. M. 2000. *Ulocladium chartarum* as the causal agent of a leaf necrosis on *Quercus pubescens*. For. Pathol. 30:297-303.
- Verkley, G. J. M. 1999. A monograph of the genus *Pezizula* and its anamorphs. Stud. Mycol. 44:1-180.
- Voigt, K., Cozijnsen, A. J., Kroymann, J., Pöggeler, S., and Howlett, B. J. 2005. Phylogenetic relationships between members of the crucifer pathogenic *Leptosphaeria maculans* species complex as shown by mating type (MAT1-2), actin and beta-tubulin sequences. Mol. Phylogenet. Evol. 37:541-557.
- Wang, C. C., Jin, J., Li, B. H., Wang, C. X., Dong, X. L., and Zhang, Z. F. 2014. Pathogens isolated from different symptoms of black dot disease and infection conditions. Acta Agric. Boreali-Sin. 29:136-144.
- Wang, Y., Pei, Y. F., O'Neill, N. R., and Zhang, X. G. 2008. Two new species of *Ulocladium* from Southwest China. Mycology 100:455-459.
- Wang, Y., Pei, Y. F., O'Neill, N. R., and Zhang, X. G. 2010. *Ulocladium cantlous* sp. nov. isolated from northwestern China: Its morphology and molecular phylogenetic position. Mycology 102:374-383.
- Woudenberg, J. H. C., Groenewald, J. Z., Binder, M., and Crous, P. W. 2013. *Alternaria* redefined. Stud. Mycol. 75:171-212.
- Woudenberg, J. H. C., Seidl, M. F., Groenewald, J. Z., De Vries, M., Stielow, J. B., Thomma, B. P. H. J., and Crous, P. W. 2015. *Alternaria* section *Alternaria*: Species, formae speciales or pathotypes? Stud. Mycol. 82:1-21.
- Xu, B. L., Wei, Z. Z., and Wang, X. L. 2000. Symptoms and identification of black dot disease on apple fruit. Plant Prot. 26:6-8.
- Xue, F., and Zhang, X. G. 2007. *Ulocladium capsicum*, a new species identified by morphological and molecular phylogenetic data. Sydowia 59: 161-178.
- Zhang, X. G., and Zhang, T. Y. 2002. Studies on the genus *Ulocladium* Preuss from China I. Mycosystema 21:25-26.
- Zhang, X. G., and Zhang, T. Y. 2006. Studies on the genus *Ulocladium* Preuss from China II. Mycosystema 25:516-520.