Morphology and Molecular Characterization of a Fungus from the *Alternaria alternata* Species Complex Causing Black Spots on *Pyrus sinkiangensis* (Koerle pear)

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ABSTRACT

A small-spored *Alternaria* was found from black spots of stored Koerle pear (*Pyrus sinkiangensis*), one of the economically important fruit in Xinjiang province, China. The morphology is similar to *A. limoniasperae* but obviously different in secondary conidiophores and conidial septa. A phylogenetic analysis using sequence datasets of ITS, GAPDH, TEF1, RPB2, Alt a1, OPA10–2, and EndoPG genes revealed that it belonged to the *Alternaria alternata* complex group. Pathogenicity tests illustrated that the fungus was the causal pathogen of black spot on Koerle pear fruit.

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Koerle pear (*Pyrus sinkiangensis* synonymy *Pyrus sp. nr. communis*) is mainly distributed in north-western China, especially in Xinjiang Autonomous Region [1]. It is one of the important agricultural fruit and primarily exported to the international market because the fruit has a distinctive nice flavor and scent, thin skin, crisp and succulence, fewer dregs, and high volume sugary [2]. In 2011, the incidence of calyx-end black spot disease of Koerle pear reached from 14.7% to 34.8% with high yield loss in some orchards of Shayidong horticultural field, Bazhou, Xinjiang, China, of which casual pathogen is identified as *Alternaria alternata* based on morphology and sequence analyses of ITS, GAPDH, and TEF1 [3].

*Alternaria* is initially described by Nees (1816), which can be found as saprophytic, endophytic, and pathogenic species not only in agricultural products but also in soil and organic matter [4–6]. Two taxonomic sections of *Alternaria* including large-spored taxa and small-spored taxa are described by Simmons [7] based on conidial morphology and sporulation patterns. Most of small-spored *Alternaria* species are challenging because some morphological characters are difficult to clearly characterize [8]. Phylogenetically, a total of 27 sections are proposed by Lawrence et al. [9] after a review of biodiversity and taxonomy on *Alternaria*. Among the phylogenetic sections, sect. *Alternaria* consists of 11 phylogenetic species and two species complexes, from which *A. alternata* species complex comprising 35 morphospecies [10]. Gannibal recommends that sec. *Alternaria* includes 59 species (1 type species, 21 phylogenetic species, and additional 37 morphospecies) [11].

Black spots of Koerle pear fruit were observed during storage in October 2017. An *Alternaria alternata*-like fungus was observed from the symptoms. The objectives of this study aim to test the pathogenicity of that fungus and clearly describe it based on morphology and sequence analyses of ITS, GAPDH, and TEF1 [3].

To determine the morphological characteristics, mycelia disks (6 mm diam.) were cut from 3-day-old colony and transferred on potato dextrose agar (PDA; Difco, Montreal, Canada) according to Luo et al. [12]. Five strains (YZU 171916, YZU 171918, YZU 171919, YZU 171920, and YZU 171921) were deposited in the Culture Collection at Yangtze University (YZU), Jingzhou, China (Table 1).

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incubated on potato carrot agar (PCA) for 7 days at 22 °C under the daily fluorescent light/dark cycle of 8/16 h to describe the conidial morphology [7]. To describe the morphology from host, diseased tissue was incubated for 4 days under the same condition as conidial description on PCA. The sporulation patterns and conidia were photographed using a compound light microscope (Nikon DS-Ri2, Tokyo, Japan). The conidia were mounted in lactophenol solution. Fifty conidia were photographed, and the GenBank accession No. was assigned.

Table 1. Strains and their accession numbers used in the study.

| Morphospecies | Strain | Host | Location | ITS | GAPDH | TEF1 | RP2B | Alt a | EndoPG | GenBank accession No. |
|---------------|--------|------|----------|-----|-------|------|-------|-------|--------|----------------------|
| A. alternata | CBS 916.96 T | Arachis hypogaea | India | AF347031 | AY278808 | CSK84634 | CSK84375 | AY536301 | JQ811978 | KP124632 |
| A. alternata | CBS 110977 T | Arachis hypogaea | India | AF347031 | AY278808 | CSK84634 | CSK84375 | AY536301 | JQ811978 | KP124647 |
| A. alstroemeriae | CBS 118808 R | Alstroemeria sp. | United States | KP124296 | KP124153 | KP125071 | KP124764 | KP123845 | KP123993 | KP124601 |
| A. alstroemeriae | CBS 118809 | Alstroemeria sp. | United States | KP124296 | KP124153 | KP125071 | KP124764 | KP123845 | KP123993 | KP124601 |
| A. arborescens | CBS 115189 | Citrus clementina | South Africa | KP124402 | KP124254 | KP125180 | KP124872 | KP123949 | KP124106 | KP124716 |
| A. arborescens | CPC 25266 | Pyrus sp. | Austria | KP124418 | KP124269 | KP125196 | KP124887 | KP123965 | KP124122 | KP124732 |
| A. arborescens | CBS 128428 | Doryza sp. | Russia | KP124416 | KP124267 | KP125194 | KP124885 | KP123963 | KP124120 | KP124730 |
| A. arborescens | CBS 102605 T | Solanum lycopersicum | United States | KP124738 | KP124234 | KP125125 | KP124835 | KP123985 | KP124100 | KP124605 |
| A. arborescens | CBS 120762 T | Astragalus bisulcatus | United States | KP124234 | KP124835 | KP123985 | KP124100 | KP124605 |
| A. betae-kenyensis | CBS 118810 T | Betula vulgaris var. cicla | Kenya | KP124419 | KP124270 | KP125197 | KP124888 | KP123966 | KP124123 | KP124733 |
| A. broussonettiae | CBS 121455 T | Broussonetia papyrifera China | KP123468 | KP124220 | KP125146 | KP124836 | KP123961 | KP124102 | KP124681 |
| A. brassicaceae | CBS 118811 T | Brassica oleracea | United States | KP124356 | KP124210 | KP125132 | KP124824 | KP123904 | KP124057 | KP124667 |
| A. bursii | CBS 107 38 T | Campanula sp. | India | KP124371 | JQ646305 | KP125198 | KP124899 | KP123967 | KP124124 | KP124734 |
| A. caudata | CBS 121544 T | Ruscus sp. | United States | KP124371 | JQ646305 | KP125198 | KP124899 | KP123967 | KP124124 | KP124734 |
| A. cerasicus | CBS 119544 T | Avena sativa | New Zealand | KP124408 | JQ646321 | KP125186 | KP124878 | KP123955 | KP124112 | KP124722 |
| A. cirtii | CBS 102.47 T | Citrus sinensis | United States | KP124304 | KP124161 | KP125080 | KP124773 | KP123855 | KP124002 | KP124610 |
| A. citricaneri | CBS 119543 T | Citrus paradisi | United States | KP124363 | KP124215 | KP125139 | KP124831 | KP123911 | KP124065 | KP124674 |
| A. mollurbusti | CBS 118810 T | Physalis alkekengi | India | KP124371 | JQ646305 | KP125198 | KP124899 | KP123967 | KP124124 | KP124734 |
| A. citricum | CBS 120762 T | Citrus x sinensis | India | KP124371 | JQ646305 | KP125198 | KP124899 | KP123967 | KP124124 | KP124734 |

T: ex-type strain; R: representative strain.

Bold contents are related to the present fungus generated in this study.
After 7 days, the colonies reached to 65–66 mm in diam. on PDA with umber to olivaceous color surrounding with white margin (Figure 1(A,B)). On PCA (Figure 1(C–F)), primary conidiophores were 15–146 × 3–5 μm producing 4–10 units catenulate conidia and the secondary conidiophores to develop lateral intra-conidia were 3–20 × 3–4 μm forming branched chains of 1–4 units. Conidia comprising 1–7 transverse septa were narrow-ellipsoid (13–50 × 6–11 μm) or ovoid (6–23 × 4–13 μm) in the initial lower part of the chains, gradually becoming ovoid (7–22 × 5–9 μm) and considerably smaller in the distal part, with apical conidia (2–12 × 2–4 μm). On the host (Figure 1(G–I)), the primary conidiophores reached 3–107 × 2–4 μm producing catenulate conidia (3–10 units) and the secondary ones to produce lateral intra-conidia (catenulate with 1–4 units) were 3–23 × 2–4 μm in size. Normally, conidia were 13–44 × 2–28 μm, with 1–6 transverse septa and false beaks 2–32 × 2–7 μm in size. The present fungus was morphologically similar to the species Alternaria limoniasperae, A. perangusta, A. interrupta, and A. turkisafria (Table 2).

To test its pathogenicity, healthy Koele pear fruits were obtained from the seller of Xinjiang market. Eighteen fruits were surface sterilized by dipping in 1% sodium hypochlorite (NaOCl) for 2 min, and then, washed with sterilized distilled water for 3 times. Each fruit was wounded two sites (one for mycelia plug and another for spore suspension) by a puncher (4 mm diam.) and placed into moist containers maintained at 25°C. Mycelia plugs (4 mm diam.) of each strain were cut from the edge of 3-day-old colonies and placed on wounded sites. Sterile PDA plugs were used as controls. Conidia were harvested from PCA to obtain the spore suspension (10^6 conidia/mL). A volume of 20 μL spore suspension was inoculated and distilled water was used as controls. Each strain was conducted with three replications and the experiment was repeated for three times. The disease development was checked daily.

Necrotic symptoms were observed obviously at 3 days in both inoculations. After 7 days, the symptoms developed up to 23 mm (diam.) inoculated with mycelia plugs (Figure 2) and 14 mm with spore suspensions. After 14 days, the symptoms turned to be rotten reaching to 30 mm in mycelium block and 22 mm in spore suspension. Any control was symptomless during the experiment. By the way, unwounded fruits were symptomless either by mycelia plug or spore suspension (data not shown). The Koch’s postulates were fulfilled by a re-isolation

Figure 1. Morphological characteristics of Alternaria sp. YZU 171921 from Pyrus sinkiangensis. Colony on PDA (A) and (B); sporulation patterns, conidiophores and conidia on the PCA (C–F); sporulation patterns, conidiophores and conidia on the host plant (G–I). Bars: (D)–(F) = 25 μm, (H) and (I) = 25 μm, (C) = 100 μm, (G) = 100 μm.
Table 2. Morphological comparison of the present fungus and its closely related species described by Simmons [7].

| Species          | Shape and size (μm)                                                                 | Septa | Conidiophore (μm) | Secondary conidiophore (μm) | Conidia per primary chain (lateral branched chain) |
|------------------|-------------------------------------------------------------------------------------|-------|-------------------|-----------------------------|---------------------------------------------------|
| A. limoniasperae | Narrow-ellipsoid 30 – 50 × 8 – 10 or ovoid 20 – 35 × 8 – 12 in the initial lower part of the chain, ovoid 8 – 12 × 4 – 8 in the distal part of the chain | 1–4   | 100               | 2 – 4 × 2 – 3               | up to 20 (4 – 10)                                  |
| YZU 171921       | Narrow-ellipsoid 13 – 50 × 6 – 11 or ovoid 6 – 23 × 4 – 13 in the initial lower part of the chain, ovoid 7 – 22 × 5 – 9 in the distal part of the chain | 1–7   | 15 – 146 × 3–5    | 3 – 20 × 3–4               | 4 – 10 (1 – 4)                                    |
| A. turkisfria    | Narrow-ovoid to long-ovoid or long-ellipsoid 20 – 50 × 6 – 8, conidia have small 1-cell secondary conidiophores | 3–8   | 30 – 60 × 4       | 3 – 5 × 2–3               | 8 – 20 (4 – 10)                                   |
| A. perangusta    | Long narrow-ellipsoid, rarely wide enough to be termed obclavate 15 – 40 × 3–7 | 3–7   | 100 – 200 × 3–4   | 3 – 5 × 2–3               | 10+ (unknown)                                     |
| A. interrupta    | Narrow-ellipsoid or narrow-obclavate 35 – 40 × 7–8                                  | 7–8   | 140 × 4           | 3 – 8 × 2–3               | 10 – 15 (unknown)                                 |

Figure 2. Pathogenicity tests on Koerle pear fruit (Pyrus sinkiangensis) inoculated with mycelia plugs of five strains (A–F) of the present fungus for 7 days (upper) and for 14 days (middle and down) at 25°C. (A) YZU 171916; (B) YZU 171918; (C) YZU 171919; (D) YZU 171920; (E) YZU 171921; (F) Control.
from inoculated fruits. The results showed that the present fungus was the causal agent of black spot of Koerle pear fruit (Figure 2).

To confirm the phylogenetic position of the present fungus, the genomic DNA was extracted using mycelia grown on PDA according to the method of Cenis [14]. Seven genes including internal transcribed spacer rDNA regions (ITS), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), partial translation elongation factor 1 alpha (TEF1), RNA
polymerase second largest subunit (RPB2), *Alternaria* major allergen gene (Alt a1), an anonymous gene region (OPA10-2), and endopolysaccharidase gene (EndoPG) were amplified using the primer pairs ITS4/ITS5 [15], gpd1/gpd2 [16], EF1-728F/EF1-968R [17], RPB2-5F [18]/RPB2-7CR [19], Alt-for/Alt-rev [20], OPA 10-2 L/OPA 10-2 R [8], and PG3/PG2 [8], respectively. PCR amplification was performed in a 25 μl reaction volume containing 8 μl ddH2O, 2 μl DNA solution, 1.25 μl each primer, and 12.5 μl 2× Taq PCR StarMix (Genstar, Beijing, China). The PCR products were checked in 1% agarose gel, run in 0.5× TBE buffer and visualized under UV illumination. Successfully amplified PCR products were sequenced by Beijing Genomics Institute (BGI, Beijing, China) using both forward and reverse primers. The resulting sequences were compared with those of morphospecies described by Simmons (2007) derived from Woudenberg et al. [10]. Each of seven gene sequences was aligned and combined using the MEGA v. 6.0.0 software [21]. The best-fit model GTRGAMMAI was selected by MrModeltest v. 2.3.6. [22]. Bayesian analyses were performed with MrBayes v. 3.1.2 [23]. The parameters including 2,000,000 Markov chain Monte Carlo (MCMC) generations and a sampling frequency of every 100 generations and a sampling frequency of every 100 generations were set to 25% after which the likelihood values were constant. A maximum-likelihood analysis was additionally run using RAXML v. 7.2.8 [24]. Bootstrap analysis was performed with 1000 replications for the combined analysis of seven loci. The *Alternaria arborescens* species complex (AASC) was used as root branch. The resulting tree was plotted and edited by FigTree v. 1.3.1 [25]. A total of 64 *Alternaria* isolates were included in the aligned sequence matrix. In the multigene phylogeny, 3454 characters were calculated including 502 of ITS, 446 of GAPDH, 240 of TEF1, 710 of RPB2, 472 of Alt a1, 633 of OPA10–2, and 442 of EndoPG. The Bayesian posterior probabilities (PP) >0.65 and RAXML bootstrap support values (BP) >65% were plotted in the phylogeny (Figure 3). Based on the seven genes, the five strains used in this study were identical to each other. The phylogenetic results showed that the present fungus was belonging to *Alternaria alternata* species complex (AALSC) group of *Alternaria* and fell into a monoclade highly supported by PP (1.00) and BP (100%) values. They were closely related to *Alternaria limoniaasperae*, *A. perangusta*, *A. interrupta*, and *A. turkisafria*. However, the present fungus was the closest to *A. limoniaasperae* with seven nucleotide position differences: Alt a 1 position 350 (C), RPB2 position 546 (G), and OPA10–2 position 369 (T), 618 (C), 624 (G), 639 (C), 648 (G). Morphologically, the present fungus was obviously different from *A. limoniaasperae* by producing more septa in conidia with shorter chains. All the previous results indicated that the species might be a new morphospecies in AALSC. All thirty-five morphospecies under one species *Alternaria alternata* [10] is not a great way for the taxonomy based on phylogeny. More works should be done to better understand the taxonomy of *Alternaria alternata* species complex based on morphology and molecular at the same times. In the present study, the fungus collected from black spot of Koerle pear fruit (*Pyrus sinkiangensis*) was found as a causal agent and illustrated clearly in morphology generated from authentic culture and host. Phylogenetically, it should be considered as a new member of AALSC. 

**Disclosure statement**

No potential conflict of interest was reported by the author(s).

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