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Identification of a novel phylogenetic lineage of *Alternaria alternata* causing citrus brown spot in China

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ABSTRACT

Alternaria alternata sensu lato, casual agent of citrus brown spot, first identified in Yunnan province in 2010 and subsequently found in Zhejiang, Hunan, Guangdong provinces, Chongqing municipality andGuangxi autonomous region in China. During 2010–2012, 86 isolates were collected from diseased citrus, of which 85 % isolates were pathogenic to Ponkan tangerine. Phylogenetic analyses of Chinese and worldwide isolates using partial sequences of an *endopolygalacturonase* gene (*endoPG*) and combined dataset of*endoPG* and two anonymous loci (OPA1-3, OPA2-1) found that Chinese isolates fell into two of three previously described clades. One clade ('clade 3') contained isolates from Turkey and Israel, and the other clade ('clade 1') contained isolates from Florida, USA. None of the isolates from China fell into the last previously described clade ('clade 2'). However, 24 isolates from Hunan, Guangdong and Guangxi fell into a fourth clade ('clade 4') not previously reported to be associated with citrus brown spot. This clade included multilocus haplotypes known to infect Japanese pear and strawberry. The observation that Chinese brown spot isolates fell into only two of three known worldwide lineages suggests that this fungus may not have co-evolved with its host in China but elsewhere in Southeast Asia and introduced to China.

Alternaria brown spot is a highly destructive disease of tangerines and tangerine hybrids with worldwide significance (Akimitsu *et al.* 2003). It attacks young leaves, twigs and fruits, typically causing brown to black lesions surrounded by a yellow halo. Severely infected leaves and fruits may drop, and entire shoots may wilt and die. Under appropriate environmental conditions, significant losses occur both in terms of yield and marketability of susceptible citrus cultivars (Peever *et al.* 2002; Akimitsu *et al.* 2003).

Alternaria brown spot was first described on Emperor mandarin of Australia in 1903 (Cobb 1903). The causal agent was originally identified as *Alternaria citri* Ellis & Pierce based on its morphological similarity to the causal agent of a post-harvest disease black rot (Pegg 1966). Later, Solel renamed this

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pathogen *Alternaria alternata* due to its morphological similarity to saprophytic *A. alternata* (Fr.:Fr.) Keissl. (Solel 1991). Two distinct pathotypes of *A. alternata* associated with *Citrus* have been described based on differences in host-specificity and toxin production. The tangerine pathotype is specific to tangerines (*Citrus reticulata* Blanco), tangelos (*C. reticulata*×*C. paradisi* Macfad.) and tangors (*C. reticulata*×*C. sinensis* (Linnaeus) Osbeck), and produces a host-specific ACT-toxin. The rough lemon pathotype is specific to rough lemon (*C. jambhiri* Lush), and produces a host-specific ACRL-toxin. Researchers demonstrated that both host-specific toxins are required for pathogenicity to each host (Ajiro *et al.* 2010; Miyamoto *et al.* 2010; Tsuge *et al.* 2013).

Since the first report in Australia (Kiely 1964; Pegg 1966; Peever *et al.* 2004), Alternaria brown spot has been identified in the USA (Whiteside 1976), Israel (Solel 1991), Colombia (Canihos *et al.* 1995), Turkey (Canihos *et al.* 1997), South Africa (Swart *et al.* 1998), Spain (Vicent *et al.* 2000), Italy (Bella *et al.* 2001), Argentina and Brazil (Peres *et al.* 2003), Peru (Marín *et al.* 2006) and Greece (Elena 2006). Previous phylogenetic analyses of 65 isolates sampled from six countries using an *endopolygalacturonase* gene (*endo*PG) showed that isolates clustered into three phylogenetic lineages, which were correlated to geographic origins (Peever *et al.* 2002). Peever *et al.* (2002) suggested at least three independent introductions of the pathogen occurred in different parts of the world, possibly associated with planting stock of the host. As the genus *Citrus* is native to the subtropical and tropical regions of Asia and the Malay Archipelago (Webber & Batchelor 1949), a hypothesis was proposed that the pathogen evolved with its host in Southeast Asia (Akimitsu *et al.* 2003; Peever *et al.* 2002). However, Alternaria brown spot was not reported from Asia until 2010 (Wang *et al.* 2010).

The first report of Alternaria brown spot from Asia was on Tangfang mandarin in Yunnan province, China in 2010 (Wang *et al.* 2010; Chen *et al.* 2011). Since this initial report, the disease has been observed on cv. 'Hongjv' (*C. reticulata* Blanco cv. Honjv) in Chongqing municipality, on cv. 'Ponkan' (*C. reticulata* Blanco) in Hunan and Yunnan provinces, on cv. 'Gonggan' (*C. reticulata* Blanco×*C. sinensis* (Linnaeus) Osbeck) in Guangxi Zhuang Autonomous Region and Guangdang province, and on 'Ougan' (*C. reticulata* Blanco cv. Ougan) in Zhejiang province and 'Bayuejv' (*C. reticulata* Blanco cv. Bayuejv) in Guangdong province (Chen *et al.* 2011; Huang *et al.* 2012; Qin *et al.* 2012). China has a long history of citrus cultivation going back approximately 4000 y (Deng *et al.* 2008; Zhou & Ye 2010). The objectives of this research were to determine if additional genetic diversity in the Alternaria brown spot pathogen could be identified in China and test the hypothesis that China is the origin of the Alternaria brown spot pathogen.

Materials and methods

Isolation and culture of isolates

Isolates of *Alternaria* spp. were obtained from infected leaves, sprouts and fruitlets of five citrus cultivars in four provinces, an autonomous region and a municipality in China where the disease was reported between 2010 and 2012 (Table 1). The sampled hosts contained tangerine cultivars including 'Hongjv', 'Ponkan', 'Ougan' and 'Bayuejv', and a tangor 'Gonggan'. Trees were randomly sampled in each grove, with one isolate sampled per tree. Fungal isolation was performed as follows. Infected young leaves, sprouts or fruitlets were washed in tap water, dried on absorbent paper, then surface-disinfested by rubbing the surface of the lesion three times with 75 % ethanol-soaked cotton ball (about 30 s). A small section (4 mm^2) of plant tissue was cut from the leading edge of lesions as the ethanol volatilized, and plated on potato dextrose agar (PDA) containing carbendazim at 10 $\mu g\ ml^{-1}$ to exclude *Colletotrichum gloeosporioides*. Isolated lesions were incubated at 25 °C under fluorescent light with an alternating 12-h photoperiod. Hyphal tips were cut directly from the agar after about 48 h, and transferred to PDA plates. Plates were incubated at 25 °C under fluorescent light with an alternating 12-h photoperiod for 7 d to induce conidia. Conidia were collected in distilled water and spread on PDA plates. Then, a single germinated conidium was harvested after 12–24 h and transferred to PDA slant for long-term storage at 4 °C.

Pathogenicity tests

Pathogenicity of sampled *Alternaria* isolates was determined by inoculating each single-conidial isolate to detached-leaves of tangerine cultivar 'Ponkan' and rough lemon (*Citrus jambhiri*). Stored isolates were recovered on PDA plates. To induce sporulation, a colony plug of each isolate, 5-mm in diameter, was transferred from a PDA plate to a thin (10 ml for a 9-cm plate) PDA plate (Peever *et al.* 1999) or a clarified V8 agar plate (Vega *et al.* 2012). After 2 weeks, conidia were harvested by adding distilled water, rubbing the colony surface with a sterile glass rod, and filtering the filtrate through three layers of cheesecloth. Conidial concentrations were adjusted to 10^4 conidia per ml with a haemocytometer. Pathogenicity of each isolate was determined by placing a conidial suspension onto the adaxial side of detached leaves of 'Ponkan' and rough lemon with a paint brush (CM-22, Jiangnan Zouming brush factory). 'Ponkan' and rough lemon trees were grown in pots and maintained in a greenhouse, they were cut back frequently to stimulate new flushes of foliage in order to obtain a constant supply of young leaves (Peever *et al.* 1999). Young leaves that were half to fully unfolded were selected for inoculation with two replicate leaves used for each isolate. Inoculated leaves were placed in 1.5-ml microfuge tubes filled with water to cover petioles (Peever *et al.* 1999). Tubes were placed in racks in humidity chambers (Haishu, Jiangnan Instrument Factory, 315012, Ningbo, China) where temperature was maintained at 25 °C, with an alternating 12 h light cycle. Nine isolates previously characterized to be pathogenic were provided by T. Peever, Washington State University (Table 1) and were used as positive controls. Distilled water was used as a negative control. Symptoms were monitored daily and an isolate was considered pathogenic if either of the inoculated leaves showed a brown to black lesion within 5 d of inoculation. In contrast, the isolate was regarded as nonpathogenic if no visible lesion was formed on both inoculated leaves by 5 d post-inoculation.