Research Papers

Biological and molecular characterization of seven *Diaporthe* species associated with kiwifruit shoot blight and leaf spot in China

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Summary. *Diaporthe* species are significant pathogens, saprobes, and endophytes, with comprehensive host association and geographic distribution. These fungi cause severe dieback, cankers, leaf spots, blights, and stem-end rot of fruits on different plant hosts. This study, explored the occurrence, diversity and pathogenicity of *Diaporthe* spp. associated with *Actinidia chinensis* and *A. delicosa* in the main kiwifruit production areas of China. *Diaporthe* isolates (284) derived from 106 diseased leaf and branch samples were examined. Multi-locus phylogenetic analyses and morphology of 43 representative isolates revealed that seven *Diaporthe* species were obtained, including *D. alangii*, *D. compactum*, *D. eres*, *D. hongkongensis*, *D. sojae*, *D. tectonae*, and *D. unshiuensis*. Pathogenicity tests were performed on kiwifruit fruits, leaves and branches. Koch’s postulates confirmed all species were pathogenic. *D. alangii* and *D. tectonae* were the most aggressive species, followed by *D. eres*, *D. sojae*, *D. hongkongensis*, and *D. unshiuensis*. Host range evaluation showed that the seven *Diaporthe* species could also infect apricot, apple, peach, pear, and plum. This is the first report of *D. alangii*, *D. compactum*, *D. sojae*, *D. tectonae*, and *D. unshiuensis* infecting kiwifruit in China, increasing understanding of the *Diaporthe* complex causing diseases of kiwifruit plants, to assist effective disease management.

Keywords. *Actinidia*, phylogeny, pathogenicity.

INTRODUCTION

Kiwifruit is known “the king of fruits” due to its rich nutritional content, abundant dietary fibres, balanced nutritional composition of minerals, high vitamin C content, antioxidant properties and other human health-beneficial metabolites, including carotenoids and flavonoids (Huang *et al*., 2013; Pan *et al*., 2013).
Diaporthe ambigua and related to (Akilli (Thomidis Diaporthe blight were 2018). In China, the major pathogens causing branch as causing cordon dieback in Chile (Díaz and Latorre, ., 2018) and bacteria (Zhang et al. 1982; Pennycook, ., 1985). Plantation of kiwifruit for 25 years has been reported to several fungi (Hawthorne blossom blight, and fruit rot. These symptoms are relat - ed to cause of these diseases is important for develop - ment of the kiwifruit industry. Accurate identifi - cation, starting from the early 20th century (Huang, 2009), which had a kiwifruit culti - vation area of 240,000 ha in 2018, producing 2.55 MT of fruit, accounting for nearly 55% of the global kiwifruit (FAO, 2018; Guo et al. 2020a).

During the past decades, with the steadily increasing duration of kiwifruit monoculture and the rapid expansion of production, diseases have become prevalent in orchards and nurseries. Branch blight and leaf spot diseases are widespread and prevalent, and these dis - eases cause serious economic losses in China, and affect development of the kiwifruit industry. Accurate identifi - cation of cause of these diseases is important for develop - ment of effective biosecurity and trade policies. The most common disease symptoms observed in kiwifruit plantations consist of branch blight, leaf spot, bacterial blossom blight, and fruit rot. These symptoms are related to several fungi (Hawthorne et al., 1982; Pennycook, 1985; Pan et al., 2018) and bacteria (Zhang et al., 2019).

Vine decline of kiwifruit in Turkey was mainly related to Phytophthora citrophthora (Akilli et al. 2011). In Greece, the pathogens which caused distinct cankers on branches of kiwifruit were Diaporthe neotheicola and Botryosphaeria dothidea (Thomidis et al., 2010, 2013). Diaporthe ambigua and D. australaficana were reported as causing cordon dieback in Chile (Díaz and Latorre, 2018). In China, the major pathogens causing branch blight were B. dothidea, D. actinidiae, D. eriantha, and D. tathiens (Li et al., 2013; Bai et al., 2017).

Leaf spot of kiwifruit, caused by Alternaria alternata, Diaporthe spp., Glomerella cingulata, Pestalotiopsis spp. and Phomopsis spp., has been previously reported in Korea and New Zealand (Jeong et al., 2008; Hawthorne and Otto, 2012). Didymella bel lids has been reported as the major cause of leaf spot in China (Zou et al., 2019).

The blossom blight of kiwifruit occurs in many countries. Several pathogens have been reported as the causal agents of this disease, including Pseudomonas viridiflava, P. fluorescens, P. syringae, P. fluorescens P. syringa pv. syringae, P. syringae pv. actinidiae, and Botrytis cinerea (Conn and Gubler, 1993; Koh et al., 2001; Shin et al., 2004; Young et al., 2009; Zhang et al., 2019).

Fruit rot of kiwifruit can be divided into field rot and postharvest fruit rot. Field rot, caused by Sclerotinia sclerotiorum, affects immature fruits on vines (Pennycook, 1985). More than seven fungi have been reported to be associated with postharvest fruit rots of kiwifruit (Beraha and O’Brien, 1979; Hawthorne et al., 1982; Pennycook, 1985). Diaporthe spp. and Botryosphaeria spp. were reported as the major causes of postharvest fruit rot. In New Zealand, Botrytis cinerea causes storage rot and B. dothidea causes ripening rot. Botryosphaeria dothidea also was the major cause of postharvest fruit rot in Iran (Nazerian et al., 2019). Diaporthe actinidiae has been reported to cause postharvest fruit rot in China, Iran, Korea, and New Zealand (Sommer and Beraha, 1975; Lee et al., 2001; Koh et al., 2005; Mousakhah et al., 2014; Li et al., 2017b). In addition, D. ambigua, D. australaficana, D. novem, and D. radis have been reported to cause postharvest fruit rot of kiwifruit during cold storage in Chile. Diaporthe ambigua was also isolated from postharvest kiwifruit rots in Greece (Thomidis et al., 2013; 2019). Diaporthe honkongensis has been reported to cause stem - end rot in Turkey (Erper et al., 2017). Diaporthe melonis and D. perniciosa have been reported as the major pathogens causing postharvest fruit rots in New Zealand (Beraha and O’Brien, 1979; Hawthorne et al., 1982).

Before the advent of molecular biology technology, identification criteria for Diaporthe species were based on the morphological characteristics (e.g., colony appearance in cultures, size and shape of ascomata and conidomata, sexual state and connections to the asexual state) and host specificity (Rehner and Uecker, 1994; Santos et al., 2011; Gomes et al., 2013; Guaraccia and Crous, 2017; Yang et al., 2018b). Previous studies demonstrated that these characters were generally not suf - ficient for species level diagnoses, because some species of Diaporthe are not host-restricted and are capable of infecting several taxonomically unrelated host genera (Rehner and Uecker, 1994; Thompson et al., 2011; Elfar et al., 2013; Huang et al., 2013; Thompson et al., 2015). Diaporthe helianthi, as the causal agent of stem canker of sunflower, was first reported in the former Yugoslavia. Subsequent studies confirmed Xanthium italicum, X. strumarium, and Arctium lappa as weed hosts of D. helianthi (Thompson et al., 2015). Three other D. species, D. gulyae, D. kochmanii, and D. kongii, have been identified as the pathogens of sunflower stem canker (Thompson et al., 2011). In addition, character plasticity and cultural variation of Diaporthe species clarification. Application of molecular data has progressed fungal species definition (Hibbett and Taylor, 2013; Yang et al., 2018a). Diaporthe species are being redefined, based on the combination of morphological,
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Cultural and phytopathogenic characteristics, mating types and DNA sequence data (Guarnaccia and Crous, 2017, 2018; Fan et al., 2018). Adoption of multi-locus phylogeny has provided clear resolution of classification and species (Udayanga et al., 2014).

China is an important kiwifruit-growing country and leader in kiwifruit cultivation. However, in recent years, the incidence and systematic identification of the *Diaporthe* species associated with branch blight of kiwifruit were only assessed in two orchards of Hubei and Anhui provinces. Only 36 strains were obtained and identified as *D. tulliensis*, *D. actinidiae* and *D. eres*, and it is unclear which species is responsible for the disease in different host varieties or species in different provinces (Bai et al., 2017). In addition, leaf spot of kiwifruit caused by *Diaporthe* species has been rarely reported in China, which makes effective prevention and control of the disease challenging. Therefore, larger scale surveys are needed to give increased understanding of the relative role of *Diaporthe* spp. in fungal branch blight and leaf spot found on kiwifruit in China.

The present sampled plants with shoot blight and leaf spot symptoms for pathogen isolation. Phylogenetic analyses based on the nuclear ribosomal internal transcribed spacer region (ITS), translation elongation factor 1-alpha (EF1-α) and beta-tubulin (TUB) genes, coupled with morphology of representative strains, were carried out to determine the diversity of pathogens. After pathogenicity determination, species associated with kiwifruit shoot and leaf blight were identified. This study has provided valuable information on pathogen ecology, as a basis for improving management strategies for these economically important diseases.

**MATERIALS AND METHODS**

**Sampling and pathogen isolation**

Surveys of incidence of shoot blight and leaf spot diseases were conducted in 16 orchards located in nine provinces of China, including Anhui, Chongqing, Henan, Hubei, Fujian, Shandong, Shanxi, Sichuan, and Zhejiang, from October 2017 to May 2019. A total of 106 samples with symptoms of shoot blight and/or leaf spot were collected from *Actinidia chinensis* ('Cuiyu', 'Donghong', 'Hongyang', 'Huangjin', 'Jinyan', and 'Longzanghong'), and *A. deliciosa* ('Cuixiang', 'Hayward', 'Jinkui', and 'Xuxiang'). Six pieces (4–5 mm²) of wood or foliage were cut from each of the diseased tissues neighbouring the asymptomatic regions with a sterile scalpel. After surface sterilization in 1% NaOCl for 45 s, the tissues were treated in 75% ethanol for 45 s, rinsed three times in sterile distilled water for 1 min each, and then dried on sterilized filter paper (Fu et al., 2018). Each tissue piece was placed on potato dextrose agar (PDA; 20% diced potato, 2% dextrose, 1.5% agar, and distilled water) plates and incubated at 25°C in the dark for 3–5 d until fungal colony formation (Bai et al., 2015). Colonies with typical characteristic of *Diaporthe* spp. were sub-cultured onto fresh PDA plates. The obtained isolates were purified using hyphal tip or single spore methods. Mycelium plugs of purified isolates were transferred to PDA tubes, or stored in 25% glycerol at -80°C for subsequent use (Zhai et al., 2014).

**DNA extraction, PCR amplification, and sequencing**

Colonies were cultivated on PDA plates where the medium was covered with sterile cellophane which was incubated at 25°C in the dark for 5–7 d. Mycelia was scraped and placed into clean tubes. Total genomic DNA was extracted using a modified CTAB method (Freeman et al., 1996). The quality and quantity of DNA were confirmed visually by staining with Gel Red after electrophoresis in 1% agarose gel and visualization under UV light (λ = 302 nm) trans illumination (Udayanga et al., 2012; Gao et al., 2017). The internal transcribed spacer (ITS) region of the nuclear ribosomal genes was amplified using the primer sets ITS1/ITS4 (White et al., 1990), the primers EF1-728F/EF1-986R (Carbone and Kohn, 1999) were used to amplify part of EF1-α, and the primers Bt-2a/Bt-2b (Glass and Donaldson, 1995) were used to amplify part of TUB. For PCR, an aliquot of 50 µL reaction solution contained 5 µL of 10 × *Taq* buffer II (Mg²⁺ Plus) (TaKaRa), 1 µL of dNTP mixture (2.5 mM each), 1 µL of each primer, 0.5 µL of Taq (5 U µL⁻¹), 2.0 µL of DNA template, and 39.5 µL of ddH₂O (Zhai et al., 2014). PCR parameters were initiated at 95°C for 5 min, followed by 35 cycles, each of denaturation at 95°C for 30 s, annealing at appropriate temperature for 30 s, extension at 95°C for 30 s, and terminated with a final elongation step at 72°C for 10 min (Guo et al., 2020b). The PCR amplicons were purified and sequenced by Sangon Biotech Company, Ltd. The obtained sequences were analyzed on DNAMAN (v. 9.0; Lynnon Biosoft), and deposited in GenBank (Table 1).

**Phylogenetic analyses**

Novel sequences generated in this study were blasted against the NCBI’s GenBank nucleotide database (http://blast.ncbi.nlm.nih.gov/) to search for closely similar relatives for a taxonomic framework of the studied
Table 1. Sources and GenBank accession numbers of isolates included in this study.

| Species               | Isolate designation* | Host                  | Country | Genbank accession numbers                  |
|-----------------------|-----------------------|-----------------------|---------|--------------------------------------------|
|                       |                       |                       |         | **ITS**  | **EF1-α** | **TUB** |
| Diaporthe alangii     | CFCC 52556*           | Alangium kurzii       | China   | MH121491 | MH121533 | MH121573 |
|                       | CFCC 52557            | Alangium kurzii       | China   | MH121492 | MH121534 | MH121574 |
|                       | CFCC 52558            | Alangium kurzii       | China   | MH121493 | MH121535 | MH121575 |
|                       | CQ155                 | Actinidia chinese     | China   | MT043825 | MT019567 | MT019603 |
|                       | FJHJB57               | Actinidia chinese     | China   | MT043831 | MT019562 | MT019615 |
|                       | HB48                  | Actinidia deliciosa   | China   | MT043835 | MT019578 | MT019619 |
|                       | SC74                  | Actinidia chinese     | China   | MT043849 | MT019592 | MT019627 |
|                       | SC83                  | Actinidia chinese     | China   | MT043850 | MT019593 | MT019628 |
|                       | D. ambigua            |                       |         |              |           |           |
|                       | CBS 114015*           | Pyrus communis        | South Africa | KC343010 | KC343736 | KC343978 |
|                       | LC3078*               | Camellia sinensis     | China   | KP267850 | KP267924 | /        |
|                       | LC3083*               | Camellia sinensis     | China   | KP267854 | KP267928 | /        |
|                       | CQ130                 | Actinidia deliciosa   | China   | MT043824 | MT019565 | MT019601 |
|                       | CQ178                 | Actinidia chinese     | China   | MT043827 | MT019570 | MT019606 |
|                       | SC42                  | Actinidia chinese     | China   | MT043846 | MT019589 | MT019624 |
|                       | SC67                  | Actinidia chinese     | China   | MT043848 | MT019591 | MT019626 |
|                       |                       |                       |         |              |           |           |
|                       | D. eres               |                       |         |              |           |           |
|                       | AR5193*               | Ulmus sp.             | Germany | KJ210529 | KJ210550 | KJ420799 |
|                       | CBS 267.55            | Laburnum × watereri 'Vossii' | Netherlands | KC343082 | KC343808 | KC344050 |
|                       | CBS 101742            | Fraxinus sp.          | Netherlands | KC343073 | KC343799 | KC344041 |
|                       | CFCC 52576            | Castanea mollissima   | China   | MH121511 | MH121553 | MH121593 |
|                       | CFCC 52578            | Sorbus sp.           | China   | MH121513 | MH121555 | MH121595 |
|                       | AH16                  | Actinidia chinese     | China   | MT043816 | MT019564 | MT019600 |
|                       | HB24                  | Actinidia chinese     | China   | MT043833 | MT019576 | MT019617 |
|                       | HB25                  | Actinidia chinensis   | China   | MT043834 | MT019577 | MT019618 |
|                       | HB25                  | Actinidia deliciosa   | China   | MT043836 | MT019579 | MT019620 |
|                       | WAI-1                 | Actinidia sp.         | China   | KX457969 | KX 457965 | /        |
|                       | MJL13                 | Actinidia chinensis   | China   | MT043839 | MT019582 | MT019632 |
|                       | MJL18                 | Actinidia chinensis   | China   | MT043841 | MT019584 | MT019634 |
|                       | FJ11                  | Actinidia chinensis   | China   | MT043828 | MT019559 | MT019612 |
|                       | SD16                  | Actinidia chinensis   | China   | MT043853 | MT019595 | MT019638 |
|                       | SX24                  | Actinidia deliciosa   | China   | MT043854 | MT019597 | MT019630 |
|                       |                       |                       |         |              |           |           |
|                       | D. ganjae             |                       |         |              |           |           |
|                       | CBS 180.91*           | Cannabis sativa       | USA     | KC343112 | KC343838 | KC344080 |
|                       | D. goulteri           | Helianthus annuus     | Australia | KJ197290 | KJ197252 | KJ197270 |
|                       | BRIP 55657a           |                       |         |              |           |           |
|                       | LC3484                | Camellia sinensis     | China   | KP267906 | KP267980 | KP293486 |
|                       | CBS 115448*           | Dichroa febrifuga     | China   | KC343119 | KC343845 | KC344087 |
|                       | CQ21                  | Actinidia chinese     | China   | MT043821 | MT019571 | MT019607 |
|                       | CQ51                  | Actinidia chinensis   | China   | MT043822 | MT019572 | MT019608 |
|                       | FJHJB53               | Actinidia chinensis   | China   | MT043830 | MT019561 | MT019614 |
|                       | MJL21                 | Actinidia deliciosa   | China   | MT043842 | MT019585 | MT019635 |
|                       |                       |                       |         |              |           |           |
|                       | D. hongkongensis      |                       |         |              |           |           |
|                       | LC3484                | Camellia sinensis     | China   | KP267906 | KP267980 | KP293486 |
|                       | CBS 115448*           | Dichroa febrifuga     | China   | KC343119 | KC343845 | KC344087 |
|                       | CQ21                  | Actinidia chinese     | China   | MT043821 | MT019571 | MT019607 |
|                       | CQ51                  | Actinidia chinensis   | China   | MT043822 | MT019572 | MT019608 |
|                       | FJHJB53               | Actinidia chinensis   | China   | MT043830 | MT019561 | MT019614 |
|                       | MJL19                 | Actinidia deliciosa   | China   | MT043842 | MT019585 | MT019635 |
|                       | MJL21                 | Actinidia deliciosa   | China   | MT043843 | MT019586 | MT019636 |
|                       |                       |                       |         |              |           |           |
|                       | D. manihotia           |                       |         |              |           |           |
|                       | CBS 505.76*           | Manihot utilisissima  | Rwanda  | KC343138 | KC343864 | KC344106 |
|                       | D. neoraonikayaporum  |                       |         |              |           |           |
|                       | MFLUCC 14-1136*       | Tectona grandis       | Tailand | KU712449 | KU749369 | KU743988 |
|                       |                       |                       |         |              |           |           |
|                       | D. raonikayaporum     |                       |         |              |           |           |
|                       | CBS 133182*           | Spondias monobin      | Brazil  | KC343188 | KC343914 | KC344156 |
|                       | FAU635*               | Glycine max           | USA     | KJ590719 | KJ590762 | KJ610875 |
|                       | FAU636                | Glycine max           | USA     | KJ590718 | KJ590761 | KJ610874 |
|                       | ZJUD68                | Citrus unshiu         | China   | KJ490603 | KJ490482 | KJ490424 |
|                       | CQ14                  | Actinidia chinensis   | China   | MT043819 | MT019566 | MT019602 |
|                       | CQ16                  | Actinidia chinensis   | China   | MT043820 | MT019568 | MT019604 |

(Continued)
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Isolates. Sequence alignments of different gene regions, including sequences obtained from this study and reference sequences based on recent studies of *Diaporthe* species, were initially carried out with the online server ([http://mafft.cbrc.jp/alignment/server/index.html](http://mafft.cbrc.jp/alignment/server/index.html)) (Katoh and Standley, 2013), and were then manually adjusted in MEGA v. 7 (Kumar et al., 2016).

An initial maximum likelihood (ML) phylogenetic analysis was conducted based on *EF1-α* sequences of 284 isolates obtained in this study and 31 reference strains including one outgroup taxon (*Diaporthella corylina* CBS121124) deposited in GenBank (Table 1) with a GTR+G substitution model by using IQ-tree v.1.6.8, to give an overview backbone phylogenetic tree for the genus *Diaporthe* (data not shown). A subset of 43 representative isolates was then selected based on the results of the general *EF1-α* analysis, and was processed through different phylogenetic analyses conducted individually for each locus and multiple sequences analyses using concatenated *ITS, EF1-α, and TUB*.

Multi-locus phylogenetic analyses were generated using Maximum likelihood (ML) and Bayesian inference (BI). The maximum-likelihood tree was inferred using the edge-linked partition model in IQ-tree (Nguyen et al., 2015; Minh et al., 2020). For the IQ-tree, the best evolutionary model for each partition was determined using ModelFinder (Minh et al., 2020). In the partition model, IQ-TREE can estimate the model parameters separately for every partition. After ModelFinder found the best partition, IQ-TREE immediately starts the

| Species                | Isolate designation* | Host                | Country | Genbank accession numbers | ITS | EF1-α | TUB |
|-----------------------|----------------------|---------------------|---------|--------------------------|-----|-------|-----|
| **Diaporthe species** |                      |                     |         |                          |     |       |     |
| *D. tectonae*         | MFLUCC 12-0777*      | *Tectona grandis*   | Thailand| KU712430 KU749359 KU743977 |
|                       | MFLUCC 12-0782       | *Tectona grandis*   | Thailand| KU712431 KU749360 KU743978 |
|                       | MFLUCC 13-0476       | *Tectona grandis*   | Thailand| KU712433 KU749362 KU743980 |
|                       | MFLUCC 14-1139       | *Tectona grandis*   | Thailand| KU712438 KU749366 KU743985 |
|                       | CQ58                 | *Actinidia chinensis* | China  | MT043823 MT09573 MT09609 |
|                       | CQ166                | *Actinidia chinensis* | China  | MT043826 MT09569 MT09605 |
| *D. tulliensis*       | BRIP 62248a*         | *Theobroma cacao*   | Australia| KR936130 KR936133 KR936132 |
| *D. unshiuensis*      | CFCC 52594           | *Carya illinoiensis* | China   | MH121529 MH121571 MH121606 |
|                       | CFCC 52595           | *Carya illinoiensis* | China   | MH121530 MH121572 MH121607 |
|                       | ZJUD51               | *Citrus japonica*   | China   | KJ490586 KJ490465 KJ490407 |
|                       | ZJUD52*              | *Citrus unshiu*     | China   | KJ490587 KJ490464 KJ490408 |
|                       | CQ7                  | *Actinidia chinensis* | China  | MT043817 MT09574 MT09610 |
|                       | CQ9                  | *Actinidia chinensis* | China  | MT043818 MT09575 MT09611 |
|                       | FJHJB22              | *Actinidia chinensis* | China  | MT043829 MT09560 MT09613 |
|                       | HN51                 | *Actinidia deliciosa* | China  | MT043837 MT09580 MT09621 |
|                       | MJL15                | *Actinidia deliciosa* | China  | MT043840 MT09583 MT09633 |
|                       | MJL35                | *Actinidia deliciosa* | China  | MT043844 MT09587 MT09637 |
|                       | SC41                 | *Actinidia chinensis* | China  | MT043845 MT09588 MT09623 |
|                       | SC65                 | *Actinidia chinensis* | China  | MT043847 MT09590 MT09625 |
| **Diaporthella corylina** | CBS121124*          | *Corylus sp.*       | China   | KC343004 KC343730 KC343972 |

* = ex-type culture.
/ = the Genbank accession number is absent.

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Table 1. (Continued).

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a ICMP: International Collection of Micro-organisms from Plants; FAU: Isolates in culture collection of Systematic Mycology and Microbiology Laboratory, USDA-ARS, Beltsville, Maryland, USA; BRIP: Queensland Plant Pathology herbarium/culture collection, Australia; CBS: Westerdijk Fungal Biodiversity Centre, Utrecht, The Netherlands; CFCC: China Forestry Culture Collection Center, China; LC: Corresponding author’s personal collection (deposited in laboratory State Key Laboratory of Mycology, Institute of Microbiology, Chinese Academy of Sciences); MFLUCC: Mae Fah Luang University Culture Collection, Chiang Rai, Thailand; ZJUD: Zhejiang University.
tree reconstruction under the best-fit partition model. Branch supports were assessed with ultrafast bootstrap approximation (UFBoot) of 1000 replicates (Hoang et al., 2017). Additionally, Bayesian inference (BI) was performed on the concatenated loci to construct phylogenies using MrBayes v. 3.2.2 (Ronquist et al., 2003) as described by Crous (2006). MrModeltest v. 2.3 (Nylander, 2004) was used to calculate the best-fit models of nucleotide substitution for each data partition with the corrected Akaike information criterion (AIC). Two analyses of four Markov Chain Monte Carlo (MCMC) chains were conducted from random trees with $8 \times 10^6$ generations. The analyses were sampled every 1000 generations, which were stopped once the average standard deviation of split frequencies was below 0.01. The first 25% of the trees were discarded as the burn-in phase, and the remaining trees were summarized to calculate the posterior probabilities (PP) of each clade being monophyletic. Phylogenetic trees were visualized in Figtree v.1.4.2 (Rambaut, 2014).

**Morphological and growth rate analyses**

Based on the results of the phylogenetic analyses, 17 representative isolates (including *D. alangii*: CQ155, FJHJB57; *D. compactum*: CQ178, SC67; *D. eres*: HN10, HB25, SX24, HB24, CQ3; *D. hongkongensis*: CQ51; *D. sojae*: CQ14, CQ78, CQ16; *D. tectonae*: CQ58, SC83; *D. unshiuensis*: CQ7, CQ9) were selected for morphological observations and growth rate assessments. Three-day-old mycelium plugs (5 mm diam.) were taken from the margins of actively growing cultures and transferred onto the centres of 9 cm diam. Petri dishes containing potato dextrose agar (PDA) or 2% tap water agar supplemented with sterile fennel stems. Cultures were incubated at 25°C with a 14 h/10 h fluorescent light/dark cycle (Guo et al., 2020b). Colony diameters were measured daily for 3 d to calculate mycelium growth rates (mm d$^{-1}$). For each representative isolate, these measurements were made in triplicate. Colony shape, density, and pigment production on PDA were noted after seven days. Generation of ascomata and conidiomata on PDA or fennel stems were examined periodically. Shape, colour, and size of asci were observed using light microscopy (Nikon Eclipse 90i or Olympus BX63), and 50 asci, ascospores, conidiophores, and conidia were measured.

**Pathogenicity and host range**

The 14 representative strains (*D. alangii*: CQ155, SC74; *D. compactum*: CQ178, SC67; *D. eres*: HN10, HB25; *D. hongkongensis*: CQ21, CQ51; *D. sojae*: CQ14, CQ16; *D. tectonae*: CQ166, CQ58; *D. unshiuensis*: CQ7, MJL15) were tested for pathogenicity on detached leaves or shoots of kiwifruit, and on branches of five other fruit tree species. One isolate of each species (*D. alangii*: CQ155; *D. compactum*: CQ178; *D. eres*: HN10; *D. hongkongensis*: CQ21; *D. sojae*: CQ14; *D. tectonae*: CQ58; *D. unshiuensis*: CQ7) was also inoculated onto fruits of kiwifruit to assess pathogenicity.

Leaves of *Actinidia chinensis* ‘Cuiyu’, wounded or unwounded, were inoculated with mycelium plugs of isolates in eight replicates, to assess the pathogenicity of representative isolates selected from the seven *Diaporthe* species. Fresh and healthy leaves were washed under running tap water followed by surface sterilization with 25% ethanol, drying with sterile tissue paper and then air-drying (Hawthorne and Otto, 2012; Mousakhah et al., 2014; Fu et al., 2018). For the wound inoculation method, the mycelium plugs (agar disks) were placed midway on each side of each leaf midrib after wounding three times by pinpricking with a sterilized needle (insect pin, 0.5 mm diam.). For the non-wound inoculation method, mycelium plugs were placed directly on the unwounded leaves. Inoculations with sterile agar plugs were used as negative controls. The experiment was conducted twice. The inoculated leaves were put into a plastic container covered with plastic film and incubated at 25 ± 1°C with a 12 h/12 h light/dark photoperiod. Symptoms and lesion lengths were recorded at 7 d after inoculation, and re-isolations were made from lesion margins to fulfil Koch’s postulates.

Pathogenicity was also determined on excised segments of 1-year-old woody shoots of *A. chinensis* ‘Cuiyu’, *A. chinensis* ‘Jinyan’, *A. chinensis* ‘Hongyang’, and *A. chinensis* ‘Huangjin’, in five replicates. Green shoots (5 – 10 mm diam.) were pruned from healthy kiwifruit vines and cut into 10 cm long segments, rinsed with tap water, surface disinfected with 75% ethanol, and then air-dried. Wounding and non-wounding inoculation methods were used. For the wounding treatment, a superficial wound (5 mm diam.) was made on each shoot segment by removing the cortex with a disinfected 5 mm diam. hole punch (Bai et al., 2015; Sessa et al., 2017). Agar plugs (5 mm diam.) from fungus cultures were inserted into the wounds, and the inoculated parts were sealed with Parafilm to maintain humidity (Mostert et al., 2001). For the non-wound inoculation method, the agar plugs were placed on the surface of unwounded shoots directly and the inoculated parts were sealed with Parafilm to maintain humidity. All inoculated shoots were kept in plastic containers covered with plastic film and maintained in the laboratory at 25°C. The experiment was conducted twice. Lesion lengths were measured at
10 d after inoculation, and pieces were excised from the xylem or phloem tissues under canker lesions neighbouring asymptomatic regions and were cultured to fulfil Koch’s postulates.

Detached fruits of *A. deliciosa* 'Hayward’ were inoculated with seven representative isolates in quadruplicate to determine isolate pathogenicity. The inoculations were conducted using wounded and non-wounded methods as previously described (Diaz et al., 2017; Li et al., 2017a). Healthy fruits were surface-sterilized with 75% ethanol prior to inoculation, washed three times with sterile water, and were air-dried (Zhou et al., 2015; Erper et al., 2017). For the wounded treatment, each mycelium plug was placed on the fruit after wounding once by pinpricking with a sterilized needle (5 mm deep) (Luongo et al., 2011). For the non-wounded method, the mycelium plug was directly placed on the surface of unwounded fruits. Inoculation with sterile agar plugs was used as controls. Inoculation points were individually wrapped with sterilized moist cotton plugs (Zhai et al., 2014; Bai et al., 2015). Fruits were placed in a sealed plastic container at 25°C with a 12 h/12 h light/dark photoperiod. The tests were repeated twice. Seven days post inoculation, symptoms on the fruit were recorded and the lengths of lesions were measured. Recovery isolations were made from the flesh at the margins of developed lesions.

The host range of the seven *Diaporthe* species was determined on detached shoots of five *Rosaceae* fruit tree species, including *Malus pumila* 'Hong Fushi', *Prunus salicina* 'Dahongpao', *Prunus armeniaca* 'Helanxiangxing', *Pyrus pyrifolia* 'Cuiguan' and *Prunus persica* 'Youtao'. Shoots of these plants were wound-inoculated (as described above). Five shoots of each host were used for each inoculation treatment.

### Data from repeated tests and among treatments in each test were analyzed using 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.

### RESULTS

#### Sampling and pathogen isolation

Investigations and analyses of the occurrence and sample collection of kiwifruit branch blight and leaf spot were conducted from October 2017 to May 2019. One hundred and six samples were collected from the surveyed orchards and nurseries for fungus isolations; 80 of the samples were diseased branches and 26 were infected leaves. In total, 284 *Diaporthe* isolates showed typical *Diaporthe* spp. cultural characteristics (Table 2), including fluffy, flattened, white, creamy, sulphur or grayish aerial mycelium, with solitary or aggregated, globose, dark pycnidia and the presence of alpha and/or beta conidia (Sessa et al., 2017; Guo et al., 2020b). Among them, 81 isolates were obtained from diseased leaf samples, and were identified as six species of *Diaporthe* (*D. alangii*, *D. compactum*, *D. eres*, *D. hongkongensis*, *D. sojae*, and *D. unshiuensis*). In total, 203 isolates were derived from infected shoots, and were identified as seven species of *Diaporthe* (*D. alangii*, *D. compactum*, *D. eres*, *D. hongkongensis*, *D. sojae*, *D. unshiuensis* and *D. tectonae*). The kiwifruit species and varieties from which these isolates were obtained included *A. chinensis* 'Cuiyu', 'Donghong', 'Hongyang', 'Huangjin', 'Jinyan',

| Sampling province | Number of isolates | *Diaporthe* unshiuensis | *D. eres* | *D. sojae* | *D. hongkongensis* | *D. compactum* | *D. alangii* | *D. tectonae* |
|-------------------|--------------------|------------------------|-----------|-----------|-------------------|---------------|-------------|--------------|
| Chongqing         | 92                 | 39                     | 19        | 22        | 7                 | 2             | 1           | 2            |
| Fujian            | 72                 | 43                     | 8         | 12        | 7                 | 1             | 1           | 0            |
| Henan             | 15                 | 3                      | 8         | 4         | 0                 | 0             | 0           | 0            |
| Hubei             | 30                 | 4                      | 11        | 8         | 4                 | 1             | 2           | 0            |
| Shanxi            | 17                 | 0                      | 17        | 0         | 0                 | 0             | 0           | 0            |
| Sichuan           | 30                 | 2                      | 17        | 7         | 0                 | 2             | 1           | 1            |
| Shandong          | 8                  | 0                      | 7         | 1         | 0                 | 0             | 0           | 0            |
| Zhejiang          | 4                  | 1                      | 0         | 3         | 0                 | 0             | 0           | 0            |
| Anhui             | 16                 | 8                      | 7         | 0         | 1                 | 0             | 0           | 0            |
| Total             | 284                | 100                    | 94        | 57        | 19                | 6             | 5           | 3            |
'Longzanghong', and A. deliciosa 'Cuixiang', 'Hayward', 'Jinkui', and 'Xuxiang'. Branch blight symptoms were commonly observed at the incision or pruned positions, with reddish-black fusiform or irregular necrotic lesions (Figure 1c); the lesions would gradually expand along each incision (Figure 1e). Under dry climate conditions, the infected branches turned brown and cracked, with internal discolourations (Figure 1d). Whole branches were withered (Figure 1f). The diseased leaves developed silvery gray or bronze spots, which were sporadically distributed on the leaves (Figure 1a). In the later stage of disease development, the spots were expanded to the edges of the leaves, and the leaves withered and curled at their margins. Scattered pycnidia were observed on the diseased leaves (Figure 1b).

**Phylogenetic analyses of isolated fungi**

The 43 representative isolates were subjected to multi-locus phylogenetic analyses with concatenated ITS, EF1-α, and TUB sequences together with 31 reference isolates from previously described species, including the outgroup sequence of *Diaporthe corylina* (culture CBS 121124). A total of 1557 characters (ITS: 1–541, EF1-α: 542–935, TUB: 936–1557) were included in the phylogenetic analyses. For the Bayesian analyses, the following priors were set in MrBayes for the different data partitions: the SYM+I+G model with invgamma-distributed rates was implemented for ITS; The GTR+G model with gamma-distributed rates was implemented for EF1-α; and the HKY +G model with propinv-distributed rates was implemented for TUB. For the IQ-tree inference, the SYM+I+G model was selected for ITS, GTR+I+G for EF1-α, and HYK+I+G for TUB. Bayesian posterior probability (PP ≥ 0.5) and Maximum likelihood bootstrap values (ML ≥50) were shown at the dendrogram nodes (Figures 2 and 3).

The multi-locus phylogenetic results showed that 43 representative isolates were assigned to seven species (Figure 2). Five isolates grouped with the type strain and other reference sequences of *D. alangii* (Bayesian posterior probability = 0.52, Maximum likelihood bootstrap
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Four isolates clustered together with the type strain and other reference strains of *D. compactum* with high support (1.00, 99). Ten isolates clustered together with the ex-type strain and other reference strains of *D. eres* with high support (1.00, 99). Five isolates grouped with the ex-type strain and other reference strains of *D. hongkongensis* with strong support (1.00, 100). Seven isolates clustered together with the type strain and other reference strains of *D. sojae* with strong support (1.00, 100). Three isolates grouped with the reference strains of *D. tectonae* with high support (0.89, 99). Nine isolates were identified as *D. unshiuensis*, forming a highly supported subclade (1.00, 100).

The individual alignments and trees of the three single loci used in the analyses were also compared with respect to their performance in species recognition. Each gene used differentiated *D. compactum*, *D. eres*, *D. hongkongensis*, *D. sojae*, and *D. unshiuensis*. Moreover, ITS and *EF1*-α gathered *D. tectonae* and *D. alangii* into one clade (data not shown). The phylogenetic analysis of TUB sequences showed that similar clades statuses compared with the phylogenetic analyses based on the concatenated ITS, *EF1*-α, and TUB sequences (Figure 3). The phylogenetic analysis of TUB sequences was conducted with a HKY+I+F substitution model using IQ-tree v.1.6.8, and 436 characters were included in the phylogenetic analysis.

**Morphological and growth rate analyses of isolates**

Morphological observations coupled with phylogenetic analyses were used to clarify the species delimi-
The morphological characteristics of the representative isolates of *Diaporthe* spp. recovered in this study were as follows:

**Diaporthe sojae.** Colonies on PDA had flattened mycelium and brown accumulation of pigment (Figure 4a), and colony diam. was 23–38 mm after 3 d at 25°C. Black or brown conidiomata were separated or aggregated on the colony surfaces of PDA (Figure 4b). Translucent spiral conidial cirri extruded from ostioles (Figure 4j). In the asexual state, two types of conidia were observed. Alpha conidia were hyaline, cylindrical, aseptate, biguttulate, rounded at each end (Figure 4s), 6–8 × 3–4 μm, mean ± SD = 7.0 ± 0.5 × 3.4 ± 0.3 μm. Beta conidia were filiform, hyaline, straight or hamate, aseptate, each with the base slightly inflated, tapering towards one apex (Figure 4s), 14–30 × 2–3 μm, mean ± SD = 23.9 ± 3.2 × 2.4 ± 0.2 μm. Conidiophores were phialidic, hyaline, terminal, cylindrical, 16–21 × 1.5–4 μm, tapered towards the apices (Figure 4l), Tapering perithecial necks protruded through substrata deeply immersed in fennel stem tissues (Figure 4 l). Asci were unitunicate, eight-spored, sessile, elongate to clavate...
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(Figure 4q), ascospores were hyaline, two-celled, often four-guttulate. *Conidiophores* phialidic, hyaline, terminal, ampulliform, 15–23 × 1.5–3.5 μm, tapered towards the apex (Figure 4I). Compared with the description of the ex-type isolate FAU 635, the asci of isolate CQ78 were slightly shorter (32.5–45.5 × 8–12 vs 38.5–46.5 × 7–9 μm; mean ± SD = 38.2 ± 2.9 × 10.0 ± 0.8 μm), and the ascospore were of similar size to the ex-type isolate (10–13 × 4–5 vs 9.5–12 × 3–4 μm), mean ± SD = 11.0 ± 0.7 × 4.3 ± 0.3 μm (Table 3). Of all species obtained from this study, only *D. sojae* produced the sexual state on fennel stems in vitro.

*Diaporthe eres*. Cultures appeared initially as white (surface) and pale yellowish to brownish at the centres with age, colony diam. 25–31 mm in 3 d at 25°C. Aerial mycelia were white, sparse and fluffy. Each colony on PDA contained no less than two wide concentric rings of conidiomata at maturity (Figure 4c). Conidiomata were subglobose to globose, dark brown to black, with spiral conidial cirri extruding from ostioles (Figure 4k). Alpha conidia were hyaline, fusiform or oval (Figure 4t), 7–11 × 3–5 μm, mean ± SD = 8.6 ± 0.9 × 3.7 ± 0.4 μm. Beta conidia were hyaline, filiform, smooth, curved, with truncate bases (Figure 4t), 30–43 × 2–3 μm, mean ± SD = 34.88 ± 3.56 × 2.35 ± 0.34 μm. Conidiophores were phialidic, hyaline, terminal, cylindrical, 9–13 × 1.5–4 μm, tapered towards the apices (Figure 4III).

*Diaporthe unshiuensis*. Aerial mycelia were white, sparse, turning to grey with age, and with light gray pigmentation at the colony centres (Figure 4d), with colony diam. 23–28 mm in 3 d at 25°C. Conidiomata were subglobose, black and solitary or aggregated on the medium surface, with opalescent, glossy conidial drops exuding from the ostioles (Figure 4l). Alpha conidia were hyaline, ellipsoidal or clavate, smooth, and aseptate (Figure 4u), 6–8 × 2–4 μm, mean ± SD = 7.0 ± 0.5 × 3.0 ± 0.3 μm. Beta conidia were filiform, hyaline, smooth, curved, with truncate bases (Figure 4u), 22–40 × 2–3 μm, mean ± SD = 28.8 ± 3.5 × 2.2 ± 0.3 μm. Conidiophores were phialidic, hyaline, terminal, and cylindrical, 15–26 × 1.5–2.5 μm, and tapered towards the apices (Figure 4IV).

*Diaporthe compactum*. Cultures were entirely white from above, and brown, and feathery from below, with neat margins (Figure 4e), and colony diam. 27–30 mm after 3 d at 25°C. Brown or pale gray and spheroidal conidiomata were semi- or fully-embedded in the media, and conidia exuded from ostioles in lustrous, yellowish drops (Figure 4m). Alpha conidia were fusiform, hya-
Table 3. Sizes of alpha and beta conidia, and growth rates in culture, of representative isolates of *Diaporthe* spp. obtained in this study.

| Species, isolate | Conidium sizes | Means ± SD of conidia | Growth rate (mm d⁻¹) |
|------------------|----------------|-----------------------|----------------------|
|                  | Alpha conidia a | Beta conidia b | α-Conidia | β-Conidia |
|                  | Length (μm) | Width (μm) | Length (μm) | Width (μm) | |
| Diaporthe eres   | HN10 | 6.56–11.23 | 3.18–4.88 | 29.43–43.23 | 1.69–3.11 | 8.59 ± 0.86 × 3.69 ± 0.35 | 34.88 ± 3.56 × 2.35 ± 0.34 | 10.1 |
|                  | HB25 | 6.85–10.27 | 3.18–4.13 | 26.26–41.96 | 1.74–3.83 | 7.91 ± 0.64 × 3.70 ± 0.24 | 34.42 ± 0.45 × 2.53 ± 0.37 | 9.2 |
|                  | SX24 | 6.06–8.52 | 2.81–4.13 | 20.91–32.30 | 1.44–3.09 | 7.30 ± 0.52 × 3.60 ± 0.28 | 27.53 ± 2.70 × 2.14 ± 0.30 | 8.3 |
|                  | HB24 | 5.86–8.48 | 2.76–4.70 | / / | 7.24 ± 0.58 × 3.67 ± 0.38 | / | / | 8.5 |
|                  | CQ3  | 5.22–7.61 | 2.75–3.53 | / / | 6.44 ± 0.60 × 3.11 ± 0.19 | / | / | 11.7 |
| D. hongkongensis  | CQ51 | 6.97–10.39 | 3.07–4.65 | / / | 8.47 ± 0.76 × 3.78 ± 0.33 | / | / | 9.8 |
| D. sojae         | CQ14 | 5.57–8.27 | 2.82–4.05 | 14.43–30.54 | 1.67–3.29 | 6.98 ± 0.52 × 3.42 ± 0.29 | 23.89 ± 3.19 × 2.38 ± 0.16 | 7.6 |
|                  | CQ78 | 6.19–9.17 | 3.10–5.00 | / / | 7.81 ± 0.76 × 3.92 ± 0.42 | / | / | 12.5 |
|                  | CQ16 | 6.16–8.11 | 2.11–3.56 | / / | 7.15 ± 0.48 × 2.95 ± 0.27 | / | / | 9.8 |
| D. unshiuensis   | CQ7  | 5.82–8.74 | 2.40–3.50 | 21.98–41.15 | 1.47–2.71 | 6.96 ± 0.52 × 3.00 ± 0.27 | 28.77 ± 3.48 × 2.15 ± 0.32 | 9.3 |
|                  | CQ9  | 5.81–8.81 | 2.59–4.05 | 18.31–30.97 | 1.85–3.19 | 7.27 ± 0.67 × 3.28 ± 0.30 | 25.48 ± 2.18 × 2.88 ± 0.28 | 7.8 |
| D. tectonae      | CQ58 | 4.38–7.09 | 2.11–3.28 | / / | 5.60 ± 0.58 × 2.64 ± 0.30 | / | / | 12.3 |
|                  | SC83 | 5.65–6.86 | 2.56–3.42 | / / | 7.91 ± 0.64 × 3.70 ± 0.24 | / | / | 10.4 |
| D. compactum     | CQ178 | 6.19–8.52 | 2.98–4.24 | / / | 7.27 ± 0.52 × 3.73 ± 0.28 | / | / | 9.6 |
|                  | SC67 | 6.19–10.01 | 2.75–4.40 | / / | 7.86 ± 0.54 × 3.53 ± 0.31 | / | / | 8.6 |
| D. alangii       | CQ155 | 5.44–9.28 | 2.33–4.13 | 22.33–40.86 | 1.42–4.00 | 7.55 ± 0.85 × 3.21 ± 0.45 | 32.67 ± 4.99 × 2.49 ± 0.49 | 9.8 |
|                  | FJHJB57 | 5.72–10.14 | 2.77–4.45 | / / | 7.20 ± 0.76 × 3.64 ± 0.35 | / | / | 8.1 |

a Minimum and maximum lengths and widths of 50 alpha conidia.

b Minimum and maximum lengths and widths of 50 beta conidia.

c Mean conidium sizes of calculated from statistical analyses. 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 Duncan’s test (at P = 0.05). SD = standard deviation. / indicates where beta conidia were not produced.
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line, usually biguttulate, straight or slightly curved, and both ends of each conidium were blunt, or one end was rounded and the other acute (Figure 4v), 6–9 × 3–4 μm, mean ± SD = 7.3 ± 0.5 × 3.7 ± 0.3 μm. Beta conidia were not observed. Conidiophores were phialidic, hyaline, terminal, ampulliform, and tapered towards their apices, 9–17 × 1–3 μm (Figure 4V).

*Diaporthe alangii*. Aerial mycelium was sparse. Colonies were white at first, and becoming light brown due to pigment formation, with neat or petaloid margins (Figure 4f), colony diam. 24–30 mm after 3 d at 25°C. Conidiomata were scattered, black or brown and irregularly distributed over agar surfaces, with yellowish conidial drops exuding from the ostioles (Figure 4n). Alpha conidia were aseptate, hyaline, biguttulate, each usually with one end obtuse and the other acute (Figure 4w), 6–10 × 3–5 μm, mean ± SD = 7.2 ± 0.8 × 3.6 ± 0.4 μm. Beta conidia were not observed. Conidiophores were phialidic, hyaline, terminal, ampulliform, tapered towards the apices, 9–18 × 1.5–2.5 μm (Figure 4VI).

*Diaporthe hongkongensis*. Colonies were snow-white, with dense aerial mycelium, which collapsed in the center, and the collapsed parts were moist and sticky (Figure 4g), colony diam. 30 mm after 3 d at 25°C. Conidiomata were spheroidal and enveloped by tangled mycelia. Irregular and yellowish conidial piles were effusing from the ostioles (Figure 4o). Alpha conidia were fusiform, hyaline, septate, and not obtuse at both ends (Figure 4x), 7–10 × 3–5 μm, mean ± SD = 8.5 ± 0.8 × 3.8 ± 0.3 μm. Beta conidia was not observed. Conidiophores were phialidic, hyaline, terminal, cylindrical, tapered towards their apices, 25–30 × 1–3 μm (Figure 4VII).

*Diaporthe tectonae*. Colonies were white at first, with gray pigmentation gradually accumulating in the centers until entire colony colonies turned brown (Figure 4h), colony diam. 31–36 mm after 3 d at 25°C. Conidiomata globose, black or brown, scattered, erumpent on PDA, with conidial droplets exuding from the centre ostioles (Figure 4p). Alpha conidia were cylindrical or fusiform, hyaline, usually biguttulate, and septate (Figure 4y), 4–7 × 2–3 μm, mean ± SD = 5.6 ± 0.6 × 2.6 ± 0.3 μm. Beta conidia were not observed. Conidiophores phialidic, hyaline, terminal, ampulliform, and tapered towards their apices, measuring 11–20 × 1.5–3 μm (Figure 4VIII).

**Pathogenicity tests**

Two typical symptoms developed on detached leaves of *A. chinensis*, which were observed at the wound sites at 7 d post inoculation. One symptom consisted of reddish-brown, round or suborbicular, small, slowly expan- sion lesions, while the other consisted of lesions with black necrotic centres surrounded by round or suborbicular brown halos (Figure 5a). The first of these symptoms developed after inoculations with *D. compactum, D. eses, D. sojae* or *D. unshiuensis*, while the second was induced by inoculations with *D. tectonae, D. alangii* or *D. hongkongensis*. Lesion diameters caused by the different fungi differed significantly. *Diaporthe tectonae*, or *D. alangii* caused large lesions (mean diam. = 10–22 mm) on all the inoculated leaves, those caused by *D. hongkongensis* or *D. eses* were smaller (5–8 mm), while those caused by isolates of *D. sojae, D. compactum*, or *D. unshiuensis* were smaller still (2–4 mm) (Figure 6a). Unwounded leaves inoculated with *Diaporthe* spp. isolates remained symptomless. In parallel, no lesions were observed on the leaves that were wound and non-wound inoculated with PDA discs as controls. Koch’s postulates were fulfilled by re-isolating each *Diaporthe* sp. isolate only from symptomatic leaves.

In the pathogenicity tests conducted on branches of four kiwifruit varieties, all the *Diaporthe* species were pathogenic to wounded branches. The symptoms induced by representative isolates were similar, as fusiform necrotic lesions and internal discolouration observed at the wound sites (Figure 5b). The lesion lengths caused by the representative isolates tested on different cultivars were diverse. *Diaporthe tectonae* and one isolate of *D. alangii* (SC74) induced large lesions (mean length = 67–83 mm) on *A. chinensis* ‘Huangjin’, *D. alangii* (CQ155) or *D. eses* caused smaller lesions (20–30 mm), and the other pathogens induced even smaller (2–18 mm) (Figure 6b). *Diaporthe tectonae, D. alangii*, and a *D. sojae* isolate (CQ16) caused large lesions (50–106 mm) on *A. chinensis* ‘Hongyang’, while the remaining isolates caused shorter lesions (4–20 mm) (Figure 6c). *Diaporthe tectonae* or *D. alangii* (SC74) induced large lesions (39–54 mm) on *A. chinensis* ‘Jinyan’, while *D. eses* (HB25) and *D. alangii* (CQ155) caused smaller lesions (5–8 mm), while *D. sojae* (SC74) induced smaller lesions (9–18 mm) on *A. chinensis* ‘Cuiyu’, *D. alangii* (CQ155), *D. compactum* (CQ178), *D. eses* (HB25), or *D. sojae* (CQ16) caused shorter lesions (15–25 mm), and those from the remaining isolates were shorter still (3–10 mm) (Figure 6e). Unwounded shoots of kiwifruit inoculated with *Diaporthe* spp. isolates remained symptomless, and no lesions developed on the shoots that were wound and non-wound inoculated with PDA discs. Each respective *Diaporthe* species was re-isolated from inoculated symptomatic shoots, fulfilling Koch’s postulates for these pathogens.
Figure 5. Pathogenesis of seven *Diaporthe* species on kiwifruit leaves and stems. a, symptoms caused after inoculation of wounded kiwi leaves (*Actinidia chinensis* 'Cuiyu') with mycelium plugs from cultures of seven *Diaporthe* spp. b, symptoms caused by inoculation of wounded kiwi branches (*Actinidia chinensis* 'Hongyang') with mycelium plugs from cultures of seven *Diaporthe* spp.
Fruits were susceptible to the representative isolates selected from each species, and all tested species caused rots on wounded fruits. Typical symptoms were some sarcocarp tissues swollen with internal softening around the inoculation wounds at early stages, transparent drops streaming from the inoculation punctures, epidermis peeling, and brownish, damp and rotted flesh (Figure 7a). Fruits inoculated with *D. alangii* (CQ155), *D. eres* (HN10), *D. sojae* (CQ14), *D. tectonae* (CQ58), or *D. hongkongensis* (CQ21) had larger lesions (mean diam. = 30–42 mm) than those inoculated with *D. unshiuensis* (CQ7) or *D. compactum* (CQ178), (13–17 mm) (Figure 7b). All the non-wounded fruits inoculated with *Diaporthe* spp. isolates remained symptomless. The negative controls of wounded and unwounded fruits did not produce lesions. Each respective *Diaporthe* species was re-isolated from the symptomatic fruits, fulfilling Koch’s postulates for these pathogens.

In the host range tests, at 10 d post-inoculation, the seven *Diaporthe* species all caused canker symptoms on detached shoots of the five different fruit crop plants. After removing phloem tissues, maroon and fusiform necrotic lesions emerged in the underlying wood below, and these extended along the inoculated branches. In most cases, the affected shoots of pear and apple showed swollen and the bark cracking at the margins, with dark-brown to reddish cankers and abundant gummosis were observed at the inoculation sites on the branch of plum and apricot. The symptoms produced on peach shoots were black depressed cankers. The *Diaporthe* isolates caused different degrees of lesioning on detached branches of the different fruit tree species.
Diaporthe tectonae or D. alangii isolates caused large lesions (mean length = 18–39 mm), and D. compactum, D. eres, D. hongkongensis, D. sojae, or D. unshiuensis caused shorter lesions (5–15 mm) on Pyrus pyrifolia ‘Cuiguan’ (Figure 8a). Lesion lengths on Prunus salicina ‘Dahongpao’ caused by the seven Diaporthe species were of length 5–20 mm, except that two isolates caused larger lesions, with those from D. sojae isolate (CQ14) being 30 mm, and those from D. alangii isolate (SC74) being 53 mm (Figure 8b). The lesion lengths on Malus pumila ‘Hong Fushi’ caused by the seven Diaporthe species were mostly 5–15 mm in length, except for those from one isolate of D. alangii (SC74) which were longer (70 mm) (Figure 8c). The lesion lengths on Prunus persica ‘Youtao’ caused by D. alangii isolate CQ155, D. compactum isolate CQ178, D. eres, D. hongkongensis, D. sojae, or D. unshiuensis were 5–10 mm long, and those from the remaining isolates were larger (15–25 mm) (Figure 8d). Diaporthe alangii caused large lesions (60 mm) on Prunus armeniaca ‘Helanxiangxing’, followed by D. hongkongensis, D. eres, D. sojae, D. tectonae isolate CQ58, or D. unshiuensis isolate CQ7 (15–30 mm), and the remaining isolates caused short lesions (5–10 mm) (Figure 8e). No lesions were induced in the branches inoculated with non-colonized PDA plugs.

**DISCUSSION**

*Diaporthe* spp. previously reported on kiwifruit have been associated with fruit stem-end rots (Sommer and Beraha, 1975; Hawthorne et al., 1982; Lee et al., 2001; Koh et al., 2005; Luongo et al., 2011; Thomidis et al., 2019), and with shoot blight and leaf spots. In the present study, a large-scale investigation of *Diaporthe* species associated with kiwifruit infections was conducted in nine major cultivation provinces of China. Multilocus phylogenetic analyses and morphological characterization of isolated fungi were employed to evaluate the diversity of *Diaporthe* species associated with shoot blight and leaf spot of kiwifruit, and pathogenicity tests was performed to fulfill Koch’s postulates for representative *Diaporthe* isolates. This study has shown that seven *Diaporthe* species, including *D. unshiuensis*, *D. eres*, *D. sojae*, *D. hongkongensis*, *D. compactum*, *D. alangii*, and *D. tectonae*, were the causal organisms of shoot blight and leaf spot diseases of kiwifruit. As well, *D. unshiuensis*, *D. sojae*, *D. compactum*, *D. alangii*, and *D. tectonae* are here first reported as causes of kiwifruit shoot blight and leaf spot. The study was comprehensive, investigating samples from 16 orchards located in nine major kiwifruit production areas in China, and used phylogenetic analyses and morphology to characterize a large number of fungus isolates.

DNA sequence data are essential for resolving taxonomic questions, redefining species boundaries, and accurate species nomenclature (Guarnaccia and Crous, 2017; Guarnaccia et al., 2017). Phylogenetic analyses individually based on ITS, EF1-α, and TUB sequence data differentiated *D. compactum*, *D. eres*, *D. hongkongensis*, *D. sojae*, and *D. unshiuensis*. However, ITS and EF1-α gathered *D. tectonae* and *D. alangii* into one
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Several studies have used three to five concatenated genes simultaneously to separate species within Diaporthe (Santos et al., 2011; Gomes et al., 2013; Gao et al., 2015; Diaz et al., 2017). As ITS and the EF1-α gene have limitations for distinguishing D. alangii and D. tectonae, the concatenated ITS, EF1-α, and TUB phylogenetic analysis was successively employed to discriminate these two fungi. The results showed that the two species clustered into two clades with high bootstrap (1.00/99). These two fungi also differed morphologically, with D. tectonae having shorter alpha conidia than D. alangii.

Prevalence analyses of the seven Diaporthe spp. showed that the most dominant species responsible for leaf spot and branch blight of kiwifruit were: D. unshiuenis (100 isolates, 35.2%, isolated from Anhui, Chongqing, Fujian, Henan, Hubei, Sichuan, and Zhejiang); and D. sojae (57 isolates, 20.1%, isolated from Chongqing, Fujian, Hubei, and Zhejiang). The other identified fungi were less common, including: D. honkongensis (19 isolates, 6.7%, isolated from Anhui, Chongqing, Fujian, Hubei, and Zhejiang), D. compactum (six isolates, 2.1%, isolated from Chongqing, Fujian, Hubei, and Sichuan); D. alangii (five isolates, 1.8%, isolated from Chongqing, Fujian, Hubei, and Sichuan), and D. tectonae (three isolates, 1.1%, isolated from Chongqing and Sichuan) (Table 2). Analysis of Diaporthe species in the sampled areas showed obvious species diversity in Anhui, Chongqing, Fujian, Henan, Hubei, Shandong, Shanxi, Sichuan, and Zhejiang. This may be attributed to the humid and warm climate in these provinces, which is suitable for survival of Diaporthe spp. In contrast, only one Diaporthe sp. was identified from Shanxi, two from

Figure 8. Mean lesion lengths on wounded shoots of pear, plum, apple, peach, and apricot, at 10 d post-inoculation, induced by mycelium plugs from cultures of representative isolates of seven Diaporthe species. a, lesion lengths on shoots of Pyrus pyrifolia 'Cuiguan', b, Prunus salicina 'Dahongpao', c, Malus pumila 'Hong Fushi', d, Prunus persica 'Youtao', or e, Prunus armeniaca 'Helanxiangxing'.

D. hongkongensis: CQ21, CQ51
D. unshiuenis: CQ7, MJL15
D. eres: HN10, HB25
D. compactum: CQ178, SC67
D. sojae: CQ14, CQ16
D. tectonae: CQ166, CQ58
D. alangii: CQ155, SC74
Shandong, and three species were identified from Henan (Figure 9a), which may be related to the dry climate of these three provinces being unsuitable for Diaporthe.

Since Diaporthe spp. have endophytic, saprobic or pathogenic lifestyles, pathogenicity to kiwifruit was assessed by inoculating leaves, shoots, and fruit of different kiwifruit species, using wound and non-wound inoculation methods. Wound inoculations showed that all the species were pathogenic and caused leaf spot and shoot blight of this host. The different fungi also showed significantly different virulence, with D. alangii and D. tectone as the most aggressive species, followed by D. compactum, D. eres, D. hongkongensis, D. unshiuensis, and D. sojae. It is significant, however, that the inoculations of these seven species on unwounded leaves, branches, and fruits did not cause disease symptoms. These species can be endophytes and opportunistic pathogens occurring in a wide range of hosts and later as saprobes on dead host tissues.

Host affiliation has been for species delimitation in Diaporthe, but this has proved uninformative because many Diaporthe spp. have been recorded on a wide range of hosts (Santos and Phillips, 2009; Udayanga et al., 2012; Gao et al., 2015; Guarnaccia et al., 2020). For example, D. lithocarpus was confirmed as the cause of diseases on five plant hosts belonging to different families, and Lithocarpus glabra was shown to host seven different species of Diaporthe (Gao et al., 2014). In the present study, the seven Diaporthe species isolated from kiwifruit were not host-specific. Diaporthe tectoneae was first reported to cause branch and twig dieback on Tectona grandis in Northern Thailand (Doilom et al., 2016), and the present study has showed that D. tectoneae could induce shoot blight of kiwifruit in China. Diaporthe alangii was originally isolated from dieback branches of Alangium in China (Yang et al., 2018b). The present study confirmed D. tectoneae as the cause of leaf spot and shoot blight of kiwifruit. Diaporthe eres, D. sojae, and D. hongkongensis are pathogens causing shoot canker of grapevine and pear (Dissanayake et al., 2014; Guo et al., 2020b), as well as kiwifruit shoot blight and leaf spot (present study). Diaporthe unshiuensis was reported from the fruit of Citrus unshiu with unidentified symptoms and non-symptomatic branches and twigs of Fortunella margarita (Huang et al., 2015), and the present study showed weak aggressiveness of this species to kiwifruit. Several Diaporthe spp. have been recently recognized as causal agents of diseases of Rosaceae fruit crop plants, including peach, pear, and apple (Bai et al., 2015; Sessa et al., 2017; Tian et al., 2018; Guo et
al., 2020b). However, kiwifruit is often in mixed plantings with apple, apricot, pear, peach, and plum in many kiwifruit production areas in China. Results of host range and virulence assessments described here have shown the seven *Diaporthe* species were pathogenic, not only to kiwifruit, but also to many other *Rosaceae* fruit crop hosts. This indicates that these pathogens have the potential to infect these alternative hosts, potentially providing pathogen inoculum across these hosts.

In conclusion, identification of these pathogens provides valuable new information to assist understanding of leaf spot and branch blight of kiwifruit. The study has also shown the *Diaporthe* species responsible for these diseases, which will assist the design of potential disease prevention and management strategies for these economically important diseases.

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