Phylogenetic Analysis and Development of Molecular Tool for Detection of *Diaporthe citri* Causing Melanose Disease of Citrus

Chingchai Chaisiri 1,2, Xiang-Yu Liu 1,3, Yang Lin 1, Jiang-Bo Li 3, Bin Xiong 3 and Chao-Xi Luo 1,2,*

1 Key Lab of Horticultural Plant Biology, Ministry of Education, Huazhong Agricultural University, Wuhan 430070, China; chaisiri.ch@gmail.com (C.C.); tj0806xy@qq.com (X.-Y.L.); yanglin@mail.hzau.edu.cn (Y.L.)

2 Department of Plant Pathology, College of Plant Science & Technology, and Key Lab of Crop Disease Monitoring & Safety Control in Hubei Province, Huazhong Agricultural University, Wuhan 430070, China

3 Nanfeng Citrus Research Institute, Nanfeng 344500, China; fzgyjljb@163.com (J.-B.L.); fzzsgyj@163.com (B.X.)

* Correspondence: cxluo@mail.hzau.edu.cn

Received: 16 February 2020; Accepted: 27 February 2020; Published: 4 March 2020

Abstract: Melanose disease caused by *Diaporthe citri* is considered as one of the most important and destructive diseases of citrus worldwide. In this study, isolates from melanose samples were obtained and analyzed. Firstly, the internal transcribed spacer (ITS) sequences were used to measure *Diaporthe*-like boundary species. Then, a subset of thirty-eight representatives were selected to perform the phylogenetic analysis with combined sequences of ITS, beta-tubulin gene (*TUB*), translation elongation factor 1-alpha gene (*TEF*), calmodulin gene (*CAL*), and histone-3 gene (*HIS*). As a result, these representative isolates were identified belonging to *D. citri*, *D. citriasiana*, *D. discoidispora*, *D. eres*, *D. sojae*, and *D. unshiuensis*. Among these species, the *D. citri* was the predominant species that could be isolated at highest rate from different melanose diseased tissues. The morphological characteristics of representative isolates of *D. citri* were investigated on different media. Finally, a molecular tool based on the novel species-specific primer pair TUBDcitri-F1/TUBD-R1, which was designed from highly conserved region of *TUB* gene, was developed to detect *D. citri* efficiently. A polymerase chain reaction (PCR) amplicon of 217 bp could be specifically amplified with the developed molecular tool. The sensitivity of the novel species-specific detection was upon to 10 pg of *D. citri* genomic DNA in a reaction. Therefore, the *D. citri* could be unequivocally identified from closely related *Diaporthe* species by using this simple PCR approach.

Keywords: Citrus; *Diaporthe citri*; geographical distribution; molecular diagnostics; multi-locus phylogenetics

1. Introduction

Citrus and their allied genera (including *Eremocitrus*, *Fortunella*, *Microcitrus*, and *Poncirus*) are widely distributed worldwide, among them, the most popular cultivars belong to the Aurantioidae subfamily of the Rutaceae family. Allegedly, the citrus was originally cultivated in Himalayas 4000 years ago [1]. Nowadays, *Citrus* is one of the most widely cultivated fruit crops with a planting area of 2.5 million ha and production of more than 38 million tons per year in China [2]. The popular citrus cultivars in China include *Citrus reticulata* (mandarin), *Citrus sinensis* (sweet orange), *Citrus grandis* or *Citrus maxima* (pummelo), and *Citrus paradisi* (grapefruit) [3].
The *Diaporthe* genus fungi are well-known as saprobic-, endophytic-, and pathogenic-plant parasites on economically significant plant cultivars [4–8]. One host species can be affected by many different *Diaporthe* species, whereas one *Diaporthe* species can infect many hosts species [9–13]. Accurate identification of *Diaporthe* species is very important for controlling the diseases caused by these fungi and making effective quarantine strategies [14–17].

*Diaporthe citri* (syn. *Phomopsis citri*) has a wide spectrum on several citrus species including mandarin, sweet orange, pumelo, grapefruit, and lemons [18]. A potential damage referred multiple symptoms e.g., wood canker, twig blight, brunch dieback, gummosis, stem-end rot, and melanose [18–24]. The melanose, one of the most serious citrus diseases caused by *D. citri* was firstly reported on citrus fruits in Florida [25]. In 1912, Fawcett [26] reported that stem-end rot was caused by *Phomopsis citri*, while Floyd and Stevens [27] provided the evidence that stem-end rot and melanose disease were infected by the same fungus. In 1914, a fungus *Diaporthe citrincola* was firstly collected and described from twigs of *Citrus nobilis* [28]. In 1917, *Phomopsis cardaba* was reported on twigs of grapefruit in Isle of Pines, Cuba [29]. In early studies, *D. citri* was reported in several names including *Diaporthe medusaea* [30], *Phomopsis californica* [31], and *Phoma cytosporella* [28]. In 1928, Bach and Wolf [32] fulfilled Koch’s postulates for *D. citri* infection on citrus. Pathogenicity test demonstrated that both conidiospore of *P. citri* and ascospore of *D. citri* could produce leaf melanose symptoms [33].

Traditional molecular barcoding for fungal species discrimination based on nuclear ribosomal internal transcribed spacer regions (ITS) is frequently used for the identification of *Diaporthe* genus [7,34–36]. The molecular phylogeny based on the combination of multi-locus DNA sequences showed better identification of *Diaporthe* species [6,20,37–40]. The combination of translation elongation factor 1-α gene (*TEF*), beta-tubulin gene (*TUB*), calmodulin gene (*CAL*), and histone-3 gene (*HIS*) showed good resolution for *Diaporthe* species discrimination [7,38,41]. Generally, molecular marker was used to detect *Diaporthe* species, and many species-specific primers were designed based on conserved ITS region such as in *Diaporthe phaseolorum* and *Diaporthe longicolla* from soybean [42], *Diaporthe azadirachtae* from neem [43,44], *Diaporthe sclerotioides* from plants and soils [45]. Also, a molecular tool based on *TEF* gene was developed to detect *Diaporthe azadirachtae* from neem [46]. However, these methods are hard to distinguish *D. citri* and its closely related species because only limited informative variations could be found in both the ITS region and *TEF* gene, thus, it is hard to design specific primers based on these sequences to distinguish *D. citri* from other *Diaporthe* species.

The aims of this study was to: (i) to define the species discrimination of *D. citri* based on phylogenetic analyses and (ii) to develop a molecular tool to simply detect *D. citri* from multiple *Diaporthe* species on citrus plants.

2. Results

2.1. Isolation of *Diaporthe* Species

Totally 140 isolates were obtained and 38 representative isolates from different tissues, i.e., leaves, fruits, and twigs were selected for further study (Table 1; Figure 1). The identification based on ITS sequence analysis showed that all these isolates belong to *Diaporthe* species (Supplementary Figure S1).
| Diaporthe Species | Isolate Number | Plant Host | Tissue | Locality | GenBank Accession Numbers 1 |
|-------------------|----------------|------------|--------|----------|-----------------------------|
| D. citri          | NFF1-1-2       | Citrus reticulata cv. Nanfengmiju | fruit | China: Jiangxi: Nanfeng | MN816394, MN894454, MN894415, MN894355, MN894380 |
|                   | NFF1-1-4       | Citrus reticulata cv. Nanfengmiju | fruit | China: Jiangxi: Nanfeng | MN816395, MN894455, MN894416, MN894356, MN894381 |
|                   | NFF1-2-5       | Citrus reticulata cv. Nanfengmiju | fruit | China: Jiangxi: Nanfeng | MN816396, MN894456, MN894417, MN894357, MN894382 |
|                   | NFF1-13        | Citrus reticulata cv. Nanfengmiju | leaf | China: Jiangxi: Nanfeng | MN816397, MN894457, MN894418, MN894358, MN894360 |
|                   | NFF1-8         | Citrus reticulata cv. Nanfengmiju | leaf | China: Jiangxi: Nanfeng | MN816398, MN894458, MN894419, MN894359, MN894358 |
|                   | NFF1-8-4       | Citrus reticulata cv. Nanfengmiju | fruit | China: Jiangxi: Nanfeng | MN816399, MN894459, MN894420, MN894360, MN894383 |
|                   | NFKL-7-11      | Citrus reticulata cv. Nanfengmiju | leaf | China: Jiangxi: Nanfeng | MN816400, MN894460, MN894421, MN894361, MN894383 |
|                   | NKDL-1-2       | Citrus sinensis | leaf | China: Jiangxi: Nanfeng | MN816401, MN894461, MN894422, MN894362, MN894384 |
|                   | NKCL-6-12      | Citrus sinensis | leaf | China: Jiangxi: Nanfeng | MN816402, MN894462, MN894423, MN894363, MN894385 |
|                   | NKCT-6-24      | Citrus sinensis | twig | China: Jiangxi: Nanfeng | MN816403, MN894463, MN894424, MN894364, MN894386 |
| D. citriamia      | XFAL-1-1       | Citrus sinensis | leaf | China: Jiangxi: Xinfeng | MN816404, MN894464, MN894425, MN894387, MN894388 |
|                   | NFF1-2-4       | Citrus reticulata cv. Nanfengmiju | leaf | China: Jiangxi: Nanfeng | MN816405, MN894465, MN894426, MN894388, MN894432 |
|                   | XFAL-15-2      | Citrus sinensis | leaf | China: Jiangxi: Xinfeng | MN816406, MN894466, MN894427, MN894389, MN894432 |
| D. discodiapora   | NFF1-1-1       | Citrus reticulata cv. Nanfengmiju | fruit | China: Jiangxi: Nanfeng | MN816407, MN894467, MN894428, MN894432, MN894434 |
|                   | NKDL-1-2       | Citrus sinensis | leaf | China: Jiangxi: Nanfeng | MN816408, MN894468, MN894429, MN894432, MN894434 |
|                   | NKDL-2-3       | Citrus sinensis | leaf | China: Jiangxi: Nanfeng | MN816409, MN894469, MN894430, MN894432, MN894434 |
|                   | NKDL-3-6       | Citrus sinensis | leaf | China: Jiangxi: Nanfeng | MN816410, MN894470, MN894431, MN894432, MN894434 |
|                   | NFKL-3-4       | Citrus reticulata cv. Nanfengmiju | leaf | China: Jiangxi: Nanfeng | MN816411, MN894471, MN894432, MN894434, MN894436 |
| D. ersonii        | NFF1-1-25      | Citrus reticulata cv. Nanfengmiju | leaf | China: Jiangxi: Nanfeng | MN816412, MN894472, MN894433, MN894435, MN894436 |
|                   | NFF1-1-36      | Citrus reticulata cv. Nanfengmiju | leaf | China: Jiangxi: Nanfeng | MN816413, MN894473, MN894434, MN894435, MN894436 |
|                   | NFKL-2-17      | Citrus reticulata cv. Nanfengmiju | leaf | China: Jiangxi: Nanfeng | MN816414, MN894474, MN894435, MN894436, MN894437 |
|                   | NFKL-2-8       | Citrus reticulata cv. Nanfengmiju | leaf | China: Jiangxi: Nanfeng | MN816415, MN894475, MN894436, MN894437, MN894438 |
|                   | NFKL-3-1       | Citrus reticulata cv. Nanfengmiju | leaf | China: Jiangxi: Nanfeng | MN816416, MN894476, MN894437, MN894438, MN894439 |
|                   | NFKL-4-5       | Citrus reticulata cv. Nanfengmiju | leaf | China: Jiangxi: Nanfeng | MN816417, MN894477, MN894438, MN894439, MN894400 |
|                   | NFFT-3-3       | Citrus reticulata cv. Nanfengmiju | twig | China: Jiangxi: Nanfeng | MN816418, MN894478, MN894439, MN894440, MN894401 |
|                   | NFFT-3-8       | Citrus reticulata cv. Nanfengmiju | twig | China: Jiangxi: Nanfeng | MN816419, MN894479, MN894440, MN894441, MN894402 |
|                   | NFF1-1-1       | Citrus reticulata cv. Nanfengmiju | fruit | China: Jiangxi: Nanfeng | MN816420, MN894480, MN894441, MN894442, MN894403 |
|                   | NFF1-1-7       | Citrus reticulata cv. Nanfengmiju | fruit | China: Jiangxi: Nanfeng | MN816421, MN894481, MN894442, MN894443, MN894404 |
|                   | NFF1-3-13      | Citrus reticulata cv. Nanfengmiju | twig | China: Jiangxi: Nanfeng | MN816422, MN894482, MN894443, MN894444, MN894405 |
|                   | NFF1-3-10      | Citrus reticulata cv. Nanfengmiju | twig | China: Jiangxi: Nanfeng | MN816423, MN894483, MN894444, MN894445, MN894406 |
|                   | NFF1-3-27      | Citrus reticulata cv. Nanfengmiju | leaf | China: Jiangxi: Nanfeng | MN816424, MN894484, MN894445, MN894446, MN894407 |
|                   | NFKL-1-7       | Citrus reticulata cv. Nanfengmiju | leaf | China: Jiangxi: Nanfeng | MN816425, MN894485, MN894446, MN894447, MN894408 |
| D. soyae          | NFGL-1-5       | Citrus reticulata cv. Nanfengmiju | leaf | China: Jiangxi: Nanfeng | MN816426, MN894486, MN894447, MN894448, MN894409 |
|                   | NFGL-1-10      | Citrus reticulata cv. Nanfengmiju | twig | China: Jiangxi: Nanfeng | MN816427, MN894487, MN894449, MN894450, MN894410 |
|                   | NFGL-1-27      | Citrus reticulata cv. Nanfengmiju | leaf | China: Jiangxi: Nanfeng | MN816428, MN894488, MN894450, MN894451, MN894410 |
|                   | NFKL-6-15      | Citrus sinensis | leaf | China: Jiangxi: Nanfeng | MN816430, MN894490, MN894451, MN894452, MN894410 |
|                   | NFKL-6-20      | Citrus sinensis | twig | China: Jiangxi: Nanfeng | MN816431, MN894491, MN894452, MN894453, MN894413 |
ITS = nuclear ribosomal internal transcribed spacer regions; TUB = beta-tubulin gene; TEF = translation elongation factor 1-α gene; HIS = histone-3 gene; and CAL = calmodulin gene.

Figure 1. Symptoms of citrus melanose caused by *Diaporthe* species. (A,B) Typical symptoms on young leaf of *Citrus reticulata* cv. Nanfengmiju. (C) Typical symptoms on old leaf of *C. sinensis*. (D,E) Typical symptoms on mature fruits of *C. sinensis* and *C. reticulata* cv. Nanfengmiju, respectively. (F) Typical symptoms on young fruit of *C. sinensis*. (G) Twig typical symptoms of *C. sinensis*. 
2.2. Geographic Distribution of D. citri

According to the Systematic Mycology and Microbiology Laboratory, ARS, USDA (SMML database), D. citri has been recorded on citrus cultivars and their allied genera worldwide. The D. citri is a dominant species in Diaporthe genus, which occurs widely in citrus-growing countries, e.g., China, Philippines, Japan, Korea, Thailand, Myanmar, Cambodia, Fiji, Mauritius, USA, Mexico, Haiti, Cuba, Dominican, Panama, Puerto Rico, Venezuela, Trinidad and Tobago, Brazil, Cyprus, Portugal (Azores Islands), New Zealand, Niue, Samoa, Tonga, Cook Islands, Cote d’Ivoire, and Zimbabwe. The detailed citrus host and their allied genera of Diaporthe spp., are shown in Figure 2 and Supplementary Table S1.

Figure 2. A global geographic distribution of D. citri associated with Citrus-host plant and available on SMML database. Blue colored dots indicate the availability of the accession numbers in the NCBI database, while red colored dots indicate the non-availability.

2.3. Phylogenetic Analysis of Diaporthe Species

Totally 3183 base pairs (bp) of combined DNA sequences were obtained for phylogenetic analysis, including 645 bp ITS sequence (1-645), 472 bp TEF gene sequence (650-1121), 893 bp TUB gene sequence (1126-2018), 617 bp CAL gene sequence (2023-2639), and 540 bp HIS gene sequence (2644-3183). Combined data set consisted of 129 taxa including the outgroup species of Diaporthella corylina (CBS 121124). Six phylogenetic trees were constructed corresponding to each single-locus analysis of ITS, TEF, TUB, CAL, HIS, and combined data of five loci (Figures 3, Supplementary Figures S1 and S2).

The combined data set comprised 56.58% (1801 bp) invariable characters, 31.32% (997 bp) phylogenetically informative characters and 12.10% (385 bp) uninformative variable characters. Each of single locus has the following invariable characters (ITS = 414, TEF = 186, TUB = 523, CAL = 310, and HIS = 352), phylogenetically informative characters (ITS = 123, TEF = 230, TUB = 263, CAL = 238, and HIS = 143) and uninformative variable characters (ITS = 108, TEF = 56, TUB = 107, CAL = 69, and HIS = 45). A comparison of alignment properties in parsimony analyses of gene/loci and nucleotide substitution models used in phylogenetic analyses are provided in Table 2. BI tree constructed with combined five-loci data was presented with annotations for isolate number, plant host, and locality. MP tree was similar to the BI tree, therefore only BI tree was shown. D. citri was dominant species and occurred on citrus hosts in countries including China, Japan, Korea, New Zealand, Portugal, and USA. D. citriasisana and D. discoidispora were found on citrus plants only. However, D. eres, D. sojae, and D. unshiuensis were found on host plants from multiple genera. Seven isolates obtained in this study clustered in the same group with three isolates from previously known as D. infertilis including ex-type strain (CBS 230.52) and several isolates known as D. citri, this group should be the D. infertilis (Figures 3, S1 and S2). Based on the similar phylogenetic analysis, all of the 140 isolates were identified (Figure S3). Results showed that D. citri was the predominant species which accounted for 44.3%, following the species of D. eres, D. unshiuensis, D. sojae, D.
discoidispora and D. citriasiana, which accounted for 11.4%, 10%, 9.3%, 6.4%, and 3.6%, respectively. There were still 15% isolates that could not be identified to the species level (Supplementary Figure S3).
Figure 3. The Bayesian inference consensus tree resulting from a combined data set of ITS, TUB, TEF, CAL, and HIS sequences. MP bootstrap support values (equal to or > 50%) and Bayesian posterior probability values (equal to or > 0.70) are indicated at the typological nodes. Ex-type, ex-isotype, and ex-epitype strains are indicated in bold. The tree was rooted to Diaporthe corylina (CBS 121124). Squares indicate isolates from leaves, circles indicate isolates from fruits, and triangles indicate isolates from twigs. The scale bar represents the expected number of nucleotide substitutions per site.

Table 2. Comparison of alignment properties in parsimony analyses of gene/locus and nucleotide substitution models used in phylogenetic analyses.

| Gene/Locus | ITS     | TEF     | TUB     | CAL     | HIS     | Combined |
|------------|---------|---------|---------|---------|---------|----------|
| No. of taxa | 129     | 124     | 124     | 68      | 112     | 129      |
| Aligned length (with gaps) | 645     | 472     | 893     | 617     | 540     | 3183     |
| Invariable characters (%) | 414 (64.19) | 186 (39.41) | 523 (38.57) | 310 (50.24) | 352 (65.19) | 1801 (56.58) |
| Phylogenetically informative characters (%) | 123 (19.07) | 230 (48.73) | 263 (29.45) | 238 (38.57) | 143 (26.48) | 997 (31.32) |
| Uninformative variable characters (%) | 108 (16.74) | 56 (11.86) | 107 (11.98) | 69 (11.18) | 45 (8.33) | 385 (12.10) |
| Tree length (TL) | 670     | 856     | 745     | 554     | 538     | 3.654    |
| Consistency index (CI) | 0.506   | 0.575   | 0.686   | 0.733   | 0.55    | 0.565    |
| Retention index (RI) | 0.901   | 0.948   | 0.94    | 0.952   | 0.926   | 0.921    |
| Rescaled consistency index (RC) | 0.456   | 0.545   | 0.645   | 0.735   | 0.509   | 0.521    |
| Homoplasy index (ID) | 0.494   | 0.425   | 0.314   | 0.257   | 0.45    | 0.435    |

Nucleotide substitution model: GTR + I + G

2.4. Morphological Characterization of D. citri

For Diaporthe species, morphological factors such as colony appearance on different media, conidiomata, conidia shape and size are important to identify and understand a specific species. Therefore, morphological observation was performed on different media. Colonies on PDA grew slowly with 0.3–1.0 mm/day in the dark at 25 °C, they were white, flat or effuse alternate to low convex; reverse mottled buff with irregular dark patches. On CMA and OMA media, sparse to moderate mycelia covered the entire plate after 10 days with numerous scattered pale mouse grey patches. Conidiomata sporulating on PDA were scattered or aggregated, black-deeply embedded in medium, becoming erumpent at maturity. Conidiomata were sub-globose and/or variable in shape and up to 200 μm diam in size with an elongated black neck. Conidial mass was initially hyaline to yellowish, becoming white to cream conidial droplets exuding from central ostioles after 25 days in light at 25 °C. Alpha conidia were aseptate, hyaline, smooth, ovate to ellipsoidal, mostly bi-guttulate, apex bluntly rounded, base sub-truncate, (5.7–) 7.2–10.1 × (1.7–) 2.1–3.1 μm ( x ± SD = 8.1 ± 1.1 × 2.6 ± 0.5). Beta conidia were aseptate, flexuous, flexible to slightly curved or hamate, smooth, hyaline, apex acutely rounded, base truncate, (11.7–) 15.7–22.7 μm ( x ± SD = (0.4–) 0.4–1.2 μm ( x ± SD = 21.7 ± 6 × 0.9 ± 0.3). Gamma conidia were not observed (Figure 4).
2.5. Specificity and Sensitivity of PCR Method for Detection of *D. citri*

As mentioned above, sequences of five loci were obtained for phylogenetic analysis (Table 4, Supplementary Figures S1 and S2), among them, TUB showed the best capability of *D. citri* distinguishing different from other *Diaporthe* species (Figure 5). Therefore, TUB gene was chosen for designing the species-specific primers by matching the forward primer in the varied region and the reverse primer in the conserved region of TUB gene (Figure 5). As the PCR reaction is performed with the commercial PCR amplification mixture, only the annealing temperature is optimized. Results showed that consistent amplification could be obtained at the annealing temperature from 50 to 60 °C for the species-specific primer pair as shown in Figure 6. Thus, 55 °C was considered as the optimized annealing temperature and used in the following experiments. For the specificity evaluation, the specific primer set TUBDcitri-F1/TUBD-R1 amplified a single product of 217 bp only from the *D. citri* isolates. The 217 bp amplicon was not observed in other five *Diaporthe* species (*D. citriasiana, D. discoidispora, D. eres, D. sojae, and D. unshiuensis*), indicating that the method has good specificity for *D. citri* (Figures 7A and S4). The sensitivity was evaluated by using a serial dilution of genomic DNA (gDNA) as templates, results showed that it could amplified the 217 bp fragment from 10 pg of isolate NFHF-8-4 gDNA in 20 μL reaction mixture, indicating very high sensitivity (Figure 7B).
Plants 2020, 9, **This Paper Peer Review**

Figure 5. A novel primer pair TURDcitri-F1 and TURD-R1 was designed based on the alignment of the partial TUB gene (from 5' to 3') of Diaportha species including *D. citri*, *D. citriasiana*, *D. discoidispora*, *D. ertes*, *D. infertilis*, *D. sojae*, and *D. unshiuensis*. Dashes (−) and dots (.) indicate the gaps and identical nucleotides in the sequences, respectively.

Figure 6. Optimization of the annealing temperature. Lane 11 are results from the annealing temperature of 50, 50.7, 51.7, 53.1, 54.7, 56.4, 58.1, 59.8, 61.4, 62.7, 63.8, and 64.4 °C, respectively in

Commented [M23]: We changed hyphen into endash. Please confirm this revision.
reactions using DNA template of *D. citri* isolate NFHF-8-4. Lane 13 is the ddH2O as the template and lane M, 100 bp ladder.

**Figure 7.** Specificity and sensitivity of the developed PCR based on *TUB* sequence for detection of *D. citri*. (A) PCR product 217 bp of *D. citri* (NFHF-8-4) was shown with 2% gel electrophoresis (lane 1). Lanes 2–6 are representatives of *D. citriasiana* (XFAL-1-1), *D. discoidispora* (NKDL-1-2), *D. vres* (NFHF-1-1), *D. sejue* (NKGL-1-5), and *D. unshiuenensis* (NFIF-1-6), respectively, Lane 7 is the double sterile water (ddH2O) as negative control, and lane M, 50 bp ladder. (B) Sensitivity was investigated with a gDNA serial dilution. Lane 1–8 are gDNA of 10^2, 10^1, 10^0, 10^-1, 10^-2, 10^-3, 10^-4, and 0 ng in 20 μL reaction mixture, respectively. Lane 9 is the ddH2O as negative control and lane M, 50 bp ladder.

3. Discussion

*D. citri*, a phytopathogenic fungus causing melanose disease has become one of the most devastating citrus pathogens. According to data recorded, the geographic distribution of *D. citri* has been documented in Asia (China, Japan, and Korea), New Zealand, Portugal (Azores Islands), and USA. Even without the DNA sequence database, *D. citri* has also been reported in many other countries, e.g., Brazil, Cambodia, Cuba, Cook Islands, Cote d’Ivoire, Dominican, Haiti, Panama, Puerto Rico, Trinidad and Tobago, Venezuela, Mexico, Fiji, Mauritius, Philippines, Thailand, Myanmar, Niue, Samoa, Tonga, Zimbabwe, and Cyprus. In China, *D. citri* has been documented in several citrus plantations, e.g., Chongqing, Guangxi, Hunan, Jiangxi, Zhejiang, Hong Kong, and Taiwan [21,47–50].

For *Diaporthe* species identification, Santos, et al. [38] suggested the combined multi-locus sequences of ITS, TEF, TUB, CAL, and HIS, which were highly effective for resolving boundaries of *Diaporthe* species. Also, a single locus *TEF* gave better delimitation for *Diaporthe* species in phylogeny analysis [38]. Nevertheless, more accurate identification could be obtained based on the combined sequences from *TEF, TUB, CAL, HIS*, and *ITS* loci [38]. It has been reported that several *Diaporthe* species could be confusing, and conflicting results could be observed if only ITS region was used to construct phylogenetic tree [6,39,51]. The *D. citri* strains were isolated from citrus in China and USA, and pathogenicity test confirmed that *D. citri* was the causal agent of melanose and stem-end rot of citrus plant [21,32,33]. However, one cluster named as *D. citri* appeared conflict demonstration with the multi-gene phylogenetic analysis [6,21]. Guarnaccia and Crous [20] analyzed *Diaporthe* species emerging on citrus in European countries and reconsidered that three isolates which were previously recognized as *D. citri*, should be the *D. infertilis* because they were obviously different from other clusters of *D. citri* based on the phylogenetic analysis. In current study, strong evidence with concatenated multi-locus sequences also showed that *D. infertilis* was distinct with *D. citri*. To date, *D. infertilis* has been found on *C. sinensis* (Suriname), *Glycine max* (Brazil), unknown host (Italy), *Citrus limon* (India), and *Mikania glomerate* (Brazil), respectively.

In earlier studies, methods based on PCR were developed for detecting fungal pathogens on citrus. For instance, Bonants, et al. [52] designed species-specific primers from the ITS region to detect *Phyllosticta citricarpa*, a black spot pathogen of orange (*Citrus sinensis*), and lemon (*C. limon*). Wang, et al. [53] also designed species-specific primer pair from ITS to detect black spot disease of pumelo (*C. maxima*). Also, simple PCR was developed to distinguish *Phyllosticta citricarpa* from *Phylllosticta mangiferae* by directly using fungal mycelia on PDA or fruit lesions [54,55]. Real-time PCR
with TaqMan probe was developed for routine quarantine of citrus black spot disease [56]. Similarly, real-time PCR based on ITS was used to distinguish Phyllosticta citricarpa from Phyllosticta citriasiana, both species could not be distinguished from each other based on morphological characterization [57].

SCAR-marker was developed to detect Pseudofabraes citricarpa, a fungus causing target spot on Satsuma mandarin (Citrus unshiu) and kumquat (Fortunella margarita) in China [58]. Similarly, SARC-marker derived from random amplified polymorphic DNA (RAPD) was used to simultaneously detect Phytophthora nicotianae and Candidatus Liberibacter asiaticus, the causal agents of citrus roots rot and greening [59]. Pereira, et al. [60] developed a multiplex real-time PCR assay to detect Colletotrichum abscissum and Colletotrichum gloeosporioides, the causal agents of citrus post-bloom fruit drop.

Latent infected D. citri may be the initial source of inoculum of melanose, and a rapid and sensitive diagnosis for detection of this pathogen is currently limited. In previously study, a conserved ITS region was used to design a molecular detection on D. longicolla, D. azadirachtae, and D. sclerotioides [42–45]. Several studies reported that molecular detection of Diaporthe species from conserved ITS region was weak and poor, thus could not distinguish the Diaporthe complex species [38]. A specific gene TEF was used to detect D. azadirachtae [46]. However, the molecular tool for D. citri detection has not been published. In present study, the novel species-specific PCR assay for detection of D. citri was established. This tool can be useful for routine diagnostic work and would be useful to monitor the prevalence of the D. citri.

4. Materials and Methods

4.1. Sample Collection and Fungal Isolation

Leaf, fruit, and twig tissues with melanose symptomatic sweet orange (Citrus sinensis) and nanfengmiju mandarin (C. reticulata cv. Nanfengmiju) were collected from Ganzhou city (Xinfeng, Nankang) and Fuzhou city (Nanfeng) Jiangxi Province, China. The samples were collected and took back to Key Lab of Horticultural Plant Biology, Ministry of Education, Huazhong Agricultural University, Wuhan, China. Photos of the diseased samples were captured by using Cannon 600D digital camera (Cannon Inc., Tokyo, Japan). Isolates of Diaporthe-like species were isolated from two citrus cultivars, sweet orange and nanfengmiju mandarin showing melanose symptoms in Jiangxi Province, China. Pure isolates were obtained by cutting off the hyphal tips growing from surface-sterilized diseased material. For fungal isolation, each sample of symptomatic tissues was cut into small pieces (5 × 5 mm) with the junction of diseased and healthy tissues. Small pieces of plant tissues were soaked in 75% ethanol solution for 1 min, surface disinfected in 1% sodium hypochlorite solution (NaClO) for 1 min, then rinsed three times with double sterilized water, and dried on sterile tissue paper. Dried small pieces of plant tissues were placed onto potato dextrose agar medium (PDA) amended with 100 μg/mL streptomycin and 100 μg/mL ampicillin (PDA-SA), then incubated for 2 –5 days at 25 °C. After that, mycelium tips growing from small pieces of plant tissues were harvested and transferred to Petri dishes with fresh PDA medium for sporulation at 25 °C for 20–30 days. Monosporic isolation was performed according to the method by Goh [61] and Yin, et al. [62]. Pure fungal isolates were kept at 4 °C whenever they are used.

4.2. Geographic Distribution of D. citri

Extensive information of D. citri with geographic distribution and host-fungus relationships were investigated in the Systematic Mycology and Microbiology Laboratory, ARS, USDA (SMML database: https://nt.ars-grin.gov/fungaldatabases) [63].

4.3. DNA Extraction from Fungal Mycelia

For genomic DNAs (gDNAs) extraction, fresh fungal mycelia were harvested from 7-day old culture on PDA [21]. A hyphal plug about 1.5 square centimeters was cut off and placed into a 2 mL micro-tube with 200 mg of sterile stainless-steel beads (1.6 mm in diameter). Next, 500 μL gDNAs

Commented [M26]: We changed hyphen into endash. Please confirm this revision.

Commented [M27]: We changed hyphen into endash. Please confirm this revision.

Commented [M28]: This URL can not be opened. Please check and edit
The PCR reaction was performed on ABI 3730xl DNA Sequencer at Wuhan Tianyi Huiyuan Biotechnology Co., Ltd. The micro-tube was vigorously homogenized at maximum speed for 10 min on the Bullet Blender® DNASTAR Lasergene Core Suite software programme (SaqMan v.7.1.0; DNASTAR Inc., Madison, WI, USA). The gDNAs supernatant were transferred to a new 1.5 mL micro-tube and 300 μL isopropyl alcohol was added. Then, the mixture was gently mixed at room temperature. The solution was centrifuged at 12,500 g for 6 min. After discarded the supernatant, gDNAs pellets were rinsed twice with 300 μL of 70% ethanol, and air dried. At last, 30 μL of sterile water (ddH2O) was added to dissolve gDNAs pellets following Chi’s protocol [64]. The gDNAs quality and quantity were measured via UV absorption at wavelength 260 and 280 nm by Thermo Scientific™ NanoDrop 2000 (Thermo Fisher Scientific Inc., Waltham, MA, USA). The gDNAs was either used or stored at −20 °C until further processing.

4.4. Sequencing of PCR Products

Fragments of nuclear ribosomal internal transcribed spacer regions (ITS), translation elongation factor 1-α gene (TEF), beta-tubulin gene (TUB), calmodulin gene (CAL), and histone-3 gene (HIS) were amplified by polymerase chain reaction (PCR) with primers described in Table 3. Twenty microliter PCR reaction volume including 1 μL gDNA, 0.8 μL (10 μM) of each primer, 7.6 μL ddH2O were amplified by polymerase chain reaction (PCR) with primers described in Table 3. Twenty microliter PCR reaction volume including 1 μL gDNA, 0.8 μL (10 μM) of each primer, 7.6 μL ddH2O and 10 μL 2 × HiFi® PCR Master Mix (Yeasen Biotech Co., Ltd., Shanghai, China), in a T100™ Thermal Cycler (Bio-Rad, Hercules, CA, USA). The PCR reaction was performed following conditions: 95 °C for 3 min, followed by 35 cycles at 95 °C for 30 s, annealing for 50 s at different temperature for different loci, 72 °C for 2 min, and 72 °C for 5 min. The PCR products were applied to electrophoresis in 1% agarose gel and visualized by staining the gel with GoldenView™ dye (Aidlab Biotechnologies Co., Ltd., Beijing, China). The Sanger sequencing of PCR products was performed on ABI 3730xl DNA Sequencer at Wuhan Tianyi Huiyuan Biotechnology Co., Ltd. (Wuhan, China). 

Table 3. Universal and species-specific primers used in PCR reactions with Diaporthe spp.

| Primer Name | Primer Sequence (5’ to 3’) | Length (nt) | Ta (°C) | %GC | Reference |
|-------------|---------------------------|-------------|---------|-----|-----------|
| ITS1        | TCCGTAGCTGGAACCTTGCGG    | 19          | 55.0    | 63.2| White, et al. [65] |
| ITS4        | TCTCCGCTTATGATCGATG     | 20          | 45.0    | 54.0| White, et al. [65] |
| EF1-728F    | CATCGAGATGAGGAGAAGG     | 20          | 58.0    | 50.0| Carbone and Kohn [66] |
| EF1-968R    | TACCTGAGGAAACCTTACC  | 20          | 45.0    | 50.0| Carbone and Kohn [66] |
| B2a         | GCTAAACCACATGCTCTCCCTCC | 24          | 58.0    | 50.0| Glass and Donaldson [67] |
| B2b         | ACCCTACATGTCATGACCTCCGC | 24          | 58.0    | 50.0| Glass and Donaldson [67] |
| TUBDini-F1  | CACTGGAGCACTGCAACAT     | 21          | 55.0    | 42.9| This study |
| TUBD-R1     | CACCGGACACCTTGTTCC    | 19          | 57.9    | 60.0| This study |
| CAL-228F    | GAGTTCAGGAGGGGCTTCCCC | 22          | 55.0    | 59.0| Carbone and Kohn [66] |
| CAL-737R    | CATCCTCTGGAATCTATGG  | 19          | 52.6    | 60.0| Carbone and Kohn [66] |
| CYLIDF      | AGGTTCCACTGCGGACAGG  | 18          | 58.0    | 61.1| Crous, et al. [68] |
| H3-1b       | GGCGGGCCAGCTGATGCTTCTGT | 21         | 66.6    | 60.0| Glass and Donaldson [67] |

1 Number of nucleotides. 2 Annealing temperature estimated by Primer Premier v.6.0.

4.5. Phylogenetic Analyses of Diaporthe Species

Phylogenetic analysis was carried out by using sequences obtained in current study and those downloaded from NCBI’s GenBank (www.ncbi.nlm.nih.gov). Diaporothella corinina (CBS 121124) was selected as an outgroup (Table 4). All unique DNA sequences were consensus and edited with DNASTAR Lasergene Core Suite software programme (SeqMan v.7.1.0; DNASTAR Inc., Madison, WI, Wisconsin, USA). Sequences combined different loci were aligned using Clustal W program with supplement software package in BioEdit v.7.2.5 [69]. Maximum parsimony (MP) analysis was done by using PAUP (Phylogenetic Analysis Using Parsimony, v.4b10) [70]. The goodness of fit values including tree length (TL), consistency index (CI), retention index (RI), rescaled consistency index (RC), and homoplasy index (HI) were calculated for parsimony and the bootstrap analyses [71]. The heuristic search function was used with 1000 random stepwise addition replicates, with tree bisection and
reconnection (TBR) branch-swapping algorithm, with all characters weighted equally weighted and alignment gaps treated as missing data. Posterior probabilities (PP) were determined using Markov chain Monte Carlo (MCMC) sampling for Bayesian inference (BI) analysis in MrBayes v.3.2.2 [72]. MrModeltest v.2.3 [73] was used to perform statistical selection of the best-fit model of nucleotide substitution with corrected Akaike information criterion (AIC). BI analyses were launched with six simultaneous Markov chains which were run for 10^5 generations, and trees were sampled every 100th generation (resulting in 10,000 total trees). The calculation of BI analyses was stopped when the average standard deviation of split frequencies fell below 0.01. The consensus trees and posterior probabilities (PP) values were calculated after discarding the first 2000 resulted trees of the analyses as burn-in phase. Finally, above 8000 trees were summarized to calculate the PP in the majority rule consensus tree. Phylogenetic trees were visualized and annotated in FigTree v.1.4.2 [74]. The concatenated alignments and phylogenetic trees were deposited in TreeBASE (study no. S25607), new sequences obtained in this study were submitted to NCBI’s GenBank nucleotide database.
| Species                    | Isolate Number | Plant Host                  | Locality                  | GenBank Accession Numbers | Reference(s) |
|---------------------------|----------------|-----------------------------|---------------------------|---------------------------|--------------|
| Diaporthe arceae          | CBS 161.64     | Acer cetelech               | Unknown                   | KC343302, KC344000, KC343758, KC343274, KC343516 | Dios, et al. [6] |
| CBS 553.75                |                | Citrus sp.                  | Suriname                  | KC343303, KC344001, KC343759, KC343275, KC343517 | Dios, et al. [6] |
| ZJUD58                    |                | Citrus limon                | China: Yunnan             | KJ490593, KJ490414, KJ490472 – KJ490535 | Huang, et al. [48] |
| ZJUD59                    |                | Citrus sinensis             | China: Jiangxi            | KJ490394, KJ490415, KJ490473 – KJ490536 | Huang, et al. [48] |
| D. bacca                  | CBS 136.797    | Vaccinium corymbosum        | Italy: Sicily, Catania    | KJ160565, MF418509, KJ160597 – MF418264 | Guarnaccia and Crous [20], Lombard, et al. [76] |
| CPC 26170                 |                | Citrus sinensis             | Italy: Catania            | MF418353, MF418510, MF418430, MF418585, MF418265 | Guarnaccia and Crous [20] |
| CPC 26465                 |                | Citrus limon                | Italy: Catania            | MF418352, MF418511, MF418431, MF418586, MF418266 | Guarnaccia and Crous [20] |
| CPC 26963                 |                | Citrus paradisi             | Italy: Vibo Valentia      | MF418353, MF418512, MF418432, MF418587, MF418267 | Guarnaccia and Crous [20] |
| CPC 27821                 |                | Citrus reticulata           | Italy: Cosenza            | MF418357, MF418516, MF418436, MF418591, MF418271 | Guarnaccia and Crous [20] |
| D. biconispora            | CGMCC3.17225   | Citrus grandis              | China: Fujian             | KJ490597, KJ490416, KJ490472 – KJ490539 | Huang, et al. [48] |
| ZJUD60                    |                | Citrus sinensis             | China: Jiangxi            | KJ490595, KJ490416, KJ490474 – KJ490537 | Huang, et al. [48] |
| ZJUD61                    |                | Fortunella margarita        | China: Guangxi            | KJ490596, KJ490417, KJ490475 – KJ490538 | Huang, et al. [48] |
| D. biguttulata             | CGMCC3.17248   | Citrus limon                | China: Yunnan             | KJ490582, KJ490403, KJ490461 – KJ490524 | Huang, et al. [48] |
| ZJUD48                    |                | Citrus limon                | China: Yunnan             | KJ490583, KJ490404, KJ490462 – KJ490525 | Huang, et al. [48] |
| D. citri                  | AR3405         | Citrus sp.                  | USA: Florida              | KC343311, KC343187, KC343175, KC343157, MF418281 | Guarnaccia and Crous [20], Udayanga, et al. [24] |
| CBS 134.239               |                | Citrus sinensis             | USA: Florida              | KC357503, KC357456, KC357522, KC357488, MF418280 | Guarnaccia and Crous [20], Huang, et al. [21] |
| ZJUD1                     |                | Citrus reticulata           | China: Zhejiang           | JQ954664, KJ490395, JQ954671 – KJ490514 | Huang, et al. [21], Huang, et al. [48] |
| CBS 144227                |                | Citrus reticulata           | Portugal: Azores          | MH06390, MH06391, MH06389, MH06389 | Guarnaccia and Crous [20] |
| CBS 135426                |                | Citrus unshiu cv. Javauntan | Korea: Oudeong-dong       | KC343324, KC343280, KC343884, KC343170 – | Udayanga, et al. [24] |
| ICMP1035                  |                | Citrus reticulata           | New Zealand: Kerikeri     | KC343314, KC343190, KC343160 – | Udayanga, et al. [24] |
| Ph-18                     |                | Citrus sinensis             | Panama: Coclé             | MK21446, – MK25870, 3 – | Aguilea-Cogly and Vicent [77] |
| FCDC2                     |                | Citrus sp.                  | Japan: Fukuoka            | AR302249, – – – – | Kanematsu, et al. [78], Kanematsu [79] |
| D. citriniscina           | CGMCC3.15224   | Citrus unshiu               | China: Shaanxi             | JQ954645, KC357459, JQ954663, KC357491, MF418282 | Guarnaccia and Crous [20], Huang, et al. [21] |
| ZJUD33                    |                | Citrus paradisi             | China: Jiangxi            | JQ954658, KC357460, JQ97216, KC357493 – | Huang, et al. [21] |
| ZJUD81                    |                | Citrus grandis cv. Shitangou | China: Zhejiang          | KJ490461, KJ490437, KJ490495 – KJ490558 | Huang, et al. [48] |
| D. citriniscina           | CGMCC3.15225   | Citrus unshiu               | China: Shaanxi             | JQ954648, MF418524, JQ954666, KC357494, KJ490516 | Guarnaccia and Crous [20], Huang, et al. [21,48] |
| ZJUDX48                   |                | Citrus unshiu               | China: Shaanxi             | KJ210599, KJ420829, KJ210562, KJ430402, KJ420879 | Udayanga, et al. [24], Udayanga, et al. [39] |
| ZJUD38                    |                | Citrus unshiu               | China: Shaanxi             | KJ357558, KC357463, KC357527, KC357498 | Huang, et al. [21] |
| ZJUD85                    | Fortunella margarita | China: Fujian               | KJ490620, KJ490441, KJ490499 – KJ490562 | Huang, et al. [48] |
| ZJUD96                    |                | Citrus unshiu               | China: Fujian               | KJ490631, KJ490452, KJ490510 – KJ490573 | Huang, et al. [48] |
| ZJUD97                    |                | Citrus grandis              | China: Fujian              | KJ490632, KJ490453, KJ490511 – KJ490574 | Huang, et al. [48] |
| D. cytosorella            | CBS 137.020    | Citrus limon                | Spain                     | KC343307, KC343221, KC343131, KC343141, MF418283 | Guarnaccia and Crous [20], Udayanga, et al. [24] |
Plants 2020, 9, x FOR PEER REVIEW

D. discoidispora
CCMCC 3.17225 1
Citrus anulata
China: Jiangxi
KJ490624 KJ490645 KJ490653
KJ490666
–
Huang, et al. [48]

D. endophytica
CBS 133.811 1
Schinus terebinthifolius
Brazil
KC343065 KC344033 KC343791
KC343307 KC343549
Gomes, et al. [6]

D. eres
ZJUD73
Citrus anulata
China: Fujian
KJ490608 KJ490649 KJ490547
–
KJ490550
Huang, et al. [48]

D. fortunella marginata
ZJUD94
Citrus limon
China: Yunnan
KJ490528 KJ490480 KJ490508
–
KJ490571
Huang, et al. [48]

D. fortunella marginata
ZJUD94
Citrus anulata
China: Jiangxi
KJ490625 KJ490504
KJ490567
Huang, et al. [48]

D. fortunella marginata
ZJUD91
Citrus sp.
China: Jiangxi
KJ490626 KJ490447
KJ490505
–
KJ490568
Huang, et al. [48]

D. fortunella marginata
ZJUD92
Citrus sp.
China: Zhejiang
KJ490528 KJ490480
KJ490508
–
KJ490569
Huang, et al. [48]

D. fuscata
CBS 135.430
Citrus limon
USA: California
KC343301 KC343215
KC343110 KC343135
MF418284
Guarnaccia and Crous [20]. Udayanga, et al. [24]

CPC 26184
Citrus maxima
Italy: Messina
MF418365 MF418525
MF418444 MF418399
MF418285
Guarnaccia and Crous [20]

CPC 26895
Citrus bergamia
Greece:
Missolonghi
MF418374 MF418534
MF418453 MF418208
MF418294
Guarnaccia and Crous [20]

CPC 26967
Citrus nilis
Italy: Messina
MF418379 MF418539
MF418458 MF418213
MF418299
Guarnaccia and Crous [20]

CPC 27985
Citrus japonica
China: Fujian
KJ490528 KJ490480
KJ490508
–
KJ490571
Huang, et al. [48]

CPC 28030
Citrus sinensis
Portugal: Evora
KC343104 KC344072
KC343830 KC343346
KC343588
Gomes, et al. [6]

CPC 28081
Citrus reticulata
Spain: Algemesi
MF418415 MF418575
MF418494 MF418249
MF418335
Guarnaccia and Crous [20]

CPC 28083
Citrus reticulata
cv. Microcitrus australasica
Italy: Catania
MF418416 MF418576
MF418495 MF418250
MF418336
Guarnaccia and Crous [20]

D. hungkiangensis
HRUC 91041 1
Dickson sphenoglossa
Hong Kong: China
KC343119 KC344072
KC343845 KC343361
KC343563
Gomes, et al. [6]

ZJUD74
Citrus anulata
China: Fujian
KJ490609 KJ490400
KJ490498
–
KJ490551
Huang, et al. [48]

ZJUD75
Citrus reticulata
cv. Nankung
China: Fujian
KJ490610 KJ490431
KJ490498
–
KJ490552
Huang, et al. [48]

ZJUD76
Citrus reticulata cv. Nankung
China: Jiangxi
KJ490611 KJ490432
KJ490498
–
KJ490553
Huang, et al. [48]

ZJUD77
Citrus anulata
China: Zhejiang
KJ490612 KJ490433
KJ490498
–
KJ490554
Huang, et al. [48]

ZJUD78
Citrus grandis
China: Fujian
KJ490613 KJ490434
KJ490492
–
KJ490555
Huang, et al. [48]

ZJUD79
Citrus grandis
China: Fujian
KJ490614 KJ490435
KJ490493
–
KJ490556
Huang, et al. [48]

D. inferfilitis
CBS 230.52 1
Citrus sinensis
Suriname: Paramaribo
KC343052 KC344020
KC343778 KC343294
KC343536
Gomes, et al. [6]

CBS 199.39
Unknown
Italy
KC343051 KC344019
KC343777 KC343293
KC343535
Gomes, et al. [6]

CPC 20122
Glycos max
Brazil
KC343053 KC344021
KC343779 KC343295
KC343537
Gomes, et al. [6]

G.01
Citrus limon
India
KJ477016
–
–
–
Mahadevavukam, et al. [81]

G.02
Mikania glomerata
Brazil
KJ534221 KJ962837
KJ962838
–
–
Polonio, et al. [82], Polonio, et al. [83]

G.03
Mikania glomerata
Brazil
KJ534220
–
–
–
Polonio, et al. [82]

D. limoncota
CBS 142.549 1
Citrus limon
Malta: Gozo
MF418422 MF418582
MF418501 MF418526
MF418342
Guarnaccia and Crous [20]

CPC 31137
Citrus limon
Malta: Zarrinq
MF418423 MF418583
MF418502 MF418527
MF418343
Guarnaccia and Crous [20]

D. melitensis
CBS 142.551 1
Citrus limon
Malta: Gozo
MF418424 MF418584
MF418503 MF418528
MF418344
Guarnaccia and Crous [20]
| CPC     | Species               | Geographic Data                  | GenBank Accession Numbers | Reference                        |
|---------|-----------------------|----------------------------------|---------------------------|----------------------------------|
| CPC 27875 | Citrus limon         | Malta: Gozo                       | MF418542 MF418585 MF418504 MF418259 MF418345 | Guarnaccia and Crous [20]       |
| D. multiguttulata | CGMCC3.17258 t      | Citrus grandis China: Fujian       | KJ490633 KJ490454 KJ490512 – KJ490575 | Huang, et al. [48]             |
| D. novem | CBS 127,270 t        | Glicine max Croatia               | KC343516 KC344124 KC343882 KC345398 KC343640 | Gomes, et al. [6]              |
| CPC 29188 | Citrus japonica      | Italy: Messina                    | MF418426 MF418586 MF418505 MF418260 MF418346 | Guarnaccia and Crous [20]       |
| CPC 28165 | Citrus aurantiifolia | Italy: Catania                    | MF418427 MF418587 MF418506 MF418261 MF418347 | Guarnaccia and Crous [20]       |
| CPC 28167 | Citrus aurantiifolia | Italy: Catania                    | MF418428 MF418588 MF418507 MF418262 MF418348 | Guarnaccia and Crous [20]       |
| CPC 28169 | Citrus aurantiifolia | Italy: Catania                    | MF418429 MF418589 MF418508 MF418263 MF418349 | Guarnaccia and Crous [20]       |
| D. ovalispora | CGMCC3.17256 t  | Citrus limon China: Yunnan       | KJ490628 KJ490449 KJ490507 – KJ490570 | Huang, et al. [48]             |
| D. sojae | CBS 139,282 t        | Glicine max USA: Ohio             | KJ490719 KJ490451 KJ490509 – KJ490572 | Udayanga, et al. [51]          |
| ZJUD68  | Citrus reticulata cv. | Zhejiang                        | KJ490605 KJ490424 KJ490482 – KJ490545 | Huang, et al. [48]             |
| ZJUD69  | Citrus reticulata cv. | Zhejiang                        | KJ490604 KJ490425 KJ490483 – KJ490546 | Huang, et al. [48]             |
| ZJUD70  | Citrus reticulata    | China: Yunnan                    | KJ490805 KJ490426 KJ490484 – KJ490547 | Huang, et al. [48]             |
| ZJUD71  | Citrus reticulata    | China: Zhejiang                  | KJ490806 KJ490427 KJ490485 – KJ490548 | Huang, et al. [48]             |
| ZJUD72  | Citrus reticulata    | China: Yunnan                    | KJ490807 KJ490428 KJ490486 – KJ490549 | Huang, et al. [48]             |
| D. subclavata | CGMCC3.17257 t   | Citrus reticulata China: Fujian   | KJ490630 KJ490451 KJ490509 – KJ490572 | Huang, et al. [48]             |
| ZJUD83  | Citrus grandis cv. Shatianyou | Guangdong                      | KJ490618 KJ490439 KJ490497 – KJ490560 | Huang, et al. [48]             |
| D. ambisiensis | CGMCC3.17569 t  | Citrus reticulata China: Zhejiang | KJ490587 KJ490488 KJ490466 – KJ490529 | Huang, et al. [48]             |
| CGMCC3.17256 | Fortunella margarita  | China: Fujian                   | KJ490594 KJ490405 KJ490463 – KJ490526 | Huang, et al. [48]             |
| CGMCC3.17257 | Fortunella margarita  | China: Fujian                   | KJ490595 KJ490406 KJ490464 – KJ490527 | Huang, et al. [48]             |
| CGMCC3.17568 | Fortunella margarita  | China: Fujian                   | KJ490586 KJ490407 KJ490465 – KJ490528 | Huang, et al. [48]             |
| D. corinina | CBS 121,124 t       | Corylus sp.                      | KJ490404 KJ490472 KJ490410 KJ490525 | Gomes, et al. [6], Vasilyeva, et al. [8] |

1 IT = ex-isotype, T = ex-type, and EP = ex-epitype. 2 AR = Corresponding author's personal collection of A.Y. Rossman; CBS = Westerdijk Fungal Biodiversity Institute (formerly CBSKNAW), Utrecht, The Netherlands; CFCC = China Forestry Culture Collection Center, China; CGMCC = China General Microbiological Culture Collection, China; CPC = Culture collection of P.W. Crous, housed at Westerdijk Fungal Biodiversity Institute, Utrecht, The Netherlands; HKUCC = University of Hong Kong Culture Collection, Department of Ecology and Biodiversity, Hong Kong, China; ICMP = International Collection of Micro-organisms from Plants, Auckland, New Zealand; and ZJUD = Diaporthe species culture collection at the Institute of Biotechnology, Zhejiang University, Hangzhou, China. 3 ITS = nuclear ribosomal internal transcribed spacer regions; TUB = beta-tubulin gene; TEF = translation elongation factor 1-α gene; HIS = histone-3 gene; and CAL = calmodulin gene.
4.6. Morphology and Culture Characteristics of D. citri

Isolates on PDA plates were incubated at 25 °C for 30 days under near-ultraviolet (UV) light (12 h light/12 h dark). The growth rate of mycelium was measured in five duplicates. Colony color on PDA, Corn meal agar (CMA), and Oatmeal agar (OMA) media incubated at 25 °C near UV light with 12 h, was investigated according to the method of Rayner [745]. Colony color images were taken using Canon 600D digital camera (Canon Inc., Tokyo, Japan) after 10 days of incubation. Conidiomata and conidia were observed under the OLYMPUS SZX16 stereomicroscope (Olympus Corporation, Tokyo, Japan), conidial length/wide ratio of 30 conidia was measured with a stage micrometer under a Motic BA200 light microscope (Motic China Group Co., Ltd., Nanjing, China). Alpha and beta conidia were measured for calculating means (\(\bar{x}\)) and standard deviations (SD). The conidia ranges were shown as (\(\bar{x} - \sigma\)) - (\(\bar{x} + \sigma\)) μm (\(\bar{x}\pm SD\)). Conidia digital images were captured using Nikon Eclipse 80i compound light microscope imaging system (Nikon Corporation, Tokyo, Japan).

4.7. Primer Design and Development of the Molecular Tool to Detect D. citri

A highly varied region in \(TUB\) gene was selected as the target for developing molecular tool based on PCR to specifically detect \(D. citri\) from other \(Diaporthe\) species. Partial \(TUB\) gene of \(D. citri\) was retrieved from NCBI GenBank database (accession no. MN894459). The obtained sequences were aligned by using Clustal W algorithm in software package BioEdit v.7.2.5 [69]. The primers were designed by analyzing hairpin-dimer potential, length of the desired amplicon, %GC content, and melting temperatures (\(T_a\)) in Primer premier 6.0 software (Premier Biosoft International, Palo Alto, California, CA, USA). The primers were synthesized by Wuhan Tianyu Huiyuan Biotechnology Co., Ltd. (Wuhan, China). All the primer sequences used in this study are listed in Table 3.

Firstly, the annealing temperature was optimized in a gradient PCR in which the annealing temperatures were set from 50 to 65 °C. For specificity evaluation, gDNAs of \(D. citri\) (NFHF-8-4), \(D. citriasiana\) (XFAL-1-1), \(D. discoidispora\) (NKDL-1-2), \(D. sojae\) (NFIF-1-1), and \(D. unshiuensis\) (NFIF-1-6) were used, because these species are the closely related \(Diaporthe\) species in the phylogenetic analysis. The PCR reaction was performed in a final volume of 20 μL with the following components: 10 μL 2 × Hieff® PCR Master Mix (Yeasen Biotech Co., Ltd., Shanghai, China), 7.6 μL ddH₂O, 0.8 μL (10 μM) of each species-specific primer (\(TUBD\)citri-F1/TUBD-R1), and 1 μL gDNA (10 ng). The T100™ Thermal Cycler (Bio-Rad, USA) was programmed for conditions as 95 °C for 3 min, followed by 35 cycles at 95 °C for 30 s, annealing temperature (\(T_a\)) of 55 °C for 2 min, and 72 °C for 5 min. Finally, 5 μL products were used to electrophoresis on 2% agarose gel and visualized by staining the gel with GoldenView™ dye (Aidlab Biotechnologies Co., Ltd., Beijing, China), along with a 50 bp ladder as molecular marker (GL DNA Marker 500; Accurate Biotechnology (Hunan) Co., Ltd., Hunan, China) and 100 bp ladder (DNA 2K plus marker; TransGen Biotech Co., Ltd., Beijing, China). Similar test was also applied for the phylogenetically analyzed 38 isolates. For sensitivity evaluation, a serial of 10-fold dilutions of gDNA from \(D. citri\) isolate NFHF-8-4 ranging from \(10^2\) to \(10^4\) ng in 20 μL reaction mixture were used under the conditions described above.

5. Conclusions

In current study, it has been documented that \(Diaporthe\) species could cause devastating citrus diseases and \(D. citri\) was the causal agent of the citrus melanose disease. Based on the phylogenetic analysis with five multi-locus sequences, \(Diaporthe\) species boundaries could be clearly delimited. We also designed species-specific primers from \(TUB\) gene to develop PCR method for detecting \(D. citri\). The PCR-based method showed high specificity and sensitivity, that could be applied for detection of \(D. citri\) efficiently in practice. In the future, efficient PCR should be developed with citrus tissues infected by \(D. citri\) and multiple PCR which can distinguish different \(Diaporthe\) species should be developed for the phytosanitary assay in plant quarantine routine work.
Supplementary Materials: The following are available online at www.mdpi.com/xxx/s1, Table S1: Checklist of Diaporthe citri and D. infertilis associated with details citrus host and their allied genera, locality and their reference(s). Figure S1: Phylogenetic trees of Diaporthe spp. by Bayesian inference (BI) analysis based on combined data set and individual locus (ITS, TUB, TEF, CAL, and HIS, respectively). Ex-type, ex-isotype, and ex-epitype strains are indicated in bold. The species Diaporthella cornigia (CBS 121124) was selected as an outgroup. Figure S2: Phylogenetic tree of Diaporthe spp. generated by Maximum Parsimony (MP) analysis based on combined data set and individual locus (ITS, TUB, TEF, CAL, and HIS, respectively). Ex-type, ex-isotype, and ex-epitype strains are indicated in bold. The species Diaporthe spp. (CBS 121124) was selected as an outgroup. Figure S3: The prevalence of Diaporthe species on citrus in Jiangxi Province, China based on phylogenetic identification. Number (%) indicate the number of obtained isolates of certain species and the percentage among the total 140 isolates. Figure S4: Species-specific 217 bp TUB gene amplified by the primer pair TUBDcitri-F1/TUBDc-R1 was shown with 2% gel electrophoresis. Thirty-eight representatives that were identified based on phylogenetic analysis were used to confirm the specificity of PCR approach. The numbers of D. citri, D. citransiana, D. discoidiopause, D. erva, D. gigas, and D. unshiuensis isolates were 10, 3, 5, 10, 5, and 5, respectively. Lane CK is the double sterile water (ddH2O) as negative control and lane M, 100 bp ladder.

Author Contributions: Conceptualization, C.C., Y.L. and C.-X.L.; Validation, C.C., X.-Y.L., Y.L. and C.-X.L.; Formal analysis, C.C. and X.-Y.L.; Investigation, C.C., X.-Y.L., J.-B.L. and B.X.; Resources, J.-B.L. and B.X.; Data curation, C.C., X.-Y.L., Y.L. and C.-X.L.; Writing, C.C. and C.-X.L.; Funding acquisition, Y.L. and C.-X.L.

Funding: This work was supported by the National Key Research and Development Program of China (No. 2017YFD02020105).

Acknowledgments: We gratefully thank Mingkhuan Doilom (Key Laboratory for Plant Diversity and Biogeography of East Asia, Kunming Institute of Botany, Chinese Academy of Sciences, China) and Sinang Hongsanan (Shenzhen Key Laboratory of Microbial Genetic Engineering, Shenzhen University, China) for technical assistance and valuable advice.

Conflicts of Interest: The authors declare no conflicts of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript; or in the decision to publish the results.

References

1. Wu, G.A.; Terol, J.; Banzer, V.; López-García, A.; Pérez-Román, E.; Borredá, C.; Domingo, C.; Tadeo, F.R.; Carborell-Caballero, J.; Alonso, R.; et al. Genomics of the origin and evolution of Citrus. Nature 2018, 554, 311–330.

2. FAO. Citrus fruit—Fresh and processed statistical bulletin 2016; Food and Agriculture Organization of the United Nations: Rome, Italy, 2017.

3. Deng, X.X.; Deng, Z.N.; Xu, J.G.; Li, J. Citrus Varieties in China. China Agriculture Press: Beijing, China, 2008.

4. Boddy, L.; Griffith, G.S. Role of endophytes and latent invasion in the development of decay communities in sapwood of angiospermous trees. Sydowia 1999, 41, 41-73.

5. Carroll, C.C. The Biology of Endophytes in Plants with Particular Reference to Woody Perennials; Cambridge University Press: Cambridge, UK, 1986.

6. Gomes, R.R.; Glenke, C.; Videira, S.I.R.; Lombard, L.; Groenewald, J.Z.; Crous, P.W. Diaporthe: A genus of endophytic, saprobioc and plant pathogenic fungi. Persoonia 2013, 31, 1-41.

7. Marin-Felix, Y.; Hernández-Restrepo, M.; Wingfield, M.J.; Akulov, A.; Carnegie, A.J.; Cheewangkoon, R.; Granado, D.; Groenewald, J.Z.; Guarnaccia, V.; Halleen, F.; et al. Genera of phytopathogenic fungi: GOLPHY 2. Stud. Mycol. 2019, 92, 47-133.

8. Suryanarayanan, T.S.; Devarajan, P.T.; Girivasan, K.P.; Govindaraju, M.B.; Kumaresan, V.; Murali, T.S.; Rajamani, T.; Thirunavukkarasu, N.; Venkatesan, G. The host range of multi-host endophytic fungi. Curr. Sci. 2011, 115, 1963–1969.

9. Guarnaccia, V.; Vitale, A.; Cirvilleri, G.; Aiello, D.; Susca, A.; Epifani, F.; Perrone, G.; Polizzi, G. Characterisation and pathogenicity of fungal species associated with branch cankers and stem-end rot of avocado in Italy. Eur. J. Plant Pathol. 2018, 146, 963–976.

10. Mostert, L.; Crous, P.W.; Kang, J.C.; Phillips, A.J.L. Species of Phomopsis and a Libertella sp. occurring on grapevines with specific reference to South Africa: Morphological, cultural, molecular and pathological characterization. Mycologia 2001, 93, 146–167.
11. Rehner, S.A.; Uecker, F.A. Nuclear ribosomal internal transcribed spacer phylogeny and host diversity in the coelomycete *Phomopsis*. *Can. J. Bot.* 1994, 72, 1666–1674.

12. Santos, J.M.; Vrandečić, K.; Ćosić, J.; Duvnjak, T.; Phillips, A.J.L. Resolving the *Diaporthe* species occurring on soybean in Croatia. *Persoonia* 2011, 27, 9–19.

13. Thompson, S.M.; Tan, Y.P.; Young, A.J.; Neate, S.M.; Atkin, E.A.B.; Shivash, R.G. Stem cankers on sunflower (*Helianthus annuus*) in Australia reveal a complex of pathogenic *Diaporthe* (*Phomopsis*) species. *Persoonia* 2011, 27, 80–89.

14. Cai, L.; Giraud, T.; Zhang, N.; Begerow, D.; Cai, G.H.; Shivash, R.G. The evolution of species concepts and species recognition criteria in plant pathogenic fungi. *Fungal Divers.* 2011, 50, 121–133.

15. Duan, W.J.; Yan, J.; Liu, F.; Cai, L.; Zhu, S.F. The list of Chinese quarantine fungi is in need of revision and renewal (in Chinese). *Mycosystema* 2015, 34, 942–960.

16. Rossman, A.Y.; Palm-Hernández, M.E. Systematics of plant pathogenic fungi: Why it matters. *Plant Dis.* 2008, 92, 1376–1386.

17. Shivash, R.G.; Cai, L. Cryptic fungal species unmasked. *Microbiol. Aust.* 2012, 33, 36–37.

18. Timmer, L.W.; Garmsey, S.M.; Graham, J.H. *Salt Diseases*, revised edition: 3–4 ed.; American Phytopathological Society Press: St. Paul, MN, USA, 2000; p. 92.

19. Whiteside, J.O.; Timmer, L.W. *Citrus Diseases: General Concepts*, revised edition: 3–4 ed.; American Phytopathological Society: St. Paul, MN, USA, 2000.

20. Guarnaccia, V.; Crous, P.W. Emerging citrus diseases in Europe caused by species of *Diaporthe*. *IMA Fungus* 2017, 8, 317–334.

21. Huang, F.; Hou, X.; Dewdney, M.M.; Fu, Y.S.; Chen, G.Q.; Hyde, K.D.; Li, H.Y. *Diaporthe* species occurring on citrus in China. *Fungal Divers.* 2013, 61, 237–250.

22. Kucharek, T.; Whiteside, J.; Brown, E. *Melanose and Stem End Rot of Citrus*; Florida Cooperative Extension Service, Institute of Food and Agricultural Sciences, University of Florida: Florida Gainesville, FL, USA, 1983.

23. Mondal, S.N.; Virent, A.; Reis, R.F.; Timmer, L.W. Saprophytic colonization of citrus twigs by *Diaporthe citri* and factors affecting pycnidal production and conidial survival. *Plant Dis.* 2007, 91, 387–392.

24. Udaiyanga, D.; Castlebury, L.A.; Rosman, A.Y.; Hyde, K.D. Species limits in *Diaporthe*: Molecular re-assessment of *D. citri*, *D. cytophthora*, *D. foeniculina* and *D. rudis*. *Persoonia* 2014, 32, 83–101.

25. Swingle, W.T.; Webber, H.J. The principal disease of citrus fruits in Florida. *USDA Div. Veg. Physiol. Pathol. Bull.* 1896, 8, 9–14.

26. Fawcett, H.S. The cause of stem-end rot of citrus fruits (*Phomopsis citri* n. sp.). *Phytopathology* 1912, 2, 109–113.

27. Floyd, B.F.; Stevens, H.E. Melanose and stem-end rot. *Res. Agr. Exp. Sta. Res.* 1912, 111, 1–16.

28. Rehm, H. Ascomycetes philippinenses VI. *Leafl. Philipp. Bot.* 1914, 6, 2258–2281.

29. Horne, W.T. A *Phomopsis* in grape fruit from the isle of Pines W. I., with notes on *Diplodia natalensis*. *Phytopathology* 1922, 12, 414–418.

30. Nitschke, T.R.J. Pyrenomycetes germanici. In *Die kernpilze Deutschlands Bearbeitet Von Dr. Th. Nitschke*; Eduard Trewendt: Breslau, Germany, 1870; Volume 2, pp. 161–320.

31. Fawcett, H.S. *A Phomopsis* of citrus in California. *Phytopathology* 1922, 12, 107.

32. Bach, W.J.; Wolf, F.A. The isolation of the fungus that causes citrus melanose and the pathological anatomy of the host. *J. Agric. Res.* 1926, 37, 243–252.

33. Ruehle, G.D.; Kunz, W.A. *Melanose of Citrus and Its Commercial Control*; Florida Agricultural Experiment Station Bulletin, University of Florida: Gainesville, FL, USA, 1940.

34. Castlebury, L. The *Diaporthe vaccinii* complex of fruit pathogens. *Incursum* 2005, 56, 12.

35. Santos, J.M.; Correia, V.G.; Phillips, A.J.L.; Spatafora, J.W. Primers for mating-type diagnosis in *Diaporthe* and *Phomopsis*: Their use in teleomorph induction in vitro and biological species definition. *Fungal Biol.* 2010, 114, 255–270.

36. Santos, J.M.; Phillips, A.J.L. Resolving the complex of *Diaporthe* (*Phomopsis*) species occurring on *Foeniculum vulgare* in Portugal. *Fungal Divers.* 2009, 34, 111–125.

37. Guarnaccia, V.; Groenewald, J.Z.; Woodhall, J.; Armengol, J.; Cinelli, T.; Eichmeier, A.; Ezra, D.; Fontaine, F.; Gramaje, D.; Gutierrez-Aguirregabiria, A.; et al. *Diaporthe* diversity and pathogenicity revealed from a broad survey of grapevine diseases in Europe. *Persoonia* 2018, 40, 135–153.
38. Santos, L.; Alves, A.; Alves, R. Evaluating multi-locus phylogenies for species boundaries determination in the genus Diaporthe. PeerJ 2017, 5, 1-26.
39. Udayanga, D.; Castlebury, L.A.; Rosman, A.Y.; Chukeatirote, E.; Hyde, K.D. Insights into the genus Diaporthe: Phylgetic species delimitation in the D. cres species complex. Fungal Divers. 2014, 67, 203–220.
40. Yang, Q.; Fan, X.L.; Guarnaccia, V.; Tian, C.M. High diversity of Diaporthe species associated with dieback diseases in China, with twelve new species described. MycoKeys 2018, 39, 97–149.
41. Hyde, K.D.; Nilsson, R.H.; Alias, S.A.; Ariyawansa, H.A.; Blair, J.E.; Cai, L.; de Cock, A.W.A.M.; Dissanayake, A.J.; Glockling, S.L.; Goonasekara, I.D.; et al. One stop shop: Backbone trees for important phytopathogenic genera: i (2014). Fungal Divers. 2014, 67, 21–125.
42. Zhang, A.W.; Hartman, G.L.; Riccioni, L.; Chen, W.D.; Ma, R.Z.; Pedersen, W.L. Using PCR to distinguish Diaporthe phaseolorum and Phomopsis longicolla from other soybean fungal pathogens and to detect them in soybean tissues. Plant Dis. 1997, 81, 1143–1149.
43. Prasad, M.K.; Bhat, S.S.; Raj, A.P.C.; Janardhana, G.R. Molecular detection of Shishido, M.; Sato, K.; Yoshida, N.; Tsukui, R.; Usami, T. PCR-based assays to detect and quantify Phomopsis azadirachtae causing dieback disease in Azadirachta indica. J. Phytopathol. 2015, 163, 818–828.
44. Shibudo, M.; Sato, K.; Yoshida, N.; Tsukui, R.; Usami, T. PCR-based assays to detect and quantify Phomopsis sclerotoides in plants and soil. J. Gen. Plant Pathol. 2010, 76, 21–30.
45. Shirahati, P.; Ramu, R.; Parshothama, C.R.A.; Prasad, M.K.N. Development of a simple and reliable species-specific detection of Phomopsis azadirachtae, using the translation elongation factor 1-alpha gene. Eur. J. Plant Pathol. 2015, 141, 769–778.
46. Anonymous. List of Plant Diseases in Taiwan; Plant Protect. Soc: Taichung, China, 1979; p. 404.
47. Huang, F.; Udayanga, D.; Wang, X.H.; Hou, X.; Mei, X.F.; Fu, Y.S.; Hyde, K.D.; Li, H.Y. Endophytic Diaporthe associated with citrus: A phylogenetic reassessment with seven new species from China. Fungal Biol. 2015, 119, 331–347.
48. Lu, B.S.; Hyde, K.D.; Ho, W.H.; Tsui, K.M.; Taylor, J.E.; Wong, K.M. Yunnan Fungi: Checklist of Hong Kong Fungi; Fungal Diversity Press: Hong Kong, China, 2000; p. 207.
49. Zhuang, W.Y. Higher Fungi of Tropical China; Mycotaxon, Ltd.: Ithaca, NY, USA, 2001; p. 485.
50. Udayanga, D.; Castlebury, L.A.; Rosman, A.Y.; Chukeatirote, E.; Hyde, K.D. The Diaporthe sojae species complex: Phylogenetic re-assessment of pathogens associated with soybean, cucurbits and other field crops. Fungal Biol. 2015, 119, 383–407.
51. Bonants, P.J.M.; Carroll, G.C.; de Weerd, M.; van Brouwershaven, I.R.; Baayen, R.P. Development and validation of a PCR-based detection method for pathogenic isolates of the citrus black spot fungus, Guignardia citricarpa. Eur. J. Plant Pathol. 2003, 109, 503–513.
52. Wang, X.H.; Chen, G.Q.; Huang, F.; Zhang, J.Z.; Hyde, K.D.; Li, H.Y. Phyllosticta species associated with citrus diseases in China. Fungal Divers. 2012, 52, 209–224.
53. Meyer, L.; Sanders, G.M.; Jacobs, R.; Korsten, L. A one-day sensitive method to detect and distinguish between the citrus black spot pathogen Guignardia citricarpa and the endophyte Guignardia mangiferae. Plant Dis. 2006, 90, 97–101.
54. Peres, N.A.; Hanakawa, R.; Carroll, G.C.; Adaskaveg, J.E.; Timmer, L.W. Comparison of molecular procedures for detection and identification of Guignardia citricarpa and G. mangiferae. Phytopathology. 2007, 91, 525–531.
55. van Gent-Peizer, M.P.E.; van Brouwershaven, I.R.; Kox, L.F.F.; Bonants, P.J.M.A. Taqman PCR method for routine diagnosis of the quarantine fungus Guignardia citricarpa on citrus fruit. J. Phytopathol. 2007, 155, 357–363.
56. Schirmacher, A.M.; Tomlinson, J.A.; Barnes, A.V.; Barton, V.C. Species-specific real-time PCR for diagnosis of Phyllosticta citricarpa on citrus species. Bull. OEPP/EPPO Bull. 2019, 49, 306–313.
57. Yang, Y.H.; Hu, J.H.; Chen, F.J.; Ding, D.K.; Zhou, C.Y. Development of a SCAR marker-based diagnostic method for the detection of the citrus target spot pathogen Pseudofabraea citricarpa. Biomed. Res. Int. 2018, 2018, 7126903.
58. Das, A.K.; Nerkar, S.; Gawande, N.; Thakre, N.; Kumar, A. Scar marker for phytophthora nicotianae and a multiplex PCR assay for simultaneous detection of P. nicotianae and Candidatus liberibacter asiaticus in citrus. J. Appl. Microbiol. 2012, 117, 1172–1183.
60. Pereira, W.V.; Bertolami, E.; Cambra, M.; Junior, N.S.M. Multiplex real-time PCR for detection and quantification of Colletotrichum abscissum and C. gloeosporioides on Citrus leaves. *Eur. J. Plant Pathol.* 2019, 155, 1–13.

61. Gob, T.K. Single-sporo isolation using a hand-made glass needle. *Fungal Divers.* 1999, 2, 47–63.

62. Yin, L.F.; Chen, S.N.; Chen, G.K.; Schnabel, G.; Du, S.F.; Chen, C.; Li, G.Q.; Luo, C.X. Identification and characterization of three *Moniliis* species from plum in China. *Plant Dis.* 2015, 99, 1775–1783.

63. Farr, D.F.; Rossman, A.Y. *Fungal Databases*, 26 December 2018 ed.; U.S. National Fungus Collections, ARS, USDA: Washington DC, USA, 2018.

64. Chi, M.H.; Park, S.Y.; Lee, Y.H. A quick and safe method for fungal DNA extraction. *Plant Pathol. J.* 2009, 25, 108–111.

65. White, T.J.; Bruns, T.; Lee, S.; Taylor, J. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In *PCR Protocols: A Guide to Methods and Applications*; Innis, M.A., Gelfand, D.H., Sninsky, J.J., White, T.J., Eds.; Academic Press: San Diego, CA, USA, 1990; pp. 315–322.

66. Carbone, I.; Kohn, L.M. A method for designing primer sets for speciation studies in filamentous ascomycetes. *Mycologia* 1999, 91, 553–556.

67. Glass, N.L.; Donaldson, G.C. Development of primer sets designed for use with the PCR to amplify conserved genes from filamentous ascomycetes. *Appl. Environ. Microb.* 1995, 61, 1323–1330.

68. Crous, P.W.; Groenewald, J.Z.; Ribeiro, M.A.S.; Sarragiotto, M.H.; Azevedo, J.L.; Pamphile, J.A. Mycology online database. *MycoBank*. 2015, 1–13.

69. Hall, A.T. Biodeit: A user-friendly biological sequence alignment editor and analysis program for windows 95/98/nt. *Nucleic Acids Res.* 1999, 11, 95–98.

70. Swoford, D.L. *PAUP*: Phylogenetic Analysis Using Parsimony, (*and Other Methods*), Version 4.0 b10; Sinauer Associates: Sunderland, MA, USA, 2003.

71. Hillis, D.M.; Bull, J.J. An empirical test of bootstrapping as a method for assessing confidence in phylogenetic analysis. *Syst. Biol.* 1993, 42, 182–192.

72. Ronquist, F.; Teslenko, M.; Van der Mark, P.; Ayres, D.L.; Darling, A.; Hînghra, S.; Larget, B.; Liu, L.; Suchard, M.A.; Huelsenbeck, J.P. MrBayes 3.2: Efficient bayesian phylogenetic inference and model choice across a large model space. *Syst. Biol.* 2012, 61, 539–542.

73. Nylander, J.A.A. Mrmodeltest v.2: Program Distributed by the Author; Evolutionary Biology Centre: Uppsala University: Uppsala, Sweden, 2004.

74. Rambaut, A. *Figtree v1.4.2*: Institute of Evolutionary Biology, Ashworth Laboratories, University of Edinburgh: Edinburgh, UK, 2014.

75. Rayner, R.W. *A Mycological Colour Chart*: Commonwealth Mycological Institute and British Mycological Society: Keyworth, Nottingham, UK, 1970.

76. Lombard, L.; van Leeuwen, G.C.M.; Guarnaccia, V.; Polozzi, G.; van Rijswick, P.C.J.; Rosendahl, C.H.M.; Gabler, J.; Crous, P.W. *Diaportha* species associated with *Vaccinium*, with specific reference to Europe. *Phytopathol. Mediterr.* 2014, 53, 287–299.

77. Aguilerá-Cogley, V.; Vicent, A. Etiology and distribution of foliar fungal diseases of citrus in Panama. *Trop. Plant Pathol.* 2019, 44, 519–532.

78. Kanenatsu, S.; Kobayashi, T.; Kudo, A.; Ohnuki, Y. Conidial morphology, pathogenicity and culture characteristics of *Phomopsis* isolates from peach, Japanese pear and apple in Japan. *Jpn. J. Phytopathol.* 1999, 65, 264–273.

79. Kanenatsu, S. Phylogeny of phomopsis species from fruit trees. In *Direct Submission Sequence of Diaportha citri Strain FCDC*; National Institute of Fruit Tree Science, Apple Research Station: Morioka, Japan, 2007, with descriptions of five new species. *Fungal Biol.* 2015, 119, 295–309.

80. Gao, Y.H.; Su, Y.Y.; Sun, W.; Cai, L. *Diaportha* species occurring on *Lithocarpus glabra* in China, with descriptions of five new species. *Fungal Biol.* 2015, 119, 295–309.

81. Mahadevakumar, S.; Yadav, V.; Tejaswini, G.S.; Sandeep, S.N.; Janardhana, G.R. First report of *Phomopsis citri* associated with dieback of *Citrus* lemon in India. *Plant Dis.* 2014, 98, 1281.

82. Polonio, J.C.; Almeida, T.T.; Garcia, D.; Mariucci, G.E.G.; Azevedo, J.L.; Rhoden, S.A.; Pamphile, J.A. Biotecnological prospective of foliar endophytic fungi of guaco (*Mikania glomerata* Spreng.) with antibacterial and antagonistic activity against phytopathogens. *Genet. Mol. Res.* 2015, 14, 7297–7309.

83. Polonio, J.C.; Ribeiro, M.A.S.; Rhoden, S.A.; Sarragiotto, M.H.; Azevedo, J.L.; Pamphile, J.A. 3-nitropropionic acid production by the endophytic *Diaportha citri*: Molecular taxonomy, chemical characterization, and quantification under ph variation. *Fungal Biol.* 2016, 120, 1600–1608.
84. Vasilyeva, L.N.; Rossman, A.Y.; Farr, D.F. New species of the Diaporthales from Eastern Asia and Eastern North America. *Mycologia* 2007, 99, 916–923.

© 2020 by the authors. Submitted for possible open access publication under the terms and conditions of the Creative Commons Attribution (CC BY) license (http://creativecommons.org/licenses/by/4.0/).