Diaporthe species causing stem gray blight of red-fleshed dragon fruit (Hylocereus polyrhizus) in Malaysia

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This study aimed to characterize the new fungal disease on the stem of red-fleshed dragon fruit (Hylocereus polyrhizus) in Malaysia, which is known as gray blight through morphological, molecular and pathogenicity analyses. Nine fungal isolates were isolated from nine blighted stems of H. polyrhizus. Based on morphological characteristics, DNA sequences and phylogeny (ITS, TEF1-α, and β-tubulin), the fungal isolates were identified as Diaporthe arecae, D. eugeniae, D. hongkongensis, D. phaseolorum, and D. tectonendophytica. Six isolates recovered from the Cameron Highlands, Pahang belonged to D. eugeniae (DF1 and DF3), D. hongkongensis (DF9), D. phaseolorum (DF2 and DF12), and D. tectonendophytica (DF7), whereas three isolates from Bukit Kor, Terengganu were recognized as D. arecae (DFP3), D. eugeniae (DFP4), and D. tectonendophytica (DFP2). Diaporthe eugeniae and D. tectonendophytica were found in both Pahang and Terengganu, D. phaseolorum and D. hongkongensis in Pahang, whereas D. arecae only in Terengganu. The role of the Diaporthe isolates in causing stem gray blight of H. polyrhizus was confirmed. To date, only D. phaseolorum has been previously reported on Hylocereus undatus. This is the first report on D. arecae, D. eugeniae, D. hongkongensis, D. phaseolorum, and D. tectonendophytica causing stem gray blight of H. polyrhizus worldwide.

Red-fleshed dragon fruit (Hylocereus polyrhizus) is one of the most highly demand varieties, grown in Malaysia owing to its nutritional value and attractive color. It belongs to the Cactaceae family. This exotic fruit is locally known as “buah naga” or “buah mata naga”. It is also known as pitaya, strawberry pear, and night-blooming cereus. In 1999, dragon fruit was first introduced in Setiawan, Perak, and Kuala Pilah, Negeri Sembilan, Malaysia. The fruit was named “dragon fruit” owing to the dragon-like scales or bracts on its surface. Aside from having an attractive color and a pleasant taste, it is considered as a healthy fruit containing excessive amounts of vitamin C and water-soluble fiber.

Like other fruit crops in Malaysia, dragon fruit has been infected with a number of fungal diseases, thus jeopardizing its future. Several cases of fungal attacks on dragon fruit have been documented worldwide, namely, Alternaria sp., Bipolaris cactivora, Botryosphaeria dothidea, Colletotrichum gloeosporioides, Colletotrichum siamense, and Colletotrichum truncatum, Diaporthe phaseolorum, Fusarium oxysporum, and Fusarium solani, Gilbertella persiciari, Lasiodiplodia theobromae, Monilinia fructicola, Neoscytalidium dimidiatum, Nigrospora sphaerica, and Sclerotium rolfsii. In Malaysia, previous studies have identified a range of fungal diseases on dragon fruit, including anthracnose, stem necrosis, stem rot, stem blight, and reddish-brown spot.

Dragon fruits with stem gray blight were found in two locations, namely, Bukit Kor, Terengganu, Malaysia, and the Cameron Highlands, Pahang, Malaysia, in November 2017 and July 2018, respectively. These fruits exhibited irregular gray chlorotic lesion on the stem surface and black pycnidia on the infected part. In both locations, of the 50 dragon fruit plants, 20 (40% disease incidence) had been infected with the stem gray blight disease, which may result in its reduced production. This study could provide insights into the management of plant diseases. This study aimed to identify the causal pathogen of the stem gray blight of H. polyrhizus via morphological, molecular, and pathogenicity analyses.

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Results

Fungal isolation and morphological identification. A total of nine fungal isolates were recovered from nine gray blighted stems obtained from the different plants of *H. polyrhizus*. Of these, three isolates (DFP2, DFP3, and DFP4) were recovered from Bukit Kor, Terengganu and six isolates (DF1, DF2, DF3, DF7, DF9, and DF12) from the Cameron Highlands, Pahang, Malaysia. A species or isolate was recovered from a single lesion.

In general, the fungal isolates produced whitish, grayish, or brownish colonies on potato dextrose agar (PDA) plates. Two types of conidia, namely, α- and β-conidia, were produced from the formation of pycnidial conidiomata on carnation leaf agar (CLA). α-conidia were characterized as aseptate, hyaline, and fusiform with bi- or multi-guttulate, meanwhile, β-conidia were characterized as aseptate, hyaline, filiform, straight, or more often hamate, and lack guttule. The conidiogenous cells of α-conidia were phialidic, cylindrical, terminal, hyaline, and slightly tapered toward the end. However, in this study, the structure of the conidiogenous cells for β-conidia was not observed. Conidiophore was characterized as hyaline, branched, multiseptate, and filiform. Based on the described characteristics, the fungal isolates were tentatively identified as *Diaporthe* species. By sorting their morphological similarities and differences, the fungal isolates were classified into five groups of *Diaporthe* species (Fig. 1, Table 1).

Figure 1. Morphological characteristics of *Diaporthe* species isolated from stem gray blight of *H. polyrhizus*. Group 1 (A1–A6): (A1) colony appearance, (A2) pigmentation, (A3) pycnidial conidiomata, (A4) α-conidia, (A5) β-conidia, (A6) conidiogenous cell for α-conidia; Group 2 (B1–B6): (B1) colony appearance, (B2) pigmentation, (B3) pycnidial conidiomata, (B4) α-conidia, (B5) β-conidia, (B6) conidiogenous cell for α-conidia; Group 3 (C1–C6): (C1) colony appearance, (C2) pigmentation, (C3) pycnidial conidiomata, (C4) α-conidia, (C5) β-conidia, (C6) conidiogenous cell for α-conidia; Group 4 (D1–D6): (D1) colony appearance, (D2) pigmentation, (D3) pycnidial conidiomata, (D4) α-conidia, (D5) β-conidia, (D6) conidiogenous cell for α-conidia; Group 5 (E1–E5): (E1) colony appearance, (E2) pigmentation, (E3) pycnidial conidiomata, (E4) α-conidia, (E5) conidiogenous cell for α-conidia. Scale bar: A3–E3 = 1000 µm; A4–A6, B4–B6, C4–C6, D4–D6, E4–E5: 0.5 µm.
### Table 1. Morphological characteristics of five different groups of *Diaporthe* isolates recovered from stem gray blight of *H. polyrhizus*.

| Group/isolate | Colony on PDA                                      | Pycnidial conidiomata on CLA | α-conidia                                                                 | β-conidia                                                                 | Conidiophore of α-conidia                  | Conidiogenous cell of α-conidia              |
|---------------|---------------------------------------------------|-----------------------------|----------------------------------------------------------------------------|----------------------------------------------------------------------------|-------------------------------------------|---------------------------------------------|
| **Group 1**   |                                                   |                             | Fusiform, slightly tapered end, asetate, and hyaline                        | Filiform to hamate, asetate, and hyaline                                   | Hyaline, branched, and slightly to straightly curve | Cylindrical phialides, terminal, hyaline, and slightly tapered towards end |
| DF1, DF3, DFP4 | Abundant and whitish-brown aerial mycelia         | Black and globose           | Conidia with size of 6.33 ± 0.68 × 1.98 ± 0.25 μm, Bi/multi-guttulate with size of 0.41 ± 0.07 μm | Conidia with size of 24.57 ± 2.77 × 1.33 ± 0.29 μm, Bi/multi-guttulate with size of 1.49 ± 0.07 μm |                                            |                                            |
| **Group 2**   |                                                   |                             | Ovoid with bluntly rounded base end, asetate, and hyaline                  | Filiform to hamate, asetate, and hyaline                                   | Hyaline, branched, and slightly to straightly curve | Cylindrical phialides, terminal, hyaline, and slightly tapered towards end |
| DF2, DF12     | Cottony and whitish-aerial mycelia Brownish-white on the lower surface | Black and globose           | Conidia with size of 6.43 ± 0.55 × 2.38 ± 0.21 μm, Bi-guttulate with size of 1.53 ± 0.17 μm | Conidia with size of 17.34 ± 2.17 × 1.49 ± 0.34 μm, Bi/guttulate with size of 1.20 ± 0.04 μm |                                            |                                            |
| **Group 3**   |                                                   |                             | Fusoid with bluntly rounded on both ends, asetate, and hyaline             | Filiform to hamate, asetate, and hyaline                                   | Hyaline, branched, and slightly to straightly curve | Cylindrical phialides, terminal, hyaline, and slightly tapered towards end |
| DFP2, DF7     | Cottony and brownish-white aerial mycelia Brownish color on the lower surface | Black and globose           | Conidia with size of 6.00 ± 0.81 × 2.39 ± 0.35 μm, Bi-guttulate with size of 1.55 ± 0.13 μm | Conidia with size of 16.29 ± 4.22 × 1.20 ± 0.44 μm, Bi/guttulate with size of 1.02 ± 0.06 μm |                                            |                                            |
| **Group 4**   |                                                   |                             | Fusiform with tapering towards both ends, asetate, and hyaline             | Filiform to hamate, asetate, and hyaline                                   | Hyaline, branched, and slightly to straightly curve | Cylindrical phialides, terminal, hyaline, and slightly tapered towards end |
| DF9           | Cottony and grayish-white aerial mycelium Whitish with gray-patches on the lower surface | Black and globose           | Conidia with size of 6.28 ± 0.64 × 2.57 ± 0.22 μm, Bi-guttulate with size of 0.58 ± 0.07 μm | Conidia with size of 18.29 ± 2.26 × 1.21 ± 0.26 μm, Bi/guttulate with size of 1.02 ± 0.06 μm |                                            |                                            |
| **Group 5**   |                                                   |                             | Fusiform with slightly pointed ends, asetate, and hyaline                  | Not observed                                                                | Hyaline, branched, and slightly to straightly curve | Cylindrical phialides, terminal, hyaline, and slightly tapered towards end |
| DFP3          | Cottony and brownish-white aerial mycelia Yellowish-brown on the lower surface | Black and globose           | Conidia with size of 7.06 ± 0.55 × 2.47 ± 0.34 μm, Bi-guttulate with size of 0.40 ± 0.07 μm |                                            |                                            |                                            |

### Molecular identification and phylogenetic analysis

The comparison of DNA sequences based on ITS, TEF1-α, and β-tubulin demonstrated that the isolates were similar to the reference sequences of *D. eugeniae*, *D. phaseolorum*, *D. tectonendophytica*, *D. hongkongensis*, and *D. arecae* from the Genbank database. The phylogenetic trees generated from each single gene had the same topology as the tree generated from the combined genes of ITS, TEF1-α, and β-tubulin (Fig. 2) (Supplementary Information). The groupings of each single tree demonstrated that all the isolates were clustered in the same clades as their respective species of *Diaporthe* (*D. eugeniae*, *D. phaseolorum*, *D. tectonendophytica*, *D. hongkongensis*, and *D. arecae*). Isolates DF1, DF3, and DFP4 were grouped with *D. eugeniae* CBS 444.82; isolates DF2 and DF12 with *D. phaseolorum* CBS113425 and BHKHADRA-2; isolates DFP2 and DFP7 with *D. tectonendophytica* MFLUCC 13-0471; and isolates DF9 and DFP3 with *H. polyrhizus* CBS 115448 and *D. arecae* CBS 161.64, respectively. The result of the phylogenetic analysis was in accordance with the molecular identification based on DNA sequences [Basic Local Alignment Search (BLAST)], thus resolving the morphological identification. The isolates from group 1 were confirmed to be *D. eugeniae*, group 2 was *D. phaseolorum*, group 3 was *D. tectonendophytica*, group 4 was *D. hongkongensis*, and group 5 was *D. arecae*. The combined sequence matrix and phylogenetic tree were deposited in TreeBASE (http://purl.org/phylo/treebase/phylows/study/TB2:S27649).

### Pathogenicity test and comparative aggressiveness among *Diaporthe* isolates

The result of pathogenicity test indicated that all isolates of the *Diaporthe* species recovered from the stem gray blight of *H. polyrhizus* were pathogenic, exhibiting similar symptoms to those in the field (Fig. 3A1–A5). The tested isolates showed typical symptoms of gray blight on the inoculated stems of *H. polyrhizus*. Initially, irregular yellowish lesion surrounded by reddish border appeared on the wounded point (Fig. 3B1), which gradually turned into a dark-brown sunken lesion and demonstrated dampening (Fig. 3B2). As the disease progressed, the lesion became apparently dry and turned gray (Fig. 3B3). Then, it expanded periodically, and tiny black pycnidia appeared on the area of the lesion (Fig. 3B3–B5). No symptoms developed on the control points.

Isolate DF1 (*D. eugeniae*) recorded the highest lesion length (10.25 ± 0.35 cm), whereas isolate DFP3 (*D. arecae*) had the lowest (3.25 ± 0.35 cm) (Table 2). The means of the length lesion of the tested isolates were...
Figure 2. Maximum-likelihood tree of *Diaporthe* species isolated from stem gray blight of *H. polyrhizus* based on combined dataset of ITS, TEF1-α, and β-tubulin using Tamura and Nei model with 1000 bootstrap replications. Isolates of the present study are presented in bold and other fungal genera are used as an outgroup. Bootstrap values are shown at the nodes and the scale bar indicates the number of substitutions per position.
Figure 3. Stem gray blight of *H. polyrhizus*. (A1–A5) Disease symptoms observed in the fields. (B1) After 2 days of inoculation, irregular yellowish lesions surrounded by reddish borders appeared. (B2) The lesions became sunken and turned darker. (B3) The lesions apparently dry and turned to gray. (B4–B5) At later stage, the lesions expanded resulting in the appearance of blighted stem with formation of tiny black pycnidia. C denotes control and P represents treatment.

| Species          | Isolate | \( ^\text{a} \text{Lesion length (cm)} \)   |
|------------------|---------|-------------------------------------------|
| *D. eugeniae*    |         |                                           |
| DF1              | 10.25 ± 0.35\(^{\text{e}}\)     |
| DF3              | 5.50 ± 0.70\(^{\text{c}}\)       |
| DF4              | 5.10 ± 0.84\(^{\text{bc}}\)      |
| *D. phaseolorum* |         |                                           |
| DF2              | 7.50 ± 0.00\(^{\text{d}}\)       |
| DF12             | 7.75 ± 0.35\(^{\text{d}}\)       |
| *D. tectomendophytica* |    |                                           |
| DF7              | 8.25 ± 0.35\(^{\text{d}}\)       |
| DFP2             | 3.45 ± 0.70\(^{\text{d}}\)       |
| *D. hongkongensis* |       |                                           |
| DF9              | 3.50 ± 0.00\(^{\text{d}}\)       |
| *D. arecae*      |         |                                           |
| DFP3             | 3.25 ± 0.35\(^{\text{d}}\)       |
| Control          | 0.00 ± 0.00\(^{\text{f}}\)       |

\(^{\text{a}}\) Mean ± standard deviation followed by different letters within the column is significantly different \((p < 0.05)\) according to Tukey’s test.

Table 2. Lesion length recorded by *Diaporthe* isolates after 3 weeks of inoculation on stems of *H. polyrhizus*. 
significant differences compared with the control at \( p < 0.05 \). The tested isolates of *Diaporthe* exhibited variability in length lesion after 3 weeks of inoculation on the stems of *H. polyrhizus*. The same *Diaporthe* species were reisolated from the symptomatic inoculated stems of *H. polyrhizus*, and their identities were reconfirmed by comparing the macroscopic and microscopic characteristics with the original cultures, thus fulfilling Koch’s postulates.

**Discussion**

The present study reported on stem gray blight, which is a new emerging disease infecting *H. polyrhizus* plantations in Malaysia. The five species of *Diaporthe*, namely, *D. eugeniae* (group 1), *D. phaseolorum* (group 2), *D. tectonendophytica* (group 3), *D. hongkongensis* (group 4), and *D. arecae* (group 5), were identified to be the causal agents of the disease. The *Diaporthe* species may act as a plant pathogen or a saprophyte or an endophytic symbiont, however, several studies have reported that it is the genus responsible for multiple destructive diseases, such as root and fruit rots, dieback, stem cankers, leaf spots, leaf and pod blights, and seed decay.

A total of nine *Diaporthe* isolates were recovered from the blighted stem of *H. polyrhizus*. Based on their morphological characteristics, all the isolates produced both \( \alpha \)-conidia and \( \beta \)-conidia, except for the *D. arecae* isolate, of which \( \beta \)-conidia was not observed. \( \alpha \)- and \( \beta \)-conidia are the key characteristics for the identification of *Diaporthe*. The formation of \( \beta \)-conidia can sometimes be rare or absent in certain species of *Diaporthe*. According to Tuset and Portilla and Diogo et al., for some *Diaporthe* species (e.g., *Phomopsis amygdali*), the formation of \( \beta \)-conidia can only be observed in pycnidia on the host but not in pycnidia in the culture plate.

Based on the similarities and differences of their macroscopic and microscopic characteristics, the isolates were assigned to five different groups. Among the groups, significant differences were observed in the number of \( \alpha \)-conidia guttules and their size (Table 1). Gomes et al. revealed that both characteristics can be varied among the *Diaporthe* species. The isolates from group 1 (*D. eugeniae*) tended to produce bi- and multi-guttules, whereas the other isolates only produced bi-guttules of \( \alpha \)-conidia. The size of the guttules of \( \alpha \)-conidia varied among the groups. The isolates from groups 1 and 5 (*D. eugeniae* and *D. arecae*) produced significantly smaller guttules compared with those produced by isolates from groups 2, 3, and 5 (*D. phaseolorum, D. tectonendophytica, and D. hongkongensis*) (Table 1). The guttule is defined as a small drop or particle in a spore resembling a nucleus.

Moreover, the morphology of \( \alpha \)-conidia of the *D. eugeniae*, *D. hongkongensis*, and *D. arecae* isolates was tapered toward the ends compared with the *D. phaseolorum* and *D. tectonendophytica* isolates, the ends of which were bluntly rounded (Fig. 1). This finding was in agreement with those of Santos et al., Dissanayake et al., and Li et al. A significant difference was also observed in the length of \( \beta \)-conidia, of which the *D. eugeniae* isolates produced longer \( \beta \)-conidia than other isolates from different groups. Conidial mass exudation can be observed in the isolates of *D. eugeniae, D. hongkongensis,* and *D. arecae*. Contrarily, it was not observed in the isolates of *D. phaseolorum* and *D. tectonendophytica*. According to Machowicz-Stefaniak et al., the *Diaporthe* species require temperatures ranging from 22 to 28 °C for the optimal growth, sporulation, and rate of conidia release of conidiomata. As applied in the present study, the addition of carnation leaves to the growing medium as substrates has been recommended to improve the sporulation of the *Diaporthe* species.

Aside from the microscopic characteristic, the cultural characteristics of all isolates in this study also varied among the groups. The color of the colonies ranged from whitish, grayish, brownish, to olive green. Due to this inconsistency, cultural characteristic is commonly considered as a less important criterion in distinguishing species within *Diaporthe* as it can be influenced by several environmental factors, such as light and temperature. Based on the results obtained, morphological characteristics alone were insufficient to identify all the isolates up to the species level due to the complexity of the genus. This finding was in agreement with that of Lim et al., who revealed that the morphological method alone is not informative for the species identification of *Diaporthe* due to pleomorphism and overlapping characteristics.

With the advances in molecular techniques, DNA sequences and multigene phylogenetic analysis of ITS, TEF1-\( \alpha \), and \( \beta \)-tubulin were employed to support the morphological identification of the *Diaporthe* isolates in this study. The result of the BLAST search and phylogenetic inference indicated that the use of all the three genes resolved identification of the *Diaporthe* isolates. Aside from the present study, ITS, TEF1-\( \alpha \), and \( \beta \)-tubulin were extensively applied to delineate species within *Diaporthe*. The ITS region served as an identification guide for the *Diaporthe* species. It was also considered as a fungal barcode in distinguishing genera and species owing to its easy amplification and ability to provide preliminary screening of fungal classification. However, the tree constructed based on ITS sequences alone may be doubtful and not demonstrate clear phylogenetic relationships due to the lack of interspecific variation or even deceptive in some fungi. Thus, TEF1-\( \alpha \) and \( \beta \)-tubulin were added to support the phylogenetic analysis of ITS in delimiting the species of the *Diaporthe* isolates. TEF1-\( \alpha \) comprises an essential part of the protein translation machinery, and highly informative at the species level; moreover, non-orthologous copies have not been detected in *Diaporthe*. \( \beta \)-tubulin was utilized as an alternative phylogenetic marker to specify *Diaporthe* as it contains fewer ambiguously aligned regions and exhibits less homoplasy among the genus. Collectively, phylogenetic analysis of a combined dataset of ITS, TEF1-\( \alpha \), and \( \beta \)-tubulin was conducted in this study to overcome the ambiguity that could have emerged in the single gene analysis. Santos et al. stated that the combined phylogenetic tree commonly provides a better resolution for the identification of the *Diaporthe* species compared with the single gene analysis.

All the tested isolates of *Diaporthe* exhibited varying lengths of lesion on the inoculated stems of *H. polyrhizus*, of which isolate DF1 (*D. eugeniae*) was found to be the most virulent. The fungus can act as a pathogen or a saprophyte and was reported to cause stem-end rot on mango (*Mangifera indica*). It also occurs as a saprophyte on cloves (*Eugenia aromatica*). This study discovered a new host and disease caused by *D. eugeniae*. The association of *D. phaseolorum* with dragon fruit was not new, because recently, this pathogen was reported to cause stem rot on *Hylocereus undatus* in Bangladesh. However, the symptoms described were slightly different from those observed in the present study. It appeared as a yellow spot with a chlorotic halo in the previous report, but

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in the present study, chlorotic halo was not observed; rather, a reddish border surrounded the lesion. Similarly, gray to black pycnidia were scattered on the surface of the lesion. Aside from the dragon fruit, D. phaseolorum was reported as a causal agent of pod and stem blight, stem canker, and seed rot on soybean and trunk disease on grapevine. It was also found to be an endophyte on Kandelia candel by Cheng et al. 

Similar to D. eugeniae, the present study highlighted H. polyrhizus as a new host associated with D. tectonendophytica as it causes stem gray blight. Contrarily, a study by Doi et al. demonstrated the role of D. tectonendophytica as an endophyte occurring on teak (Tectona grandis) in Thailand. The capability of D. hongkongensis to act as a pathogen is undeniable as the fungus has been reported to cause severe diseases on a number of host plants, such as stem-end rot on kiwifruit, dieback on grapevine, and shoot canker on pear. Meanwhile, D. arecae has been reported to be pathogenic on M. indica, Areca catechu and Citrus. D. hongkongensis and D. arecae were first reported on H. polyrhizus worldwide especially in Malaysia.

The occurrence of the disease in two different locations in Malaysia indicates its possibility to spread worldwide. Aside from Diaporthe, dragon fruits in Malaysia also suffer from multiple diseases caused by other fungi. Among these diseases are anthracnose caused by C. gloeosporioides and C. truncatum; stem necrosis by Curvularia lunata; stem canker by N. dimidiatum; stem rot by Fusarium proliferatum and F. fujikuroi; reddish brown spot by Nigrospora lacticolonia and N. sphaerica; and stem blight by F. oxysporum. 

This study provides overview of the five different species of Diaporthe causing stem gray blight on H. polyrhizus in Malaysia. It improves our knowledge on the symptomatology of the disease and identity of the pathogens through morphological and molecular analyses. The findings may be essential to strategize effective disease management for stem gray blight on H. polyrhizus and for quarantine restrictions.

Materials and methods

Fungal isolation. In November 2017 and July 2018, nine gray blighted stems from the different plants of H. polyrhizus were collected from Bukit Kor, Terengganu, Malaysia, and the Cameron Highlands, Pahang, Malaysia. The symptomatic samples were brought back to the laboratory for isolation. One lesion per stem exhibiting the same symptom was selected for fungal isolation. The lesion consisting of diseased and healthy parts was excised (1.5 cm²) and surface-sterilized with 70% ethanol for 3 min. Then, the samples were soaked in 10% sodium hypochlorite (1% NaOCl) for 3 min and rinsed with sterile distilled water three times consecutively for 1 min each. The sterilized samples were air-dried on the sterile filter papers before being transferred to PDA plates. The inoculated plates were incubated at 25 °C ± 2 °C for 2 to 3 days. Pure cultures of fungal isolates were obtained via hyphal tip isolation and were used for morphological and molecular analyses.

Morphological identification. Each fungal isolate obtained was cultured on PDA and incubated at 25 °C ± 2 °C for 7 days. Macroscopic characteristics, such as colony appearance and pigmentation, were recorded. CLA was utilized to induce the formation of pycnidial conidiomata, and the inoculated plates were incubated at 25 °C ± 2 °C for 7 days. The morphology of α- and β-conidia was observed from the pycnidal conidiomata. The other microscopic characteristics observed were conidiophores and conidiogenous cells. The length and width of 30 randomly selected conidia and the size of the gullets of 30 randomly selected α-conidia were measured and recorded. The differences in the length and width of conidia and the size of the gullets of α-conidia were evaluated via one-way ANOVA. In addition, the means of both parameters were compared via Tukey's test (p < 0.05) using the IBM SPSS Statistics software version 24.

Molecular identification and phylogenetic analysis. The identity of all the fungal isolates was further confirmed by molecular characterization. The isolates were grown in potato dextrose broth (PDB) and incubated at 25 °C ± 2 °C for 7 days. Fungal mycelia from PDB were homogenized under liquid nitrogen to obtain fine powder. A total of 60 mg fine powder was transferred into a 1.5 mL microcentrifuge tube, and the genomic DNA of the fungal isolates was extracted using the Invisorb Spin Plant Mini Kit (Stratec Biomedical AG, Birkenfeld, Germany), following the manufacturer's protocols. The primers of ITS5/ITS4, EF1-728/EF1-986 and BT2a/b were used for PCR amplification. The obtained sequences were aligned using the Molecular Evolutionary Genetic Analysis software (MEGA7).

The obtained sequences were aligned using the Molecular Evolutionary Genetic Analysis software (MEGA7). After pairwise alignment, the BLAST algorithm (https://blast.ncbi.nlm.nih.gov/Blast.cgi) was used to compare the generated consensus sequences with other sequences in the GenBank database. The sequences obtained were deposited in the GenBank database.

The isolates in the present study and reference sequences used in the phylogenetic analysis are presented in Table 3. Multiple sequence alignments of fungal isolates and reference isolates were generated using the MEGA7 software. Phylogenetic analysis was conducted using the maximum likelihood (ML) method in MEGA7.
| Species                  | Isolate                  | Host                        | Locality                | GenBank accession no. | References          |
|--------------------------|--------------------------|----------------------------|-------------------------|-----------------------|---------------------|
| D. amygdali              | CBS 126679                  | Prunus dulcis               | Portugal                | KC343022              | KC343748            |
| D. amygdali              | CBS 111811                | Vitis vinifera             | South Africa            | KC343019              | KC343745            |
| D. amygdali              | CBS 115620                | Prunus persica             | USA                     | KC343020              | KC343746            |
| D. arecae                | CBS 161.64                | Areca catechu              | India                   | KC343032              | KC343758            |
| D. arecae                | CBS 535.75               | Citrus sp.                 | Suriname                | KC343033              | KC343759            |
| Diaporthe sp. (Group 5) | DFP3                     | Hylocleroces polyhizatus   | Bukit Kor, Terengganu,  | MN862382              | MN889938            |
|                          |                          |                            | Malaysia                |                       | MN889947            |
| D. arengae               | CBS 114979                | Arenga engleri             | Hong Kong               | KC343034              | KC343760            |
| D. brasiliensis          | CBS 133183                | Aspisperma tomentosum      | Brazil                  | KC343042              | KC343768            |
| D. caulivora             | CBS 127268                | Gycine max                 | Croatia                 | KC343043              | KC343769            |
| D. caulivora             | CBS 178.55               | Gycine soja                | Canada                  | KC343046              | KC343772            |
| D. eugenieae             | CBS 444.82               | Eugenia aromatica          | Indonesia               | KC343098              | KC343824            |
| Diaporthe sp. (Group 1) | DF1                      | Hylocleroces polyhizatus   | Cameron Highlands,     | MN862375              | MN889932            |
|                          |                          |                            | Pahang, Malaysia        |                       | MN889940            |
| Diaporthe sp. (Group 1) | DFP4                     | Hylocleroces polyhizatus   | Bukit Kor, Terengganu,  | MN862383              | MN889939            |
|                          |                          |                            | Malaysia                |                       | MN889948            |
| D. fuscini-asongstofliae | BRIP 54791                | Fuscinae angustifolia      | Australia               | IX862528              | IX862534            |
| D. helianthi             | CBS 592.81                | Helianthus annuus          | Serbia                  | KC343115              | KC343841            |
| D. helianthi             | CBS 344.94               | Helianthus annuus          | -                       | KC343114              | KC343840            |
| D. hongkongensis         | CBS 115448                | Dichroa febrifuga          | Hong Kong               | KC343119              | KC343845            |
| D. hongkongensis         | ZJUD74                   | Citrus unshiu              | China                   | KJ490609              | KJ490488            |
| D. hongkongensis         | ZJUD78                   | Citrus unshiu              | China                   | KJ490613              | KJ490492            |
| Diaporthe sp. (Group 4) | DP9                      | Hylocleroces polyhizatus   | Cameron Highlands,     | MN862379              | MN889933            |
|                          |                          |                            | Pahang, Malaysia        |                       | MN889941            |
| D. litchica              | BRIP 54900                | Litchi chinensis           | Australia               | IX862533              | IX862539            |
| D. mastrevicci           | BRIP 57892                | Helianthus annuus          | Australia               | KJ197276              | KJ197239            |
| D. mastrevicci           | BRIP 57330               | Chrystanthesoides montifera| Australia               | KJ197275              | KJ197237            |
| D. miricae               | BRIP 54736                | Helianthus annuus          | Australia               | KJ197282              | KJ197244            |
| D. miricae               | BRIP 55662c              | Gycine max                 | Australia               | KJ197283              | KJ197245            |
| D. miricae               | BRIP 56918a              | Vigna radiata              | Australia               | KJ197284              | KJ197246            |
| D. musigena              | CBS 129519               | Musa sp.                   | Australia               | KC343143              | KC343869            |
| D. novem                 | CBS 127270               | Gycine max                 | Croatia                 | KC343156              | KC343882            |
| D. novem                 | CBS 127269               | Gycine max                 | Croatia                 | KC343155              | KC343881            |
| D. novem                 | CBS 127271               | Gycine max                 | Croatia                 | KC343157              | KC343883            |
| D. oncostoma             | CBS 589.78               | Robinia pseudoacacia       | Australia               | KC343162              | KC343888            |
| D. oncostoma             | CBS 100454               | Robinia pseudoacacia       | Germany                 | KC343160              | KC343886            |
| D. oncostoma             | CBS 109741               | Robinia pseudoacacia       | Russia                  | KC343161              | KC343887            |
| D. pseudeiorum           | CBS 133186               | Maytenus ilicifolia        | Brazil                  | KC343164              | KC343890            |
| D. ramosa                | BRIP 54847               | Passerea americana         | Australia               | IX862532              | IX862538            |
| D. persae                | CBS 151.73               | Passerea americana         | Netherlands             | KC343173              | KC343899            |
| D. pescicola             | MFLUCC 16-0105            | Prunus persica             | China                   | KU557555              | KU557623            |
| D. pescicola             | MFLUCC 16-0106            | Prunus persica             | China                   | KU557556              | KU557624            |
| D. pescicola             | MFLUCC 16-0107            | Prunus persica             | China                   | KU557557              | KU557625            |
| D. phaseolorum           | CBS 139281               | Phaseolus vulgaris         | USA                     | KS907387              | KS907393            |
| D. phaseolorum           | CBS 113425               | Olearia cl. rani           | New Zealand             | KC343174              | KC343900            |
| D. phaseolorum           | BRDKHADRA-2              | Hylocleroces undatus       | Bangladesh              | MH174560              | KC343902            |
| Diaporthe sp. (Group 2) | DF2                      | Hylocleroces polyhizatus   | Cameron Highlands,     | MN862376              | MN889931            |
|                          |                          |                            | Pahang, Malaysia        |                       | MN889942            |
| Diaporthe sp. (Group 2) | DF12                     | Hylocleroces polyhizatus   | Cameron Highlands,     | MN862380              | MN889936            |
|                          |                          |                            | Pahang, Malaysia        |                       | MN889945            |
| D. pseudomangiferae      | CBS 101339               | Mangifera indica           | Dominican Republic      | KC343181              | KC343907            |
| D. pseudomangiferae      | CBS 388.89               | Mangifera indica           | Mexico                  | KC343182              | KC343908            |
| D. pseudophoenicicola    | CBS 462.69               | Phoenix dactylifera        | Spain                   | KC343184              | KC343910            |
| D. pseudophoenicicola    | CBS 176.77               | Mangifera indica           | Iraq                    | KC343183              | KC343909            |

Continued
| Species          | Isolate           | Host               | Locality               | GenBank accession no.       | References                  |
|------------------|-------------------|--------------------|------------------------|----------------------------|-----------------------------|
| D. schini        | CBS 133181TF      | Schinus terebinthifolius | Brazil                 | KC343191, KC343197, KC344159 | Gomes et al.                |
| D. schini        | LGMPF 910         | Schinus terebinthifolius | Brazil                 | KC343192, KC343198, KC344160 | Gomes et al.                |
| D. sennae        | CFCC 51636TF      | Senecio baccapiarius | China                  | KY203724, KY228885, KY228891 | Yang et al.                |
| D. sennae        | CFCC 51637         | Senecio baccapiarius | China                  | KY203725, KY228886, KY228892 | Yang et al.                |
| D. sojae         | FAU 599TF         | Glycine max        | USA                    | KJ590728, KJ590767, KJ610883 | Udayanga et al.            |
| D. sojae         | FAU 644           | Glycine max        | USA                    | KJ590730, KJ590769, KJ610885 | Udayanga et al.            |
| D. tectonendophytica | MFLUCC 13-0471TF | Tectona grandis | Thailand              | KU712439, KU749567, KU714396 | Doolom et al.              |
| Diaporthe sp. (Group 3) | DF7               | Hyllocereus polyrhizus | Cameron Highlands, Pahang, Malaysia | MN862387, MN889934, MN889946 | This study                  |
| Diaporthe sp. (Group 3) | DFP2            | Hyllocereus polyrhizus | Bukit Kor, Terengganu, Malaysia | MN862381, MN889937, MN889946 | This study                  |
| D. veckerae      | FAU 656TF         | Cucumis melo      | USA                    | KJ590726, KJ590747, KJ610881 | Udayanga et al.            |
| D. veckerae      | FAU 659           | Cucumis melo      | USA                    | KJ590724, KJ590745, KJ610879 | Udayanga et al.            |
| D. veckerae      | FAU 658           | Cucumis melo      | USA                    | KJ590725, KJ590746, KJ610880 | Udayanga et al.            |
| D. unshiuensis   | ZIUD 52           | Citrus unshiu     | China                  | KI490585, KJ490466, KJ490408 | Huang et al.               |
| D. unshiuensis   | ZIUD 50           | Citrus japonica   | China                  | KI490585, KJ490466, KJ490406 | Huang et al.               |
| D. unshiuensis   | ZIUD 51           | Citrus japonica   | China                  | KI490586, KJ490465, KJ490407 | Huang et al.               |
| D. vaccinii      | CBS 160.32TF      | Oxyccoccus macrocarpos | USA                   | KC343228, KC343954, KC344196 | Gomes et al.                |
| D. vaccinii      | CBS 118571        | Vaccinium corymbosum | USA                   | KC343223, KC343949, KC344191 | Gomes et al.                |
| D. vaccinii      | CBS 122112        | Vaccinium macrocarpus | USA                   | KC343224, KC343950, KC344192 | Gomes et al.                |
| Diaphorthea corylina | CBS 12112        | Corylus sp.       | China                  | KC343004, KC343730, KC343972 | Gomes et al.                |
| Lasiotrichia pseudotheobromae | CBS 116459TF | Gmelina arborea | Costa Rica            | EF622077, EF620507, EU673111 | Alves et al.               |
| Nigrospora musae | CBS 319.34TF      | Musa paradisitica | Australia             | KX986076, KY019419, KY019455 | Wang et al.                |
| Arthrinium obovatum | CGMCC 3.18331TF | Lithocarpus sp. | China                  | KY494696, KY705095, KY705166 | Wang et al.                |
| Paraphoma chloromycolicola | BRIP 65168TF | Tannacetum cinerariifolium | Australia            | KU999072, KU999080, KU999084 | Moslemi et al.              |

Table 3. Isolates in the present study and reference isolates used in the phylogenetic analysis. EP ex-epitype culture, EI ex-isotype culture, ET ex-type culture, EN ex-neotype culture, EH ex-holotype culture.

Tamura-Nei model\(^7\) was used to generate the ML trees based on a single and combined genes of ITS, TEF1-α, and β-tubulin with 1000 bootstrap replications\(^\)\(^2\)\.

**Pathogenicity test.** The pathogenicity test was conducted on 18 healthy stems of *H. polyrhizus* for all the obtained fungal isolates. Conidial suspension was prepared by flooding the 7-day-old PDA culture with sterile distilled water, and the concentration was adjusted to \(1 \times 10^6\) conidia/mL using a hemocytometer (Weber, Teddington, UK). The stems were surface-sterilized with 70% ethanol, and 0.1 mL of conidial suspension was utilized for inoculation using a disposable needle and syringe. Likewise, the control points were treated with sterile distilled water. On each stem, three points were used to inoculate fungal isolate and one point for control. Each fungal isolate was tested in three replicates, and the pathogenicity tests were conducted twice. All the inoculated plants were placed in a plant house in the School of Biological Sciences, USM, and incubated at 26–32 °C for 21 days. The progression of the disease symptom was observed daily. The lesion length was measured and recorded after 3 weeks of inoculation. The differences in the lesion length were evaluated via one-way ANOVA, and the means were compared via Tukey’s test \((p < 0.05)\) using the IBM SPSS Statistics software version 24. For the fulfillment of Koch’s postulates, the fungal isolates were reisolated from symptomatic inoculated stems and reidentified by morphological characteristics.

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Competing interests
The authors declare no competing interests.

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