Diaporthe species in south-western China

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Abstract

Three strains of the genus Diaporthe were isolated from different plant hosts in south-western China. Phylogenetic analyses of the combined ITS, β-tubulin, tef1 and calmodulin dataset indicated that these strains represented three independent lineages in Diaporthe. Diaporthe millettiae sp. nov. clustered with D. hongkongensis and D. arecae, Diaporthe osmanthi sp. nov. grouped with D. arengae, D. pseudomangiferae and D. perseae and Diaporthe strain GUCC9146, isolated from Camellia sinensis, was grouped in the D. eres species complex with a close relationship to D. longicicola. These species are reported with taxonomic descriptions and illustrations.

Keywords

Diaporthe, phylogeny, taxonomy, 2 new taxa

Introduction

Genus Diaporthe has been well-studied in recent years by Udayanga et al. (2011, 2012), incorporating morphological and molecular data and recommending appropriate genes to resolve species limitations in the genus. Since these revolutionary papers, 43 novel Diaporthe species have been described from China with morphological and
phylogenetic evidence (Huang et al. 2013, 2015; Fan et al. 2016; Gao et al. 2014, 2015, 2016, 2017; Yang et al. 2017a,b, 2018; Yang et al. 2016; Du et al. 2016; Dis-sanayake et al. 2017a). Dissanayake et al. (2017b) provided an update of the genus with additional 15 species (7 new and 8 known species) from Italy based on molecular evidence. New records and species have also been reported by Hyde et al. (2016), Ross-man et al. (2016), Chen and Kirschner (2017), Guarnaccia et al. (2018), Perera et al. (2018), Tibpromma et al. (2018) and Wanasinghe et al. (2018).

Three strains of *Diaporthe* were isolated from different medicinal plants collected in Guizhou and Guangxi during a survey of fungal diversity in south-western China. All the strains produced conidiomata containing alpha- and beta-conidia, typical of *Diaporthe*. This paper describes these three collections using molecular evidence, based on the analysis of combined ITS, β-tubulin, *tef1* and calmouduulin datasets, as *Diaporthe millettiae* sp. nov. and *D. osmanthi* sp. nov. and *D. longicicola* with a new host record from *Camellia sinensis*.

**Materials and methods**

**Isolation and morphological studies**

The samples were collected from Guizhou and Guangxi provinces. The *Diaporthe* strains were isolated using the single-spore method (Chomnunti et al. 2014). Colonies, growing from single spores, were transferred to potato-dextrose agar (PDA) and incubated at room temperature (28 °C). Following 2–3 weeks of incubation, morphological characters were recorded as in Udayanga et al. (2011, 2015). Conidia and conidiophores were observed using a compound microscope (Olympus BX53). The holotype specimens are deposited in the Herbarium of Department of Plant Pathology, Agricultural College, Guizhou University (HGUP). Ex-type cultures are deposited in the Culture Collection at the Department of Plant Pathology, Agriculture College, Guizhou University, China (GUCC). Taxonomic information of the new taxa was submitted to MycoBank (http://www.mycobank.org) and Facesoffungi (http://www.facesoffungi.org).

**DNA extraction and sequencing**

Fungal cultures were grown on PDA medium until they nearly covered the whole Petri-dish (90 mm diam.) at 28 °C. Fresh fungal mycelia were scraped from the surface with sterilised scalpels. A BIOMIGA Fungus Genomic DNA Extraction Kit (GD2416) was used to extract fungal genome DNA. DNA amplification was performed in a 25 μl reaction volume system which contained 2.5 μl 10 × PCR buffer, 1 μl of each primer (10 μM), 1 μl template DNA and 0.25 μl Taq DNA polymerase (Promega, Madison, WI, USA). Primers ITS4 and ITS5 (White et al. 1990) were used to amplify the ITS region. Three protein-coding gene fragments (β-tubulin, *tef1* and
calmodulin) were amplified by the primers Bt2a/Bt2b (Glass and Donaldson 1995), CAL228F/CAL737R and EF1-728F/EF1-986R (Carbone and Kohn 1999). Gene sequencing was performed with an ABI PRISM 3730 DNA autosequencer using either a dRhodamine terminator or Big Dye Terminator (Applied Biosystems Inc., Foster 19 City, California). The sequences of both strands of each fragment were determined for sequence confirmation. The DNA sequences were submitted to GenBank and their accession numbers were provided in Table 1.

Table 1. GenBank accession numbers of isolates include in this study.

| Species                  | Culture no. | GenBank no.  | ITS | tel/β-tubulin | calmodulin |
|--------------------------|-------------|--------------|-----|--------------|------------|
| Diaporthe alleghanensis  | CBS 495.72  | KC343007     | KC343733 | KC343975     | KC343249   |
| D. ambigua               | CBS 114015  | AF230767     | GQ250299 | KC343798     | KC343522   |
| D. anacardii             | CBS 720.97* | KC343024     | KC343750 | KC343992     | KC343266   |
| D. arecae                | CBS 161.64  | KC343032     | KC343758 | KC344000     | KC343274   |
| D. araneae               | CBS 114979  | KC343034     | KC343760 | KC344002     | KC343276   |
| D. baccae                | CBS 136972  | KJ160565     | KJ160597 | MF418509     | MG281695   |
| D. betulicola            | BRIP 54792  | JX862529     | JX862535 | KF170921     | –          |
| D. betulae               | CFCC 50470  | KT732951     | KT733017 | KT733021     | KT732998   |
| D. bidentata             | CBS 121004  | KC343134     | KC343860 | KC344102     | KC343376   |
| D. bicincta              |              |              |       |              |            |
| D. biguttuis             | CGMCC 3.17081 | KF576282  | KF576257 | KF576306     | –          |
| D. celatrina             | CBS 139.27  | KC343047     | KC343773 | KC344015     | KC343289   |
| D. celeris               | CBS 143349  | MG281107     | MG281538 | MG281190     | MG281712   |
| D. charlesworthii        | BRIP 54884m* | KJ197288     | KJ197250 | KJ197268     | –          |
| D. cinerascens           | CBS 719.96  | KC343050     | KC343776 | KC344018     | KC343292   |
| D. cotoneastri           | CBS 439.82  | FJ889450     | GQ250341 | JX275437     | JX197429   |
| D. decedens              | CBS 109772  | KC343059     | KC343785 | KC344027     | KC343301   |
| D. elaeagni              | CBS 504.72  | KC343064     | KC343790 | KC344032     | KC343306   |
| D. ellipicola            | CGMCC 3.17084 | KF576270  | KF576245 | KF576291     | –          |
| D. evers                 | CBS 138594  | KJ210529     | KJ210550 | KJ420799     | KJ434999   |
| D. foeniculina           | CBS 187.27  | KC343107     | KC343833 | KC344075     | KC343349   |
| D. goulteri              | BRIP 55657a | KJ197289     | KJ197252 | KJ197268     | –          |
| D. helianthi             | CBS 592.81  | KC343115     | GQ250308 | KC343841     | JX197454   |
| D. hongkongensis         | CBS 115448  | KC343119     | KC343845 | KC344087     | KC343361   |
| D. inoniscia             | CBS 138313  | KC343123     | KC343849 | KC344091     | KC343365   |
| D. longicola             | GUCC9146    | MK398676     | MK480611 | MK502091     | MK502088   |
| D. longicola             | CGMCC 3.17091 | KF576267  | KF576242 | KF576291     | –          |
| D. macinthoshii          | BRIP 55064a* | KJ197290    | KJ197251 | KJ197269     | –          |
| D. millelta              | GUCC9167    | MK398674     | MK480609 | MK502089     | MK502086   |
| D. ocntostoma            | CBS 589.78  | KC343162     | KC343888 | KC344130     | KC343404   |
| D. osmanthiis            | GUCC9165    | MK398675     | MK480610 | MK502090     | MK502087   |
| D. persae                | CBS 151.73  | KC343173     | KC343899 | KC344141     | KC343415   |
| D. phragmatitis          | CBS 138897  | KP004445     | KP004507 | –            | –          |
| D. pseudomangiferae      | CBS 101339  | KC343181     | KC343907 | KC344149     | KC343423   |
| D. pseudophoenicicola    | CBS 462.69  | KC343184     | KC343910 | KC344152     | KC343426   |
| D. rosiola               | MFLU 17.0646| NR157515     | MG829270 | MG838377     | MG829274   |
| D. saccharata            | CBS 116311  | KC343190     | KC343916 | KC344158     | KC343432   |
| D. sticta                | CBS 370.54  | KC343212     | KC343936 | KC344180     | KC343454   |
| D. vaccinii              | CBS 160.32  | AF317578     | GQ250326 | KC344196     | KC343470   |
| Valsa ambiens           | CFCC 8894   | KR045617     | KU710912 | KR045658     | –          |

Ex-type isolates were labeled with bold.
Phylogenetic analyses

DNA sequences from our three strains and reference sequences downloaded from GenBank (Dissanayake et al. 2017a, b), Guarnaccia et al. (2018) and Wanasinghe et al. (2018) were analysed by maximum parsimony (MP) and maximum likelihood (ML). Sequences were optimised manually to allow maximum alignment and maximum sequence similarity, as detailed in Manamgoda et al. (2012). MP analyses were performed in PAUP v. 4.0b10 (Swofford 2003), using the heuristic search option with 1,000 random taxa additions and tree bisection and re-connection (TBR) as the branch swapping algorithm. Maxtrees = 5000 was set to build the phylogenetic tree. The characters of the alignment document were ordered according to ITS+tef1+β-tubulin+CAL for GUCC9165 and GUCC9167 and tef1+β-tubulin for GUCC9146 with equal weight and gaps were treated as missing data. The Tree Length (TL), Consistency Indices (CI), Retention Indices (RI), Rescaled Consistency Indices (RC) and Homoplasy Index (HI) were calculated for each tree generated. The resulting Phylip file was used to make ML and Bayesian trees by the CIPRES Science Gateway (https://www.phylo.org/portal2/login.action) and RAxML-XSEDE with 1000 bootstrap inferences.

Results

Phylogenetic analyses

Three Diaporthe strains isolated from different plant hosts were sequenced. PCR products of 456–465 bp (ITS), 292–303 bp (tef1), 666–690 bp (β-tubulin) and 336–345 bp (CAL) were obtained. By alignment with the single gene region and then combination according to the order of ITS, tef1, β-tubulin and CAL with Valsa ambiens (CFCC 89894), only 1833 characters were obtained, viz. ITS: 1–492, tef1: 493–801, β-tubulin: 802–1469, CAL: 1470–1833, with 500 parsimony-informative characters. This procedure yielded eleven parsimonious trees (TL = 2169, CI = 0.58, RI = 0.71, RC = 0.41 and HI = 0.42), the first one being shown in Figure 1. All Diaporthe species clustered together, although without credible support for bootstrap and BPP values (Figure 1). Phylogenetic analysis of strains GUCC9165 and GUCC9167, using the four gene loci, confirmed them as well-resolved species (Figure 1). Strain GUCC9165 formed an independent branch adjacent to D. arecae and D. hongkongensis (MP: 100%, ML: 94% and BPP: 1). Strain GUCC 9167 grouped with the branch which included D. arengae, D. perseae and D. pseudomangiferae (MP: 92%, ML: 98% and BPP: 1). Strain GUCC 9146 was aligned to the branch having D. longicicola and D. rosicola in the Diaporthe eres species-complex (Figure 2), with high statistical support (MP: 84%, ML: 93% and BPP: 1). This strain also showed a close relationship to D. eres and D. cotoneastri. In addition, we also compared the DNA base pair differences between our strains and related species in different gene regions (Suppl. material 1: Table S1). In Diaporthe strain GUCC9165, the four genes had 64 base pair differ-
Figure 1. Parsimonious tree obtained from a combined analyses of an ITS, β-tubulin, calmodulin and tef1 sequence dataset. MP, ML above 50% and BPP values above 0.90 were placed close to topological nodes and separated by “/”. The bootstrap values below 50% and BPP values below 0.90 were labelled with “-”. The tree is rooted with *Valsa ambiens* (CFCC89894). The branch of our new *Diaporthe* species is in pink.
Figure 2. Parsimonious tree obtained from a combined analyses of a β-tubulin and tef1 sequence dataset (TL = 265, CI = 0.89, RI = 0.76, RC = 0.68 and HI = 0.11). MP, ML above 50% and BPP values above 0.90 were placed close to topological nodes and separated by “/”. The bootstrap values below 50% and BPP values below 0.90 were labelled with “-”. The tree is rooted with *Diaporthe decedens* (CBS 109772).

ences with *D. arecae* and 119 with *D. hongkongensis*, the main differences being with β-tubulin and tef1. Strain GUCC9167 had 52 base pair differences with *D. arencae*, 61 with *D. perseae* and 64 with *D. pseudomangiferae*, wherein the base distinction was
primarily in the β-tubulin and tef1 gene region. The β-tubulin sequences of D. eres and D. longicicola were apparently shorter than in strain GUCC 9146. The CAL sequences of D. rosicola were shorter than GUCC 9146. The DNA sequence of CAL for Diaporthe longicicola was not available (Gao et al. 2015). Integrating available DNA information, we discovered that 28 base pair differences were shown between GUCC 9146 and D. eres, 51 between GUCC 9146 and D. cotoneastri, 26 between GUCC 9146 and D. rosicola and 22 (only three genes) between GUCC 9146 and D. longicicola. Meanwhile, the phylogenetic analysis, based on only tef1 and β-tubulin for the D. eres species-complex (Figure 2), also indicated that GUCC 9146 clustered with D. longicicola and D. rosicola which obtained support values of MP: 99%, ML: 100% and BPP: 1 and maintained a closer relationship with D. longicicola.

### Taxonomy

**Diaporthe millettiae** H. Long, K.D. Hyde & Yong Wang bis, sp. nov.

Mycobank MB 829563

Figure 3

**Diagnosis.** Characterised by larger J-shaped β-conidia.

**Type.** China, Guangxi Province, Nanning City, from leaves of Millettia reticulata, 20 September 2016, Y. Wang, HGUP 9167, holotype, ex-type living culture GUCC 9167.

**Description.** Colonies on PDA attaining 9 cm diam. after 10 days; coralloid with feathery branches at margin, adpressed, with apparent aerial mycelium, with numerous irregularly zonate dark stromata, isabelline becoming lighter towards the margin; reverse similar to surface, with zonations. Conidiomata pycnidial, multilocular, scattered, abundant on PDA after 3 wks, subglobose to irregular, 1.5–1.8 mm diam., ostiolate, with up to 1 mm necks when present. Conidiophores formed from the inner layer of the locular wall, sometimes reduced to conidiogenous cells, when present 1-septate, hyaline to pale yellowish-brown, cylindrical, 10–23 × 1–2.5 μm. Conidiogenous cells cylindrical to flexuous, tapered towards apex, hyaline, 8–18 × 1.5–3 μm. Alpha conidia abundant, fusiform, narrowed towards apex and base, mostly biguttulate, hyaline, 4.5–9 × 2–3.5 μm. Beta conidia scarce to abundant, flexuous to J-shaped, hyaline, 17.5–32 × 1–2 μm. Perithecia not seen.

**Habitat and distribution.** Isolated from leaves of Millettia reticulata in China.

**Etymology.** Species epithet millettiae, referring to the host, Millettia reticulata from which the strain was isolated.

**Notes.** Phylogenetic analysis combining four gene loci showed that Diaporthe millettiae (strain GUCC 9167) displayed a close relationship with D. arengae, D. pseudomangiferae and D. perseae with high bootstrap values (Figure 1). We compared the DNA base pair differences of the four gene regions, the main differences being in the β-tubulin and tef1 genes, especially tef1. Diaporthe millettiae produced two types of conidia (α, β), whereas D. pseudomangiferae only produced alpha conidia and D. perseae...
produced three types of conidia (α, β, γ). The β-conidia of *D. arengae* were smaller (20–25 × 1.5 μm) than those of *Diaporthe milletiae* (17.5–32 × 1–2 μm). The shape of β-conidia was also different. Conidiophores of *D. arengae* (10–60 μm) with more septa (0–6), were longer than those of *D. milletiae* (10–23 × 1–2.5 μm; 0-1-septate) (Gomes et al. 2013).

*Diaporthe osmanthi* H. Long, K.D. Hyde & Yong Wang bis, sp. nov.
MycoBank MB 829564
Figure 4

**Diagnosis.** Characterised by size of α-conidia and β-conidia.

**Type.** China, Guangxi province, Nanning City, from leaves of *Osmanthus fragrans*, 20 September, 2016, Y. Wang, HGUP 9165, holotype, ex-type living culture GUCC 9165.

**Description.** Colonies on PDA attaining 9 cm diam. after 10 days; coralloid with feathery branches at margin, adpressed, without aerial mycelium, with numerous irregularly zonated dark stromata, isabelline becoming lighter towards the margin; re-
figure similar to the surface with zonations more apparent. **Conidiomata** pycnidial and multilocular, scattered, abundant on PDA after 3 wks, globose, subglobose or irregular, up to 1–1.5 mm diam., ostiolate, necks absent or up to 1 mm. **Conidiophores** formed from the inner layer of the locular wall, reduced to conidiogenous cells or 1-septate, hyaline to pale yellowish-brown, cylindrical, 20.5–61 × 1–3 μm. **Conidiogenous cells** cylindric to flexuous, tapered towards apex, hyaline, 10–15 × 1.5–3 μm. **Alpha conidia** abundant, fusiform, narrowed towards the apex and base, apparently biguttulate, hyaline, 5.5–8.5 × 2–3 μm. **Beta conidia** scarce to abundant, flexuous to J-shaped, hyaline, 20–31.5 × 1–2.5 μm. **Perithecia** not seen.

**Habitat and distribution.** Isolated from leaves of *Osmanthus fragrans* in China.

**Etymology.** Species epithet *osmanthi*, referring to the host, *Osmanthus fragrans* from which our strain was isolated.

**Notes.** *Diaporthe osmanthi* (strain GUCC9165) formed an independent lineage, but was also related to *D. arecae* and *D. hongkongensis* (Figure 1). The sequences of β-tubulin and tef1 included about two-three differences between *D. osmanthi* (GUCC9165) and *D. arecae* (42) and *D. hongkongensis* (78) and thus they were different species according to the guidelines of Jeewon and Hyde (2016). Additionally, *Diaporthe hongkongensis* produced three types of conidia, but *Diaporthe osmanthi* did...
not produce γ-conidia. In addition, β-conidia of _D. hongkongensis_ (18–22 μm) were shorter than those of _Diaporthe osmanthi_ (Gomes et al. 2013). According to original description Srivastava et al. (1962), _D. arecae_ also produced two types of conidia. The α-conidia (7.2–9.6 × 2.4 μm) were longer than in _Diaporthe osmanthi_, but its β-conidia (14.4–24 × 1.2 μm) were shorter and their shape also had some differences.

**Diaporthe longicicola** Y.H. Gao & L. Cai, Fungal Biology 119(5): 303 (2015)

**Figure 5**

**Description.** Colonies on PDA attaining 9 cm diam. in 10 days; coralloid with feathery branches at margin, adpressed, without aerial mycelium, without numerous irregularly zonated dark stromata, isabelline becoming lighter towards the margin; reverse similar to the surface with zonations more apparent. _Conidiomata_ pycnidial and multilocular, scattered, abundant on PDA after 20 d, subglobose or irregular, 1.5–1.8 mm diam., ostiolate and up to 1 mm long. _Conidiophores_ formed from the inner layer of the locular wall, densely aggregated, hyaline to pale yellowish-brown, cylindrical, tapering towards the apex, 15–25 × 1.5–2 μm. _Alpha conidia_ abundant, ellipsoid to fusiform, apparently biguttulate, hyaline, 6–9 × 2–3 μm. _Beta conidia_ scarce to abundant, flexuous to J-shaped, hyaline, 25.5–35.5 × 1–2.5 μm.

**Habitat and distribution.** Isolated from leaves of _Camellia sinensis_ in Duyun, Guizhou Province, China.
Notes. Phylogenetic analyses (Figures 1, 2) indicated that GUCC 9146 has a close relationship with *D. longicicola*, *D. rosicola*, *D. eres* and *D. cotoneastri*. Morphological comparison indicated that this strain was most similar to *D. longicicola* but not a related species by the width of alpha conidia and length of beta conidia (Udayanga et al. 2014; Gao et al. 2015).

Discussion

Phylogenetic analysis and morphology provide evidence for the introduction of *Diaporthe millettiae* and *D. osmanthi* as new species. In order to support the validity of these new species, we followed the guidelines of Jeewon and Hyde (2016) in comparing base pair differences (Suppl. material 1: Table S1). In accordance with Udayanga et al. (2014), we also believed that the ITS fragment was problematic for the *D. eres* species-complex. When not considering ITS, integration with morphological comparison was helpful and we concluded that GUCC 9146 is *D. longicicola*. *Diaporthe longicicola* was firstly reported on *Lithocarpus glabra* in Zhejiang Province, but our strain (GUCC 9146) was recovered from *Camellia sinensis* in Guizhou Province. Thus, this is the report of a new host and new location in China for *D. longicicola*.

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Supplementary material 1

The DNA bases difference between our strains and related taxa on four gene regions
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Data type: molecular data

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