Characterization of *Diaporthe* species on *Camellia oleifera* in Hunan Province, with descriptions of two new species

Qin Yang¹², Jie Tang¹, Guo Y. Zhou¹²

¹ Forestry Biotechnology Hunan Key Laboratories, Central South University of Forestry and Technology, Changsha 410004, China ² The Key Laboratory for Non-Wood Forest Cultivation and Conservation of the Ministry of Education, Central South University of Forestry and Technology, Changsha 410004, China

Corresponding author: Guo Y. Zhou (zgyingqq@163.com)

Abstract

Tea-oil tree (*Camellia oleifera* Abel.) is an important edible oil woody plant with a planting area over 3,800,000 hectares in southern China. Species of *Diaporthe* inhabit a wide range of plant hosts as plant pathogens, endophytes and saprobes. At present, relatively little is known about the taxonomy and genetic diversity of *Diaporthe* on *C. oleifera*. Here, we conducted an extensive field survey in Hunan Province in China to identify and characterise *Diaporthe* species associated with tea-oil leaf spots. As a result, eleven isolates of *Diaporthe* were obtained from symptomatic *C. oleifera* leaves. These isolates were studied by applying a polyphasic approach including morphological and phylogenetic analyses of partial ITS, *cal, his3, tef1* and *tub2* gene regions. Two new *Diaporthe* species (*D. camelliae-oleiferae* and *D. hunanensis*) were proposed and described herein, and *C. oleifera* was revealed to be new host records of *D. hubeiensis* and *D. sojae*. This study indicated there is a potential of more undiscovered *Diaporthe* species from *C. oleifera* in China.

Keywords

*Camellia oleifera*, DNA phylogeny, systematics, taxonomy, two new taxa
Introduction

Tea-oil tree, *Camellia oleifera* Abel., is a unique woody edible oil species in China, mainly distributed in the Qinling-Huaihe River area. It has a long history of cultivation and utilization for more than 2300 years since ancient China (Zhuang 2008). Camellia oil, obtained from *C. oleifera* seeds, is rich in unsaturated fatty acids and unique flavors, and has become a rising high-quality edible vegetable oil in China. The edible of tea-oil is also conducive to preventing cardiovascular sclerosis, anti-tumor, lowering blood lipid, protecting liver and enhancing human immunity (Wang et al. 2007). Hunan Province leads the country in *C. oleifera* production with the average of 3.3–40,000 hm² to expand the cultivation area every year (Tan et al. 2018). By the end of 2017, the cultivation area of *C. oleifera* reached 1.4 million hm², tea oil 290100 tons, and output value of 35 billion yuan (Tan et al. 2018). Thus, the development of *C. oleifera* industry is of great significance for the economic development of Hunan Province and the poverty alleviation of local farmers.

Diseases are a major constraint to *C. oleifera* production. Anthracnose disease caused by *Colletotrichum* species is one of the foremost diseases in southern China, which can infect leaves and fruits of *C. oleifera*, causing up to 40% fruit drop and up to 40% camellia seeds loss (Wang et al. 2020). During July and August of 2020, new leaf spots were detected on tea-oil tree with irregular, brownish-grey lesions, often associated with leaf margins. Infected leaves cultured on medium had dark pycnidia producing ellipsoid guttulate conidia, similar to that of *Diaporthe* species (Yang et al. 2020, 2021). *Diaporthe* species are responsible for diseases on a wide range of plant hosts, including agricultural crops, forest trees and ornaments, some of which can cause substantial yield losses (Santos et al. 2011; Gomes et al. 2013; Udayanga et al. 2015; Gao et al. 2016; Guarnaccia and Crous 2017, 2018; Yang et al. 2018, 2020, 2021). For instance, *D. ampelina*, the causal agent of Phomopsis cane and leaf spot, is known as a severe pathogen of grapevines (Hewitt and Pearson 1988), infecting all green tissues and causing yield reductions of up to 30% in temperate regions (Erincik et al. 2001). *Diaporthe citri* is another well-known pathogen exclusively found on *Citrus* spp. causing melanose, stem-end rot and gummosis in all the citrus production area except Europe (Mondal et al. 2007; Udayanga et al. 2014a; Guarnaccia and Crous 2017, 2018).

Species identification criteria in *Diaporthe* has mainly relied on host association, morphology and culture characteristics (Mostert et al. 2001; Santos and Phillips 2009; Udayanga et al. 2011), which resulted in the description of over 200 species. Some species of *Diaporthe* were reported to colonise a single host plant, while other species were found to be associated with different host plants (Santos and Phillips 2009; Diogo et al. 2010; Santos et al. 2011; Gomes et al. 2013). In addition, considerable variability of the phenotypic characters was found to be present within a species (Rehner and Uecker 1994; Mostert et al. 2001; Udayanga et al. 2011). During the past decade, a polyphasic approach, based on multi-locus DNA data, morphological, phytopathological and phylogenetical analyses, has been employed for species boundaries in the
genus *Diaporthe* (Huang et al. 2015; Gao et al. 2016, 2017; Guarnaccia and Crous 2017; Guarnaccia et al. 2018; Yang et al. 2018, 2020, 2021).

The classification of *Diaporthe* has been ongoing; however, little is known about species able to infect *C. oleifera*. Thus, the objective of the present study was to identify the prevalence of *Diaporthe* spp. associated with tea-oil tree leaf spot in the major plantations in Hunan Province based on morphological and phylogenetic features.

### Materials and methods

**Fungal isolation**

Leaves of *C. oleifera* with typical symptoms of leaf spots were collected from the main tea-oil camellia production fields in Hunan Province. Small sections (3 × 3 mm) were cut from the margins of infected tissues, and surface-sterilised in 75% ethanol for 30 s, then sterilised in 5% sodium hypochlorite for 1 min, followed by three rinses with sterilised water and finally dried on sterilised filter paper. The sections were then plated on to PDA plates and incubated at 25 °C. Fungal growth was examined daily for up to 7 d. Isolates were then transferred aseptically to fresh PDA and purified by single-spore culturing. All fungal isolates were placed on PDA slants and stored at 4 °C. Specimens and axenic cultures are maintained in the Central South University of Forestry and Technology (CSUFT).

**Morphological and cultural characterization**

Agar plugs (6 mm diam.) were taken from the edge of actively growing cultures on PDA and transferred on to the centre of 9 cm diam. Petri dishes containing 2% tap water agar supplemented with sterile pine needles (PNA; Smith et al. 1996) and potato dextrose agar (PDA), and incubated at 25 °C under a 12 h near-ultraviolet light/12 h dark cycle to induce sporulation as described in recent studies (Gomes et al. 2013; Lombard et al. 2014). Colony characters and pigment production on PNA and PDA were noted after 10 d. Colony colours were rated according to Rayner (1970). Cultures were examined periodically for the development of ascomata and conidiomata. The morphological characteristics were examined by mounting fungal structures in clear lactic acid and 30 measurements at ×1000 magnification were determined for each isolate using a Leica compound microscope (DM 2500) with interference contrast (DIC) optics. Descriptions, nomenclature and illustrations of taxonomic novelties are deposited in MycoBank (Crous et al. 2004a).

**DNA extraction, PCR amplification and sequencing**

Genomic DNA was extracted from colonies grown on cellophane-covered PDA using a CTAB [cetyltrimethylammonium bromide] method (Doyle and Doyle 1990).
DNA was estimated by electrophoresis in 1% agarose gel, and the quality was measured using the NanoDrop 2000 (Thermo Scientific, Waltham, MA, USA), following the user manual (Desjardins et al. 2009). PCR amplifications were performed in a DNA Engine Peltier Thermal Cycler (PTC-200; Bio-Rad Laboratories, Hercules, CA, USA). The primer set ITS1/ITS4 (White et al. 1990) was used to amplify the ITS region. The primer pair CAL228F/CAL737R (Carbone and Kohn 1999) was used to amplify the calmodulin gene (cal), and the primers CYLH4F (Crous et al. 2004b) and H3-1b (Glass and Donaldson 1995) were used to amplify part of the histone H3 (his3) gene. The primer pair EF1-728F/EF1-986R (Carbone and Kohn 1999) was used to amplify a partial fragment of the translation elongation factor 1-α gene (tef1). The primer set T1 (O’Donnell and Cigelnik 1997) and Bt2b (Glass and Donaldson 1995) was used to amplify the beta-tubulin gene (tub2); the additional combination of Bt2a/Bt2b (Glass and Donaldson 1995) was used in case of amplification failure of the T1/Bt2b primer pair. The PCR amplifications of the genomic DNA with the phylogenetic markers were done using the same primer pairs and conditions as in Yang et al. (2018). PCR amplification products were assayed via electrophoresis in 2% agarose gels. DNA sequencing was performed using an ABI PRISM 3730XL DNA Analyzer with a BigDye Terminator Kit v.3.1 (Invitrogen, USA) at the Shanghai Invitrogen Biological Technology Company Limited (Beijing, China).

Phylogenetic analyses

The quality of the amplified nucleotide sequences was checked and combined using SeqMan v.7.1.0 and reference sequences were retrieved from the National Center for Biotechnology Information (NCBI), based on recent publications on the genus *Diaporthe* (Guarnaccia et al. 2018; Yang et al. 2018, 2020, 2021). Sequences were aligned using MAFFT v. 6 (Katoh and Toh 2010) and corrected manually using Bioedit 7.0.9.0 (Hall 1999). The best-fit nucleotide substitution models for each gene were selected using jModelTest v. 2.1.7 (Darriba et al. 2012) under the Akaike Information Criterion.

The phylogenetic analyses of the combined gene regions were performed using Maximum Likelihood (ML) and Bayesian Inference (BI) methods. ML was conducted using PhyML v. 3.0 (Guindon et al. 2010), with 1000 bootstrap replicates while BI was performed using a Markov Chain Monte Carlo (MCMC) algorithm in MrBayes v. 3.0 (Ronquist et al. 2003). Two MCMC chains, started from random trees for 1,000,000 generations and trees, were sampled every 100th generation, resulting in a total of 10,000 trees. The first 25% of trees were discarded as burn-in of each analysis. Branches with significant Bayesian Posterior Probabilities (BPP) were estimated in the remaining 7500 trees. Phylogenetic trees were viewed with FigTree v.1.3.1 (Rambaut and Drummond 2010) and processed by Adobe Illustrator CS5. The nucleotide sequence data of the new taxa were deposited in GenBank (Table 1). The multilocus sequence alignments were deposited in TreeBASE (www.treebase.org) as accession S28703 and S22703.
Table 1. Isolates and GenBank accession numbers used in the phylogenetic analyses of *Diaporthe*.

| Species             | Isolate     | Host            | Location          | GenBank accession numbers                  |
|---------------------|-------------|-----------------|-------------------|--------------------------------------------|
|                      |             |                 |                   | **ITS** | **cal** | **his3** | **tef1** | **tub2** |
| *D. acercola*       | MFLUCC 17-0956 | *Acer negundo* | Italy             | KY964224 | KY964137 | NA       | KY964180 | KY964074 |
| *D. acerigena*      | CFCC 52554  | *Acer tataricum* | China             | MH121489 | MH121413 | MH121449 | MH121531 | NA       |
| *D. alangii*        | CFCC 52556  | *Alangium kurzii* | China            | MH121491 | MH121415 | MH121451 | MH121533 | MH121573 |
| *D. alnea*          | CBS 146.46  | *Alnus* sp.     | Netherlands       | KC343008 | KC343250 | KC343492 | KC343734 | KC343976 |
| *D. amygdali*       | CBS 126679  | *Prunus dulcis* | Portugal          | KC343022 | KC343264 | KC343506 | AY343478 | KC343990 |
| *D. angicola*       | CBS 111592  | *Hericium sp.*  | Austria           | KC343027 | KC343269 | KC343511 | KC343753 | KC343995 |
| *D. apiculatum*     | CGMCC 3.17533 | *Camellia sinensis* | China        | KP267896 | NA       | NA       | KP267970 | KP293476 |
| *D. arecae*         | CBS 161.64  | *Aarea catechu* | India             | KC343032 | KC343274 | KC343516 | KC343758 | KC344000 |
| *D. arengae*        | CBS 114979  | *Avena engleri* | Hong Kong         | KC343034 | KC343276 | KC343518 | KC343760 | KC344002 |
| *D. arecae*         | CBS 120679  | *Prunus dulcis* | Portugal          | KC343022 | KC343264 | KC343506 | AY343478 | KC343990 |
| *D. aseana*         | MFLUCC 12-0299 | Unknown dead leaf | Thailand     | KT459414 | KT459464 | NA       | KT459448 | KT459432 |
| *D. bigotractula*   | CGMCC 3.17248 | *Citrus limon*  | China             | KJ490582 | NA       | KJ490524 | KJ490461 | KJ490403 |
| *D. camelliae-oleiferae* | HNZZ027 | *Camellia oleifera* | China       | MZ509555 | MZ504685 | MZ504696 | MZ504702 | MZ504718 |
| *D. camelliae-oleiferae* | HNZZ030 | *Camellia oleifera* | China       | MZ509556 | MZ504686 | MZ504697 | MZ504708 | MZ504719 |
| *D. camelliae-oleiferae* | HNZZ032 | *Camellia oleifera* | China       | MZ509557 | MZ504687 | MZ504698 | MZ504709 | MZ504720 |
| *D. celeris*        | CPC 28262   | *Vitis vinifera* | Czech Republic    | MG281017 | MG281712 | MG281363 | MG281538 | MG281190 |
| *D. eulata*         | CBS 139.27  | *Celastrus* sp. | USA                | KC343047 | KC343289 | KC343516 | KC343758 | KC344015 |
| *D. eres*           | AR5193      | *Ulmus* sp.     | Germany           | KJ210529 | KJ434999 | KJ420850 | KJ210550 | KJ420799 |
| *D. fraxini-angustifoliae* | BRIP 54781 | *Fraxinus angustifolia* | Australia | KC343057 | KC343299 | KC343541 | KC343783 | KC344025 |
| *D. fructicola*     | MAFF 246408 | *Passiflora edulis* × *P. edulis f. flavicarpa* | Japan         | LC342734 | LC342738 | LC342737 | LC342735 | LC342736 |
| *D. fusocarpa*      | CGMCC 3.17087 | *Listerastrum glabrum* | China     | KF576281 | KF576233 | NA       | KF576256 | KF576305 |
| Species            | Isolate         | Host                   | Location       | GenBank accession numbers                                      |
|--------------------|-----------------|------------------------|----------------|----------------------------------------------------------------|
| D. ganzhouensis     | CFCC 53087      | Unknown                | China          | MK432665 MK442985 MK443010 MK578139 MK578065                   |
| D. garethjonesii    | MFLUCC 12-0542a | Unknown dead leaf      | Thailand       | KT459423 KT459470 NA KT459457 KT459441                       |
| D. guangxiensis     | JZB320094       | Vitis vinifera         | China          | MK355772 MK36727 NA MK523566 MK500168                        |
| D. helicis         | AR5211          | Hedera helix           | France         | KJ210538 KJ450943 KJ420875 KJ210559 KJ420828                |
| D. heterostemmatis  | SAUCC194.85     | Heterostemma grandiflorum | China         | MT822613 MT855692 MT85581 MT859295 MT855810                 |
| D. kuboensis       | JZB320123       | Vitis vinifera         | China          | MK355809 MK500235 NA MK523570 MK500148                        |
| D. kadsuense       | CFCC 52586      | Kadsura longipedunculata | China         | MH121521 MH121439 MH121479 MH121563 MH121600                 |
| D. litchicola      | BRIP 54900      | Litchi chinensis       | Australia      | JX862533 NA NA JX862539 KF170925                           |
| D. licoriceae      | MFLUCC 17-0963  | Lonicera sp.           | Italy          | KY964190 KY964116 NA KY964146 KY964073                       |
| D. maisirevicii    | BRIP 57892a     | Helianthus annuus      | Australia      | KJ197277 NA NA KJ197239 KJ197257                           |
| D. miricinae       | BRIP 54736j     | Helianthus annuus      | Australia      | KJ197282 NA NA KJ197244 KJ197262                           |
| D. monica          | MFLUCC 16-0113  | Prunus persica         | China          | KU557563 KU557611 NA KU557631 KU557578                       |
| D. musigena        | CBS 129519      | Musa sp.               | Australia      | KC343143 KC343385 KC343627 KC343869 KC344111                 |
| D. neilliae        | CBS 144.27      | Spiraea sp.            | USA            | KC343144 KC343384 KC343628 KC343870 KC344112                 |
| D. nobilis         | CBS 113470      | Castanea sativa        | Korea          | KC343146 KC343388 KC343630 KC343872 KC344114                 |
| D. oraccinii       | CGMCC 3.17531   | Camellia sinesis       | China          | KP267863 NA KP293517 KP267937 KP293443                       |
| D. ovicola         | CGMCC 3.17093   | Citrus sp.             | China          | KF576265 KF576223 NA KF576240 KF576289                       |
| D. pandaniola      | MFLUCC 18-0006  | Pandanus sp.           | Thailand       | MG6460974 NA NA NA MG646930                                 |
| D. pataei          | BRIP 58487      | Persea americana       | Australia      | JX862532 NA NA JX862538 KF170924                           |
| D. passiflorica    | CBS 141329      | Passiflora foetida     | Malaysia       | KX228292 NA KX228367 NA KX228387                           |
| D. penetriteum     | CGMCC 3.17532   | Camellia sinesis       | China          | KF714505 NA KP714493 KP714517 KP714529                       |
| D. pereae          | CBS 151,73      | Persea gratissima      | Netherlands    | KC343173 KC343415 KC343657 KC343899 KC344141                 |
| D. pecilica        | MFLUCC 16-0105  | Prunus persica         | China          | KU557555 KU557605 NA KU557623 KU557579                       |
| D. pseudomangiferae| CBS 101339      | Mangifera indica       | Dominican Republic | KC343181 KC343423 KC343665 KC343907 KC344149              |
| D. pseudophoenixioi| CBS 462,69      | Phoenix dactylifera    | Spain          | KC343184 KC343426 KC343668 KC343910 KC344152                 |
| D. paulei          | CBS 338,89      | Hedera helix           | Yugoslavia     | KC343152 KC343394 KC343636 KC343878 KC344120                 |
| D. racemosae       | CBS 145770      | Eucaolia racemos        | South Africa   | MG600223 MG600219 MG600221 MG600225 MG600227               |
| D. schima           | CGMCC 3.17532   | Schima superba         | China          | MK432640 MK442962 MK442987 MK578116 MK578043                 |
| D. schini          | CBS 135181      | Schinus terebinthifolius | Brazil        | KC343191 KC343433 KC343675 KC343917 KC344159                |
| D. schoeni         | MFLU 15-1279    | Schoenus nigricans     | Italy          | KY964226 KY964139 NA KY964182 KY964109                       |
| D. saureri         | BRIP 66528      | Macadamia sp.          | South Africa   | MN708231 NA NA NA MN696540                                   |
## Results

### Phylogenetic analyses

The five-gene sequence dataset (ITS, cal, his3, tef1 and tub2) was analysed to infer the interspecific relationships within *Diaporthe*. The dataset consisted of 96 sequences including the outgroup taxon, *Diaporthella corylina* (CBS 121124). A total of 2520 characters including gaps (510 for ITS, 518 for cal, 533 for his3, 460 for tef1 and 499 for tub2) were included in the phylogenetic analysis. The best nucleotide substitution
model for ITS, *his3* and *tub2* was *TrN*+I+G, while HKY+I+G was selected for both *cal* and *tef1*. The topologies resulting from ML and BI analyses of the concatenated dataset were congruent (Fig. 1). According to the phylogenetic tree, two known species, *D. hubeiensis* and *D. sojae*, were part of *Diaporthe*. *Diaporthe camelliae-oleiferae* and *D. hunanensis* are new to science based on the distinct and well-supported molecular phylogenetic placement with their closest described relatives. Phylogenetically, *D. camelliae-oleiferae* clustered together with *D. pandanicola* and *D. viniferae*. *Diaporthe hunanensis* clustered together with *D. chrysalidocarpi* and other species, including *D. drenthii*, *D. searlei* and *D. spinosa*.

**Taxonomy**

*Diaporthe camelliae-oleiferae* Q. Yang, sp. nov.

MycoBank No: 840451

**Figure 2**

**Diagnosis.** Distinguished from the phylogenetically closely-related species, *D. pandanicola* and *D. viniferae* based on DNA sequence data.

**Etymology.** Named after the host species, *Camellia oleifera*.

**Description.** Asexual morph: *pycnidia* on PDA 500–660 μm in diam., superficial, scattered on PDA, dark brown to black, globose, solitary, or clustered in groups of 3–5 pycnidia. Pale yellow conidial drops exuding from ostioles. *Conidiophores* reduced to *conidiogenous cells*. *Conidiogenous cells* (7.5–)10–14(–15.5) × 1.5–2.3 μm (n = 30), aseptate, cylindrical, straight, densely aggregated, terminal, slightly tapered toward the apex. Alpha conidia 5–6.5(–7.5) × 1.9–2.3 μm (n = 30), aseptate, hyaline, ellipsoidal to fusiform, biguttulate. Beta conidia (26.5–)28.5–31(–33) × 0.8–1.2 μm (n = 30), hyaline, aseptate, filiform, sinuous at one end, eguttulate.

**Culture characters.** Culture incubated on PDA at 25 °C, originally flat with white fluffy aerial mycelium, becoming brown to black in the centre, with yellowish-cream conidial drops exuding from the ostioles after 20 days.

**Specimens examined.** China. Hunan Province: Zhuzhou City, on leaves of *Camellia oleifera*, 27°2’41"N, 113°19’17"E, 14 Aug. 2020, Q. Yang (holotype CSUFT027; ex-type living culture: HNZZ027; other living cultures: HNZZ030 and HNZZ032).

**Notes.** Three isolates representing *D. camelliae-oleiferae* cluster in a well-supported clade (ML/BI=100/1) and appear most closely related to *D. pandanicola* on *Pandanus* sp. and *D. viniferae* on *Vitis vinifera*. *Diaporthe camelliae-oleiferae* can be distinguished from *D. pandanicola* based on ITS and *tub2* loci (24/462 in ITS and 11/401 in *tub2*); from *D. viniferae* based on ITS, *cal*, *tef1* and *tub2* loci (13/453 in ITS, 42/448 in *cal*, 7/339 in *tef1* and 26/402 in *tub2*). Morphologically, *D. camelliae-oleiferae* differs from *D. viniferae* in having shorter alpha conidia (5–6.5 μm vs. 5–8.3 μm) (Manawasinghe et al. 2019); from *D. pandanicola* in having narrower alpha conidia (1.9–2.3 μm vs. 2.5–3.2 μm) (Huang et al. 2021).
Figure 1. Phylogram of Diaporthe resulting from a maximum likelihood analysis based on combined ITS, cal, his3, tef1 and tub2. Numbers above the branches indicate ML bootstraps (left, ML BS ≥ 50%) and Bayesian Posterior Probabilities (right, BPP ≥ 0.75). The tree is rooted with Diaporthella corylina. Isolates in current study are in blue. “-” indicates ML BS < 50% or BI PP < 0.75.
***Diaporthe hubeiensis*** Dissanayake, X.H. Li & K.D. Hyde

Figure 3

Manawasinghe, Dissanayake, Li, Liu, Wanasinghe, Xu, Zhao, Zhang, Zhou, Hyde, Brooks & Yan, Frontiers in Microbiology 10(no. 1936): 20 (2019)

**Description.** Asexual morph: *pycnidia* on PDA in culture, 700–885 μm in diam., superficial, scattered, dark brown to black, globose or subglobose. *Conidiophores* reduced to conidiogenous cells. *Conidiogenous cells* (6.5–)7–10(–11.5) × 2–3.5 μm (n = 30), aseptate, cylindrical, phialidic, straight or slightly curved. *Alpha conidia* 5.8–8(–8.5) × 2.5–3.2 μm (n = 30), aseptate, hyaline, ellipsoidal to cylindrical, biguttulate, blunt at both ends. *Beta conidia* not observed.
Taxonomy of *Diaporthe* on *Camellia oleifera*

Culture characters. Culture incubated on PDA at 25 °C, originally flat with white felted aerial mycelium, becoming dark brown mycelium due to pigment formation, conidiomata irregularly distributed over agar surface after 20 days.

**Figure 2.** *Diaporthe camelliae-oleiferae* (HNZZ027) A Culture on PDA B conidiomata C conidiogenous cells D–F alpha and beta conidia. Scale bars: 200 μm (B); 10 μm (C–D); 20 μm (E, F).
Specimens examined. China. Hunan Province: Zhuzhou City, on leaves of *Camellia oleifera*, 27°2’35"N, 113°19’20"E, 14 Aug. 2020, Q. Yang (CSUFT019; living cultures: HNZZ019 and HNZZ009).

Notes. *Diaporthe hubeiensis* was originally described as pathogen of grapevines in Hubei Province, China (Manawasinghe et al. 2019). In the present study, two isolates (HNZZ019 and HNZZ009) are closely related to *D. hubeiensis* in the combined phylogenetic tree (Fig. 1). The differences of nucleotides in the concatenated alignment (1/460 in ITS, 3/458 in *cal*, 1/320 in *his3* and 3/433 in *tub2*) are minor. Morphological comparison indicated that the isolates were similar to *D. hubeiensis* by the size of alpha conidia. We therefore identify the isolates as belonging to *D. hubeiensis*.

*Diaporthe hunanensis* Q. Yang, sp. nov.
MycoBank No: 840452
Figure 4

Diagnosis. Distinguished from its phylogenetically closely-related species, *D. chrysalidocarpi*, *D. drenthii*, *D. searlei* and *D. spinosa* based on DNA sequence data.
Taxonomy of Diaporthe on Camellia oleifera

Etymology. In reference to the Hunan province, from where the fungus was first collected.

Description. Asexual morph: pycnidia on PDA 180–300 μm in diam., superficial, scattered, black, globose, solitary in most. Conidiophores reduced to conidiogenous cells. Conidiogenous cells (8–)9–15(–16.5) × 1.7–2.1 μm (n = 30), aseptate, cylindrical, phialidic, straight or slightly curved. Alpha conidia 6.5–7.5(–8.5) × 2.4–2.9 μm (n = 30), aseptate, hyaline, ellipsoidal, biguttulate, both ends obtuse. Beta conidia not observed.

Culture characters. Culture incubated on PDA at 25 °C, originally flat with white fluffy aerial mycelium, becoming pale brown with age, with visible solitary conidiomata at maturity after 18 days.

Specimens examined. China. Hunan Province: Zhuzhou City, on leaves of *Camellia oleifera*, 27°2’41”N, 113°19’17”E, 14 Aug. 2020, Q. Yang (holotype CSUFT 023; ex-type living culture: HNZZ023; living cultures: HNZZ025 and HNZZ033).

Figure 4. *Diaporthe hunanensis* (HNZZ023) A Culture on PDA B conidiomata C conidiogenous cells D alpha conidia. Scale bars: 500 μm (B); 10 μm (C–D).
**Notes.** Three isolates representing *D. hunanensis* cluster in a well-supported clade (ML/BI=100/1) and appear most closely related to *D. chrysalidocarpi* on *Chrysalidocarpus lutescens*, *D. drenthii* and *D. searlei* on *Macadamia* sp., and *D. spinosa* on *P. pyrifolia* cv. Cuiguan. *Diaporthe hunanensis* can be distinguished from *D. chrysalidocarpi* based on ITS, *cal*, *his3* and *tub2* loci (7/457 in ITS, 8/455 in *his3* and 5/401 in *tub2*); from *D. drenthii* based on ITS, *tef1* and *tub2* loci (9/457 in ITS, 13/328 in *tef1* and 23/401 in *tub2*); from *D. searlei* based on ITS and *tub2* loci (10/457 in ITS and 12/401 in *tub2*); from *D. spinosa* based on ITS, *cal*, *his3*, *tef1* and *tub2* loci (8/458 in ITS, 31/448 in *cal*, 5/455 in *his3*, 8/328 in *tef1* and 19/401 in *tub2*). Morphologically, *D. chrysalidocarpi* produces only beta conidia, while *D. hunanensis* produces alpha conidia (Huang et al. 2021); *D. hunanensis* differs from *D. drenthii* and *D. searlei* in wider alpha conidia (2.4–2.9 μm in *D. hunanensis* vs. 1.5–2.5 μm in *D. drenthii* vs. 1.5–2 μm in *D. searlei*) (Wrona et al. 2020); from *D. spinosa* in shorter alpha conidia (6.5–7.5 × 2.4–2.9 μm vs. 5.5–8 × 2–3.5 μm) (Guo et al. 2020). Therefore, we establish this fungus as a novel species.

**Diaporthe sojae** Lehman, *Ann. Mo. bot. Gdn* 10: 128 (1923)

**Figure 5**

**Description.** Sexual morph: *perithecia* on pine needles in culture, black, globose, 250–500 μm in diam., densely clustered in groups, deeply immersed with elongated, tapering perithecial necks protruding through substrata, 525–800 μm. *Asci* unitunicate, 8-spored, sessile, elongate to clavate, (35–)37–42 (–44.5) × (8–)10–11.5 μm (n = 30). *Ascospores* hyaline, two-celled, often 4-guttulate, with larger guttules at centre and smaller one at ends, elongated to elliptical, slightly or not constricted at septum, (9–) 9.5–11.5 × 2.7–4 μm (n = 30). Asexual morph not observed.

**Culture characters.** Culture incubated on PNA at 25 °C, originally white, fluffy aerial mycelium, reverse yellowish pigmentation developing in centre, later becoming dark brown, with yellowish-cream drops exuding from the perithecia after 15 days.

**Specimens examined.** China. Hunan Province: Zhuzhou City, on leaves of *Camellia oleifera*, 27°2’41”N, 113°19’17”E, 14 Aug. 2020, Q. Yang (USUFT 022; living cultures: HNZZ022, HNZZ008 and HNZZ010).

**Notes.** *Diaporthe sojae* was first reported on pods and stems of soybean, and subsequently reported on a wide range of hosts (Dissanayake et al. 2015; Udayanga et al. 2015; Guo et al. 2020). It was also reported on some fruit trees in China, such as *Vitis* spp. (Dissanayake et al. 2015) and *Citrus* spp. (Huang et al. 2015). In the present, three isolates (HNZZ008, HNZZ010 and HNZZ022) are closely related to *D. sojae* in the combined phylogenetic tree (Fig. 1). The differences of nucleotides in the concatenated alignment (1/460 in ITS, 3/458 in *cal*, 1/320 in *his3* and 3/433 in *tub2*) are minor. Compared with the description of the ex-type isolate FAU635, the isolate has wider asci (10–11.5 μm vs. 7–9 μm) (Udayanga et al. 2015). We therefore identify the isolates as belonging to *D. sojae*. 
Discussion

In this study, an important oil-tea tree species, *Camellia oleifera* was investigated and *Camellia* leaf disease was found as a common disease in plantations in Hunan Province. Identification of our collections was conducted, based on isolates from symptomatic leaves of *C. oleifera* using five combined loci (ITS, *cal, his3, tef1* and *tub2*), as well as morphological characters. It includes *D. hubeiensis*, *D. sojae*, as well as two new species named *D. camelliae-oleiferae* and *D. hunanensis*.

The expanding cultivation of *C. oleifera* over the last several decades has attracted increasing attention from plant pathologists to infectious diseases on this crop. Therein, diseases caused by *Diaporthe* species have becoming the emerging Camellia leaf diseases in southern China (Gao et al. 2016; Guarnaccia et al. 2018; Yang et al. 2018; Zhou and Hou 2019). Understanding the diversity of *Diaporthe* species and the genetic variation within pathogen populations could help in developing sustainable disease management strategies.

According to the USDA Fungal–host interaction database, there are two records of *Diaporthe* species associated with *C. oleifera* (https://nt.ars-grin.gov/fungaldatabases/fungushost/fungushost.cfm) (accessed 9 September 2021). These records are related
to the following two *Diaporthe* species: *D. eres* and *D. huangshanensis* (Zhou and Hou 2019). *Diaporthe eres*, the type species of the genus, was described by Nitschke (1870) on *Ulmus* sp. collected in Germany, which has a widespread distribution and a broad host range as pathogens, endophytes or saprobes (Udayanga et al. 2014b). *Diaporthe eres* differs from *D. camelliae-oleiferae* and *D. hunanensis* in having wider alpha conidia (3–4 μm in *D. eres* vs. 1.9–2.3 μm in *D. camelliae-oleiferae* vs. 2.4–2.9 μm in *D. hu- nanensis*) (Gomes et al. 2003); *D. huangshanensis* differs from *D. camelliae-oleiferae* in having larger alpha conidia (5.7–8.4 × 2.7–4.5 μm vs. 5–6.5 × 1.9–2.3 μm); from *D. hunanensis* in having wider alpha conidia (2.7–4.5 μm vs. 2.4–2.9 μm) and longer conidiophores (12.1–23.5 μm vs. 9–15 μm) (Zhou and Hou 2019).

As the species concept of *Diaporthe* has been improved a lot by using molecular data (Huang et al. 2015; Gao et al. 2016, 2017; Guarnaccia and Crous 2017; Guarnaccia et al. 2018; Yang et al. 2018, 2020, 2021; Manawasinghe et al. 2019; Guo et al. 2020), many new species have been discovered and reported in recent years. In this study, the *Diaporthe* isolates from *C. oleifera* were identified based on sequence analysis and morphological characteristics. Future studies should focus on pathogenicity, epidemiology and fungicide sensitivity of the important plant fungal pathogen to develop effective management of *C. oleifera* disease and on the pathogenic molecular mechanism.

**Acknowledgements**

This study is financed by the Research Foundation of Education Bureau of Hunan Province, China (Project No.: 19B608) and the introduction of talent research start-up fund project of CSUFT (Project No.: 2019YJ025).

**References**

Carbone I, Kohn LM (1999) A method for designing primer sets for speciation studies in filamentous ascomycetes. Mycologia 3: 553–556. https://doi.org/10.2307/3761358

Crous PW, Gams W, Stalpers JA, Robert V, Stegehuis G (2004a) MycoBank: an online initiative to launch mycology into the 21st century. Studies in Mycology 50: 19–22.

Crous PW, Groenewald JZ, Risède JM, Simoneau P, Hywel-Jones NL (2004b) *Calonectria* species and their *Cylindrocladium* anamorphs: species with sphaeropedunculate vesicles. Studies in Mycology 50: 415–430.

Darriba D, Taboada GL, Doallo R, Posada D (2012) jModelTest 2: more models, new heuristics and parallel computing. Nature Methods 9: 772–772. https://doi.org/10.1038/nmeth.2109

Desjardins P, Hansen JB, Allen M (2009) Microvolume protein concentration determination using the NanoDrop 2000c spectrophotometer. Journal of Visualized Experiments: JoVE 33: 1–3. https://doi.org/10.3791/1610

Diogo E, Santos JM, Phillips AJ (2010) Phylogeny, morphology and pathogenicity of *Diaporthe* and *Phomopsis* species on almond in Portugal. Fungal Diversity 44: 107–115. https://doi.org/10.1007/s13225-010-0057-x
Doyle JJ, Doyle JL (1990) Isolation of plant DNA from fresh tissue. Focus 12: 13–15. https://doi.org/10.2307/2419362

Erincik O, Madden L, Ferree D, Ellis M (2001) Effect of growth stage on susceptibility of grape berry and rachis tissues to infection by *Phomopsis viticola*. Plant Disease 85: 517–520. https://doi.org/10.1094/PDIS.2001.85.5.517

Gao YH, Liu F, Cai L (2016) Unravelling *Diaporthe* species associated with Camellia. Systematics and Biodiversity 14: 102–117. https://doi.org/10.1080/14772000.2015.1101027

Gao YH, Liu F, Duan W, Crous PW, Cai L (2017) *Diaporthe* is paraphyletic. IMA Fungus 8: 153–187. https://doi.org/10.5598/imafungus.2017.08.01.11

Gomes RR, Glienke C, Videira SIR, Lombard L, Groenewald JZ, Crous PW (2013) *Diaporthe*: a genus of endophytic, saprobic and plant pathogenic fungi. Persoonia 31: 1–41. https://doi.org/10.3767/003158513X666844

Guarnaccia V, Crous PW (2017) Emerging citrus diseases in Europe caused by species of *Diaporthe*. IMA Fungus 8: 317–334. https://doi.org/10.5598/imafungus.2017.08.02.07

Guarnaccia V, Crous PW (2018) Species of *Diaporthe* on *Camellia* and *Citrus* in the Azores Islands. Phytopathologia Mediterranea 57: 307–319.

Guarnaccia V, Groenewald JZ, Woodhall J, Armengol J, Cinelli T, Eichmeier A, Ezra D, Fontaine F, Gramaje D, Gutierrez-Aguirregabiria A, Kaliterna J, Kiss L, Larignon P, Luque J, Mugnai L, Naor V, Raposo R, Sándor E, Váczy KZ, Crous PW (2018) *Diaporthe* diversity and pathogenicity revealed from a broad survey of grapevine diseases in Europe. Persoonia 40: 135–153. https://doi.org/10.3767/persoonia.2018.40.06

Guindon S, Dufayard JF, Lefort V, Anisimova M, Hordijk W, Gascuel O (2010) New algorithms and methods to estimate maximum-likelihood phylogenies: assessing the performance of PhyML 3.0. Systematic Biology 59: 307–321. https://doi.org/10.1093/sysbio/syq010

Guo YS, Crous PW, Bai Q, Fu M, Yang MM, Wang XH, Du YM, Hong N, Xu WX, Wang GP (2020) High diversity of *Diaporthe* species associated with pear shoot canker in China. Persoonia 45: 132–162. https://doi.org/10.3767/persoonia.2020.45.05

Hall T (1999) BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucleic Acids Symposium Series 41: 95–98.

Hewitt W, Pearson R (1988) Phomopsis cane and leaf spot. Compendium of grape diseases: 17–18. APS Press, St Paul, Minnesota.

Huang F, Udayanga D, Wang X, Hou X, Mei X, Fu Y, Hyde KD, Li HY (2015) Endophytic *Diaporthe* associated with *Citrus*: A phylogenetic reassessment with seven new species from China. Fungal Biology 119: 331–347. https://doi.org/10.1016/j.funbio.2015.02.006

Huang ST, Xia JW, Zhang XG, Sun WX (2021) Morphological and phylogenetic analyses reveal three new species of *Diaporthe* from Yunnan, China. MycoKeys 78: 49–77. https://doi.org/10.3897/mycokeys.78.60878

Katoh K, Toh H (2010) Parallelization of the MAFFT multiple sequence alignment program. Bioinformatics 26: 1899–1900. https://doi.org/10.1093/bioinformatics/btq224

Li H, Zhu XD, Liu JA, Xu JP (2014) Population genetic structure of *Colletotrichum gloeosporioides* causing anthracnose of *Camellia oleifera* in China. Acta Phytopathologica Sinica 44: 620–628.

Lombard L, Van Leeuwen GCM, Guarnaccia V, Polizzi G, Van Rijswick PC, Karin Rosendahl KC, Gabler J, Crous PW (2014) *Diaporthe* species associated with *Vaccinium*, with specific reference to Europe. Phytopathologia Mediterranea 53: 287–299.
Manawasinghe IS, Dissanayake AJ, Li X, Liu M, Wanasinghe DN, Xu J, Zhao W, Zhang W, Zhou Y, Hyde KD, Brooks S, Yan J (2019) High genetic diversity and species complexity of Diaporthe associated with grapevine dieback in China. Frontiers in Microbiology 10: e1936. https://doi.org/10.3389/fmicb.2019.01936

Mondal SN, Vincent A, Reis RF, Timmer LW (2007) Saprophytic colonization of citrus twigs by Diaporthe citri and factors affecting pycnidial production and conidial survival. Plant Disease 91: 387–392. https://doi.org/10.1094/PDIS-91-4-0387

Mostert L, Crous PW, Kang JC, Phillips AJ (2001) Species of Phomopsis and a Libertella sp. occurring on grapevines with specific reference to South Africa: morphological, cultural, molecular and pathological characterization. Mycologia 93: 146–167. https://doi.org/10.2307/3761612

O’Donnell K, Cigelnik E (1997) Two divergent intragenomic rDNA ITS2 types within a monophyletic lineage of the fungus Fusarium are nonorthologous. Molecular Phylogenetics and Evolution 7: 103–116. https://doi.org/10.1006/mpev.1996.0376

Rambaut A, Drummond A (2010) FigTree v.1.3.1. Institute of Evolutionary Biology, University of Edinburgh, Edinburgh.

Rayner RW (1970) A mycological colour chart. Commonwealth Mycological Institute, Kew.

Rehner SA, Uecker FA (1994) Nuclear ribosomal internal transcribed spacer phylogeny and host diversity in the coelomycete Phomopsis. Canadian Journal of Botany 72: 1666–1674. https://doi.org/10.1139/b94-204

Ronquist F, Huelsenbeck JP (2003) MrBayes 3: Bayesian phylogenetic inference under mixed models. Bioinformatics 19: 1572–1574. https://doi.org/10.1093/bioinformatics/btg180

Santos JM, Phillips AJL (2009) Resolving the complex of Diaporthe (Phomopsis) species occurring on Foeniculum vulgare in Portugal. Fungal Diversity 34: 111–125.

Santos JM, Vrandečić K, Ćosić J, Duvnjak T, Phillips AJL (2011) Resolving the Diaporthe species occurring on soybean in Croatia. Persoonia 27: 9–19. https://doi.org/10.3767/003158511X603719

Smith H, Wingfeld MJ, Coutinho TA, Crous PW (1996) Sphaeropsis sapinea and Botryosphaeria dothidea endophytic in Pinus spp. and Eucalyptus spp. in South Africa. South African Journal of Botany 62: 86–88. https://doi.org/10.1016/S0254-6299(15)30596-2

Tan XF, Guan TQ, Yuan J (2018) Report on upgrading output value of oil-tea industry to 100 billion RMB in Hunan. Non-wood Forest Research 36: 1–4.

Tibpromma S, Hyde KD, Bhat JD, Mortimer PE, Xu J, Promputtha I, Doilom M, Yang JB, Tang AMC, Karunaratha SC (2018) Identification of endophytic fungi from leaves of Pandanaceae based on their morphotypes and DNA sequence data from southern Thailand. MycoKeys 33: 25–67. https://doi.org/10.3897/mycokeys.33.23670

Udayanga D, Castlebury LA, Rossman AY, Chukeatirote E, Hyde KD (2014b) Insights into the genus Diaporthe: phylogenetic species delimitation in the D. eres species complex. Fungal Diversity 67: 203–229. https://doi.org/10.1007/s13225-014-0297-2

Udayanga D, Castlebury LA, Rossman AY, Chukeatirote E, Hyde KD (2015) The Diaporthe sojae species complex: Phylogenetic re-assessment of pathogens associated with soybean, cucurbits and other field crops. Fungal Biology 119: 383–407. https://doi.org/10.1016/j.funbio.2014.10.009
Udayanga D, Castlebury LA, Rossman AY, Hyde KD (2014a) Species limits in *Diaporthe*: molecular re-assessment of *D. citri*, *D. cytopsporella*, *D. fueniculina* and *D. rudis*. Persoonia 32: 83–101. https://doi.org/10.3767/003158514X679984

Udayanga D, Liu X, McKenzie EH, Chukenatirote E, Bahkali AH, Hyde KD (2011) The genus *Phomopsis*: biology, applications, species concepts and names of common phytopathogens. Fungal Diversity 50: 189–225. https://doi.org/10.1007/s13225-011-0126-9

Wang WJ, Chen CG, Cheng J (2007) The medicinal active role of tea oil in health care. Food and Nutrition in China 9: 48–51.

Wang Y, Chen JY, Xu XW, Cheng JY, Zheng L, Huang JB, Li DW (2020) Identification and characterization of *Colletotrichum* species associated with anthracnose disease of *Camellia oleifera* in China. Plant Disease 104: 474–482. https://doi.org/10.1094/PDIS-11-18-1955-RE

White TJ, Bruns T, Lee S, Taylor J (1990) Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. PCR Protocols: A Guide to Methods and Applications 18: 315–322. https://doi.org/10.1016/B978-0-12-372180-8.50042-1

Wrona CJ, Mohankumar V, Schoeman MH, Tan YP, Shivs GR, Jeff-Ego OS, Akinsanmi OA (2020) Phomopsis husk rot of macadamia in Australia and South Africa caused by novel *Diaporthe* species. Plant Pathology 69: 911–921. https://doi.org/10.1111/ppa.13170

Yang Q, Fan XL, Guarnaccia V, Tian CM (2018) High diversity of *Diaporthe* species associated with dieback diseases in China, with twelve new species described. MycoKeys 39: 97–149. https://doi.org/10.3897/mycokeys.39.26914

Yang Q, Jiang N, Tian CM (2020) Three new *Diaporthe* species from Shaanxi Province, China. MycoKeys 67: 1–18. https://doi.org/10.3897/mycokeys.67.49483

Yang Q, Jiang N, Tian CM (2021) New species and records of *Diaporthe* from Jiangxi Province, China. MycoKeys 77: 41–64. https://doi.org/10.3897/mycokeys.77.59999

Zhou H, Hou CL (2019) Three new species of *Diaporthe* from China based on morphological characters and DNA sequence data analyses. Phytotaxa 422: 157–174. https://doi.org/10.11646/phytotaxa.422.2.3

Zhuang RL (2008) *Camellia oleifera*. 2nd Edn. China Forestry Press, Beijing.