



Diaporthe nobilis, a new record on *Camellia sinensis* in Guizhou Province, China

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Abstract

One *Diaporthe* strain was isolated from diseased leaves of *Camellia sinensis* in Guizhou Province. It is characterized by the production of two types conidia (α , β -conidia). Morphologically the species is very similar to *Phomopsis laurella* (syn. *Diaporthe nobilis*) in producing cylindrical or ellipsoidal α -conidia with two oil drops. Phylogenetic analysis of combined ITS, β -tubulin and *tefl* sequence data shows that these strains are placed in *D. eres* species complex. Critical examination of the phenotypic characters indicated that this strain represents *Diaporthe nobilis*, a new record in *Camellia sinensis*.

Key words – coelomycetes – phylogeny – taxonomy – tea

Introduction

Tea (*Camellia sinensis*) is most popular beverages in the world. More than three million hectares of the world's arable land is covered by tea plantations (Li et al. 2011). In Guizhou Province, the area of tea plantations has achieved 0.29 million ha in 2011, which centralizes in Zunyi and Qiannan cities. During 2011–2012, we investigated the fungal diversity of *C. sinensis* in Guizhou provinces, *Colletotrichum* spp., *Phoma* spp., *Alternaria* spp., *Diaporthe* spp., *Pestalotiopsis* spp. and *Exobasidium* spp. were discovered in the leaves of *C. sinensis*. Among them, they also include important or new tea pathogens.

Diaporthe Nitschke is the sexual state of *Phomopsis* with more than 800 names included in *Index fungorum* mostly independent of any anamorphic affinities (Udayanga et al. 2011). A single name must now be applied for the different morphs of *Phomopsis* / *Diaporthe* (Shenoy et al. 2007, Hawksworth 2011, Hyde et al. 2011, 2014). Santos & Phillips (2009) proposed to give preference

to the older *Diaporthe* (1870) names, rather than the younger anamorphic genus, *Phomopsis* (1905) and this has been followed by subsequent authors (Udayanga et al. 2011, 2012, 2014, Liu et al. 2015, Hyde et al. 2016). The coelomycetes strain only producing anamorph was discovered from *C. sinensis* leaves in Guizhou province, whose morphological characters of conidia and conidiophores are very similar to *D. fukushii*. For phylogeny, Udayanga et al. (2011) proposed “the rDNA ITS phylogenetic tree generated here is based on the phylogenetic backbone tree as a rough and quick identification guide for fresh isolates of *Diaporthe* species”. Meanwhile, the combined analysis of more than one gene provides higher resolution than a single gene (Udayanga et al. 2012).

Diaporthe eres Nitschke is the type species of *Diaporthe*, which was described by Nitschke (1870). Wehmeyer (1933) listed a number of synonyms under this species with approximately 70 plant host associations based on only morphological observations. Following individual and combined analyses of multi-gene DNA sequences, Udayanga et al. (2014) accept only nine *Diaporthe* species belonging to *D. eres* species complex and epitypified six species, which provide powerful convenience to identify species in this complex. Fortunately, Gao et al. (2016) investigated *Diaporthe* species with *Camellia* in China, and in this paper four novel species and three known species were reported by morphological comparison and multi-gene analyses, which provided abundant reference information for our study. This paper based on morphological observation and three gene regions (ITS, β -tubulin and *tef1*) analysis indicated our coelomycetous strain differed to other *Diaporthe* species in *D. eres* species complex, but very close to *Diaporthe nobilis*. Finally, we determined our strain should be *D. nobillis*, which is also the first record on *Camellia sinensis* in China.

Materials & Methods

Isolation and morphological studies

Infected tea leaves were collected from Huishui, Guizhou Province. Single-spore isolation was used to obtain pure culture of pathogens (Choi et al. 1999). For the growth study the culture was grown on Potato Dextrose Agar (PDA), Corn Meal Agar (CMA) and Czapek Solution Agar (CZ) at 25°C for 8 d. Plates were inoculated with 5 mm plugs taken from an actively growing colony on CM. The plugs were placed at the edge of a 90 cm petri dish. A colony diameter of 7 cm indicates that the colony has reached the edge of the petri dish. Cultural characteristics and morphology were determined on tea leaf agar (TLA) [1 % water agar (10 g/L) with autoclaved carnation leaves placed onto the medium] and incubated for 7 days at 25°C under continuous near-UV light, to promote sporulation. A water solution of 60% (v/v) lactic acid without a color dye was used as the mounting medium. Slides were examined under oil immersion with a Nikon 80i microscope (Nikon Corporation, Japan) at 1000 \times magnification. The specimens and living culture were deposited in the Herbarium of the Department of Plant Pathology, Agricultural College, Guizhou University (HGUP).

DNA extraction, PCR and sequencing

Total genomic DNA was extracted from the pure culture using modified CTAB protocol described in Guo et al. (2000). Primer pairs ITS1/ITS4 (White et al. 1990), Bt2a/Bt2b (Glass & Donaldson 1995), and EF1-728F/EF1-986R (Carbone et al. 1999) were used to amplify partial

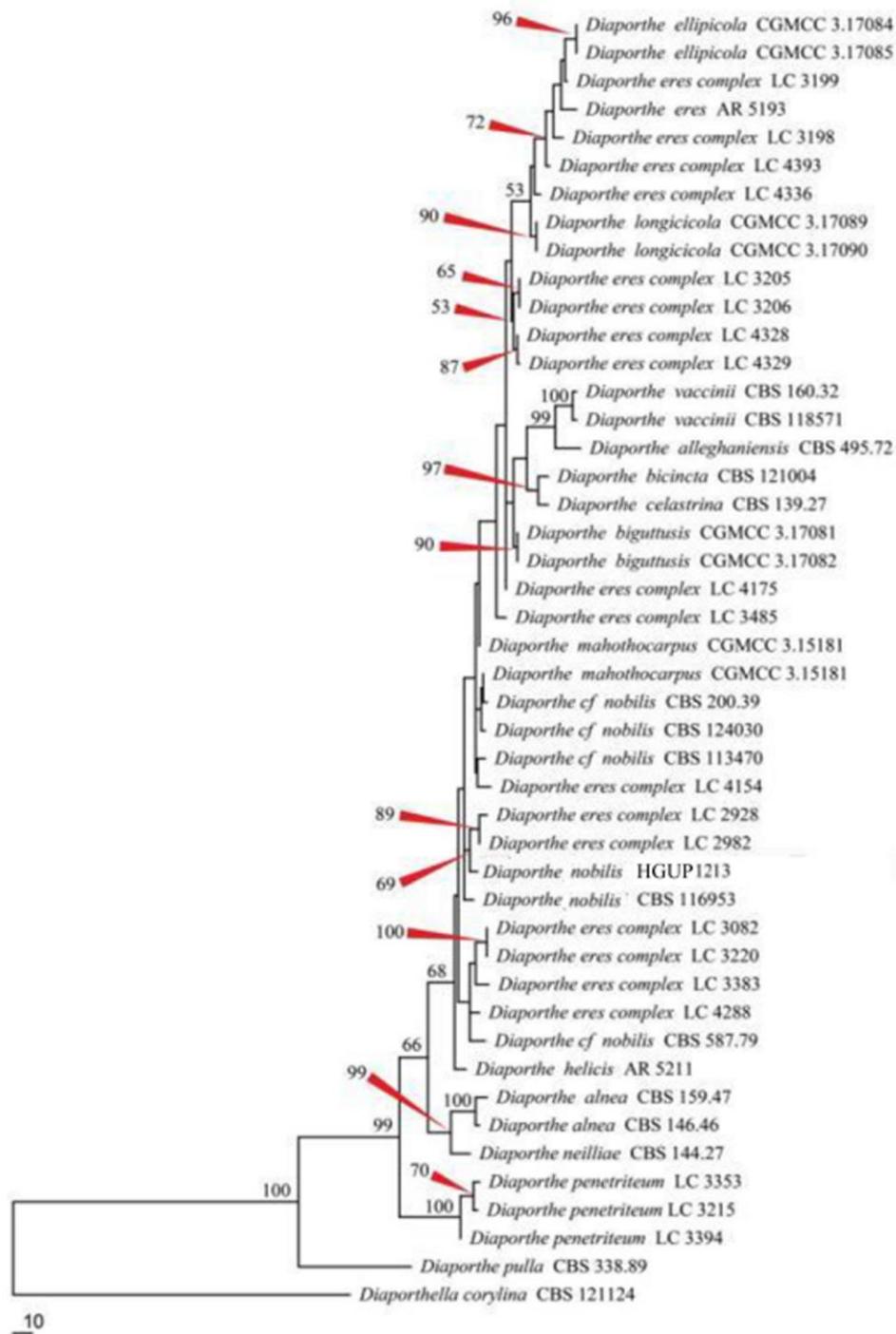


Fig. 1 – Topology showing the most parsimonious tree, inferred from combined ITS, beta-tubulin and *tef1* gene regions. Bootstrap values smaller than 50% are not shown. The tree was rooted with *Diaporthe citri* (AR3405).

internal transcribed spacer (ITS), beta-tubulin and *tef1* gene regions. Sequencing was performed with an ABI PRISM 3730 DNA autosequencer using either dRhodamine terminator or Big Dye Terminator (Applied Biosystems Inc., Foster 19 City, California). The sequences of both strands of each fragment determined for sequence confirmation. The DNA sequences of HGUP1213 in ITS, beta-tubulin and *tef1* regions generated in this study were submitted to GenBank (ITS: KC252992, beta-tubulin: KC252993 and *tef1*: KC252991).

Sequence alignment and phylogenetic analyses

Sequences of our strain, along with references obtained from Udayanga et al. (2014), were aligned by Clustal X (Thompson et al. 1997). Alignments were optimized manually in Bioedit (Hall 1999). Phylogenetic and molecular evolutionary analyses were conducted using PAUP* (Swofford 2002) combined with ITS, β -tubulin and *tefl* dataset. Ambiguously aligned regions were excluded from all analyses. Unweighted parsimony (UP) analysis was performed. Trees were inferred using the heuristic search option with TBR branch swapping and 1000 random sequence additions. Maxtrees were 5000, branches of zero length were collapsed and all multiple parsimonious trees were saved. Clade stability was assessed in a bootstrap analysis with 1000 replicates, each with ten replicates of random stepwise addition of taxa (Felsenstein 1985).

Results

Phylogenetic analysis

In order to accurately identify our fungal isolate, ITS, beta-tubulin and *tefl* sequences of 44 strains of *Diaporthe* species (mainly ex-type or epitype cultures) and important strains associated with *Camellia* (Gao et al. 2016) were aligned with our sequence data, for phylogenetic analysis. Sequences were obtained a 1324 aligned characters file. Among them, 180 characters were parsimony-informative. *Diaporthella corylina* (CBS 121124) was selected as outgroup following Gao et al. (2016).

The most parsimonious tree is used to represent the topology of the *Diaporthe* strains (Fig. 1), which indicated that all 45 isolates grouped as a single large clade with high bootstrap support (100%). Tree length (TL) is 805 steps, consistency index (CI) is 0.73, retention index (RI) is 0.78 rescaled consistency index (RC) is 0.57. In Fig. 1, HGUP1213 kept a closer relationship with CBS 116953 as *D. nobilis*, LC2982 and LC2928 as *Diaporthe eres* complex supported by a moderate bootstrap support (69%), but relatively distant to other members of the *D. eres* species complex.

Morphological description

Diaporthe nobilis Sacc. & Speg., *Michelia* 1 (4): 386 (1878)

Figs. 2–3

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Habit leaves of *Camellia sinensis*. *Substratum* covered by a thin stromata of 3–4 layers of dark brown, thick-walled cells, epidermis evident below the stromata. Sexual morph: Undetermined. Asexual morph: *Conidiomata* are pycnidial, subcuticular, scattered to confluent, uniloculate, dark brown to black, uniloculate, broadly spherical to flattened, 650–700 μm high and 400–500 μm wide (Fig. 2f). *Conidiomatal wall* is dark-brown to black with *textura angularis*; ostiole is single, circular (Fig. 2g). *Conidiophores* thin walled, brown, vertically aligned, multicellular, cells 2–6 μm wide, elongate, tightly packed, *textura prismatica*, lining pycnidial base and sides up to apex (Fig. 2h–j). *Conidiogenous cells* formed at the apex of the conidiophores, 8–18 \times 0.5–1.2 μm , obclavate to cylindrical, straight or curved, developing from the apex of columnar cells (Fig. 2h–j). *Alpha conidia* 7–9 \times 3–5 μm (av. 8 \times 3.8 μm), no septate, straight or curved, cylindrical or ellipsoidal, obtuse at the both ends, hyaline, generally biguttulate. *Beta conidia* 20–30 \times 0.3–0.8 μm (av. 24 \times 0.5 μm), filiform, blunt at one end, pointed and usually curved at the other, hyaline, one-celled (Fig. 2 k–n). *Colony morphology*: On CZ 3 cm diameter, dark brown to greyish orange at margin, surface mycelium appressed-felty, margin feathery, reverse dark brown to greyish orange at margin,

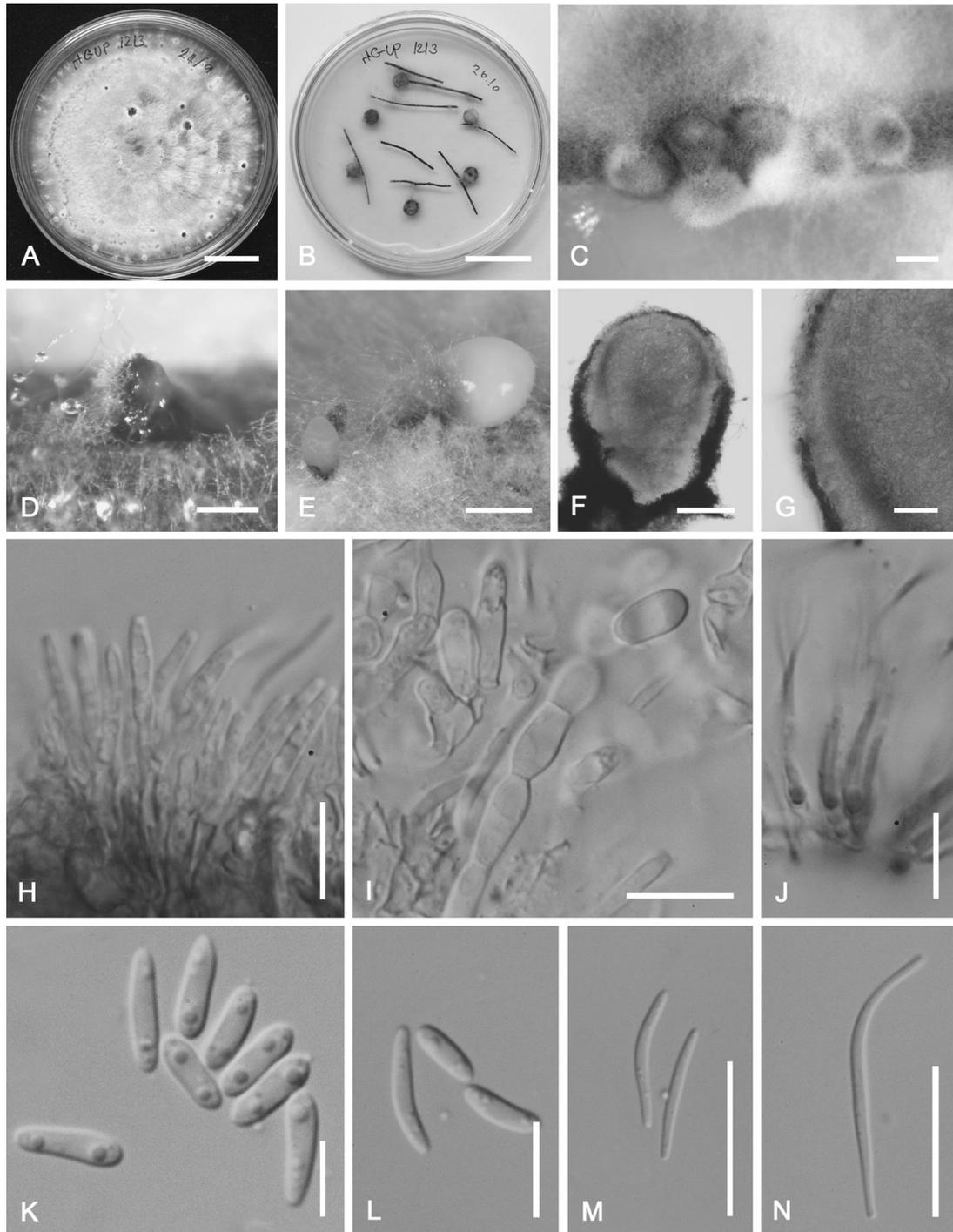


Fig. 2 – *Diaporthe nobilis* (HGUP 1213). **a.** Colony on PDA. **b.** Asexual morph on PDA with pine needles. **c.** Conidiomata. **d–e.** Pycnidia on PDA. **f.** Section of conidioma. **g.** Peridial wall. **h–j.** Conidiophores. **k–n.** Conidia. Scale bars: a, b = 2 cm., c = 500 μ m, d = 200 μ m, e = 500 μ m, f = 200 μ m, g=50 μ m, h–j = 10 μ m, k = 5 μ m, l–n = 10 μ m.

without zones; on CMA 3 cm diameter, translucent, no surface mycelium, margin lobate, without zones; on PDA 6.3 cm after 7 days, white to olive grey surface mycelium cottony, radiate growth pattern, reverse black to dark brown, without zones (Fig. 3).

Material examined – CHINA, Guizhou Province, Huishui, on leaf spots on living leaves of *Camellia sinensis*, July 2011, P. Tan (GT11-4), living culture HGUP 1213.

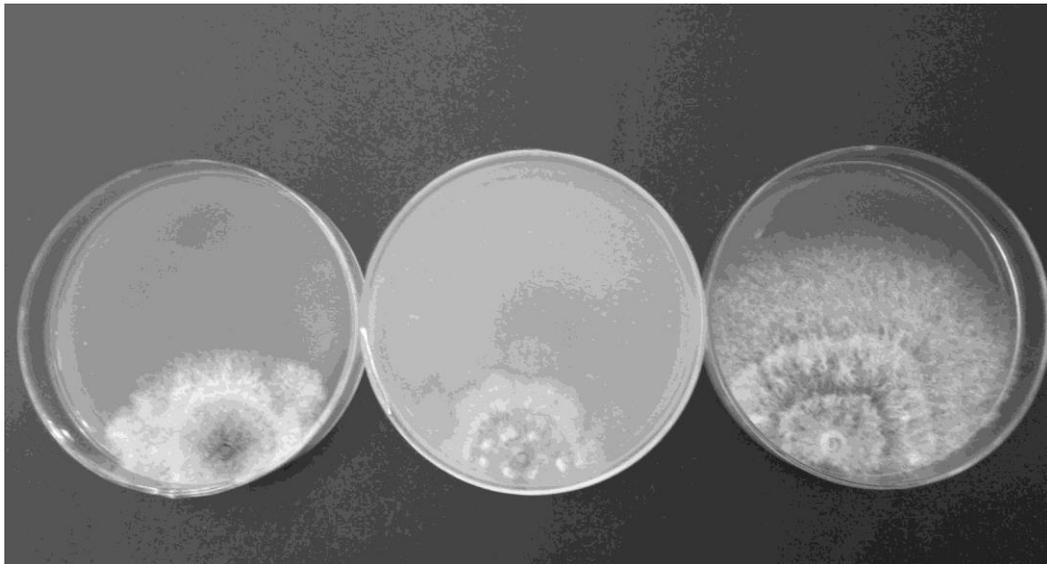


Fig. 3 – *Diaporthe nobilis* (HGUP1213) on CZ, CM and PDA media (from left to right).

Discussion

Placement of our *Diaporthe* species is suggested by a number of morphological observations as well as the molecular data analyses. It is placed in *D. eres* species complex by the phylogenetic tree based on three gene loci (ITS, beta-tubulin and *tef1*), and then keeps a closer relationship with *D. nobilis* (CBS 116953) and two *Diaporthe eres* complex strains (LC2928 and LC2982) in Fig. 1. According to Udayanga et al. (2014), *P. fukushii*, *D. castaneae-mollissimae* and *D. cotoneastri* were synonyms of *D. eres* mainly based on multi-gene analyses. However, in this paper, we still looked them as different species for full morphological comparison. Beta conidia of our strain (0.3–0.8 μm) were obviously narrower than those of *D. eres* (1–1.5 μm). Between HGUP1213 and *D. heliciis*, we could easily observe the beta conidia from HGUP1213, but *D. heliciis* was not. *Diaporthe cotoneastri* produced fusiform α -conidia, but *D. theae-guizhouense* produced smaller cylindrical or ellipsoidal α -conidia (Abreo et al. 2012). Beta conidia of *D. castaneae-mollissimae* are straight or somewhat curved, but those of *D. theae-guizhouense* are hamate. *D. castaneae-mollissimae* was also reported by Chinese mycologists (Jiang & Ma 2010) as *P. castaneae-mollissimae* and then combined by Udayanga et al. (2012) into *Diaporthe* genus. For *D. vaccinii*, its α -conidia were bigger than those of our strain (Farr et al. 2002). Alpha conidia size of our strain were nearly identical to *D. nobilis* (7.5–10 \times 2.5–4 μm). *Phomopsis theae* Petch could cause tea stem canker, which has been reported in Zhejiang and Anhui provinces of China (Jiang et al. 2006). In the beginning, we hypothesized our strain might belong to this tea pathogen. However, their ITS sequences were obviously different by NCBI-BLAST, but could not discover *P. theae*'s beta-tubulin and *tef1* sequences. The phylogenetic results have determined that our strain was different to *Diaporthe* strains obtained from *Camellia* in China, except for LC2928 and LC2982. In Gao et al. (2016), they did not give a clear answer about their taxonomic placement but only as *Diaporthe eres* complex. Regretfully, not morphological description of these two isolates for taxonomic determination. Thus, combined the analyses of morphology and phylogeny, we described our strain, HGUP1213 as *Diaporthe nobilis*. This was also the first time to report it on *Camellia sinensis*. In view of the phylogenetic placements of LC2928 and LC2982, we thoughtfully proposed they was the same species with our strain.

Although this strain was isolated from diseased leaves of *Camellia sinensis*, we re-inoculated by conidial suspension ($1 \times 10^5 \text{ mL}^{-1}$) and mycelium cake (1 cm diam.) to healthy tea leaves in three pots in my lab. However, the inoculation test confirmed it was not a pathogen but only an opportunity pathogen or endophyte. It was very common because *Diaporthe* spp. were important endophytes in many plant hosts. Thus, we did not provide any information about inoculation experiment and photographs of diseased leaves.

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