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RESEARCH ARTICLE

Diaporthe eleutharrhenae sp. nov. Associated with a Critically Endangered Liana in China

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ABSTRACT

A new fungus isolated from the leaves of *Eleutharrhena macrocarpa* in southern Yunnan, China is described using morphological and molecular evidence. Phylogenetic trees based on the combined nuclear ribosomal DNA internal transcribed spacer (ITS), translation elongation factor-1α (TEF1), and β-tubulin gene (TUB2) sequences showed that *Diaporthe eleutharrhenae* sp. nov. is sister to *Diaporthe chinensis* N.I. de Silva, Lumyong & K.D. Hyde and morphologically differs in shorter alpha conidia (5–8.5 × 1.5–2 μm) and the presence of beta conidia. This study also resolves a nomenclatural problem, as two taxa were published using the same name. To avoid confusion, the unrelated *D. chinensis* H. Dong, J. W. Xia & X. G. Zhang is here renamed as *D. dongii* (H. Dong, J. W. Xia & X. G. Zhang) S. J. Song & Landrein, sp. nov. in honor of the author that described this species. Study and description of fungi associated with threatened tropical species could help to understand their ecology as well as the potential spread of fungi onto cultivated crop species.

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1. Introduction

With more than 19,000 plant species, Yunnan is a biodiversity hotspot [1]. The southern Yunnan flora, including *Eleutharrhena macrocarpa* (Diels) Forman, has strong affinities with the southeast flora of Asia [2] and is threatened by climate change and loss of native forests. *E. macrocarpa* is a critically endangered species in the Menispermaceae family and also a plant species with extremely small populations (PSESP) with less than 100 individuals remaining [3]. Menispermaceae species have high alkaloid and tannin content and are frequently used as medicinal plants including a promising treatment for cancer [4]. Few associated fungi have been reported from this family [5,6], and to date, no fungi associated with *E. macrocarpa* have been described.

Diaporthe is a very large genus including more than 1000 published species names (http://www.indexfungorum.org/Names/Names.asp). The genus is widely distributed throughout the world, includes many pathogens, epiphytes, endophytes, and saprobes and is associated with a wide range of plant hosts [7]. Some pathogenic *Diaporthe* species are responsible for serious economic damage and can cause severe cases of leaf spot, blight, wilt, dieback, or canker [8].

Diaporthe classification was previously based solely on morphological characters resulting in many duplicate names and nomenclature problems. A combination of morphology and phylogenetic data has recently become prevalent and the nomenclature within the genus is now more stable [9]. Internal transcribed spacer (ITS), translation elongation factor-1α (TEF1), and β-tubulin gene (TUB2) sequences are widely used for identification within *Diaporthe* [10].

During an investigation of the *E. macrocarpa* phyllosphere in southern Yunnan, we found a new species that is published here. *Diaporthe eleutharrhenae* sp. nov. is described and compared to sister taxa, using morphology and phylogenetic results based on ITS, TEF1, and TUB2 sequences.

2. Material and methods

2.1. Morphological observations

The specimens used in this study were deposited in the herbarium of China Forestry Culture Collection Center and China General Microbiological Culture Collection Center. Sterilized potato dextrose agar (PDA) produced by Acmec Tech. Co., Ltd (Shanghai, China) was prepared (46 g/L) for isolating fungi and purifying fungi from *E. macrocarpa*.

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Synthetic low-nutrient agar medium (SNA) produced by Coolaber biochemical Tech. Co., Ltd (Beijing, China) was prepared (3.15 g/L) for generating conidia. Observations were carried out using a 1000X microscope (Eclipse Ci, Nikon, Co., Ltd, Tokyo, Japan) after 15 d growth on a SPH-310A shaker (Shiping Tech. Co., Ltd, Beijing, China) at 150 rpm and in the dark.

2.2. DNA extraction, DNA sequencing, and phylogenetic analysis

Mycelium DNA was collected and extracted using the DNA extraction kit (SK8259, Sangon Biotech. Co., Ltd, Shanghai, China). The nuclear rDNA ITS was amplified with primers ITS1/ITS4 [11]; Nuclear rDNA TEF-1, and TUB2 were amplified using the primers EF1–728/EF1– 986 [12] and BT2a/BT2b [13], respectively. PCR was carried out in a 25 μL volume, including 12.5 μL PCR mix, 1 μL of each primer, 1 μL template DNA, and 9.5 μL ddH2O, following the protocol described here: one 5 min cycle of initial denaturation at 95 °C, 30 cycles of denaturation (94 °C) and annealing (57 °C), one cycle of extension at 72 °C, and a 10 min final extension cycle at 72 °C. PCR products were purified and sequenced by Sangon Biotechnology Co., Ltd (Shanghai, China) and sequences were then uploaded to the NCBI database (Table 1).

Taxa for phylogenetic analysis and outgroups were chosen according to previous studies [14,15] and additional related species were added according to the ITS, TEF1, and TUB2 blast results. Sequences were then downloaded from the NCBI database (Table 1).

The sequences were aligned and trimmed in Geneious and then concatenated in PhyloSuite [16]. *Diaporthella corylina* was used as the outgroup [14,17]. The best nucleotide substitution model was chosen using ModelFinder [17] and a Bayesian analysis was performed using MrBayes [18] with two parallel runs of 500,000 generations. Maximum likelihood analysis was performed using IQ-tree [19] with 5000 bootstraps.

3. Taxonomy

Diaporthe eleutharrhenae S. J. Song & Landrein, sp. nov.

Table 1. ITS, TEF1, and TUB2 sequences used for the identification of *Diaporthe eleutharrhenae*.

Name	ITS	TEF1	TUB2
Diaporthe alangii CFCC 52556	MH121491	MH121533	MH121573
Diaporthe alangii CFCC 52557	MH121492	MH121534	MH121574
Diaporthe ambigua CBS 114015	MH862953	GQ250299	KC343978
Diaporthe ambigua CBS 117167	KC343011	KC343737	KC343979
Diaporthe aspalathi CBS 117169	NR_165951	KC343762	KC344004
Diaporthe biguttulata ZJUD47	KJ490582	KJ490461	KJ490403
Diaporthe brasiliensis CBS 133183	NR_111844	KC343768	KC344010
Diaporthe caatingaensis URM7486	KY085926	KY115603	KY115600
Diaporthe caulivora CBS 127268	NR_111845	KC343771	KC344013
Diaporthe chinensis S16/MFLUCC:19-0101	MW187324	MW205017	MW245013
Diaporthe chinensis S16/MFLUCC:19-0106	MW187325	MW219603	MW245014
Diaporthe chinensis SAUC194.29	MT822557	MT855870	MT855754
Diaporthe chinensis SAUC194.30	MT822558	MT855871	MT855755
Diaporthe clematidina MFLUCC 17-2060	MT310657	MT394669	MT394623
Diaporthe compactum LC3083	KP267854	KP267928	KP293434
Diaporthe conferta SAUC194.16	MT822544	MT855858	MT855741
Diaporthe conferta SAUC194.9	MT822537	MT855851	MT855734
Diaporthella corylina CBS 121124	KC343004	KC343730	KC343972
Diaporthe crotalariae CBS 162.33	NR_103697	KC343782	KC344024
Diaporthe discoidispora ZJUD89	KJ490624	KJ490503	KJ490445
Diaporthe eleutherrhenae 01	OK017069	OK017070	OK017071
Diaporthe eleutherrhenae 02	OK648457	OK648458	OK648459
Diaporthe ellipsoidea GZCC19_0231	MT385949	MT424684	MT424704
Diaporthe ganjae CBS 180.91	NR_120259	KC343838	KC344080
Diaporthe goulteri BRIP 55657a	NR_147540	KJ197252	KJ197270.1
Diaporthe hubeiensis JZB320123	MK335809	MK523570	MK500147
Diaporthe incompleta LC6754	KX986794	KX999186	KX999226
Diaporthe longispora CBS 194.36	KC343135	KC343861	KC344103
Diaporthe malorum CAA734	KY435638	KY435627	KY435668
Diaporthe manihotia MFLU:19-2826	MT012296	MW014359	MW018927
Diaporthe oxe CBS 133186	NR_111856	KC343890	KC344132
Diaporthe paranensis CBS 133184	KC343171	KC343897	KC344139
Diaporthe passiflorae CBS132527	NR_120155	KY435633	KY435674
Diaporthe rhoina CBS 146.27	KC343189	KC343915	KC344157
Diaporthe sclerotioides CBS 296.67	KC343193	KC343919	KC344161
Diaporthe siamensis MFLUCC 12-0300	KT459417	KT459451	KT459435
Diaporthe tectonae MFLUCC 12-0777	NR_147590	KU749359	KU743977
Diaporthe tectonae MFLUCC 14-1138	KU712437	KU749365	KU743984
Diaporthe tulliensis BRIP 62248a	NR_147574	KR936133	KR936132
Diaporthe woodii CBS 558.93	KC343244	KC343970	KC344212
Diaporthe yunnanensis LC8106	KY491541	KY491551	KY491561
Diaporthe yunnanensis LC8107	KY491542	KY491552	KY491562

Etymology: The epithet *eleutharrhenae* refers to the free stamens in the flowers of the genus *Eleutharrhena*, the host of *Diaporthe eleutharrhenae*.

Diagnosis: *Diaporthe eleutharrhenae* can be distinguished from its closest related sister species *D. chinensis* N. I. de Silva, Lumyong & K. D. Hyde [14] by the shorter alpha conidia (5–8.5 µm, 10–14 in *D. chinensis*) and the presence of beta conidia (beta conidia are absent in *D. chinensis*) (Table 2; Figure 1).

Description: Conidiomata pycnidia, globose or irregular, solitary or aggregated together, dark brown to black. Conidia exuding from the pycnidia in white to cream drops. Conidiophores cylindrical, straight or slightly curved. Alpha conidia 5–8.5 × 1–2.8 µm ($n = 60$), hyaline, obovate to clavate with both ends obtuse. Beta conidia 16.5–56.1 × 0.67–3.33 µm ($n = 0$) curved and hyaline, one end obtuse, another end acute.

Cultures: Colonies can reach 60 mm in diameter after 7 d at 25 °C. Colonies on PDA are flat, with abundant dirty white and yellowish pigmented mycelium showing in the later stages. Pale to dark brownish pigmented rings can be seen. Brown secretion can be observed after ca.14 d PDA.

Typus: Yunnan Province, China, 1.5 km from Manbian road (21°57′N, 101°15′E) Menglun town, Xishuangbanna Dai Autonomous prefecture, May 2021.

Known distribution: Currently known from a single site in Xishuangbanna Dai Autonomous prefecture in Yunnan, China.

Host: Currently only known from *E. macrocarpa*.

Table 2. Synoptic characters of *Diaporthe eleutharrhenae* and its most closely related species.

Name	Host	Conidiophores (µm)	Alpha conidia (µm)	Beta conidia (µm)	Location	References
D. eleutharrhenae	*Eleutharrhena macrocarpa*	–	5–8.5	16.5–56.1	Yunnan, China	Our study
D. chinensis	*Magnolia candolli*	12-15	10–14	–	Yunnan, China	[14]
D. yunnanensis	*Coffea* spp.	-	3–6.5 × 1– 2.5	13.5–33.5 × 1–1.5	Yunnan, China	[8]
D. siamensis	*Thunbergia* spp.	5-20	–	(14–)15-17(–18)	Chiang Rai, Thailand	[20]
D. biguttulata	*Citrus limon*	12-5.5	5.7–6.8	23.7–31.6	Yunnan, China	[21]
D. discoidispora	*Citrus unshiu*	8.9-23.4	5.6–8	21.2–38.7	Jiangxi, China	[21]

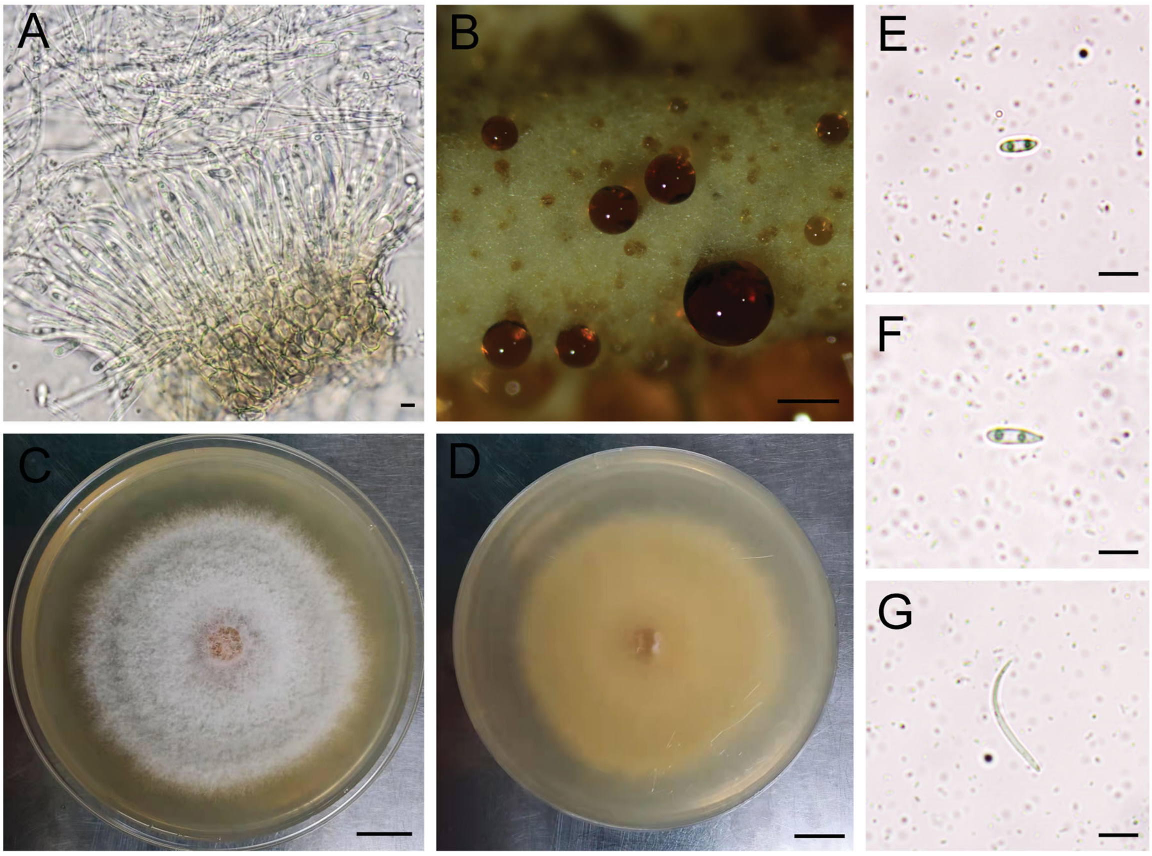

Figure 1. (A) Pycnidia of *Diaporthe eleutharrhenae* isolated from synthetic low-nutrient agar medium (SNA) on day 15. (B) Secretion of *D. eleutharrhenae* on the PDA Petri dish and on day 20. (C and D) Colony morphology of *D. eleutharrhenae* observed from the top and below the PDA Petri dish on day 7. (E and F) Alpha conidia. (G) beta conidia. Conidia were isolated from synthetic low-nutrient agar medium (SNA). Scale bars in A, E, F, G are 5 µL. Scale bar in B is 50 µL. Scale bars in C and D are 1 cm. Shorter alpha conidia (5–8.5 µm) and the presence of beta conidia are a key character for distinguishing *D. eleutharrhenae* from the most closely related species *D. chinensis N. I. de Silva, Lumyong* & K. D. Hyde.

4. Result and discussion

The combined ITS, TEF1, and TUB2 matrix is 1452 bp in length and the phylogenetic tree included 42 samples. TIMEF + G (ITS), TVM + I +G (TEF1), and HKY + G (TUB2) substitution models were selected for maximum likelihood and Bayesian analyses. Phylogenetic trees based on each region were congruent and indicate that *Diaporthe eleutharrhenae* is sister to *Diaporthe chinensis* N. I. de Silva, Lumyong & K.D. Hyde with high support (Maximum likelihood bootstrap = 94%, Bayesian posterior probability = 1) (Figure 2).

During the preparation of sequences for phylogenetic analysis, we found two different fungi taxa had the same name, *Diaporthe chinensis* N. I. de Silva, Lumyong & K.D. Hyde [14] and *Diaporthe chinensis* H. Dong, J. W. Xia & X. G. Zhang [15].

D. chinensis N. I. de Silva, Lumyong & K. D. Hyde is sister to *Diaporthe yunnanensis* Y. H. Gao & L. Cai isolated from healthy leaves of *Magnolia candolli* and published on March 25 2021 [14]. It was described based on large alpha conidia (10–14 × 3–6 µm) and its phylogenetic position. *Diaporthe chinensis* H. Dong, J. W. Xia & X. G. Zhang is sister to *D. conferta* isolated from *Litchi chinensis* leaves and published on March 22 2021 [15]. Its description was based on the host identity, distribution, the existence of beta conidia and gamma conidia, as well as its phylogenetic position. To avoid confusion and following the International Code of Nomenclature for algae, fungi, and plants [22], we propose the renaming of *Diaporthe chinensis* H. Dong, J. W. Xia & X. G. Zhang to *Diaporthe dongii* (H. Dong, J. W. Xia & X. G. Zhang) S. J. Song & Landrein.

Diaporthe species are associated with diverse hosts. Their life cycle is variable, and their distribution can be narrow or wide. This variability is reflected by our results; *D. dongii* is a pathogenic species found on cultivated *Litchi chinensis*, and *D. eleutharrhenae* is an epiphytic species growing on the leaves of the native and endangered liana *E. macrocarpa*. It is not known if *D. eleutharrhenae*

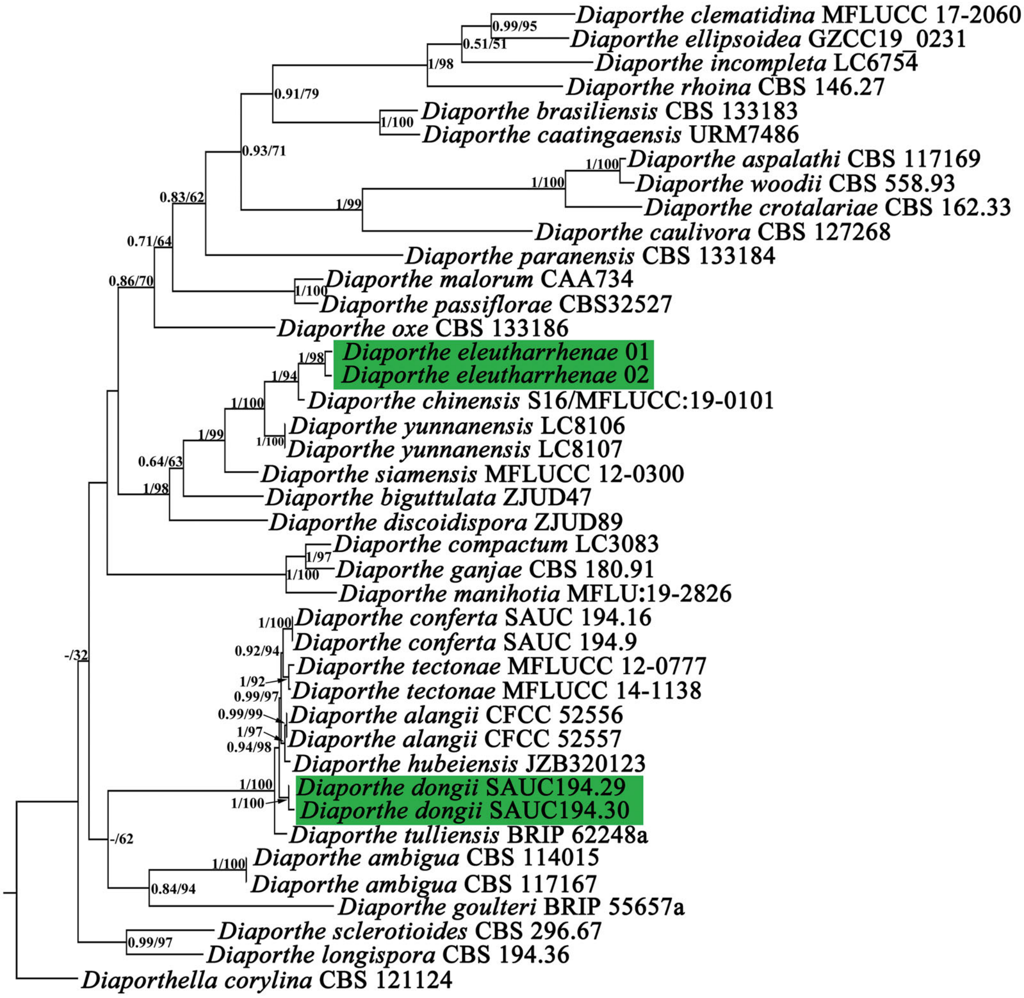

Figure 2. Maximum likelihood tree based on two collections of *Diaporthe eleutharrhenae* and its closest related species using ITS, TEF1, and TUB2 sequences. "–" means no support. Maximum likelihood bootstrap value and Bayesian posterior probabilities are shown next to the branch node. *Diaporthe dongii* (H. Dong, J. W. Xia & X. G. Zhang) S. J. SONG & Landrein is the suggested new name of *Diaporthe chinensis* H. Dong, J. W. Xia & X. G. Zhang. Results strongly support *Diaporthe chinensis* N. I. de Silva, Lumyong & K. D. Hyde as the closest related species of *Diaporthe eleutharrhenae*.

occurs onto cultivated species. The population of *E. macrocarpa* is located at the margin of a fragmented forest and is surrounded by grapefruit and rubber plantations. It is not known at present if *D. eleutharrhenae* is specific to its host or it could be more widespread and be associated with other rainforest species. To our knowledge, no fungi have been reported to be associated with *E. macrocarpa* before. These results will contribute to the research in the conservation of *E. macrocarpa* as well as our understanding of the potential spread of native fungi onto cultivated crops.

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Disclosure statement

The authors declare no conflict of interest.

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