Phytopathologia Mediterranea (2018), 57, 2, 307–319
DOI: 10.14601/Phytopathol_Mediterr-23254

RESEARCH PAPERS

Species of *Diaporthe* on *Camellia* and *Citrus* in the Azores Islands

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Summary. Species of *Diaporthe* are important plant pathogens, saprobes, and endophytes on a wide range of plant hosts. Species such as *D. citri* are well-known on *Citrus*, as agents of pre- or post-harvest infections, causing dieback, melanose and stem-end rot on fruit. In this study we explored the occurrence and diversity of *Diaporthe* associated with tropical and sub-tropical plants. In particular, species of *Camellia* and *Citrus* were sampled. Surveys were carried out during 2017 in the Azores Islands, Portugal. Ten *Diaporthe* strains were isolated from symptomatic twigs and leaves. Five representative isolates were subjected to morphological characterization and multi-locus phylogeny based on five genomic loci (ITS, *tef1*, *cal*, *his3* and *tub2*). *Diaporthe citri* was found associated with shoot blight on *Citrus reticulata*, which represents a new record for Europe. A new species, *Diaporthe portugallica* sp. nov. was isolated and described from leaf spots on *Camellia sinensis*.

Key words: *Phomopsis*, tea, mandarin, leaf spot, multi-locus sequence typing, shoot blight.

Introduction

Species of *Diaporthe* are present worldwide as plant pathogens, endophytes in healthy plant tissues, or as saprobes of a wide range of hosts (Muralli *et al*., 2006; Udayanga *et al*., 2011). They are well-known as the causal agents of many important plant diseases, including fruit and root rots, dieback, stem cankers, leaf spots, leaf and pod blights, and seed decay (Mostert *et al*., 2001a, 2001b; Van Rensburg *et al*., 2006; Santos *et al*., 2011; Udayanga *et al*., 2011; Guarnaccia *et al*., 2018). Species of the genus have also been used in secondary metabolite research due to their production of a large number of polyketides and unique low- and high- molecular-weight metabolites with different activities (Gomes *et al*., 2013), and for biological control of fungal pathogens (Santos *et al*., 2016).

The generic names *Diaporthe* and *Phomopsis* are no longer used to distinguish different morphs of this genus, and a recent study (Rossman *et al*., 2015) recommended that the genus name *Diaporthe* be retained over *Phomopsis*, because it is the older name.

Several studies revisited the taxonomy of *Diaporthe* (Thompson *et al*., 2011; Gomes *et al*., 2013; Udayanga *et al*., 2014a, 2014b, 2015). Almost 2,000 species names are available for both *Diaporthe* and *Phomopsis* (Index Fungorum; http://www.indexfungorum.org). Recently, Marin-Felix *et al*. (2019) accepted 213 species based on their DNA barcodes. Some species of *Diaporthe* occur on diverse hosts while others occur only on one host genus, often as different morphs (Mostert *et al*., 2001a; Guarnaccia *et al*. 2016). As a consequence, identification of species based only on host association is no longer tenable within *Diaporthe* (Gomes *et al*., 2013; Udayanga *et al*., 2014a, 2014b). Previously, morphological characters were the basis on which to study the taxonomy of *Diaporthe*/*Phomopsis* (Udayanga *et al*., 2011). However, recent studies have demonstrated that these characters are not always reliable for species level identification due to their variability under changing environmental conditions (Gomes *et al*., 2013).

Following the adoption of DNA sequence-based methods, the polyphasic protocols for studying the

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genus significantly changed the classification and species concepts, resulting in rapid proliferation of new species descriptions. Therefore, genealogical concordance methods, based on multi-gene DNA sequence data, provide clearer resolution of the *Diaporthe* taxonomy (Gomes *et al*., 2013).

Recent plant pathology studies have revealed several *Diaporthe* species to be associated with diseases on a wide range of economically important agricultural crops, such as *Camellia*, *Citrus*, *Glycine*, *Helianthus*, *Persea*, *Vaccinium*, *Vitis*, vegetables, fruit crops and forest plants (Van Rensburg *et al*., 2006; Santos and Phillips, 2009; Santos *et al*., 2011; Thompson *et al*., 2011; Huang *et al*., 2013; Lombard *et al*., 2014; Gao *et al*., 2016; Udayanga *et al*., 2015; Guarnaccia *et al*., 2016, 2018; Guarnaccia and Crous, 2017).

Guarnaccia and Crous (2017) revealed a large diversity of *Diaporthe* spanning several clades, recovered from *Citrus* in European countries such as Greece, Italy, Malta, Portugal and Spain. These include two newly described species *D. limonicola* and *D. melitensis* associated with severe cankers. In total, 22 species of *Diaporthe* are now known to be associated with *Citrus*. *Diaporthe citri* is known as an important pathogen of *Citrus*, causing stem-end rot and melanose of fruits, young leaf and shoot gummosis, and blight of perennial branches and trunks (Kucharek *et al*., 1983; Timmer and Kucharek, 2001; Mondal *et al*., 2007; Udayanga *et al*., 2014b). This species occurs in many *Citrus* growing regions of the world (Timmer *et al*., 2000). Udayanga *et al*. (2014b) re-assessed *D. citri* based on molecular phylogenetic analysis of conserved ex-type and additional strains, collected exclusively from symptomatic *Citrus* tissues in different geographic locations worldwide. They showed that *D. citri* is unknown from Europe. This was confirmed following a broad survey by Guarnaccia and Crous (2017).

Recently, Gao *et al*. (2016; 2017) investigated the taxonomic and phylogenetic diversity of *Diaporthe* associated with *Camellia* spp. in China, based on morphological characteristics and sequence data. They demonstrated high diversity of *Diaporthe* species with the identification of 17 species on *Camellia*.

In 2017, shoot blight on *Citrus reticulata* trees and a leaf spot disease on *Camellia sinensis* were observed in two orchards in San Miguel Island (Azores, Portugal), so a study was conducted to identify the causative agents. This aimed to identify the strains of *Diaporthe* associated with disease symptoms on *Citrus* and *Camellia* using morphological characterization and multi-locus DNA sequence data, and to compare the results with data from other phylogenetic studies of the genus.

Materials and methods

Sampling and isolation

Diseased twig and leaf samples were collected from of tropical plants during collecting trips in the Azores Islands, Portugal in July 2017. Shoot blight and leaf spot symptoms were observed and sampled, respectively, in a 40-year old *Citrus reticulata* orchard and a 20-year old *Camellia sinensis* plantation. Both sites are located in Ponta Delgada Province (Portugal). Fragments (5 × 5 mm) of symptomatic tissues were cut from the margins of lesions, surface-sterilised in a sodium hypochlorite solution (10%) for 20 s, followed by 70% ethanol for 30 s, and rinsed three times in sterilised water. Tissue fragments were dried on sterilised filter paper, placed on 2% potato dextrose agar (PDA) amended with 100 μg mL^{-1} penicillin and 100 μg mL^{-1} streptomycin (PDA-PS), and then incubated at 25°C until characteristic diaporthe-like colonies were observed. Pure cultures were obtained by transferring germinating single conidia to fresh PDA dishes with the aid of a stereomicroscope (Nikon SMZ1000). Isolates used in this study are maintained in the culture collection of the Westerdijk Fungal Biodiversity Institute (CBS), Utrecht, The Netherlands, and in the working collection of Pedro Crous (CPC), housed at the Westerdijk Institute (Table 1).

DNA extraction, PCR amplification and sequencing

Genomic DNA was extracted using a Wizard® Genomic DNA Purification Kit (Promega) following the manufacturer's instructions. Partial regions of five loci were amplified. The primers EF1-728F and EF1-986R (Carbone and Kohn, 1999) or EF2 as reverse (O'Donnell *et al*., 1998) were used to amplify part of the translation elongation factor 1-α gene (*tef1*). The primers CAL-228F and CAL-737R (Carbone and Kohn, 1999) or CL1/CL2A (O'Donnell *et al*., 2000) were used to amplify part of the calmodulin (*cal*) gene. The partial histone H3 (*his3*) region was amplified using the CYLH3F and H3-1b primer set (Glass and Donaldson, 1995; Crous *et al*., 2004a), and the beta-tubulin (*tub2*) region was amplified using the Bt2a

Table 1. Collection details and GenBank accession numbers for isolates included in this study.

Species	Culture No.[a]	Host	Locality	GenBank number[b]				
				ITS	*tub2*	*his3*	*tef1*	*cal*
D. anacardii	**CBS 720.97**	*Anacardium occidentale*	Kenya	KC343024	KC343992	KC343508	KC343750	KC343266
D. angelicae	**CBS 111592**	*Heracleum sphondylium*	Austria	KC343026	KC343994	KC343511	KC343752	KC343268
D. arecae	**CBS 161.64**	*Areca catechu*	India	KC343032	KC344000	KC343516	KC343758	KC343274
	CBS 535.75	*Citrus* sp.	Suriname	KC343033	KC344001	KC343517	KC343759	KC343275
D. arengae	**CBS 114979**	*Arenga engleri*	Hong Kong	KC343034	KC344002	KC343518	KC343760	KC343276
D. baccae	**CBS 136972**	*Vaccinium corymbosum*	Italy	KJ160565	MF418509	MF418264	KJ160597	MG281695
	CPC 26170 = CBS 142545	*Citrus sinensis*	Italy	MF418351	MF418510	MF418265	MF418430	MF418185
	CPC 27831 = CBS 142546	*Citrus sinensis*	Italy	MF418358	MF418517	MF418272	MF418437	MF418192
D. biconispora	**ICMP20654**	*Citrus grandis*	China	KJ490597	KJ490418	KJ490539	KJ490476	-
D. biguttulata	**ICMP20657**	*Citrus limon*	China	KJ490582	KJ490403	KJ490524	KJ490461	-
D. citri	CBS 134237	*Citrus reticulata*	China	JQ954660	KC357426	MF418279	JQ954676	KC357465
	CBS 135423	*Citrus* sp.	USA	KC843321	KC843197	-	KC843081	KC843167
	CBS 135424	*Citrus paradisi*	USA	KC843327	KC843203	-	KC843087	KC843173
	CBS 135425	*Citrus unshiu*	Korea	KC843326	KC843202	-	KC843086	KC843172
	CBS 135426	*Citrus unshiu*	Korea	KC843324	KC843200	-	KC843084	KC843170
	CBS 135427	*Citrus reticulata*	China	KC843323	KC843199	-	KC843083	KC843169
	CBS 135767	*Citrus reticulata*	China	KC843322	KC843198	-	KC843082	KC843168
	CBS 134239	*Citrus sinensis*	Florida, USA	KC357553	KC357456	MF418280	KC357522	KC357488
	CBS 135422	*Citrus* sp.	USA	KC843311	KC843187	MF418281	KC843071	KC843157
	CPC 34227	*Citrus reticulata*	Portugal	*MH063902*	*MH063914*	*MH063896*	*MH063908*	*MH063890*
	CPC 34229	*Citrus reticulata*	Portugal	*MH063903*	*MH063915*	*MH063897*	*MH063909*	*MH063891*
	CPC 34235 = CBS 144227	*Citrus reticulata*	Portugal	*MH063904*	*MH063916*	*MH063898*	*MH063910*	*MH063892*
D. citriasiana	**CBS 134240**	*Citrus unshiu*	China	JQ954645	KC357459	MF418282	JQ954663	KC357491
D. citrichinensis	**CBS 134242**	*Citrus* sp.	China	JQ954648	MF418524	KJ420880	JQ954666	KC357494
D. cuppatea	**CBS 117499**	*Aspalathus linearis*	South Africa	AY339322	JX275420	KC343541	AY339354	JX197414
D. cytosporella	**CBS 137020**	*Citrus limon*	Spain	KC843307	KC843221	MF418283	KC843116	KC843141

(Continued)

Table 1. (Continued).

Species	Culture No.[a]	Host	Locality	GenBank number[b]				
				ITS	*tub2*	*his3*	*tef1*	*cal*
D. discoidispora	**ICMP20662**	*Citrus unshiu*	China	KJ490624	KJ490445	KJ490566	KJ490503	-
D. endophytica	ZJUD73	*Citrus unshiu*	China	KJ490608	KJ490429	KJ490550	KJ490487	-
D. eres	**CBS 439.82**	*Cotoneaster* sp.	Scotland	KC343090	KC344058	KC343574	KC343816	KC343332
D. foeniculina	CBS 187.27	*Camellia sinensis*	Italy	KC343107	KC344075	KC343591	KC343833	KC343349
	CBS 111553	*Foeniculum vulgare*	Spain	KC343101	KC344069	KC343585	KC343827	KC343343
	CBS 111554	*Foeniculum vulgare*	Portugal	KC343102	KC344070	KC343586	KC343828	KC343344
	CBS 123208	*Foeniculum vulgare*	Portugal	KC343104	KC344072	KC343588	KC343830	KC343346
	CBS 123209	*Foeniculum vulgare*	Portugal	KC343105	KC344073	KC343589	KC343831	KC343347
	CBS 135430	*Citrus limon*	USA	KC843301	KC843215	MF418284	KC843110	KC843135
	CPC 28033 = CBS 142547	*Citrus sinensis* 'Valencia'	Portugal	MF418402	MF418562	MF418322	MF418481	MF418236
D. helianthi	CBS 344.94	*Helianthus annuus*	-	KC343114	KC344082	KC343598	KC343840	KC343356
	CBS 592.81	*Helianthus annuus*	Serbia	KC343115	KC344083	KC343599	KC343841	JX197454
D. hongkongensis	**CBS 115448**	*Dichroa febrifuga*	China	KC343119	KC344087	KC343603	KC343845	KC343361
D. inconspicua	**CBS 133813**	*Maytenus ilicifolia*	Brazil	KC343123	KC344091	KC343607	KC343849	KC343365
D. infertilis	CBS 199.39	Unknown	Italy	KC343051	KC344019	KC343535	KC343777	KC343293
	CBS 230.52	*Citrus sinensis*	Suriname	KC343052	KC344020	KC343536	KC343778	KC343294
	CPC 20322	*Glycine max*	Brazil	KC343053	KC344021	KC343537	KC343779	KC343295
D. limonicola	**CPC 28200 = CBS 142549**	*Citrus limon*	Malta	MF418422	MF418582	MF418342	MF418501	MF418256
	CPC 31137 = CBS 142550	*Citrus limon*	Malta	MF418423	MF418583	MF418343	MF418502	MF418257
D. melitensis	**CPC 27873 = CBS 142551**	*Citrus limon*	Malta	MF418424	MF418584	MF418344	MF418503	MF418258
	CPC 27875 = CBS 142552	*Citrus limon*	Malta	MF418425	MF418585	MF418345	MF418504	MF418259
D. multigutullata	**ICMP20656**	*Citrus grandis*	China	KJ490633	KJ490454	KJ490575	KJ490512	-
D. novem	CBS 127270	*Glycine max*	Croatia	KC343156	KC344124	KC343640	KC343882	KC343398
	CBS 127271	*Glycine max*	Croatia	KC343157	KC344125	KC343641	KC343883	KC343399
	CPC 26188 = CBS 142553	*Citrus japonica*	Italy	MF418426	MF418586	MF418346	MF418505	MF418260
	CPC 28165 = CBS 142554	*Citrus aurantiifolia*	Italy	MF418427	MF418587	MF418347	MF418506	MF418261

(Continued)

Table 1. (Continued).

Species	Culture No.[a]	Host	Locality	GenBank number[b]				
				ITS	*tub2*	*his3*	*tef1*	*cal*
D. ovalispora	**ICMP20659**	*Citrus limon*	China	KJ490628	KJ490449	KJ490570	KJ490507	-
D. portugallica	**CPC 34247 = CBS 144228**	*Citrus reticulata*	Portugal	*MH063905*	*MH063917*	*MH063899*	*MH063911*	*MH063893*
	CPC 34248	*Citrus reticulata*	Portugal	*MH063906*	*MH063918*	*MH063900*	*MH063912*	*MH063894*
D. pseudomangiferae	**CBS 101339**	*Mangifera indica*	Dominican Republic	KC343181	KC344149	KC343665	KC343907	KC343423
D. pseudophoenicicola	**CBS 462.69**	*Phoenix dactylifera*	Spain	KC343184	KC344152	KC343668	KC343910	KC343426
D. rudis	CBS 113201	*Vitis vinifera*	Portugal	KC343234	KC344202	KC343718	KC343960	KC343476
D. saccarata	**CBS 116311**	*Protea repens*	South Africa	KC343190	KC344158	KC343674	KC343916	KC343432
D. sojae	**FAU 635**	*Glycine max*	USA	KJ590719	KJ610875	KJ659208	KJ590762	-
	ZJUD68	*Citrus unshiu*	China	KJ490603	KJ490424	KJ490545	KJ490482	-
D. sterilis	**CBS 136969**	*Vaccinium corymbosum*	Italy	KJ160579	KJ160528	MF418350	KJ160611	KJ160548
D. subclavata	**ICMP20663**	*Citrus unshiu*	China	KJ490630	KJ490451	KJ490572	KJ490509	-
D. unshiuensis	**CGMCC3.17569**	*Citrus unshiu*	China	KJ490587	KJ490408	KJ490529	KJ490466	-
D. velutina	CGMCC 3.18286	*Neolitsea* sp.	China	KX986790	KX999223	KX999261	KX999182	-
	LC 4641	*Callerya cinerea*	China	KX986792	KX999225	KX999263	KX999184	KX999287
Diaporthella corylina	**CBS 121124**	*Corylus* sp.	China	KC343004	KC343972	KC343488	KC343730	KC343246

[a] CPC: Culture collection of P.W. Crous, housed at Westerdijk Fungal Biodiversity Institute; CBS: Westerdijk Fungal Biodiversity Institute, Utrecht, the Netherlands; CGMCC: China, General Microbiological Culture Collection, Beijing, China; FAU: Isolates in culture collection of Systematic Mycology and Microbiology Laboratory, USDA-ARS, Beltsville, Maryland, USA; ICMP: International Collection of Microorganisms from Plants, Landcare Research, Auckland, New Zealand; LC: Working collection of Lei Cai, housed at Institute of Microbiology, CAS, China; ZJUD, Diaporthe strains collected in Zhejiang University, China. Ex-type and ex-epitype cultures are indicated in bold.

[b] ITS: internal transcribed spacers 1 and 2 together with 5.8S nrDNA; *tub2*: partial beta-tubulin gene; *his3*: histone3; *tef1*: partial translation elongation factor 1-α gene; *cal*: partial calmodulin gene. Sequences generated in this study indicated in italics.

and Bt2b primer set (Glass and Donaldson, 1995) or 2Fd/4Rd (Woudenberg *et al*., 2009). The PCR products were sequenced in both directions using the BigDye® Terminator v. 3.1 Cycle Sequencing Kit (Applied Biosystems Life Technologies), after which amplicons were purified through Sephadex G-50 Fine columns (GE Healthcare) in MultiScreen HV plates (Millipore). Purified sequence reactions were analyzed on an Applied Biosystems 3730xl DNA Analyser (Life Technologies). The DNA sequences generated were analysed and consensus sequences were computed using the program SeqMan Pro (DNASTAR).

Phylogenetic analyses

Novel sequences generated in this study were blasted against the NCBIs GenBank nucleotide database, to determine the closest relatives for a taxonomic framework of the studied isolates. Alignments of different gene regions, including sequences obtained from this study and those downloaded from GenBank, were initially performed by using the MAFFT v. 7 online server (http://mafft.cbrc.jp/alignment/server/index.html) (Katoh and Standley, 2013), and then manually adjusted in MEGA v. 7 (Kumar *et al*., 2016).

To establish the identity of the isolates at species level, phylogenetic analyses were conducted, first individually for each locus (data not shown) and then as combined analyses of five loci. Additional reference sequences were selected based on recent studies of *Diaporthe* species (Gomes *et al*., 2013; Udayanga *et al*., 2014a, 2014b; Gao *et al*., 2016, 2017; Guarnaccia and Crous, 2017). Phylogenetic analyses were based on Maximum Parsimony (MP) for all the individual loci and on MP and Bayesian Inference (BI) for the multi-locus analyses. For BI, the best evolutionary model for each partition was determined using MrModeltest v. 2.3 (Nylander, 2004) and incorporated into the analyses. MrBayes v. 3.2.5 (Ronquist *et al*., 2012) was used to generate phylogenetic trees under optimal criteria per partition. The Markov Chain Monte Carlo (MCMC) analysis used four chains and started from a random tree topology. The heating parameter was set to 0.2, and trees were sampled every 1,000 generations. Analyses stopped once the average standard deviation of split frequencies was below 0.01. The MP analyses were performed using PAUP (Phylogenetic Analysis Using Parsimony, v. 4.0b10; Swofford, 2003). Phylogenetic relationships were estimated by heuristic searches with 100 random addition sequences. Tree bisection-reconnection was used, with the branch swapping option set on 'best trees' only with all characters weighted equally and alignment gaps treated as fifth state. Tree length (TL), consistency index (CI), retention index (RI) and rescaled consistence index (RC) were calculated for parsimony and bootstrap analyses (Hillis and Bull, 1993), which were based on 1,000 replications. Sequences generated in this study are deposited in GenBank (Table 1).

Taxonomy

Agar plugs (6 mm diam.) were taken from the margins of actively growing cultures on malt extract agar (MEA) and transferred onto the centre of 9 cm diam. Petri dishes containing 2% tap water agar supplemented with sterile pine needles (PNA; Smith *et al*., 1996), potato dextrose agar (PDA), oatmeal agar (OA) or MEA (Crous *et al*., 2009), and incubated at 21–22°C under a 12 h near-ultraviolet light /12 h dark cycle to induce sporulation, as described by Lombard *et al*. (2014). Colony characters and pigment production on MEA, OA or PDA were noted after 15 d. Colony colours were described according to Rayner (1970). Cultures were examined periodically for the development of ascomata and conidiomata. Colony diameters were measured after 7 and 10 d. The morphological characteristics were examined by mounting fungal structures in clear lactic acid and 30 measurements at ×1,000 magnification were determined for each isolate using a light microscope (Zeiss Axioscope 2) with interference contrast (DIC) optics. Descriptions, nomenclature and illustrations of taxonomic novelties were deposited in MycoBank (www.MycoBank.org; Crous *et al*., 2004b).

Results

Isolates

Several *Diaporthe* spp. were associated with symptoms of tropical and subtropical plants during the survey. We focussed on *Citrus reticulata* shoot blight and *Camellia sinensis* leaf spot diseases. The *Citrus* plants presented twigs with dieback and wither-tip, and occasionally gummosis. In contrast, necrotic lesions with reddish to purple margins were detected on *Camellia* leaves (Figure 1). Pycnidium formation on dead tissue was observed in both cases. Ten mono-

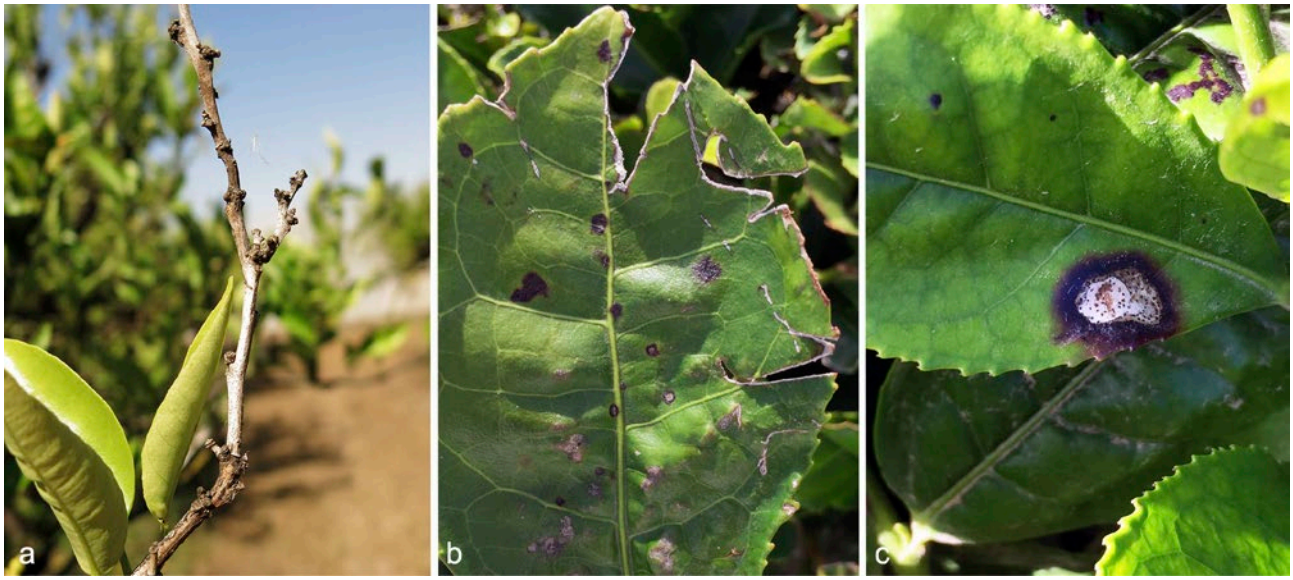

Figure 1. Symptoms on plant tissues with associated *Diaporthe* spp. (a) Shoot blight on *Citrus reticulata* with conidiomata of *D. citri*. (b–c) Leaf spot of *Camellia sinensis* with visible *D. portugallica* conidiomata.

sporic isolates resembling those of the genus *Diaporthe* were collected and, based on preliminary ITS sequencing, five representative strains were selected for phylogenetic analyses and further taxonomic study (Table 1).

Phylogenetic analyses

Six alignments were analysed representing single gene analyses of ITS, *tub2*, *his3*, *tef1*, *cal* and a combined alignment of the five genomic loci. The alignments produced topologically similar trees. The combined species phylogeny of the *Diaporthe* isolates consisted of 67 sequences, including the outgroup sequences of *Diaporthella corylina* (culture CBS 121124). A total of 2,797 characters (ITS: 1–581, *tub2*: 588–1,198, *his3*: 1,205–1,741, *tef1*: 1,748–2,221, *cal*: 2,228–2,797) were included in the phylogenetic analysis; 1,137 characters were parsimony-informative, 489 were variable and parsimony-uninformative, and 1,147 were constant. A maximum of 1,000 equally most parsimonious trees were saved (Tree length = 5,017, CI = 0.561, RI = 0.834 and RC = 0.468). Bootstrap support values from the parsimony analysis are plotted on the Bayesian phylogenies in Figure 2. For the Bayesian analyses, MrModeltest suggested that all partitions should be analysed with dirichlet state frequency distributions. The following models were recommended by MrModeltest and used: GTR+I+G for ITS, *his3*, *tef1* and *cal*, HKY+I+G for *tub2*. In the Bayesian analysis, the ITS partition had 188 unique site patterns, the *tub2* partition had 346, the *his3* partition had 239, the *tef1* partition had 369, and the *cal* partition had 340 unique site patterns. The analysis ran for 516,000 generations, resulting in 1,042 trees of which 782 were used to calculate the posterior probabilities.

In the combined analysis, three representative isolates from *Citrus* clustered with nine reference strains and the ex-type of *D. citri*. Two isolates from *Camellia sinensis*, identified as the novel taxon *D. portugallica*, formed a highly supported subclade (1.00/100) close to *D. anacardii*.

The individual alignments and trees of the five single loci used in the analyses were compared with respect to their performance in species recognition. *Diaporthe portugallica* and *D. citri* could be differentiated based on each gene used.

Taxonomy

Descriptions and illustrations of the species resolved in this study, based on multi-gene phylogenetic analyses and morphological characters, are provided below. *Diaporthe citri* occurred only on *Citrus* while

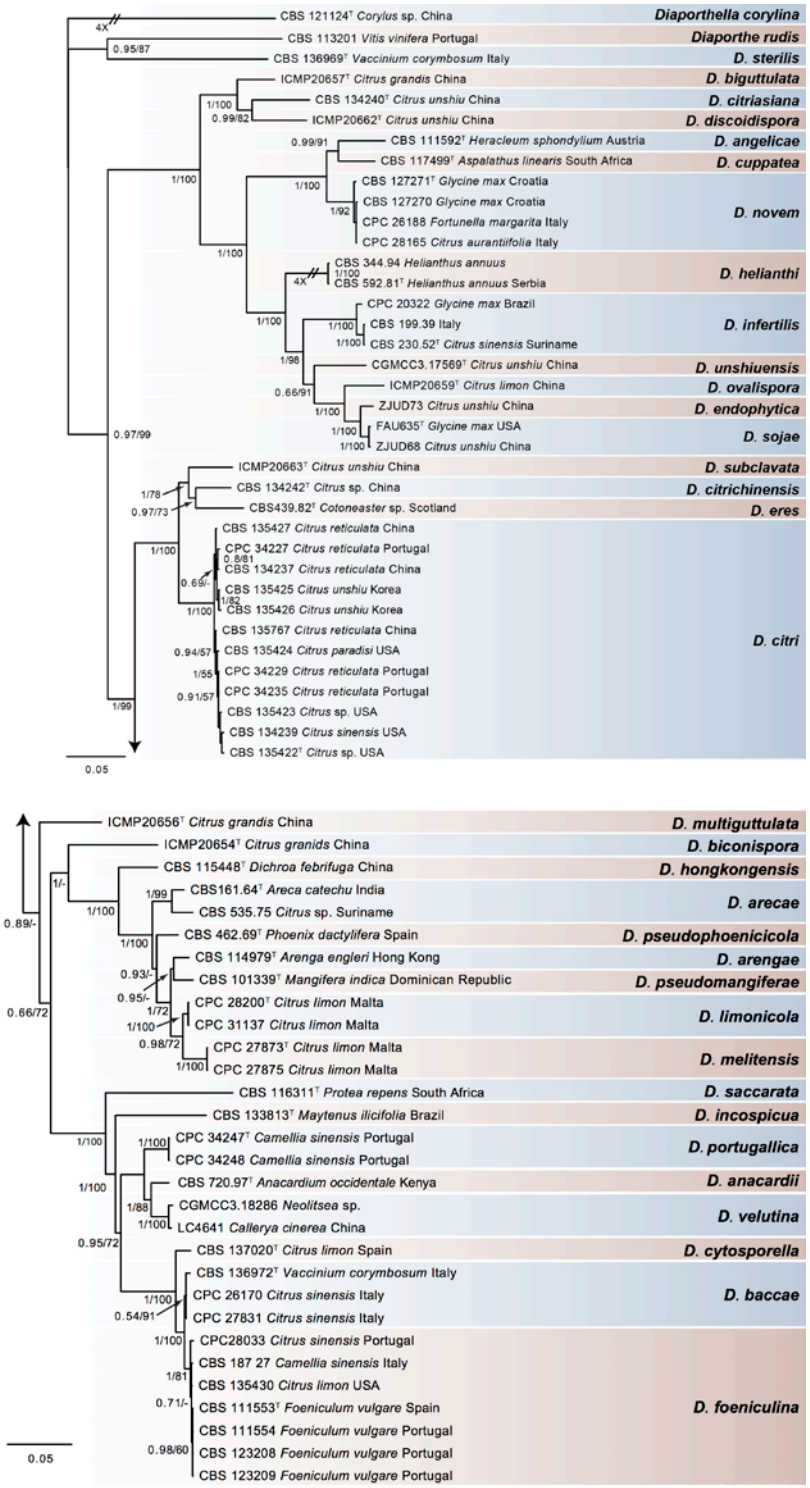

Figure 2. Consensus phylogram of 1,042 trees resulting from a Bayesian analysis of the combined ITS, *tub2*, *his3*, *tef1* and *cal* sequences. Bootstrap support values and Bayesian posterior probability values are indicated at the nodes. Substrate and country of origin are listed next to the strain numbers. ^{T} indicates ex-type strains. The tree was rooted to *Diaporthella corylina* (CBS 121124).

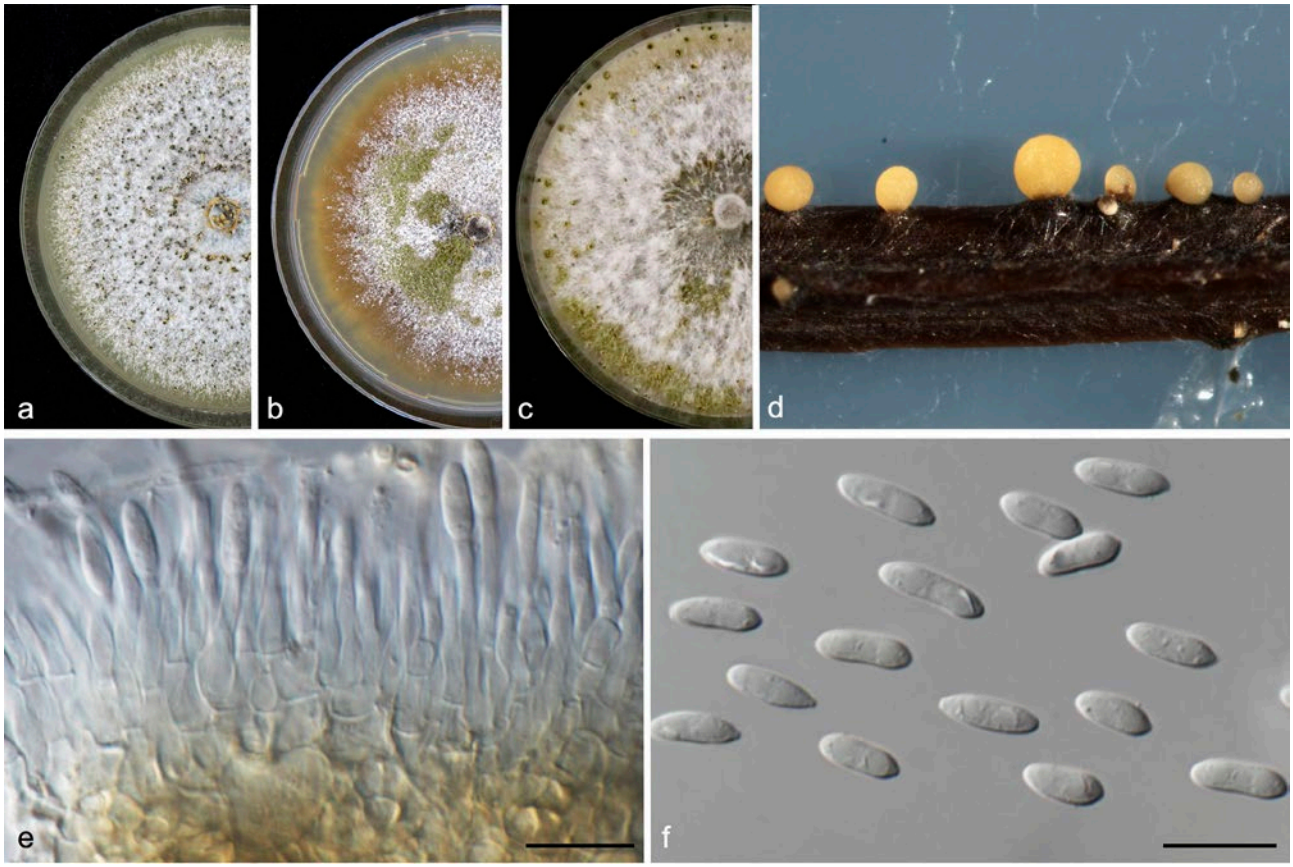

Figure 3. *Diaporthe citri* (CBS 144227). (a–c) Colonies after 7 d at 21°C on MEA, OA and PDA. (d) Conidiomata sporulating on PNA. (e) Conidiogenous cells. (f) Alpha conidia. Scale bars = 10 μm.

D. portugallica occurred on *Camellia sinensis*. *Diaporthe portugallica* is described based on specimens, and extype and other cultures linked to specimens.

Diaporthe citri (H.S. Fawc.) F.A. Wolf, J. Agric. Res. 33: 625. 1926. – Figure 3

Decaying twigs showing abundant conidiomatal production. Conidiomata pycnidial, solitary or aggregated under moist conditions, developing on twigs and on PNA, OA and MEA, deeply embedded in OA, erumpent, dark brown to black, up to 400 μm diam., yellowish translucent to cream spiral conidial cirrus or drops exuding from ostioles. Conidiophores hyaline, smooth, 1-septate, densely aggregated, cylindrical to ampuliform, straight to sinuous, 10–14 × 1.5–2 μm. Conidiogenous cells phialidic, hyaline, terminal, cylindrical, 5–10 × 1–1.5 μm, tapered towards apex. Paraphyses abundant among conidiophores, 20–30 × 1.5–1 μm. Alpha conidia aseptate, ovoid to ellipsoid, hyaline, smooth, mono- to biguttulate and acute at both ends, 7.5–10 × 2.5–3.5 μm, mean ± SD = 8.5 ± 0.8 × 2.9 ± 0.3 μm, L/B ratio = 2.9. Beta or gamma conidia not observed.

Culture characteristics: Colonies covering medium after 15 d at 21°C, surface mycelium flattened, dense and felt-like. Colonies on MEA and OA white, flat, with dense and felted mycelium, reverse cream to yellowish with age, with visible solitary or aggregated sporulating conidiomata at maturity. On PDA cream to brown with greenish sectors, reverse pale brown.

Materials examined: Portugal, Azores Islands, Sao Miguel, from shoot blight of *Citrus reticulata*, 17

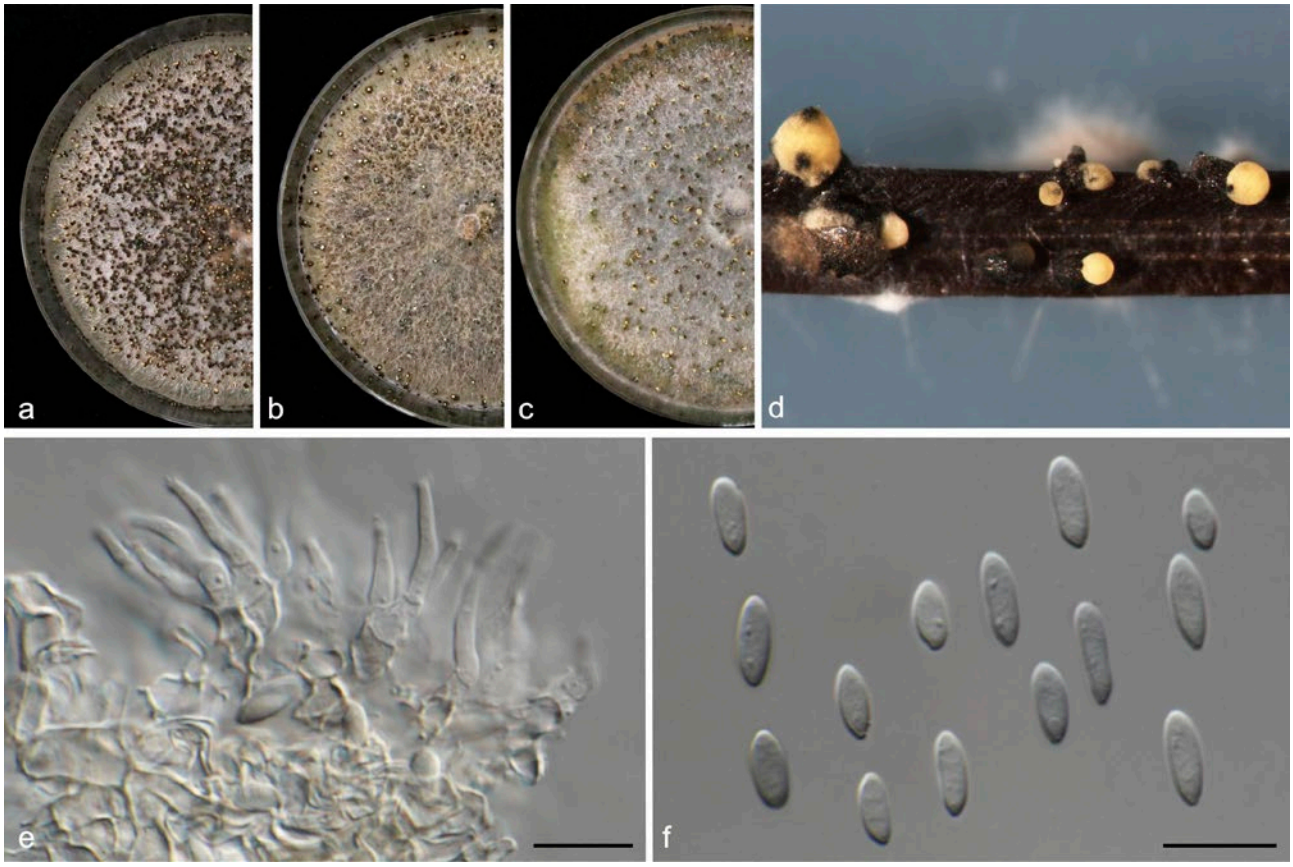

Figure 4. *Diaporthe portugallica* (CBS 144228). (a–c) Colonies after 7 d at 21°C on MEA, OA and PDA. (d) Conidiomata sporulating on PNA. (e) Conidiogenous cells. (f) Alpha conidia. Scale bars = 10 μm.

July 2017, *V. Guarnaccia* (CBS 144227 = CPC 34235); additional cultures from the same host and origin (cultures: CPC 34227, CPC 34229).

Notes: Perithecial ascomata and conidiomata of *D. citri* are commonly found on dead twigs, stems and fruits of *Citrus* affected by melanose and stem end rot (Fawcett, 1922). The fungus generally propagates on dead twigs. Fungal structures such as conidiomata or perithecia are never visible in these melanose lesions, and therefore the fungus cannot be observed in the infected leaves or fruit. In this study, several decayed twigs with conidiomata were observed during the sampling. *Diaporthe citri* is considered a key pathogen of *Citrus* species and has been confirmed from Brazil, China, Korea, New Zealand, and USA and is also reported widely throughout Asia, Australasia, and South America (Timmer *et al*., 2000; Mondal *et al*., 2007; Udayanga *et al*., 2014b). However, *D. citri* was never been reported from Europe before this study.

Diaporthe portugallica Guarnaccia, **sp. nov.**
MycoBank MB827265 – Figure 4

Etymology: Named after the country where it was collected, Portugal (ancient Latin name, *Portugallia*).

Lesions on leaves small, circular or irregular, brownish to purple, initially appearing on fully developed leaves, gradually enlarging, coalescing and becoming dark purple. Conidiomata pycnidial observed developing on lesions under moist conditions. Conidiomata solitary or aggregated in cultures on PNA, PDA, OA and MEA, deeply embedded in PDA,

erumpent, dark brown to black, 250–700 μm diam., yellowish translucent to cream conidial drops exuded from the ostioles.

Conidiophores hyaline, smooth, 1-septate, densely aggregated, cylindrical to ampulliform, straight or slightly curved, 5–22 × 1.5–4 μm. Conidiogenous cells phialidic, hyaline, terminal, cylindrical, 5–14 × 1–2 μm, tapered towards apex. Paraphyses not observed. Alpha conidia aseptate, fusoid, hyaline, mono- to biguttulate and acute at both ends, 5.5–8.5 × 1.5–3 μm, mean ± SD = 6.6 ± 0.8 × 2.2 ± 0.3 μm, L/B ratio = 3. Beta or and gamma conidia not observed.

Culture characteristics: Colonies covering medium after 10 d at 21°C, surface mycelium flattened, dense and felt-like. Colonies on MEA or OA at first white, becoming cream to yellowish, flat, with dense and felted mycelium, reverse pale brown with brownish dots with age, with visible solitary or aggregated sporulating conidiomata at maturity. On PDA cream to yellowish, reverse pale brown.

Materials examined: Portugal, Sao Miguel, Azores Islands, from leaf lesions of *Camellia sinensis*, 17 July 2017, *V. Guarnaccia* (CBS H-23474 – **holotype;** CBS 144228 = CPC 34247 – culture ex-type); additional culture from the same host and origin: (culture CPC 34248).

Notes: *Diaporthe portugallica* is only known from *Camellia sinensis* in Portugal. This species clusters in a subclade with *D. anacardii* and *D. velutina,* and can be identified by its unique *tub2*, *his3*, *tef1* and *cal* sequences. Morphologically, *D. portugallica* differs from *D. anacardii* and *D. velutina* in its shorter alpha conidia (5.5–8.5 *vs*. 6.5–9 μm for *D. anacardii* and 5.5–8.5 *vs*. 5.5–10 μm for *D. velutina*) and the absence of beta conidia, which are known in both *D. anacardii* and *D. velutina* (Gomes *et al*., 2013; Gao *et al*., 2017). Moreover, *D. portugallica* differs from the above described *D. citri* in its shorter alpha conidia (5.5–8.5 *vs* 7.5–10) and in its faster growing colonies on media.

Discussion

Diaporthe citri is a well-known pathogen causing serious melanose and stem-end rots of *Citrus* species (Timmer, 2000; Mondal *et al*., 2007). Several *Diaporthe* (or *Phomopsis*) species have been reported associated with *Citrus* and have previously been considered as synonyms of *D. citri*, such as *D. citrincola* and *P. californica*, *P. caribaea* and *P. cytosporella*, described from the Philippines, California, Cuba and Italy, respectively (Rehm, 1914; Fawcett, 1922). Using a polyphasic approach, several species have been determined to occurring on *Citrus*. Huang *et al*. (2013) reported *D. citri* as the predominant species in China and described two new taxa: *D. citriasiana* and *D. citrichinensis*. In another study, Huang *et al*. (2015) identified various *Diaporthe* species known as *Citrus* endophytes, such as *D. endophytica*, *D. eres*, *D. hongkongensis*, *D. sojae*, and different taxa clustering in the *D. arecae* species complex. They also described *D. biconispora*, *D. biguttulata*, *D. discoidispora*, *D. multigutullata*, *D. ovalispora*, *D. subclavata*, and *D. unshiuensis* as new species associated with *Citrus*. Udayanga *et al*. (2014b) re-assessed strains from China, Korea, New Zealand, and the USA within the *D. citri* clade, but no European strains were found clustering with this group.

After a major screening of fungal diseases of *Citrus* in Europe (Guarnaccia *et al*., 2017a, 2107b), molecular phylogenetic and morphological analyses were used to evaluate the diversity of several fungal genera, including *Diaporthe*. The results revealed a large diversity of species spanning several clades and species complexes. These included *D. baccae*, *D. infertilis*, *D. novem*, and two newly described species, *D. limonicola* and *D. melitensis*, causing severe cankers on host plants.

Similarly, recent studies have revealed a high diversity of *Diaporthe* species associated with *Camellia* spp. (Gao *et al*., 2016, 2017), demonstrating that 17 species occur on this host as endophytes and pathogens.

Considering these findings, the changes in species concepts and the poor investigation of *Diaporthe* species in Europe, new surveys were required to study the diversity within this genus related to tropical and sub-tropical hosts.

According to recent studies supported by molecular approaches, *D. citri* appeared to be absent from Europe (Udayanga *et al*., 2014b; Guarnaccia and Crous, 2017). However, based on the new samples investigated in the present study, this key pathogen of *Citrus* is confirmed from the Azores Islands. Thus, the present study represents the first report of *D. citri* associated with *Citrus* disease in Europe. Furthermore, this fungal species might threaten *Citrus* production, and could become a major limiting factor for future production.

This study has also identified two isolates from *Camellia sinensis* as belonging to a new species, described as *D. portugallica*.

Despite the increasing European distribution and economical importance of tropical and subtropical crops such as citrus and tea, knowledge of the fungal species associated with these species is still incomplete. Further studies are required to fully elucidate the host ranges, specificity, distribution and pathogenicity of these *Diaporthe* species.

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Accepted for publication: August 6, 2018