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RESEARCH ARTICLE

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Species limits in *Diaporthe*: molecular re-assessment of *D. citri*, *D. cytosporella*, *D. foeniculina* and *D. rudis*

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Abstract Species of *Diaporthe* are important plant pathogens of a wide range of hosts worldwide. In the present study the species causing melanose and stem end rot diseases of *Citrus* spp. are revised. Three species of *Diaporthe* occurring on *Citrus* are characterised, including *D. citri*, *D. cytosporella* and *D. foeniculina*. Morphology and phylogenetic analyses of the complete nuclear ribosomal internal transcribed spacer regions and partial sequences of actin, beta-tubulin, calmodulin and translation elongation factor 1-α were used to resolve species on *Citrus* and related *Diaporthe* species. *Diaporthe citri* occurs on *Citrus* throughout the *Citrus*-growing regions of the world. *Diaporthe cytosporella* is found on *Citrus* in Europe and California (USA). *Diaporthe foeniculina*, including the synonym *D. neotheicola*, is recognised as a species with an extensive host range including *Citrus*. *Diaporthe medusaea*, a name widely used for *D. citri*, was determined to be a synonym of *D. rudis*, a species with a broad host range. *Diaporthe citri* is delimited based on molecular phylogenetic analysis with the inclusion of the conserved ex-type and additional collections from different geographic locations worldwide. *Diaporthe cytosporella*, *D. foeniculina* and *D. rudis* are epitypified, fully described and illustrated with a review of all synonyms based on molecular data and morphological studies. Newly designed primers are introduced to optimise the amplification and sequencing of calmodulin and actin genes in *Diaporthe*. A discussion is provided of the utility of genes and the need for multi-gene phylogenies when distinguishing species of *Diaporthe* or describing new species.

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INTRODUCTION

The genus *Diaporthe* is an economically important group of plant pathogenic fungi causing diseases on a wide range of crops, ornamentals and forest trees (Farr et al. 2002a, b, Crous 2005, Udayanga et al. 2011). Accurate species identification is vital for controlling the diseases caused by these fungi as well as for implementing quarantine regulations (Rossman & Palm-Hernández 2008, Cai et al. 2011, Shivas & Cai 2012). Until recently, species of *Diaporthe* have been defined based on morphology and host association. However, patterns of host association and speciation have yet to be fully understood within *Diaporthe*. Multiple species of *Diaporthe* can often be found on a single host and a single species of *Diaporthe* can be associated with many different hosts (Crous 2005, van Niekerk et al. 2005, Santos & Phillips 2009, Diogo et al. 2010, Gomes et al. 2013). Using molecular data, much progress has been made towards identifying and characterising emerging pathogens, prevalent endophytes and saprobes in the genus *Diaporthe* (Santos & Phillips 2009, Diogo et al. 2010, Luongo et al. 2011, Udayanga et al. 2012a, b, Thomidis et al. 2013).

Modern systematic accounts of *Diaporthe* have used DNA sequence data as the most accurate means to circumscribe species within this genus (Rehner & Uecker 1994, Castlebury et al. 2003, van Rensburg et al. 2006). Markers used in contemporary phylogenetic revisions include the complete nuclear ribosomal internal transcribed spacer regions (ITS) and more recently partial sequences of actin (ACT), beta-tubulin (TUB), calmodulin (CAL), histone H3 (HIS), mating type genes (MAT 1-1-1 and MAT 1-2-1) and translation elongation factor 1-alpha (EF1-α) (van Niekerk et al. 2005, Diogo et al. 2010, Santos et al. 2010, Udayanga et al. 2012a, b, Gomes et al. 2013). Multi-gene phylogenetic species delineation has become the most effective tool for taxonomic studies of fungi compared to traditional mating experiments and morphology (Taylor et al. 2000, Dettman et al. 2003). Although the ITS region is often useful for identification of *Diaporthe* species, multi-gene phylogenetic analyses are required for accurate reconstruction of species boundaries and relationships (Udayanga et al. 2012a, Gomes et al. 2013). Intraspecific variation observed in ITS sequences in several species of *Diaporthe* can cause confusion in species recognition when used alone (Farr et al. 2002a, b, Santos et al. 2010).

Diaporthe citri is a pathogen that causes melanose and stem end rot disease of *Citrus* spp. throughout the world (Whiteside & Timmer 2000a, Mondal et al. 2007). Melanose disease can affect young leaves and fruits of different species and varieties of *Citrus* causing black blemishes on fruit rind and small, black, raised lesions often surrounded by yellow necrotic halos (Timmer & Kucharek 2001). Symptoms of the disease may vary with host variety, geographic location, seasonal occurrence, ecophysiological factors and severity of infection (Timmer & Fucik 1976, Whiteside 1977, Kucharek et al. 1983). The range of symptoms varies from small spots, scab lesions and mudcake to star melanose on different tissues of *Citrus* spp. (Timmer 2000, Whiteside & Timmer 2000a, Agostini et al. 2003). Perithecia and pycnidia are only produced on dead and dying twigs and on fruit affected by stem end rot. Because perithecia are rarely formed, conidia produced by pycnidia are the primary source of inoculum (Bach & Wolf 1928, Kuhara 1999).

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Table 1 Isolates used and genes sequenced in this study.

Species	Isolate no.[1]	Host	Origin	Collector / contributor	GenBank accession no.				
					ITS	EF 1-α	TUB	ACT	CAL
D. alleghaniensis	CBS 495.72*	*Betula alleghaniensis*	Canada: Ontario	R. Arnold	FJ889444	GQ250298	KC843228	JQ807299	KC343249
D. australafricana	CBS 113487*	*Vitis vinifera*	South Africa	L. Mostert	KC343039	KC843099	JX275457	KC843265	JX197448
	CBS 111886	*Vitis vinifera*	Australia	R.W.A. Schepers	KC343038	KC343764	KC344006	–	KC343280
	AR5209 = CBS 135771	*Persea americana*	USA: California	Akif Eskalen	KF199875	KF199877	KF199879	KF199883	KF199881
	AR5210 = CBS 135772	*Persea americana*	USA: California	Akif Eskalen	KF199876	KF199878	KF199880	KF199884	KF199882
D. canthii	CBS 132533*	*Canthium inerme*	South Africa	P.W. Crous	JX069864	KC843120	KC843230	KC843291	KC843174
D. citri	AR3405* = CBS 135422	*Citrus* sp.	USA: Florida	L.W. Timmer	KC843311	KC843071	KC843187	KC843234	KC843157
	AR3404	*Citrus* sp.	USA: Florida	L.W. Timmer	KC843316	KC843076	KC843192	KC843239	KC843162
	AR3406	*Citrus* sp.	USA: Florida	L.W. Timmer	KC843320	KC843080	KC843196	KC843243	KC843166
	AR4469 = CBS 135423	*Citrus* sp.	USA: Florida	L.W. Timmer	KC843321	KC843081	KC843197	KC843244	KC843167
	AR4470	*Citrus* sp.	USA: Florida	L.W. Timmer	KC843318	KC843078	KC843194	KC843241	KC843164
	AR4471	*Citrus* sp.	USA: Florida	L.W. Timmer	KC843317	KC843077	KC843193	KC843240	KC843163
	FAU583 = CBS 135424	*Citrus paradisi*	USA: Florida	F.A. Uecker	KC843327	KC843087	KC843203	KC843250	KC843173
	AR3403	*Citrus* sp.	USA: Florida	L.W. Timmer	KC843310	KC843070	KC843186	KC843233	KC843156
	AR4473	*Citrus* sp.	USA: Florida	L.W. Timmer	KC843319	KC843079	KC843195	KC843242	KC843165
	AR3407	*Citrus* sp.	USA: Florida	L.W. Timmer	KC843313	KC843073	KC843189	KC843236	KC843159
	AR4472	*Citrus* sp.	USA: Florida	L.W. Timmer	KC843312	KC843072	KC843188	KC843235	KC843158
	AR4364 = CBS 135425	*Citrus* unshiu cv. juwadeun	Korea: Odeung-dong	S.K. Hong	KC843326	KC843086	KC843202	KC843249	KC843172
	AR4370 = CBS 135426	*Citrus* unshiu cv. juwadeun	Korea: Odeung-dong	S.K. Hong	KC843324	KC843084	KC843200	KC843247	KC843170
	AR4350	*Citrus* unshiu cv. juwadeun	Korea: Odeung-dong	S.K. Hong	KC843325	KC843085	KC843201	KC843248	KC843171
	CBS 135767	*Citrus reticulata*	China	D. Udayanga	KC843322	KC843082	KC843198	KC843245	KC843168
	DA103 = CBS 135427	*Citrus reticulata*	China	D. Udayanga	KC843323	KC843083	KC843199	KC843246	KC843169
	ICMP 10355	*Citrus reticulata*	New Zealand: Kerikeri	G.J. Samuels	KC843314	KC843074	KC843190	KC843237	KC843160
	ICMP 6981	*Citrus* sp.	USA: Texas	G.J. Samuels	KC843315	KC843075	KC843191	KC843238	KC843161
D. cotoneastri	CBS 439.82*	*Cotoneaster* sp.	UK: Scotland	H. Butin	FJ889450	GQ250341	JX275437	KC843231	JX197429
	DP0667 = CBS 135428	*Juglans cinerea*	USA: North Carolina	S. Anagnostakis	KC843328	KC843121	KC843229	KC843232	KC843155
D. cynaroidis	CBS 122676*	*Protea cynaroidis*	South Africa	P.W. Crous	EU552122	EU552093	KC344026	–	KC343300
D. cytosporella	FAU461 = CBS 137020	*Citrus limon*	Spain	M. Palm	KC843307	KC843116	KC843221	KC843285	KC843141
	AR5148	*Citrus sinensis*	USA: California	A. Eskalen	KC843308	KC843117	KC843222	KC843286	KC843142
	AR5149*	*Citrus sinensis*	USA: California	A. Eskalen	KC843309	KC843118	KC843222	KC843287	KC843143
D. foeniculina	FAU460	*Citrus limon*	Spain	M. Palm	KC843304	KC843113	KC843218	KC843282	KC843138
	MEP1289-1	*Citrus limon*	Spain	D. Grenier	KC843305	KC843114	KC843219	KC843283	KC843139
	FAU462 = CBS 135429	*Citrus limon*	Spain	M. Palm	KC843292	KC843101	KC843206	KC843270	KC843126
	ICMP 6986	*Citrus limon*	New Zealand: Hope	–	KC145897	KC145989	–	–	–
	ICMP 6970	*Acacia* sp.	New Zealand: Auckland	G.J. Samuels	KC145896	KC145984	–	–	–
	ICMP 12285	*Juglans regia*	New Zealand	K. Knight	KC145853	KC145937	–	–	–
	ICMP 6987	*Malus domestica*	New Zealand: Nelson	G.J. Samuels	KC145894	KC145990	–	–	–
	ICMP 17058	*Paraserianthes lophantha*	New Zealand: Auckland	C.F. Hill	KC145842	KC145977	–	–	–
	ICMP 11892	*Fuchsia excorticata*	New Zealand: Taupo	J.M. Young	KC145898	KC145931	–	–	–
	DP0454	*Ribes nigrum*	New Zealand: Nelson	C.F. Hill	KC843297	KC843106	KC843211	KC843275	KC843131
	AR3607 = STE-U2654	*Vitis vinifera*	South Africa	L. Mostert	AF230743	JQ807419	KC843204	JQ807344	KC843123
	DP0392 = CBS 111554	*Foeniculum vulgare*	Portugal: Marcos	A.J.L. Phillips	KC843296	KC843105	KC843210	KC843274	KC843130
	DP0391 = CBS 111553*	*Foeniculum vulgare*	Portugal: Marcos	A.J.L. Phillips	KC843295	KC843104	KC843209	KC843273	KC843129
	AR5151	*Citrus latifolia*	USA: California	A. Eskalen	KC843303	KC843112	KC843217	KC843281	KC843137

D. foeniculina (cont.)	AR5142 = CBS 135430	*Citrus limon*	USA: California	A. Eskalen	KC843301	KC843110	KC843215	KC843279	KC843135
	AR5145	*Citrus limon*	USA: California	A. Eskalen	KC843306	KC843115	KC843220	KC843284	KC843140
	AR5147	*Citrus limon*	USA: California	A. Eskalen	KC843299	KC843108	KC843213	KC843277	KC843133
	AR5144	*Citrus limon*	USA: California	A. Eskalen	KC843302	KC843111	KC843216	KC843280	KC843136
	AR5143	*Citrus limon*	USA: California	A. Eskalen	KC843294	KC843103	KC843208	KC843294	KC843128
	AR5146	*Citrus limon*	USA: California	A. Eskalen	KC843298	KC843107	KC843212	KC843298	KC843132
	AR5150 = CBS 135431	*Citrus latifolia*	USA: California	A. Eskalen	KC843293	KC843102	KC843207	KC843293	KC843127
	AR5152	*Citrus latifolia*	USA: California	A. Eskalen	KC843300	KC843109	KC843214	KC843300	KC843134
D. foeniculina (syn. *P. theicola*)	CBS 187.27*	*Camellia sinesis*	Italy	M. Curzi	DQ286287	DQ286261	JX275463	JQ807298	KC843122
D. foeniculina (syn. *D. neotheicola*)	CBS 123208*	*Foeniculum valgare*	Portugal	A.J.L. Phillips	EU814480	GQ250315	JX275464	KC843269	KC843125
D. foeniculina (syn. *D. rhusicola*)	CBS 129528*	*Rhus pendulina*	South Africa	P.W. Crous	JF951146	KC843100	KC843205	KC843268	KC843124
D. helianthi	CBS 592.81	*Helianthus annuus*	Serbia	M Muntanola-Cvetkovic	AY705842	GQ250308	JX275465	KF199885	JX197454
D. pterocarpi	MFLUCC10-588	*Magnolia* sp.	Thailand: Chiang Rai	D. Udayanga	JQ619900	JX275417	JX275461	KC843289	JX197452
	MFLUCC10-575 = CBS 137021	*Pterocarpus indicus*	Thailand: Chiang Rai	N.F. Wulandari	JQ619901	JX275418	JX275462	KC843288	JX197453
	MFLUCC10-571* = CBS 135768	*Pterocarpus indicus*	Thailand: Chiang Rai	D. Udayanga	JQ619899	JX275416	JX275460	KC843290	JX197451
D. pterocarpicola	MFLUCC10-580a* = CBS 135432	*Pterocarpus indicus*	Thailand: Chiang Rai	D. Udayanga	JQ619887	JX275403	JX275441	KF214779	JX197433
	MFLUCC10-580b	*Pterocarpus indicus*	Thailand: Chiang Rai	N.F. Wulandari	JQ619889	JX275405	JX275443	KF214780	JX197435
D. rudis	AR3654 = CBS 135433	*Rosa canina*	Austria	W. Jaklitsch	KC843338	KC843097	KC843184	KC843262	KC843153
	DP0423	*Pyrus* sp.	New Zealand	W. Kandula	KC843335	KC843094	KC843181	KC843258	KC843150
	DP0350 = CBS 135434	*Castanea* sp.	New Zealand: Churchill	H. Smith	KC843338	KC843098	KC843185	KC843264	KC843154
	ICMP 12522	*Ileostylis micranthus*	New Zealand: Central Otago	P.R. Johnston	KC145906	KC145940	–	–	–
	ICMP 15267	*Alnus* sp.	New Zealand: Mid Canterbury	K. Eade	KC145839	KC145998	–	–	–
	ICMP 16419	*Castanea sativa*	New Zealand: Mid Canterbury	H.C. Smith	KC145904	KC145976	–	–	–
	ICMP 7025	*Vaccinium corymbosum*	New Zealand: Waikato	P.R. Johnston	KC145885	KC145995	–	–	–
	AR3422 = CBS 109292*	*Laburnum anagyroides*	Austria	W. Jaklitsch	KC843331	KC843090	KC843177	KC843254	KC843146
	AR3646	*Epilobium angustifolium*	Canada: British Columbia	M. Barr	KC843330	KC843089	KC843176	KC843253	KC843145
	AR3478 = CBS 109768	*Epilobium angustifolium*	Canada: British Columbia	M. Barr	KC843329	KC843088	KC843175	KC843252	KC843144
	DA243 = CBS 135435	*Brugmansia* sp.	Germany	R. Schumacher	KC843332	KC843091	KC843178	KC843255	KC843147
	DA244	*Brugmansia* sp.	Germany	R. Schumacher	KC843334	KC843093	KC843180	KC843257	KC843149
	ER285A = CBS 135437	*Acer opalus*	Italy	E. Camporesi	KC843336	KC843095	KC843182	KC843259	KC843151
	ER286C	*Acer opalus*	Italy	E. Camporesi	KC843337	KC843096	KC843183	KC843260	KC843152
	ER286D	*Acer opalus*	Italy	E. Camporesi	KC843333	KC843092	KC843179	KC843256	KC843148
	DPG01	*Vitis viniferra*	Italy	X.Z. Liu	JQ619896	JX275412	JX27545	KC843261	JX197446
	DPG02	*Vitis viniferra*	Italy	X.Z. Liu	JQ619897	JX275413	JX275456	KC843263	JX197447
D. rudis (syn. *D. viticola*)	CBS 113201*	*Vitis vinifera*	Portugal	A.J.L. Phillips	AY485750	GQ250327	JX275454	KC843251	JX197445
D. thunbergii	MFLUCC10-576a* = CBS 135769	*Thunbergia laurifolia*	Thailand: Chiang Mai	D.S. Manamgoda	JQ619893	JX275409	JX275449	KF199886	JX197440
	MFLUCC10-576b	*Thunbergia laurifolia*	Thailand: Chiang Mai	S.C. Karunaratne	JQ619894	JX275410	JX275450	KF199887	JX197441
	MFLUCC10-576c	*Thunbergia laurifolia*	Thailand: Chiang Mai	D. Udayanga	JQ619895	JX275411	JX275451	KF199888	JX197442
D. vaccinii	CBS 160.32*	*Vaccinium macrocarpon* (as *Oxycoccus macrocarpos*)	USA: Massachusetts	C. Shear	AF317578	GQ250326	JX275436	JQ807297	KC343470
	FAU446 = CBS 122113	*Vaccinium macrocarpon*	USA: Massachusetts	F. Caruso	U11317,U11367	JQ807398	KC843224	JQ807322	KC849455
	DF5032 = CBS 135436	*Vaccinium corymbosum*	USA: North Carolina	D. Farr	AF317570	JQ807380	KC843225	JQ807303	KC849456
	FAU633	*Vaccinium* sp.	USA: Michigan	–	U11360,U11414	JQ807413	KC843226	JQ807338	KC849457
	FAU468	*Vaccinium macrocarpon*	USA: New Jersey	–	U113327,U11377	JQ807399	KC843227	JQ807323	KC849458

[1] AR, DA, DF, DLR, DP, DPG, ER, FAU, MEP: isolates in SMML culture collection, USDA-ARS, Beltsville, MD, USA; CBS: CBS-KNAW Fungal Biodiversity Centre, Utrecht, The Netherlands; ICMP: International Collection of Microorganisms from Plants, Landcare Research, New Zealand; MFLUCC: Mae Fah Luang University Culture Collection; STE-U: University of Stellenbosch culture collection, Stellenbosch, South Africa; * = ex-type cultures.

Although the biology and epidemiology of melanose are well studied, the phylogenetic relationships of the causal organisms, genetic variability and population structure have not been investigated (Burnett 1962, Moherek 1970, Mondal et al. 2004, 2007). *Diaporthe* pathogens of *Citrus* are usually identified as *D. citri* in taxonomic and plant pathological studies and regional checklists (Timmer & Kucharek 2001, Udayanga et al. 2011). In addition to *D. citri*, several other species of *Diaporthe* have been reported from *Citrus*, often as *Phomopsis*. These include *D. citrincola* described from the Philippines, *Phomopsis californica* from California, *P. caribaea* from Cuba and *P. cytosporella* (as *Phoma cytosporella*) from Italy, which have all previously been considered synonyms of *D. citri* (Rehm 1914, Fawcett 1922, 1936, Horne 1922). Yamato (1976) recognised four unidentified morphological species on *Citrus* spp. in Japan. *Diaporthe citri* was also considered a synonym of *D. medusaea* by Wehmeyer (1933) who also listed *D. californica*, *P. citri* and *P. citrincola* as host or ecological forms of *D. medusaea*. Others followed this synonymy including Punithalingam & Holliday (1973) and Whiteside & Timmer (2000a). The name *D. medusaea* is used in several articles and checklists for the fungus causing melanose and stem end rot, therefore, the true host range and geographic distribution of *D. citri* are difficult to determine (Kobayashi 1970, Pantidou 1973, French 1987).

Given the vague species concept of *D. citri* and its broad application, a modern taxonomic and phylogenetic reappraisal of *D. citri* and other *Diaporthe* species on *Citrus* is necessary. In this study, we analyse DNA sequence data from recent collections of *Diaporthe* isolated from *Citrus* and other hosts in Asia, Europe and the United States to accurately identify the taxa associated with *Citrus*. The objectives of this study are: 1) to define the species of *Diaporthe* on *Citrus* worldwide based on phylogenetic analysis of multi-gene sequence data, the genealogical sorting index and morphological characters; 2) to resolve taxonomic and nomenclatural uncertainty by providing modern descriptions for *D. citri* and designating epitypes for *D. cytosporella*, *D. foeniculina* and *D. rudis* and their synonyms; 3) to evaluate their host range and geographic distribution; and 4) to assess the utility of individual genes for accurate circumscription of these species.

MATERIALS AND METHODS

Isolates and morphology

Strains of *Diaporthe* from *Citrus* hosts were obtained from China, Korea, New Zealand, Spain and the United States (California, Florida and Texas). These strains have been isolated from specimens with typical symptoms of *Citrus* melanose and stem end rot as well as saprobes on twigs and branches. Isolates from other hosts were obtained from culture collections including CBS (The Netherlands), Fawcett Laboratory, University of California, Riverside (CA, USA), ICMP (New Zealand), MFLUCC (Thailand) and the SMML, USDA-ARS (MD, USA) and various contributors listed in Table 1. Morphological descriptions are based on sporulating pycnidia from inoculated alfalfa stems placed on 1.5 % water agar (WA) for living cultures as well as type and other specimens. Digital images of fruiting bodies were captured using a Discovery V20 stereomicroscope and AxioCam digital camera (Carl Zeiss Microscopy, Thornwood, NY, USA) imaging system. Whenever possible, 20–30 measurements were made of the structures mounted in 5 % KOH using a Carl Zeiss Axioplan2 compound light microscope using the 40× or 100× objectives. The extreme measurements are given in parentheses with mean and standard deviation. Three sets of duplicate cultures of each isolate were used for determining colony characters on potato-dextrose agar (PDA, Becton, Dickinson and Company, Franklin Lakes, NJ, USA) at 25 °C in the dark following the methods of Brayford (1990). Colony diameters on PDA were recorded at intervals of 24 h for 1 wk and used to calculate the growth rate of eight replicates per isolate. After 1 wk, colony size and colour of the colonies (Rayner 1970) and zonation were recorded.

Table 2 Primers used and alternative new primers designed for current study and optimised PCR protocols.

Locus	Primers	Optimised PCR protocols	Approximate sizes of the PCR amplicons obtained	References for primers[a] & protocols[b]
ACT	ACT-512F: ATGTGCAAGGCCGGTTTCGC	(95 °C: 30 s, 55 °C: 50 s, 72 °C: 1 min) ×39 cycles for ACT512F/ACT783R	280bp (ACT512F/ACT783R)	Carbone & Kohn 1999[a]
	ACT-783R (5'-TACGAGTCCTTCTGGCCCAT-3')	(95 °C: 30 s, 58 °C: 50 s, 72 °C: 1 min) ×39 cycles for ACT512F/ACT878R	380bp (ACT512F/ACT878R)	This study[a,b]
	ACT878R (new): ATCTTCTCC ATGTCGTCCCAG			
TUB	Bt-2a: GGTAACCAAATCGGTGCTGCTTTC	(95 °C: 30 s, 58 °C: 50 s, 72 °C: 1 min) ×39 cycles	500bp	Glass & Donaldson 1995[a] Udayanga et al. 2012a[b]
	BT-2b: ACCCTCAGTGTAGTGACCCTTGGC			
CAL	CAL-228F: GAGTTCAAGGAGGCCTTCTCCC	(95 °C: 30 s, 55 °C: 50 s, 72 °C: 1 min) ×39 cycles for CAL228F/CAL737R	500bp (CAL228F.CAL737R)	Carbone & Kohn 1999[a]
	CAL-737R: CATCTTCTGGCCATCATGG	(95 °C: 30 s, 52 °C: 50 s, 72 °C: 1 min) ×39 cycles for CL1/CL2A	800bp (CL1/CL2A)	O'Donnell et al. 2000[a]; Udayanga et al. 2012a[b]
	CL1F: GARTWCAAGGAGGCCTTCTC	(95 °C: 30 s, 51 °C: 50 s, 72 °C: 1 min) ×39 cycles for CAL563F/CL2A	570bp (CAL563F/CL2A)	This study[a,b]
	CL2A : TTTTTGCATCATGAGTTGGAC			
	CAL563F (new): GACAAATCA CCACCAARGAGC			
EF1-α	EF1-728F: CATCGAGAAGTTCGAGAAGG	(95 °C: 30 s, 58 °C: 50 s, 72 °C: 1 min) ×39 cycles	350bp	Carbone & Kohn 1999[a] Udayanga et al. 2012a[b]
	EF1-986R: TACTTGAAGGAACCCTTACC			
ITS	ITS1: TCCGTAGGTGAACCTGCGG	(95 °C: 30 s, 55 °C: 50 s, 72 °C: 1 min) ×39 cycles	600bp	White et al. 1990[a] Udayanga et al. 2012a[b]
	ITS4: TCCTCCGCTTATTGATATGC			

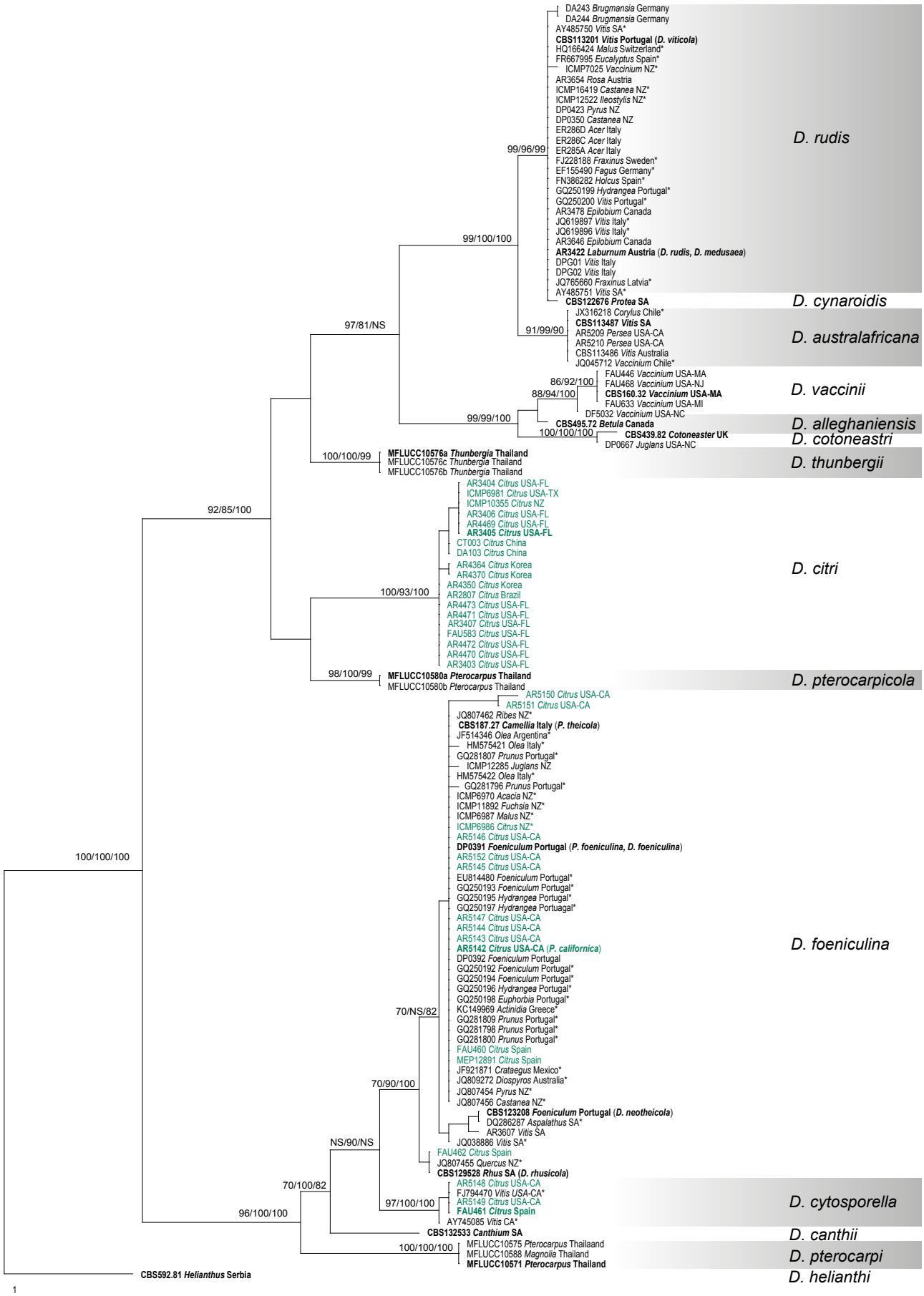

Fig. 1 One of the 45 equally parsimonious trees generated from the analysis of the ITS sequence alignment. MP/RAxML bootstrap values/Bayesian posterior probabilities ≥ 70 % are displayed above or below each branch. Ex-type and ex-epitype culture numbers are in **bold**. GenBank accessions are given for downloaded sequences and isolate codes for the newly generated sequences annotated with host and location. Isolates from *Citrus* are indicated in green. ITS sequences obtained from GenBank verified as *D. cytosporella*, *D. foeniculina* and *D. rudis* are indicated with an asterisk. The tree is rooted with *D. helianthi* (CBS 592.81).

DNA extraction, PCR and sequencing

Mycelial scrapings (50–60 mg) from the leading edge of cultures on PDA, incubated for 4–5 d at 25 °C were harvested and lysed in tubes containing 500 µm garnet media and a 6 mm zirconium bead (OPS Diagnostics, Lebanon, NJ, USA) with the Fast Prep FP120 (Fischer Scientific Inc, Waltham, MA, USA) for 20 s. Genomic DNA was extracted with the DNeasy Plant Mini Kit (Qiagen, Inc., Valencia, CA, USA) according to the manufacturer's instructions. The ACT, CAL, EF1-α, ITS and TUB gene regions were amplified following the conditions outlined in Table 2 on a Bio-Rad Dyad Peltier thermal cycler in a 25 µL reaction volume: 10–15 ng genomic DNA, 12.5 µL Quick load Taq 2x Master Mix (New England BioLabs, Ipswich, MA, USA), 1 µL 10 mM of each primer and 1 % DMSO with volumes adjusted to 25 µL with nuclease-free water.

PCR products were visualised by electrophoresis in 1 % agarose gels stained with SYBR Safe DNA Gel Stain (Invitrogen, Eugene, OR, USA). Excess primers and dNTPs were removed from PCR amplification mixtures with ExoSAP-IT (USB Corp., Cleveland, OH, USA) according to the manufacturer's instructions. Amplicons were sequenced using the BigDye Terminator v. 3.1 cycle sequencing kit (Life Technologies, Grand Island, NY, USA) on an Applied Biosystems 3130xl Genetic Analyzer using the primers used to amplify each of the gene regions (Table 2).

New primer design and PCR optimisation

Complete failure of amplification of the isolates in the *D. foeniculina* clade (Fig. 1) and evidence of non-specific priming in the sequences of the CAL gene region was observed when using the CAL-228F/CAL-737R (Carbone & Kohn 1999) or CL1/CL2A (O'Donnell et al. 2000) primer sets. Additionally frequent failures of sequencing when using ACT-512F/ACT-783R (Carbone & Kohn 1999) were encountered in this clade. On closer inspection of ACT and CAL multiple sequence alignments for *Diaporthe*, non-specific binding sites were observed for both ACT-783R and CAL-228F primers (Carbone & Kohn 1999).

A sequence alignment consisting of both complete and partial sequences of CAL from *Neurospora crassa* (L02964), *Pyricularia grisea* (AF089808), *Apiognomonia errabunda* (DQ313615, DQ313596), *Ophiognomonia clavigignenti-juglandacearum* (GU993756), *Diaporthe lusitanicae* (JX197416), *D. melonis* (JX197417), *D. ampelina* (as *Phomopsis viticola* in GenBank) (AY745032), *D. phaseolorum* (JX197418, JX197419), *D. rudis* (JX197447), *D. sclerotiodes* (JX197420) and *D. eres* (as *Phomopsis* sp. OH-48 in GenBank, AY745025) was generated to design a new internal forward primer (CAL563F) located in the region corresponding to exon 4 in the *N. crassa* calmodulin gene (Table 2).

A sequence alignment of both complete and partial sequences of the actin gene from *Neurospora crassa* (U78026), *Gaeumannomyces graminis* (AY424309), *Hypocrea orientalis* (JQ238613), *Magnaporthe oryzae* (XM003719823), *Fusarium oxysporum* f. *cubense* (JQ965663), *Thielavia terrestris* (XM003649706), *Nectria haematococca* (XM003050001), *Colletotrichum gloeosporioides* f. sp. *malvae* (AF112537), *Cleistogenes songorica* (FJ972820), *Verticillium alboatrum* (XM003008431), *Phaeosphaeria nodorum* (XM001791742), *Pyrenophora teres* f. *teres* (XM003298028), *Gibberella zeae* (XM387511), *Diaporthe neotheicola* (JQ807344), *D. vaccinii* (JQ807322) and *D. ampelina* (as *Phomopsis viticola* in GenBank, JN230390) revealed that non-specific binding sites for the ACT-783R (Carbone & Kohn 1999) primer exist in *Diaporthe* resulting in the frequent failures of amplification and sequencing. To eliminate these problems a new reverse primer (ACT-878R) was designed. The primer combination of ACT-512F/ACT-878R was used for amplification with isolates in which amplification failed with the primer combination ACT-512F/783R in this study (Table 1).

Gradient PCR and reagent optimisations were used to develop the standard protocols for amplification of ACT and CAL genes (Table 2). Twelve reactions across an annealing temperature gradient of 50–65 °C for each of the test isolates were performed in three replicates. Optimal annealing temperatures were determined by the intensity of the amplicons visualized by agarose gel electrophoresis. Also the addition of 1 % DMSO to the PCR mix was used to enhance the reaction. Existing and newly designed primers used to amplify ACT and CAL were evaluated for thermal properties, hairpin formation and self-complementarities using the online platforms of OligoCalc (http://www.basic.northwestern.edu/biotools/oligocalc.html) and the Sequence Manipulation Suite (http://www.bioinformatics.org/sms2/pcr_primer_stats.html).

Sequence alignment and phylogenetic analysis

Raw sequences were assembled with Sequencer v. 4.9 for Windows (Gene Codes Corp., Ann Arbor, MI, USA). The assembled consensus sequences were initially aligned with Clustal W and optimised with MAFFT v. 7 using default settings (http://mafft.cbrc.jp/alignment/server/) and adjusted manually where necessary (Katoh & Standley 2013). Newly generated ITS sequences were analysed with all available type-derived sequences listed in Udayanga et al. (2011, 2012a) to determine the preliminary identifications of the isolates. Sequences from isolates recognised as *D. citri*, *D. cytosporella*, *D. foeniculina* and *D. rudis* were analysed with a selected set of additional ITS sequences available in GenBank identified using the NCBIs BLAST search and authenticated by the publications where sequences were reported. To more fully resolve closely related species, single gene phylogenies were inferred for ACT, CAL, EF1-α, ITS and TUB and a selected set of isolates were subjected to a multigene combined analysis. Trees were rooted with *D. helianthi* (CBS 592.81), which was determined to fall outside of the clades included in this study (trees not shown).

PAUP v. 4.0b10 (Swofford 2002) was used to perform maximum parsimony analyses. Trees were inferred using the heuristic search option with 1 000 random sequence additions. Maxtrees were unlimited, branches of zero length were collapsed and all multiple equally parsimonious trees were saved. Descriptive tree statistics for parsimony (Tree Length (TL), Consistency Index (CI), Retention Index (RI), Related Consistency Index (RC) and Homoplasy Index (HI)) were calculated for trees generated in a parsimony analysis. Evolutionary models for phylogenetic analyses were selected independently for each locus using MrModeltest v. 2.3 (Nylander 2004) under the Akaike Information Criterion (AIC) implemented in both PAUP v. 4.0b10 and MrBayes v. 3. Phylogenetic reconstructions of concatenated and individual gene-trees were performed using both Bayesian Inference (BI) Markov Chain Monte Carlo and Maximum Likelihood (ML) criteria. Bayesian reconstructions were performed using MrBayes v. 3.1.2 (Huelsenbeck & Ronquist 2001). Two simultaneous analyses, each consisting of six Markov chains, were run for 1 000 000 generations with trees sampled every 100 generations resulting in 20 000 total trees. The first 2 000 trees, representing the burn-in phase of the analyses were discarded from each run and the 16 000 remaining trees were used for calculating posterior probabilities (PP).

Maximum likelihood trees were generated using the software RAxML v 7.4.2 Black Box (Stamatakis 2006, Stamatakis et al. 2008) in the CIPRES Science Gateway platform (Miller et al. 2010). For the combined dataset all free modal parameters were estimated by RAxML with ML estimate of 25 per site rate categories. The combined five-gene dataset was partitioned by gene region. The RAxML software accommodated the GTR model of nucleotide substitution with the additional options of modelling rate heterogeneity (Γ) and proportion invariable sites (I).

These analyses utilised the rapid bootstrapping algorithm in RAxML in XSEDE high performance online computing service. Phylogenetic trees and data files were viewed in MEGA v. 5 (Tamura et al. 2011), TreeView v. 1.6.6 (Page 1996) and FigTree v. 1.4 (Rambaut & Drummond 2008).

Genealogical sorting index

The rooted gene genealogies resulting from each of the single gene analyses of ACT, CAL, EF1-α, ITS and TUB were submitted to the genealogical sorting index (*gsi*) parallel computing resource (http://www.genealogicalsorting.org/) for analysis. The *gsi* estimates the degree of exclusive ancestry of individuals in labelled predefined groups in a rooted tree (Cummings et al. 2008). Values range from 0 to 1 with 0 corresponding to a lack of genealogical divergence from other groups and 1 corresponding to monophyly for the predetermined clade (or species). Each isolate was assigned to a predetermined species based on Genealogical Concordance Phylogenetic Species Recognition (GCPSR) and the *gsi* was calculated for the best tree selected in parsimony analysis and for all trees using 10 000 permutations (Cummings et al. 2008). The assignment of each tip to groups representing the recognised species was identical for

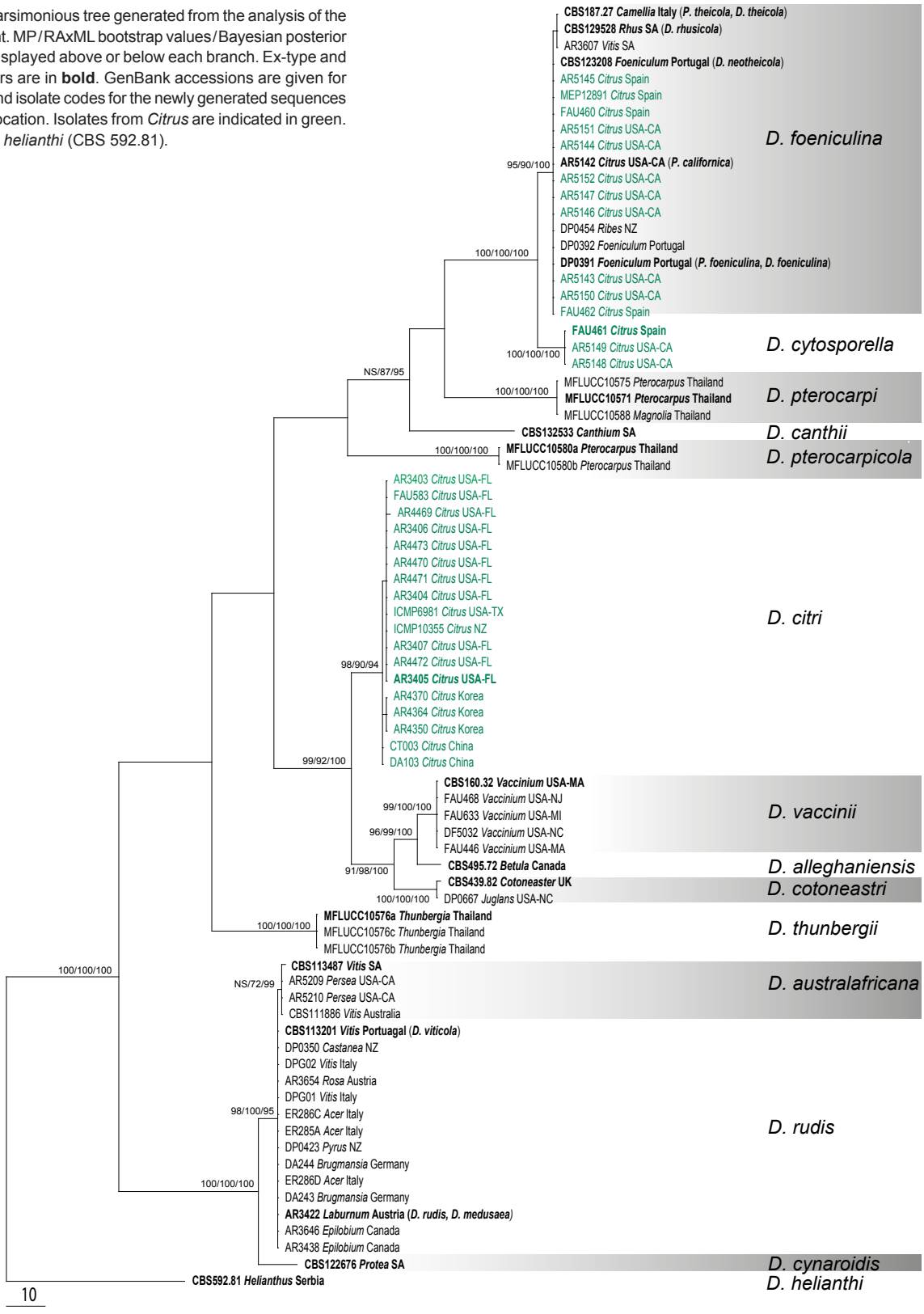

Fig. 2 The single most parsimonious tree generated from the analysis of the EF1-α sequence alignment. MP/RAxML bootstrap values/Bayesian posterior probabilities ≥ 70 % are displayed above or below each branch. Ex-type and ex-epitype culture numbers are in **bold**. GenBank accessions are given for downloaded sequences and isolate codes for the newly generated sequences annotated with host and location. Isolates from *Citrus* are indicated in green. The tree is rooted with *D. helianthi* (CBS 592.81).

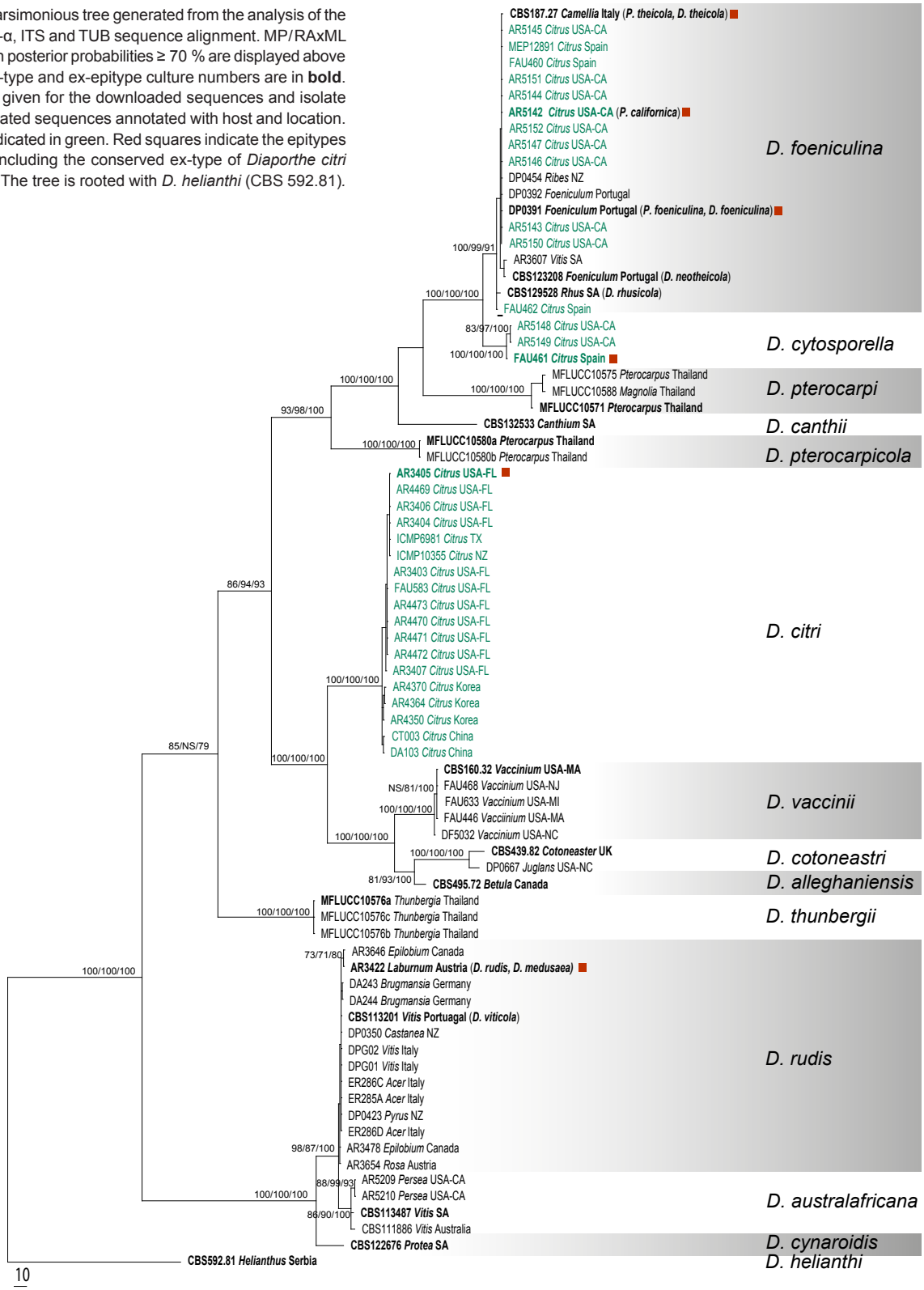

Fig. 3 The single most parsimonious tree generated from the analysis of the combined ACT, CAL, EF1-α, ITS and TUB sequence alignment. MP/RAxML bootstrap values/Bayesian posterior probabilities ≥ 70 % are displayed above or below each branch. Ex-type and ex-epitype culture numbers are in **bold**. GenBank accessions are given for the downloaded sequences and isolate codes for the newly generated sequences annotated with host and location. Isolates from *Citrus* are indicated in green. Red squares indicate the epitypes designated in this study including the conserved ex-type of *Diaporthe citri* in Rossman et al. (2013). The tree is rooted with *D. helianthi* (CBS 592.81).

the EF1-α and combined phylogenetic trees (Fig. 2, 3). Taxa in the ITS tree that were not present in EF1-α and combined trees were not included in the calculation of *gsi*. The *gsi* and each of the probability values (P) corresponding to the species represented by more than one isolate were tabulated (Table 3). Species with one representative isolate including the outgroup were not subjected to *gsi* analysis. The ensemble genealogical sorting index (gsi_T) is the sum of the *gsi* values calculated for all individual gene trees (Table 3).

All the novel sequences were deposited in GenBank and the sequence alignments were submitted to TreeBASE (www.treebase.org) as S14141 (ITS), S14146 (EF1) and S14147 (combined alignment). Taxonomic novelties (MB) and typifications (MBT175959–MBT175968) were registered in MycoBank (www.mycobank.org) (Crous et al. 2004).

RESULTS

Phylogenetic analysis

Three hundred new sequences were generated in this study from 77 cultures (Table 1). Other available sequences were obtained from GenBank. Six alignments were analysed corresponding to single gene analyses of ACT, CAL, EF1-α, ITS and TUB and a combined alignment of the five genes. Comparison

Table 3 Comparison of alignment properties in parsimony analysis of genes and nucleotide substitution models used in phylogenetic analysis.

Genes / loci	ITS	EF1-α	TUB*	ACT*	CAL*	Combined ITS / EF / ACT / CAL
Characters included (with gaps)	508	347	454	279	232	2033
Invariable characters	360	155	318	205	129	1337
Parsimony informative characters (%)	97 (20 %)	189 (55 %)	132 (29 %)	68 (24 %)	95 (40 %)	659 (32 %)
Uninformative polymorphic characters	14	3	4	6	8	37
Alignment strategy (MAFFT v6)	FFT-NS-I+manual	FFT-NS-I+manual	FFT-NS-I	FFT-NS-I	FFT-NS-I+manual	–
Number of branches > 70 % bootstrap MP / BI and ML analysis	18	17	15	11	11	20
Nucleotide substitution models for Bayesian analysis (determined by MrModeltest)	SYM+I+G	GTR+I+G	HKY+G	GTR+G	HKY+G	GTR+I+G

* Phylogenetic trees not shown.

of the alignment properties and nucleotide substitution models are provided in Table 3. Phylogenetic trees inferred from EF1-α and ITS to show the phylogenetic placement of species and a combined alignment of five genes are presented with annotations for species, host and geographic origin (Fig. 1–3). Individual gene trees for ACT, CAL and TUB did not markedly differ from the EF1-α and ITS gene trees and are not shown.

ITS phylogenetic analysis

The ITS sequence alignment contained 126 sequences including the outgroup taxon (Table 3). Maximum parsimony analysis resulted in 45 equally most parsimonious trees (TL = 209, CI = 0.684, RI = 0.977, RC = 0.668, HI = 0.316). BI and ML trees were identical to the MP tree presented in Fig. 1. A total of 12 clades were resolved corresponding to the species recognised as *D. alleghaniensis*, *D. australafricana*, *D. canthii*, *D. citri*, *D. cotoneastri*, *D. cytosporella*, *D. foeniculina*, *D. pterocarpi*, *D. pterocarpicola*, *D. rudis*, *D. thunbergii* and *D. vaccinii*. *Diaporthe cynaroidis* was not resolved as distinct from *D. rudis*. Among the major clades of interest in this study, the *D. foeniculina* clade consists of 48 isolates derived from 21 different hosts in ten countries representing the geographic regions of Australia, Europe, New Zealand, northern South America and South Africa. The isolates from *Citrus* in this clade originated from California (USA), Spain and New Zealand. The ex-type of *D. rhusicola* is also placed within the *D. foeniculina* clade. *Diaporthe cytosporella* is represented by four isolates from *Citrus* in Spain and *Citrus* and *Vitis* in California (USA). *Diaporthe rudis* comprises 34 isolates derived from 18 different hosts from 13 countries representing the geographic regions of Canada, Europe, New Zealand, South America and South Africa, including the epitype culture of *D. viticola*. No isolates of *D. rudis* were reported from *Citrus*. Isolates identified here as *D. citri* include 19 from various *Citrus* spp. in China, Korea, New Zealand and the United States (Florida and Texas).

EF1-α phylogenetic analysis

The EF1-α data matrix contained 77 sequences including the outgroup and consisted of 429 characters including gaps (Table 3). Maximum parsimony analysis yielded a single most parsimonious tree and is presented here as Fig. 2 (TL = 442, CI = 0.742, RI = 0.964, RC = 0.715, HI = 0.258). The MP, BI and ML trees generated were identical. The closely related taxa *D. foeniculina* and *D. cytosporella* were clearly distinguished and *D. rhusicola* was placed within *D. foeniculina*. Isolates of *D. australafricana* including the ex-type isolate were placed within the *D. rudis* clade, whereas *D. cynaroidis*, represented by the ex-type isolate, formed a distinct branch. Inspection of EF1-α sequences of *D. australafricana* vs *D. rudis* isolates revealed two base changes including one insertion and one transversion between the two taxa.

Combined analysis of five genes

The combined data matrix consisted of 74 isolates including the outgroup with 2 033 characters included in the maximum parsimony analysis (Table 3). The maximum parsimony analysis of the alignment yielded a single most parsimonious tree presented here as Fig. 3 (TL = 1302, CI = 0.720, RI = 0.961, RC = 0.692, HI = 0.280). The MP, BI and ML trees generated were identical. A total of 13 clades were resolved in the combined phylogenetic tree. *Diaporthe citri* forms a sister clade to a clade containing *D. cotoneastri* and *D. vaccinii*. *Diaporthe citri* occurs only on *Citrus* in the United States and elsewhere while *D. vaccinii* occurs only on *Vaccinium* in North America. The *D. rudis* clade includes the taxon previously known as *D. viticola* represented by an ex-epitype culture (CBS 113201) and several authentic isolates previously known as *D. medusaea*. *Diaporthe australafricana* forms a well-supported clade closely related to *D. rudis*. The multi-gene phylogenetic tree resolves the closely related taxa *D. canthii*, *D. cytosporella*, *D. foeniculina* and *D. pterocarpi*. The ex-type of *D. rhusicola* is placed within the *D. foeniculina* clade. *Diaporthe cytosporella*, *D. foeniculina* and *D. pterocarpi* are all found to occur on multiple, unrelated hosts. *Diaporthe canthii*, represented by a single isolate, is known only from its type host.

Table 4 Genealogical Sorting Index (*gsi*) and probability values (P) for gene trees of species resolved in this study.

Species	ITS gsi[1] P	EF1-α gsi[1] P	TUB gsi[1] P	CAL gsi[1] P	ACT gsi[1] P	ALL gsi^1_T P_T
D. australafricana	1* 0.0004	1* 0.0001	0.4051* 0.001	0.2712* 0.0078	0.1187 0.0483	0.7353* 0.0001
D. citri	1* 0.0004	1* 0.0001	1* 0.0001	1* 0.0001	1* 0.0001	1* 0.0001
D. cotoneastri	1* 0.0005	1* 0.0019	1* 0.0008	1* 0.0079	1* 0.0007	1* 0.0001
D. cytosporella	1* 0.0001	1* 0.0002	0.4492* 0.0003	1* 0.0003	1* 0.0004	1* 0.0001
D. foeniculina	0.869* 0.0001	1* 0.0001	0.9476* 0.0001	1* 0.0001	0.9301 0.0001	0.9598* 0.0001
D. pterocarpi	1* 0.0001	1* 0.0002	1* 0.0001	1* 0.0003	1* 0.0002	1* 0.0001
D. pterocarpicola	1* 0.0014	1* 0.0020	1* 0.0010	1* 0.0088	1* 0.0002	1* 0.0001
D. rudis	1* 0.0001	1* 0.0001	0.9138* 0.0001	0.8392 0.0001	0.7719 0.0001	0.9332* 0.0001
D. thunbergii	1* 0.0001	1* 0.0001	1* 0.0001	1* 0.0002	1* 0.0001	1* 0.0001
D. vaccinii	1* 0.0004	1 0.0001	1* 0.0001	1* 0.0001	1* 0.0001	1* 0.0001

[1] The *gsi* statistic is based on a continuum of 0–1, with 0 = lack of genealogical divergence from other groups and 1 = monophyly; (*) = statistically significant P-value ≤ 0.05. The *gsi* is calculated under the null hypothesis that the gene copies labeled as each species assigned are a single group of mixed genealogical ancestry. gsi_T = ensemble *gsi* of 5 gene trees. Species represented by single isolate are excluded in calculation of *gsi*.

Analysis of gsi data

All *gsi* values were in range of 0.5–1.0 with the exception of TUB (0.4482) for *D. cytosporella* and TUB (0.4051), CAL (0.2712) and ACT (0.1187) for *D. australafricana* (Table 4). Despite minor variation within the ITS1 region in both *D. foeniculina* and *D. rudis*, the *gsi* recognised each as monophyletic for each of the genes, confirming the placement of the ex-type culture of *D. rhusicola* with *D. foeniculina*. Therefore, the observed variation in the ITS regions of these two species is not considered meaningful in terms of species distinction and does not conflict with the other gene regions. Individually, the ITS and EF1-α genes estimated significant measures of exclusive ancestry for all the species including *D. australafricana* and *D. cytosporella*. The ACT gene resolved all species as monophyletic except *D. australafricana*. The ensemble *gsi* value (gsi_T) for all species included indicated significant genealogical divergence from all other species in spite of the conflict observed among genes for *D. australafricana* and *D. cytosporella*. All other species resolved in the combined phylogeny were supported without conflict.

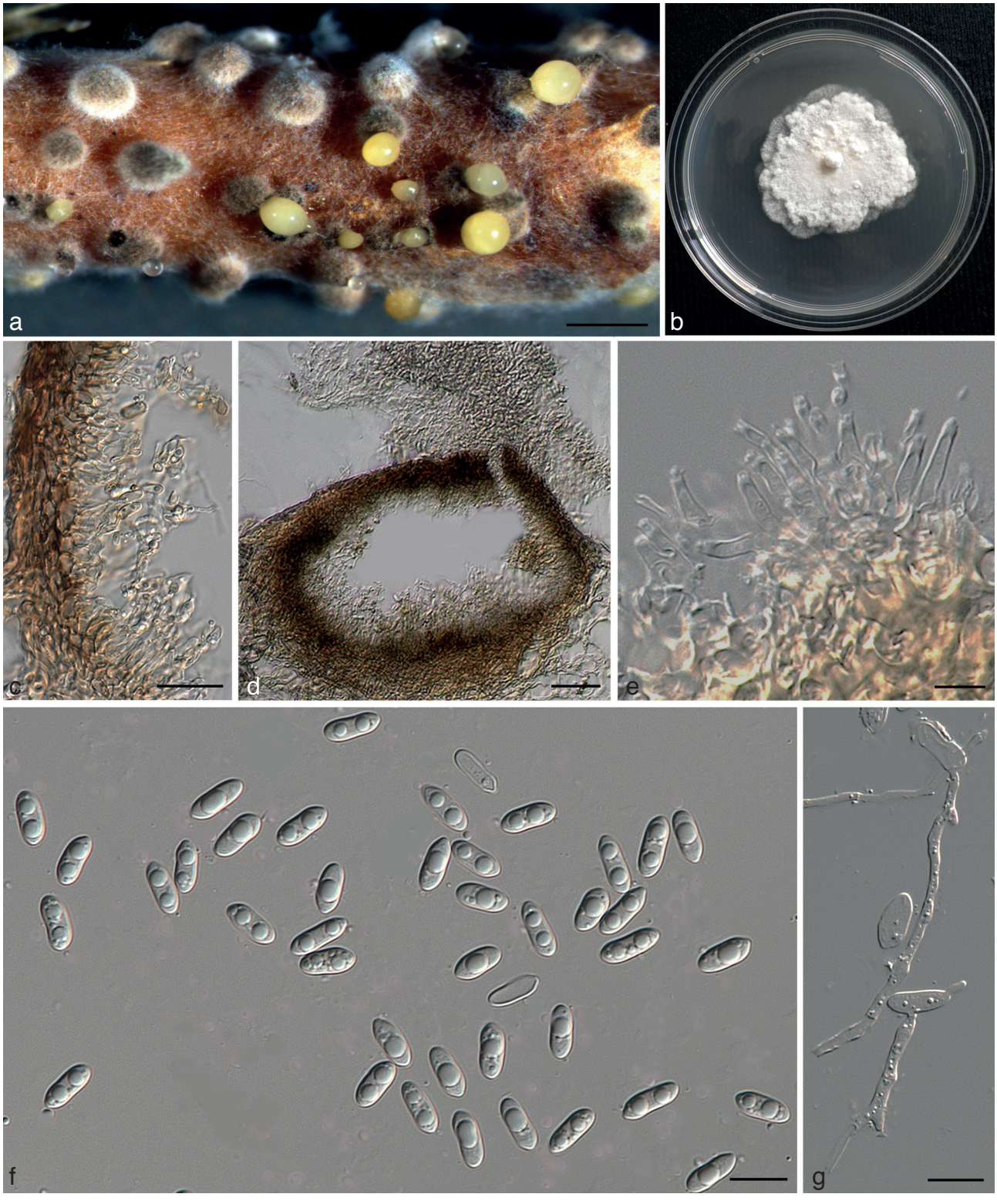

Fig. 4 *Diaporthe citri* (ex-epitype culture AR3405 = CBS 135422). a. Sporulation on alfalfa stem in WA; b. culture on PDA (25 °C, dark, 7 d); c. pycnidial walls lined with paraphyses and conidiophores; d. section through conidiomata; e. conidiophores; f. alpha conidia; g. germinating conidia on a slide. — Scale bars: a = 500 µm; c = 20 µm; d–g = 10 µm.

New primers for Diaporthe and protocols for amplification

The evaluation of the thermal properties of the primers by OligoCalc and Sequence Manipulation Suite revealed that the forward CAL primers, CAL-228F (Carbone & Kohn 1999) and CL1 (O'Donnell et al. 2000), showed potential for self-annealing in each of the tests in addition to issues with non-specific binding sites in the targeted gene region. The newly designed CAL-563F primer and the existing CL2A reverse primer (O'Donnell et al. 2000) were determined to be a suitable primer pair under the criteria given including percentage GC, self-annealing, GC clamp, hairpin formation and length. They were used to eliminate the problems in amplification and sequencing encountered in this study. Use of this primer pair resulted in an amplicon overlapping c. 300 bp of the 500-bp CAL-228F/CAL-737R fragment. However, two additional introns, each c. 60–100 bp in length, are found in the extended sequence obtained using the primers CAL-563F/CL2A. One of these informative introns in *Diaporthe* is not found in either of the *N. crassa* or *P. grisea* reference sequences used as references for primer design.

The newly designed reverse primer for actin (ACT-878R) worked well in combination with ACT-512F for isolates that failed with the ACT-512F/ACT-783R primer combination and resulted in an amplicon of c. 350 bp in length. The extended 3' region of the newly generated amplicons was not included in the analyses as the majority of the sequences were generated with primer pairs ACT-512F/ACT-783R and it consisted entirely of exon sequence with little variation among isolates.

Taxonomy

In this section we provide modern descriptions and illustrations of the species resolved here based on multi-gene phylogenetic analyses and morphological characters. *Diaporthe citri* occurs only on *Citrus* while *D. cytosporella* and *D. foeniculina* occur on *Citrus* and other woody and herbaceous hosts including high value crops. *Diaporthe rudis* is not known from *Citrus* but was previously confused with those species especially as *D. medusaea* and has a broad host range. Each species is described based on type and other specimens as well as ex-epitype cultures. Synonymous names of *Diaporthe* or *Phomopsis* are reviewed based on protologues, type and other specimens and cultures. When specimens with cultures from similar substrates and localities are available, epitype specimens with ex-epitype cultures are designated for both accepted and synonymous names.

Diaporthe citri (H.S. Fawc.) F.A. Wolf, J. Agric. Res. 33: 625. 1926. — Fig. 4

Basionym. *Phomopsis citri* H.S. Fawc., Phytopathology 2: 109. 1912 nom. conserv. prop. non *Phomopsis citri* (Sacc.) Traverso & Spessa 1910.
= *Diaporthe citrincola* Rehm, Leafl. Philipp. Bot. 6: 2269. 1914.
= *Phomopsis caribaea* W.T. Horne, Phytopathology 12: 417. 1922.

Perithecia on decaying twigs, black, globose to conical, 130–200 µm diam, scattered, solitary or in groups, immersed deep in bark with tapering perithecial necks 190–700 µm long. *Asci* unitunicate, 8-spored, sessile, elongate to clavate, (37.3–)40.5–50.5(–55) × (9–)10.5–12(–12.2) µm. *Ascospores* hyaline, 2-celled, often 4-guttulate, with larger guttules at centre and smaller one at ends, elongated to elliptical, (12–)12.4–14(–14.2) × 3.2–3.6(–3.8) µm (av. ± SD = 13.2 ± 0.8 × 3.3 ± 0.2, n = 30). *Pycnidia* on alfalfa twigs on WA: globose, 200–250 µm diam, later conical, embedded in tissue, erumpent at maturity, up to 450 µm diam, 65–100 µm high, with an elongated black neck, often with a yellowish, spiral conidial cirrus extruding from ostiole; walls parenchymatous, consisting of 3–4 layers of medium brown *textura angularis*. *Conidiophores* hyaline, smooth, unbranched, ampulliform, straight to sinuous, 10–15 × 1–2 µm. *Conidiogenous cells* phialidic, cylindrical, terminal, slight tapering towards apex, 0.5–1 µm diam. *Paraphyses* abundant among conidiophores, 20–40 × 1–2 µm. *Alpha conidia* aseptate, hyaline, smooth, ovate to ellipsoidal, mono- to biguttulate, rarely 3-guttulate, base subtruncate, (7.6–)8–9(–10.2) × 3–4.2 µm (av. ± SD = 8.5 ± 0.8 × 3.7 ± 0.2 , n = 30). *Beta* and *gamma conidia* not observed on alfalfa twigs or in culture.

Culture characteristics — In dark at 25 °C for 1 wk, colonies on PDA slow growing, 4.2 ± 0.2 mm/day (n = 8), white, fluffy aerial mycelium, reverse centre greenish yellow pigmentation developing in centre.

Host range — Causing melanose and stem end rot disease, associated with dying or dead twigs of *Citrus* spp. and closely related hosts including *C. aurantiifolia*, *C. aurantium*, *C. maxima* (= *C. grandis*), *C. nobilis*, *C. paradisi*, *C. reticulata*, *C. sinensis*, *C. sudachi*, *C. unshiu*, × *Citrofortunella microcarpa* (= × *C. mitis*), *Fortunella japonica* (Kobayashi 2007), *F. margarita*, *Poncirus trifoliata*.

Distribution — Probably throughout *Citrus*-growing regions of the world. Reported from Brazil, China, Cuba, Japan, Korea, New Zealand, The Philippines, Puerto Rico and the United States (Florida, Texas).

Type specimens examined. USA, Florida, Lake Alfred, Ana, on twigs of *Citrus* sp, 26 Apr. 2000, *L.W. Timmer*, dried specimen from culture sporulating on alfalfa stem (type of *Phomopsis citri* proposed for conservation in Rossman et al. (2013) (BPI 892456, ex-type culture AR 3405 = CBS 135422). – Philippines, Los Baños, on dead twigs of *Citrus nobilis*, Oct. 1913, coll. *M.B. Raimundo*, comm. C.F. Baker, no. 1875 (holotype of *Diaporthe citrincola* S-F52860). – Cuba, Isle of Pines, on *Citrus paradisi*, 30 Oct. 1917, *Fredrick Maskew*, intercepted in San Francisco, derived culture sporulating on *Citrus* twig (lectotype of *Phomopsis caribaea* designated here BPI 358328, isolectotype NY01097305; MBT175959).

Additional materials examined. Brazil, Escola Agr., Vicosa, Minas Gerais, on peel of *Citrus* sp., 17 May 1932, *P.H. Rolfs* (BPI 615855); intercepted New York #pi 7163, on fruit of *Citrus sinensis*, 22 June 1924, *A.C. Hill*, det. A.J. Bruman (BPI 358408). – Japan, Yokohama, intercepted Seattle Washington #pi 4780, on fruit of *Citrus sinensis*, 14 Jan. 1940, *A.G. Webb*, det. J.A. Stevenson (BPI 358405). – Mexico, intercepted Brownsville Texas #692229, on leaves of *Citrus* sp., 30 Jan. 1930, *Mueller*, det. D.J. Smith, A.E. Jenkins, J.A. Watson (BPI 615856); intercepted Laredo Texas #50818, on leaves of *Citrus* sp., 23 Jan. 1951, *Trotter*, det. A.H. Lewis, J.A. Watson (BPI 615857). – Puerto Rico, Bayamon, on leaves of *Citrus grandis*, 22 Aug. 1933, *C.G. Anderson* (BPI 358392). – USA, Florida, Orlando, on dead stems of *Citrus aurantifolia*, July 1925, *F.A. Wolf* (BPI 615860); Florida, Orlando, on dead stems of *Citrus* sp., Jan. 1926, *F.A. Wolf* (BPI 615959); Florida, Orlando, on leaves of *Citrus sinensis*, Mar. 1922, *J.R. Winston* (BPI358409); Florida, Eustis, on leaves of *Citrus grandis*, 8 Jan. 1932, *H.S. Fawcett* (BPI 358391); Florida, St. Nicholas, on stem of *Citrus sinensis*, 28 Nov. 1895, det. F. Albert, W.W. Diehl (BPI 358404); Florida, Fort Myers, on stems of *Citrus sinensis*, 16 Feb. 1924, *J.A. Stevenson* (BPI 358407); Florida, Gainesville, Florida Agricultural Experiment Station, on stems of *Citrus sinensis*, 16 Mar. 1910, *H.S. Fawcett* (BPI 358406); Florida, Winter Park, on stems of *Citrus grandis*, 21 Feb. 1923, *C.L. Shear* (BPI 615868); Florida, Winter Park, on stems of *Citrus grandis*, 20 Jan. 1925, *H.E. Stevens*, det. C.L. Shear (BPI 615869); Florida, on dead stem of *Citrus* sp., 1913, *J.G. Grossenbacker*, det. C.L. Shear (BPI 615858); Florida, on dead stem of *Citrus* sp., 8 July 1929, *F.A. Wolf*, det. C.L. Shear (BPI 615854); Florida, on leaves of *Citrus grandis*, 6 Jan. 1932, *H.S. Fawcett* (BPI 358393).

Notes — The name *D. citri* is based on *Phomopsis citri* H.S. Fawc. 1912, a later homonym of *Phomopsis citri* (Sacc.) Traverso & Spessa 1910. A conservation proposal has been published to continue the use of the widely used name for the species associated with melanose or stem end rot of *Citrus* as *D. citri* (Rossman et al. 2013). *Diaporthe citri* based on the basionym *Phomopsis citri* H.S. Fawc. has priority over the later synonyms *D. citrincola* and *P. caribaea*. This is also in accordance with the change to unit nomenclature with the older genus *Diaporthe* serving as the correct name for all species in *Diaporthe-Phomopsis* (McNeill et al. 2012). No type specimen for *P. citri* could be located at BPI or FLAS, leaving only an illustration (Fawcett 1912) as a potential, but unsatisfactory,

iconotype, thus *P. citri* is proposed for conservation with a new type specimen (Rossman et al. 2013). The type specimens of *D. citrincola* and *P. caribaea* were examined and contributed to the conclusions that these names are synonyms of *D. citri*. A lectotype of *P. caribaea* is designated.

The fruiting structures of *D. citri* are found on dead twigs, stems and fruits of *Citrus* affected by melanose and stem end rot (Wolf 1926, Fawcett 1932, Whiteside & Timmer 2000b). The fungus generally propagates itself on dead twigs of *Citrus*. A few days after infecting leaf tissue or fruit, the melanose symptoms appear as small, brown, discrete or confluent, sunken spots. A few

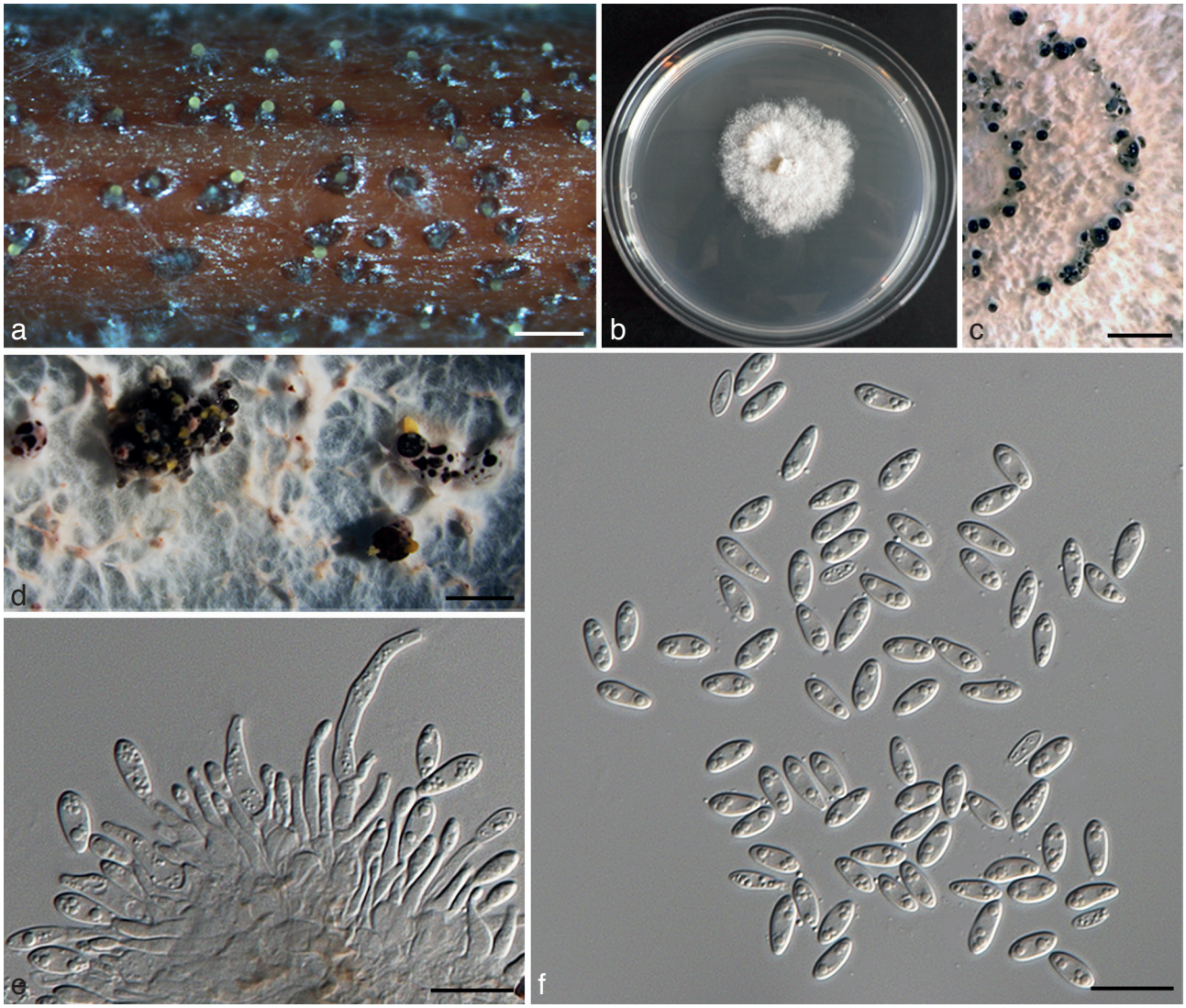

Fig. 5 *Diaporthe cytosporella* (AR5149). a. Sporulating pycnidia on alfalfa stem; b. culture on PDA (25 °C, dark, 7 d); c. concentric pycnidial in rings on culture; d. pycnidia on culture; e. conidiophores; f. alpha conidia. — Scale bars: a, c = 2 000 µm; d = 3 000 µm; e, f = 20 µm.

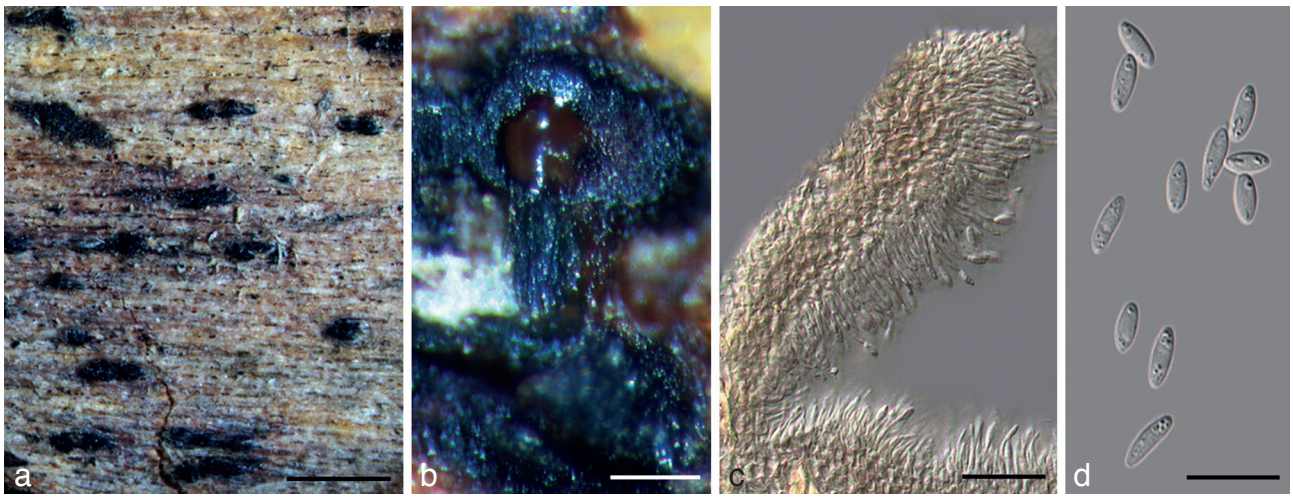

Fig. 6 Holotype specimen of *Diaporthe cytosporella* (BPI 798526). a. Pycnidia-bearing bark of *Citrus* sp.; b. branched stroma and sporulating pycnidia; c. section through pycnidia with pycnidial wall and conidiophores; d. alpha conidia. — Scale bars: a = 1 000 µm; b = 50 µm; c, d = 15 µm.

epidermal cell layers on infected tissue are killed and become impregnated with reddish brown gum that later become raised black pustules (Timmer 2000). Although pustules on leaves are initially surrounded by yellow halos, they recover and become green again and corky pustules are often the only symptoms (Bach & Wolf 1928, Nelson 2008). Fungal structures such as pycnidia or perithecia are never visible in these melanose lesions, therefore, the fungus cannot be observed in the infected leaves or fruit. When the fruiting structures are present on dead twigs or bark of the stems, the pycnidia or ascomata are abundant deep in the tissue.

Diaporthe cytosporella (Penz. & Sacc.) Udayanga & Castl., *comb. nov.* — MycoBank MB803986; Fig. 5, 6

Basionym. *Phoma cytosporella* Penz. & Sacc., Fung. Agron.: 361. 1887.
≡ *Phomopsis cytosporella* (Penz. & Sacc.) H.S. Fawc. & H.A. Lee, Citrus diseases and their control, Ed. 1: 407. 1926.

Perithecia unknown. *Pycnidia* on alfalfa twigs on WA: globose, 150–200 µm diam, mostly embedded in tissue and erumpent at maturity, up to 450 µm diam, 65–100 µm high, with an elongated black neck, often with a yellowish conidial cirrus extruding from ostiole; walls parenchymatous consisting of 3–4 layers of medium brown *textura angularis*. *Paraphyses* lacking. *Conidiophores* 7–18 × 1–2 µm, hyaline, smooth, branched or unbranched, ampulliform, cylindrical, wider at base, occurring in dense clusters. *Conidiogenous cells* phialidic, cylindrical, terminal, with slight tapering towards apex, 0.5–1 µm diam. *Alpha conidia* (6.9–)8–9(–12.6) × (2.3–)2.6–3.2 µm (av. ± SD = 8.8 ± 0.9 × 3.0 ± 0.3, n = 30), aseptate, hyaline, smooth, ovate to ellipsoidal, biguttulate or multi-guttulate, base subtruncate, occasionally larger alpha conidia present in culture and on alfalfa stems. *Beta* and *gamma conidia* not observed on alfalfa twigs or in culture.

Culture characteristics — In dark at 25 °C for 1 wk, colonies on PDA relatively slow growing, 4.0 ± 0.2 mm/day (n = 8). On PDA white, fluffy aerial mycelium, reverse with ash colour pigmentation developing in centre. In a 2-wk-old culture, clusters of black, branched stromata occurring in concentric rings with sporulating pycnidia.

Host range — *Citrus limon*, *C. sinensis* and *Vitis vinifera*.

Distribution — Europe (Spain, Italy), United States (California).

Type specimens examined. Italy, Rome, Modena, on *Citrus limonia*, Jan. 1886 (holotype of *Phoma cytosporella* BPI 798526). – Spain, on *Citrus limon*, *M.E. Palm*, dried culture (epitype of *Phoma cytosporella* designated here: BPI 892459, living culture FAU461 = CBS 137020; MBT 175960).

Additional material examined. USA, California, on twigs of *Citrus sinensis*, 4 Oct. 2011, *A. Eskalen* UCR1751, dried culture with pycnidia sporulating on alfalfa stems (BPI 892457, living culture AR5149); ibid., UCR1750, dried culture with pycnidia sporulating on alfalfa stems (BPI 892458, living culture AR5148).

Notes — *Diaporthe cytosporella* is phylogenetically closely related to *D. foeniculina* but clearly distinguished based on ITS and EF1-α sequences (Fig. 1, 2). The species was first described from Italy and later synonymised under *D. citri.* Although in this study this species is primarily recognized using isolates from *Citrus limon* in Europe (Spain) and the United States (California), two ITS sequences (FJ94470, AY745085) from GenBank are 100 % identical suggesting that this species may also occur on *Vitis* and other host species in California (retrieved on 1 Feb. 2013). At maturity cultures of *D. cytosporella* (AR5148 and AR5149) on PDA produce distinctive black, branched stromata. Perithecia were not observed in culture. Morphological characters were highly similar among the type specimen (Fig. 6) and the isolates used in epitype and genetically similar additional materials examined.

Diaporthe foeniculina (Sacc.) Udayanga & Castl., *comb. nov*. — MycoBank MB803929; Fig. 7

Basionym. *Phoma foeniculina* Sacc., Michelia 2: 95. 1880.
≡ *Phomopsis foeniculina* (Sacc.) Sousa da Câmara, Agron. Lusit. 9: 104. 1947.
= *Diaporthe theicola* Curzi, Atti Ist. Bot. Lab. Crittog. Univ. Pavia 3 Sér. 3: 60. 1927.
= *Phomopsis theicola* Curzi, Atti Ist. Bot. Lab. Crittog. Univ. Pavia 3 Sér. 3: 64. 1927.
= *Phomopsis californica* H.S. Fawc., Phytopathology 12: 419. 1922.
= *Diaporthe neotheicola* A.J.L. Phillips & J.M. Santos, Fung. Diversity 34: 120. 2009.
= *Diaporthe rhusicola* Crous, Persoonia 26: 135. 2011.

Perithecia on decaying twigs black, globose to subglobose (200–)360 × 200 µm, scattered, solitary or in groups, with tapering perithecial necks barely protruding through epidermis. *Asci* unitunicate, 8-spored, sessile, cylindrical to clavate, (40–)50.5–60.5(–65) × 8–10(–12.2) µm. *Ascospores* hyaline, 2-celled, often with four guttules, larger guttules near centre and smaller ones at ends, elongated to elliptical, (9.0–)12.4–14(–15.2) × (3.2–)3.4–3.6(–5.2) µm (av. ± SD = 13.2 ± 0.8 × 3.5 ± 0.1, n = 30). *Pycnidia* on alfalfa twigs on WA: globose to subglobose 400–700 µm diam, erumpent at maturity, (300–)500–800(–930) µm high, with an elongated, black neck, mostly embedded in tissue, often with a yellowish, drop-like conidial cirrus extruding from ostiole; walls parenchymatous, consisting of 2–3 layers of medium brown *textura angularis*. *Paraphyses* lacking. *Conidiophores* hyaline, smooth, unbranched, cylindrical, straight to sinuous, 9–15(–18) × 1–2 µm. *Conidiogenous cells* phialidic, cylindrical, terminal, with slight taper towards apex, 0.5–1 µm diam. *Alpha conidia* aseptate, hyaline, smooth, ellipsoidal or fusiform, with none, two or many guttules, rarely with subtruncate base, (7.5–)8.5–9(–9.2) × (2–)2.3–2.5(–2.7) µm (av. ± SD = 8.8 ± 0.3 × 2.4 ± 0.1 µm, n = 30). *Beta conidia* hyaline, aseptate, eguttulate, hamate or slightly curved, abundant, base subtruncate, acute apex, (20–)22–28(–29) × (1.1–)1.4–1.6(–2) µm (av. ± SD = 25.1 ± 3.3 × 1.5 ± 0.1 µm, n = 30).

Culture characteristics — In dark at 25 °C for 1 wk, colonies on PDA slow growing, 5.2 ± 0.2 mm/day (n = 8), white, sparse aerial mycelium, greenish yellow pigmentation developing in reverse centre.

Host range — *Acacia*, *Acer*, *Actinidia deliciosa*, *Aspalathus linearis*, *Bougainvillea spectabilis*, *Camellia sinensis*, *Castanea*, *Citrus limon*, *C. limonia*, *Crataegus*, *Diospyros*, *Foeniculum vulgare*, *Fuchsia*, *Hydrangea*, *Juglans*, *Malus*, *Olea*, *Prunus*, *Pyrus*, *Quercus*, *Rhus*, *Ribes*, *Vitis vinifera* and *Wisteria sinensis.* In addition to the hosts on the specimens listed below, these hosts are represented in Fig. 1 based on the ITS phylogeny and Gomes et al. (2013) as *D. foeniculacea*.

Distribution — Argentina, Australia, Europe (Greece, Portugal, Spain, Italy), New Zealand, South Africa and USA (California).

Type specimens examined. France, on stems of *Foeniculum 'arvensis'*, *Brunaud*, cited in Phillips (2003) with illustration (holotype of *Phoma foeniculina* PAD 281 – unavailable, not examined). – Portugal, Madeira, Serra da Agua, at base of 2-yr-old stem of *Foeniculum vulgare*, Aug. 2001, *A.J.L. Phillips* (epitype of *Phoma foeniculina* designated here LISE 94791, ex-epitype culture from single ascospores CBS 111553 = DP0391; MBT175961). – USA, California, Santa Barbara County, on dead outer bark and decaying fruit of *Citrus limonia*, 3 Mar. 1922, *H.S. Fawcett* (holotype of *Phomopsis californica* BPI0358313); California, San Diego, on branch of *Citrus limonia*, 16 Nov. 2012, *Akif Eskalen* (epitype of *Phomopsis californica* designated here BPI 892460, ex-epitype culture AR5142 = CBS135430; MBT 175962). – Italy, on *Camellia sinensis*, *Curzi*, dried culture specimen (epitype of *Diaporthe theicola* designated here BPI 892462, ex-epitype culture CBS 187.27, same as ex-isotype culture of *Phomopsis theicola*; MBT175963); Illustration in Atti dell'Istituto Botanica della Universita e Laboratoria Crittogamico di Pavia 3 Sér. 3: 60 (1926) [1927] (lectotype of *Phomopsis theicola* designated here; MBT175964). – Portugal, Évora, on *Foeniculum vulgare*, Nov. 2007, *A.J.L.*

Phillips (holotype of *Diaporthe neotheicola* CBS-H 20131, ex-holotype culture (Di-C004/5 = CBS 123208).

Additional specimens examined. Portugal, Madeira, Serra da Agua, at base of 2-yr-old stem of *Foeniculum vulgarae*, Aug. 2001, *A.J.L. Phillips* (LISE 94792 as *Diaporthe foeniculacea*, culture from single ascospores DP0392 = CBS 111554. – Spain, on fruit of *Citrus limon*, intercepted at Elizabeth, New Jersey, 20 Mar. 1987, *C. Markham* 001514, *M.E. Palm* (BPI 1107900, living culture MEP1289); ibid. (BPI 747926); ibid. (BPI 747927); on peel of *Citrus limonia*, intercepted New York #87452, 13 Nov. 1940, *Hodson*, *E.A. Jenkins* (BPI 615878); ibid., on fruit of *Citrus limon*, *M.E. Palm* (BPI 892461, culture FAU460 = CBS).

Notes — *Diaporthe foeniculina* is known to occur on *Citrus* and many other woody plants hosts in temperate and tropical regions. This species causes a stem end rot of lemandarin (*Citrus limonia*) in Europe and the United States (California) and was observed as a saprobe on branches of this host. As *D. neotheicola*, this species has been reported to cause diseases of temperate and tropical fruits from Australia, Europe and South Africa. Our results indicate that isolates from *Citrus* in Spain are conspecific with the type isolate of the recently described *D. neotheicola* from *Foeniculum* as well as isolates from other hosts now considered to be *D. foeniculina*. We reviewed the possible synonyms of this species based on available molecular data, living cultures and type specimens. The specimen deposited in LISE 94792 corresponding to the living culture CBS 111554 was selected as the epitype specimen for *Phoma foeniculina*, now recognised as *D. foeniculina.* Molecular data derived from the epitype of *Phoma foeniculina*, now *D. foeniculina*, and additional isolates show that this taxon is conspecific with the ex-type isolates of *D. neotheicola* and *P. theicola* (Phillips 2003, Santos & Phillips 2009, Gomes et al. 2013) as well as isolates from *Citrus* in Spain. The name *D. neotheicola* has been widely used for this taxon (Santos & Phillips 2009, Udayanga et al. 2012a, Thomidis et al. 2013).

Phomopsis foeniculina (syn. *Phoma foeniculina*) was considered a synonym of *D. foeniculacea* (syn. *Sphaeria foeniculacea*) by Phillips (2003). We examined the type specimen of *Sphaeria foeniculacea* and agree with von Arx & Müller (1954) who recognised this species as *Guignardia foeniculacea* (Mont.) Arx & E. Müll. (as *G. foeniculata*). Gomes et al. (2013) used the name *D. foeniculacea* to refer to this species based on Phillips (2003). However, observation of type specimens of *Sphaeria foeniculacea* confirmed that this name cannot be applied to a species of *Diaporthe*. This is further explained under the excluded species.

Although type specimens of *Phomopsis theicola* and *D. theicola* could not be located, an ex-type culture of *P. theicola* exists as mentioned by Santos & Phillips (2009). They stated that *P. theicola* was not the same as *D. theicola* based on the illustrations in the protologues of these taxa and described the name *D. neotheicola* for the sexual state of *P. theicola*, with an ex-holotype culture from *Foeniculum*. However, measurements of asci and ascospores in Curzi's (1927) original description of *D. theicola* are consistent with those of *D. foeniculina* specimens examined in this study. We agree with the opinion of Curzi (1927) that *D. theicola* and *P. theicola* are known sexual and asexual states of the same fungus, simultaneously described from the same specimen and therefore here we epitypify the name *D. theicola* with Curzi's ex-type culture of *P. theicola*.

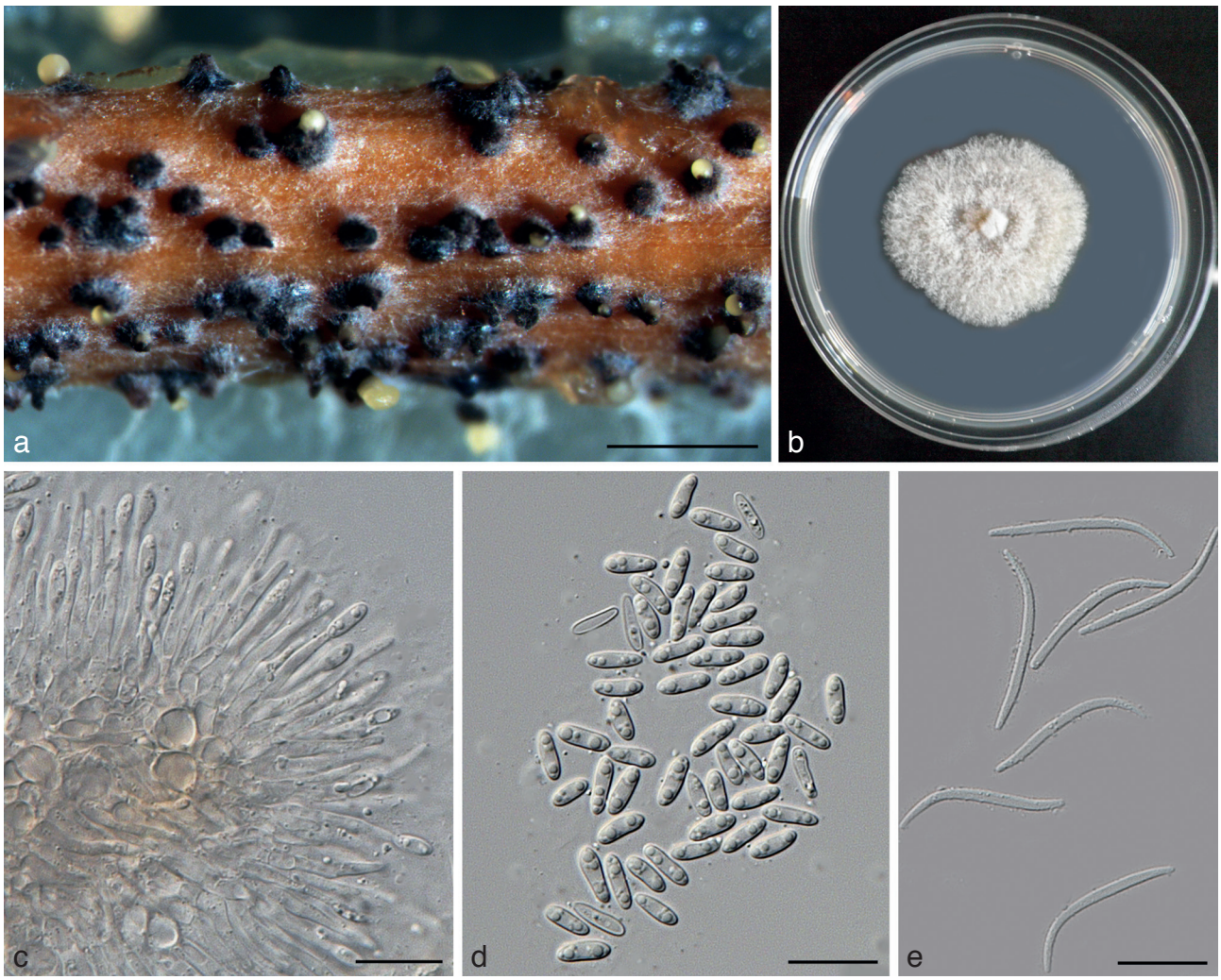

Fig. 7 *Diaporthe foeniculina* (ex-epitype culture DP0391 = CBS 111553). a. Sporulation on alfalfa stem in WA; b. culture on PDA (25 °C, dark, 7 d); c. conidiophores; d. alpha conidia; e. beta conidia. — Scale bars: a = 2 000 µm; all others = 10 µm.

Diaporthe rudis (Fr.) Nitschke, Pyrenomycetes Germanici 2: 282. 1870. — Fig. 8

Basionym. *Sphaeria rudis* Fr., Elench. Fung. (Griefswald) 2: 98. 1828.
≡ *Rabenhorstia rudis* (Fr.) Fr., Summa Veg. Scand., Section Post. (Stockholm): 410. 1849.
≡ *Aglaospora rudis* (Fr.) Tul. & C. Tul., Select. Fung. Carpol. (Paris) 2: 165. 1863.
= *Phoma rudis* Sacc., Michelia 1: 257. 1878.
≡ *Phomopsis rudis* (Sacc.) Höhn., Sitzungsber. Kaiserl. Akad. Wiss., Math.-Naturwiss. Cl., Abt. 1, 115: 680. 1906.
= *Diaporthe faginea* Sacc., Syll. Fung. 1: 619. 1882.
= *Diaporthe macrostoma* Nitschke, Pyrenomycetes Germanici 2: 284. 1870.
= *Diaporthe medusaea* Nitschke, Pyrenomycetes Germanici 2: 251. 1870.
= *Diaporthe viticola* Nitschke, Pyrenomycetes Germanici 2: 264. 1870.
= *Diaporthe silvestris* Sacc. & Berl., Atti Rev. Instit. Venet. Ser. II, 6: 737. 1885.

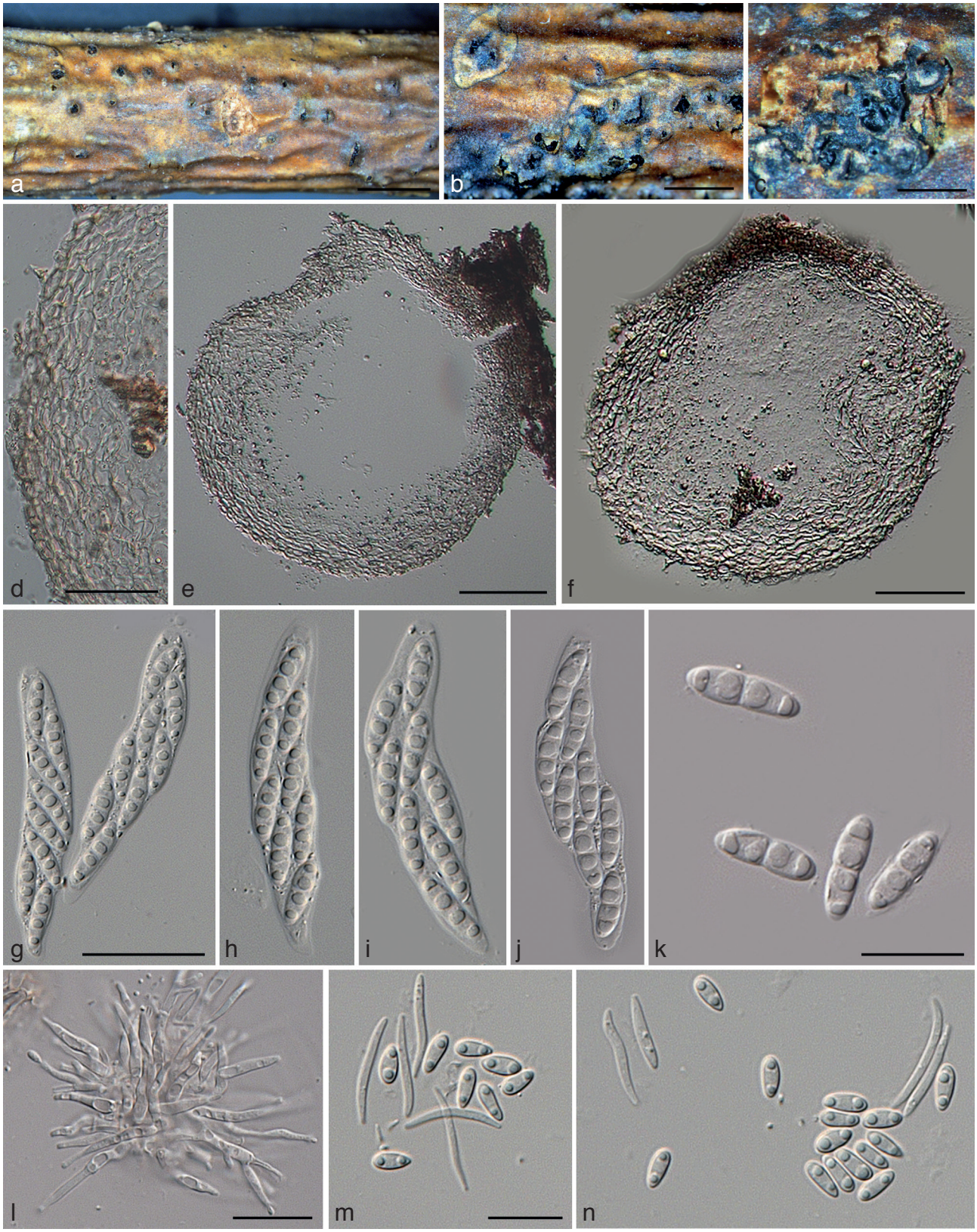

Fig. 8 *Diaporthe rudis*. a, b. Ectostroma and perithecia on *Laburnum anagyroides*; c. perithecia in transverse section; d. perithecial wall in longitudinal section; e, f. perithecia in longitudinal section; g–j. asci; k. ascospores; l. conidiophores developing on alfalfa stem in culture; m. alpha and beta conidia developing on alfalfa stem in culture (a–k. Epitype specimen BPI 748231; l–n. ex-epitype culture AR3422 = CBS 109292). — Scale bars: a = 2 000 µm; b, c = 1 000 µm; d = 50 µm; e, f = 100 µm; g–k = 25 µm; l–n = 15 µm.

Perithecia black, clustered, globose, 300–350 µm, with tapering perithecial necks, 200–700 µm long. Asci unitunicate, sessile, elongate to clavate, (50.3–)53.5–58.5(–59.6) × (8.9–)10.6–12(–12.3) µm. *Ascospores* hyaline, 2-celled, often tetra-guttulate, with larger guttules at centre and smaller at ends, elongated to clavate, (11.6–)12–14.2(–15) × (2.8–)3.5–3.7(–3.8) µm (av. ± SD = 13.2 ± 1.1 × 3.6 ± 0.1, n = 30). *Pycnidia* on alfalfa twigs on WA: globose 200–250 µm diam, erumpent at maturity, up to 400–500 µm diam; walls 60–150 µm diam, parenchymatous, consisting of 3–4 layers of medium brown *textura angularis*. *Conidiophores* cylindrical, hyaline, smooth, branched, ampulliform, straight to sinuous, 20–45 × 2–2.4 µm. *Conidiogenous cells* phialidic, cylindrical, terminal, with slight tapering towards apex, 0.5–1 µm diam. *Paraphyses* abundant among conidiophores 20–40 × 1–2 µm. *Alpha conidia* aseptate, hyaline, smooth, ovate to ellipsoidal, biguttulate, base subtruncate (6.3–)7–8(8.7) × 2–2.5 µm (av. ± SD = 7.5 ± 0.4 × 2.2 ± 0.2, n = 30). *Beta conidia* aseptate, hyaline, smooth, fusiform or hooked, base subtruncate, 27–31(–35.2) × (3–)3.4–3.8(–4.2) µm (av. ± SD = 29.5 ± 2 × 3.6 ± 2, n = 30). *Gamma conidia* aseptate, hyaline, smooth, fusiform, mostly biguttulate, base subtruncate (10–)14–15 × 1–2 µm (av. ± SD = 14.4 ± 0.2 × 1.7 ± 0.24, n = 30).

Culture characteristics — In dark at 25 °C for 1 wk, colonies on PDA relatively slow growing, 4.2 mm/day, white, fluffy aerial mycelium, reverse with yellow pigmentation developing in centre.

Host range — *Acer*, *Asphodelus albus*, *Aucuba japonica*, *Brugmansia*, *Castanea*, *Corylus*, *Dipsacus fullonum*, *Epilobium*, *Eucalyptus*, *Fagus*, *Fraxinus*, *Holcus*, *Hydrangea*, *Ileostylis*, *Laburnum*, *Lupinus*, *Malus*, *Protea*, *Pyrus*, *Rosa*, *Sambucus*, *Salix*, *Vaccinium* and *Vitis vinifera.* In addition to the hosts on the specimens listed below, these hosts are represented in Fig. 1 based on ITS phylogeny and Gomes et al. (2013) as *D. viticola*.

Distribution — Australia, Canada, Chile, Europe (Austria, Germany, Italy, Latvia, Portugal, Spain, Sweden, Switzerland), New Zealand and South Africa.

Type specimens examined. France, on a dead branch of *Laburnum anagyroides* (as *Cytisus laburnum)*, ex herb. Guépin no. 163 (holotype of *Sphaeria rudis* UPS F-004948). – Austria, Vienna, 19. 7763/2, Reisenbergbach-Weg, on stem of *Laburnum anagyroides*, 8 Apr. 2000, *W. Jaklitsch* (epitype of *Sphaeria rudis* designated here BPI 748231, ex-epitype culture AR 3422 = CBS 109292; MBT175965). – Germany, Nordrhein-Westfalen, Landkreis Unna, Cappenberg, Schloßgarten zu Cappenberg, on twigs of *Laburnum anagyroides* (syn. *Cytisus laburnum*), 18 Aug. 1866, *T. Nitschke* (holotype of *Diaporthe medusaea* B 70 0009168). – Austria, Vienna, 19. 7763/2, Reisenbergbach-Weg, on stem of *Laburnum anagyroides*, 8 Apr. 2000, *W. Jaklitsch* (epitype of *Diaporthe medusaea* designated here BPI 748231, ex epitype culture AR3422 = CBS 109292; MBT175966). – Germany, Nordrhein-Westfalen, Munsterland, Munster Botanischer Garten, on thin branch of *Fagus sylvatica*, 18 May 1866, *T. Nitschke* (holotype of *Diaporthe macrostoma* B 70 0009167). – Germany, Westfalen, Munster, bei der Wienburg, on *Vitis vinifera*, Feb. 1866, *Nitschke* (holotype of *Diaporthe viticola* B: not seen), ibid. (isotype BPI 797316). – Portugal, Santo Tirso, Burgaes, on *Vitis vinifera*,16 Feb. 1998, *A.J.L. Phillips* (epitype of *D. viticola* designated in van Niekerk et al. (2005) CBS-H 7950 not seen, ex-epitype culture STE-U 5683 = CBS 113201). – Italy, "In sarmentis *Vitis viniferae silvestris*, *Cervarese*" (holotype of *Diaporthe silvestris*: PAD 228 not seen). The synonymy of this name is based on van Niekerk et al. (2005) in which the holotype specimen was observed and considered to be *D. viticola.*

Additional specimens examined. Austria, Vienna, stem of *Rosa canina*, 13 May 2001, *W. Jaklitsch* (BPI 840948, living culture AR3654); Vienna, stem of *Acer pseudoplatanus*, 31 Mar. 2001, *W. Jaklitsch* (BPI840940, living culture AR3634). – Germany, urban residential area, container plant, dead stem of *Brugmansia* sp., 31 Oct. 2012, *R. Schumacher* (BPI 892463, living culture DA243 = CBS135435). – Italy, on dead stem of *Acer opalus*, 2 May 2012, *E. Camporesi* ER285 (BPI 892464, living culture ER285A = CBS 135437); ibid., (ER 286, BPI 892465, living culture ER286D).

Notes — The name *D. rudis* is based on the oldest epithet of the many synonyms for this species including *D. medusaea*. *Diaporthe medusaea*, originally described from *Laburnum anagyroides* in Germany, has been used as the name for the fungus causing melanose and stem end rot of *Citrus* in North America. We observed holotype material as well as isolates on the same host from Austria in order to recognise the similarities or differences as discussed herein. Wehmeyer (1933) listed a number of synonyms for *D. medusaea* including *D. citri*, *D. citrincola*, *D. faginea*, *D. rudis* and *D. viticola*. *Diaporthe citrincola* is here recognised as a synonym of *D. citri. Diaporthe faginea* was established as a legitimate name for *Sphaeria faginea* Curr., Trans. Linn. Soc. London 22: 281. 1859 nom. illeg. non *S. faginea* Pers. 1794. No specimen as *D. faginea* exists in PAD. Based on an ITS sequence (EF155490) of the isolate from *Fagus* in Germany and a morphological comparison of the protologue, this name is accepted as a synonym of *D. rudis*. Although *D. viticola* was recognised as a distinct taxon and characterised and epitypified using a specimen on *Vitis* by van Niekerk et al. (2005), it is here determined to be a synonym of *D. rudis*.

EXCLUDED SPECIES

Phoma citri Sacc., Nuovo Giorn. Bot. Ital. 8: 200. 1876.

≡ *Phomopsis citri* (Sacc.) Traverso & Spessa, Bol. Soc. Brot. 25: 100. 1910.

Italy, Traviso, a Vittorio, on branches of *Citrus limon*, Oct. 1873 (lectotype specimen of *Phoma citri* designated here, Mycotheca Veneto no. 332, FH labelled *Diplodia citri*; MBT175967).

Notes — *Phoma citri* has been confused with *Phomopsis citri*, now regarded as *Diaporthe citri*. Examination of type material of *Phoma citri* confirms that this taxon is not a *Phomopsis* and should be treated as a distinct taxon in the genus *Phoma*.

Diaporthe foeniculacea (Mont.) Niessl, in Thüm., Inst. Rev. Sci. Litt. Coimbra 27: 250. 1879.

Basionym. *Sphaeria foeniculacea* Mont., Ann. Sci. Nat., Bot., sér. 3, 11: 40. 1849.

≡ *Physalospora foeniculacea* (Mont.) Sacc. (as '*foeniculata*'), Syll. Fung. (Abellini) 1: 445. 1882.

≡ *Sphaerella foeniculacea* (Mont.) Cooke (as '*foeniculata*'), J. Bot. London 21: 70. 1883.

≡ *Guignardia foeniculacea* (Mont.) Arx & E. Müll. (as '*foeniculata*'), Beitr. Kryptogamenfl. Schweiz 11 (no. 1): 48. 1954.

Portugal, Coimbra, on stem of *Foeniculum officinalis*, June 1881, (lectotype specimen of *Sphaeria foeniculacea* designated here: in Thümen, Mycotheca Universalis 2260, bound collection in BPI; MBT175968). Isolectotypes: ibid. (BPI 616247, BPI 797288 Shear Types and Rarities).

Notes — One of the names mentioned in Phillips (2003) is *Diaporthe foeniculacea* (Mont.) Niessl (basionym *Sphaeria foeniculacea* Mont.), a name that has been confused with *Phomopsis foeniculina* (basionym *Phoma foeniculina*). We observed three isotype specimens of *D. foeniculacea* in BPI and confirmed the status of this species as a *Guignardia* (sexual morph of *Phyllosticta*) as suggested by von Arx & Müller (1954), unrelated to *Diaporthe*. One of these specimens is here designated the lectotype.

DISCUSSION

Melanose and stem end rot of *Citrus* have been reported from the United States since the late 18th century killing twigs and causing a minor form of gummosis by latent infection (Floyd & Stevens 1912, Fawcett 1936). A disease of *Citrus* to which the common name melanose is applied was first recognised near Citra, Florida, by Swingle & Webber in 1892 (Floyd & Stevens 1912). The stem end rot disease was investigated in Florida by Burger (1923) and Winston et al. (1923). *Phomopsis citri*

was first described from the United States (Florida) as a pycnidial fungus on dead branches and decayed fruits of *Citrus aurantium*, *C. decumana* and *C. nobilis* (Fawcett 1912). This name is a later homonym of *Phomopsis citri* (Sacc.) Traverso & Spessa (1910) based on *Phoma citri* Sacc. (1876) originally described from *Citrus limonia* in Italy and now considered to belong in *Phoma*. *Diaporthe citri* (H.S. Fawc.) F.A. Wolf, described as sexual morph of *Phomopsis citri* H.S. Fawc., was originally collected from the United States (Florida) and has since been reported as saprobic or parasitic on leaves, stems and fruits of *Citrus* spp. throughout the world. Fisher (1972) used the concept of Wehmeyer (1933) and considered *Phomopsis cytosporella* as the valid name for *D. citri* based on the chronology of names. However, this interpretation was not adopted by plant pathologists or taxonomists. In our study *P. cytosporella* is determined to be a distinct taxon in *Diaporthe*, *D. cytosporella*, and epitypified based on fresh collections from Europe.

In addition to the association with *Citrus* in the United States, *Diaporthe citri* is confirmed here in Brazil, China, Korea and New Zealand and appears to be widespread in Asia, Australasia and South America. Based on sampling in this study, we did not find *D. citri* to occur in Europe or any sequences in public databases corresponding to *D. citri* from Europe. However, our results suggest that this species may be pantropical. The genetic similarities of the isolates of *D. citri* from Asia where *Citrus* originated with those found worldwide suggest a long standing co-existence and the probable widespread movement of the pathogen with its host. *Diaporthe citri* was the dominant species causing melanose and stem end rot symptoms among the recent collections from *Citrus* spp. throughout China (Huang et al. 2013). Two newly described species discovered in north central China (Shaanxi Province) in the same study, *Diaporthe citiasiana* and *D. citrichinensis*, were primarily associated with dead wood of *Citrus unshiu* (satsuma mandarin) and not with melanose and stem end rot diseases. Gomes et al. (2013) included two species from *Citrus* from Suriname in their analysis, *Diaporthe arecae* and an unidentified species. None of these species were encountered in this study. This indicates that numerous species are associated with *Citrus* worldwide and it is likely that more will be discovered.

Diaporthe foeniculina (referred to as *D. foeniculacea* in Gomes et al. 2013, see taxonomy section) is found to be a pathogen of diverse hosts ranging from crops to temperate woody plants and fruit trees. The recent reports of *D. foeniculina* (as *D. neotheicola*) causing diseases of temperate cultivated fruit trees including shoot blight of persimmon in Australia and kiwifruit disease in Greece suggest potential for this species to infect a wide range of fruits as an opportunistic pathogen (Golzar et al. 2012, Thomidis et al. 2013). Although a number of isolates from *Citrus* in California are identified in this study as *D. foeniculina*, its pathogenicity on *Citrus* in California is unknown. Herbarium specimens previously identified as *D. citri* intercepted at ports in the United States on the fruits of *Citrus limonia* from Spain were identified morphologically as *D. foeniculina* (BPI 615878: intercepted in New York, 1940; BPI 747926, BPI 747927, BPI 1107900: intercepted in New Jersey, 1987). A living culture (MEP1289-1) from BPI 1107900 was used in the phylogenetic analyses in this study and is confirmed as *D. foeniculina*.

Phylogenetic analysis of ITS sequence data was able to resolve the closely related species *D. canthii*, *D. cytosporella*, *D. foeniculina* and *D. pterocarpi*. Although the ITS sequence analysis resolved a clade corresponding to the recently described *D. rhusicola*, this clade was not supported by the ACT, CAL, EF1-α or TUB gene regions, individually or in the multi-gene phylogeny. Inspection of the ITS sequences for this group of isolates (*D. foeniculina* and *D. rhusicola*) indicated that the ITS differences consisted of two deletions and one transition in the ITS1 and two transitions in the ITS2 region. Alternatively, in the case of *D. cynaroidis* and *D. rudis*, ITS sequences do not definitively distinguish the two species although EF1-α, CAL, HIS and TUB do distinguish the two species.

In the case of *D. rudis* (referred to as *D. viticola* in van Niekerk et al. 2005 and Gomes et al. 2013) and *D. australafricana*, the ITS1 region differs by a single transition while the ITS2 region shows minor differences over a span of c. 7 bp consisting of three transitions, one transversion and one deletion. The CAL gene differs by one transition and isolates of *D. rudis* share a 9 bp insertion in ACT not found in *D. australafricana*. Data from Gomes et al. (2013) show a 3 bp insertion in isolates of *D. australafricana* not shared by *D. rudis* in HIS. Isolates with ITS sequences matching that of *D. australafricana* were previously only known from Africa and Australia on *Vitis* but have recently been found on *Vaccinium* and *Corylus* in Chile and *Persea* from California in the United States (Latorre et al. 2012, Elfar et al. 2013). Although closely related, the low but consistent variation found in the sampled genes is considered sufficient to recognise *D. australafricana* as a distinct phylogenetic species in agreement with van Niekerk et al. (2005), Udayanga et al. (2012a) and Gomes et al. (2013). However, additional isolates of both taxa would be desirable in order to further investigate population structure and species boundaries.

Much confusion in the literature exists in how to interpret the ITS sequences of closely related species in *Diaporthe* and authors treat observed variation in ITS sequences in different ways (Farr et al. 2002a, b, Murali et al. 2006, Santos et al. 2010). This can result in superfluous, multiple terminal branches in combined gene analyses, even when other gene regions do not support these distinctions. Additionally Santos et al. (2010) showed the occurrence of two phylogenetically distinct ITS populations within an unidentified species of *Diaporthe* based on the sequencing of single ascospore-derived isolates from the same perithecium. Sequence differences were confined to the ITS1 region over a span of c. 40 bp and are more extensive than those differences noted among isolates of a single species in this study. Sequence differences were not noted in the EF1-α and mating type genes in their analyses and the isolates were fully reproductively compatible (Santos et al. 2010).

While we consider ITS rDNA sequences to be useful as barcodes for identification of known, circumscribed *Diaporthe* species, we suggest that caution is warranted when differences are noted in the absence of other data and that at the minimum EF1-α should be used to confirm identities of *Diaporthe* species. The EF1-α gene region amplified by primers EF1-728F/EF1-986R has given the most consistent results when analysing available ex-type sequences of reliably identified and vouchered species (Castlebury 2005, Santos et al. 2010, 2011, Udayanga et al. 2012a). Phylogenetic signals observed in protein coding genes are generally considered superior to rRNA genes, although there is less standardisation in terms of universal primers, genes or even regions of genes sequenced for fungi (Schoch et al. 2012). However, results from this study suggest that use of CAL and TUB genes can be problematic with the potential for incorrect species identification in *Diaporthe* due to non-specific priming and the resulting poor sequence quality (CAL) or potential paralogy (TUB), necessitating critical evaluation of sources of conflict in gene trees.

Epitypification is recognised as the best approach to resolve long standing taxonomic and phylogenetic issues of known taxa that are poorly circumscribed, thus providing a modern interpretive type (Hyde & Zhang 2008). There is an unprecedented need for mycologists to return to the field to recollect species, fully characterise taxa with DNA sequences and morphological descriptions and epitypify species in *Diaporthe*, which includes a large number of names not linked to DNA sequence data or ex-

type cultures (Hyde et al. 2010a, b, Ko-Ko et al. 2011, Udayanga et al. 2011). Several modern studies aimed at epitypification of important pathogens in *Diaporthe* provide clarification and knowledge of the phylogeny and species boundaries within the genus (Castlebury et al. 2003, van Niekerk et al. 2005, Rensburg et al. 2006, Diogo et al. 2010, Udayanga et al. 2012b, Gomes et al. 2013). Application of genealogical concordance and/or *gsi* in combination with epitypification can provide critical insights into speciation processes, ecology and host associations (Sakalidis et al. 2011, Gazis et al. 2011, Mejia et al. 2011, Silva et al. 2012, Walker et al. 2012).

Species circumscription should only be undertaken in conjunction with rigorous application of multi-gene analyses and genealogical concordance. In addition best practices for introduction of new names should require a thorough investigation of existing names and type specimens. This is particularly important to prevent additional superfluous names in a genus such as *Diaporthe* with an abundance of existing names including those in *Phomopsis.* A total of 2 453 results are returned when searching for *Diaporthe* and *Phomopsis* ITS sequences in the NCBI GenBank databases. A search using the terms 'Phomopsis sp.' (Organism) AND 'internal transcribed' (All Fields) returned 922 sequences; substituting 'Diaporthe sp.' returned 341 sequences (http://www.ncbi.nlm.nih.gov, retrieved 3 April 2013), which suggests that c. 51 % of all *Diaporthe* and *Phomopsis* ITS sequences in GenBank are unidentified. Of the 49 % that do have species names, many do not include culture or specimen vouchers and it is likely that the majority are misidentified or named without a proper taxonomic revision. As sequence data accumulate in public databases, the need for correctly identifying and vouchering sequences, particularly from ex-type or epitype cultures, becomes pressing as these data are being used for identification of pathogens by plant pathologists and in public health and quarantine situations.

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