

Insights into the genus *Diaporthe*: phylogenetic species delimitation in the *D. eres* species complex

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Abstract The genus *Diaporthe* comprises pathogenic, endophytic and saprobic species with both temperate and tropical distributions. Cryptic diversification, phenotypic plasticity and extensive host associations have long complicated accurate identifications of species in this genus. The delimitation of the generic type species *Diaporthe eres* has been uncertain due to the lack of ex-type cultures. Species limits of *D. eres* and closely related species were evaluated using molecular phylogenetic analysis of eight genes including nuclear ribosomal internal transcribed spacer (ITS), partial sequences of actin

(ACT), DNA-lyase (Apn2), translation elongation factor 1- α (EF1- α), beta-tubulin (TUB), calmodulin (CAL), 60s ribosomal protein L37 (FG1093) and histone-3 (HIS). The occurrence of sequence heterogeneity of ITS within *D. eres* is observed, which complicates the analysis and may lead to overestimation of the species diversity. The strict criteria of Genealogical Concordance Phylogenetic Species Recognition (GCPSR) were applied to resolve species boundaries based on individual and combined analyses of other seven genes except the ITS. We accept nine distinct phylogenetic species including *Diaporthe alleghaniensis*, *D. alnea*, *D. bicincta*, *D. celastrina*, *D. eres*, *D. helicis*, *D. neilliae*, *D. pulla* and *D. vaccinii*. Epitypes are designated for *D. alnea*, *D. bicincta*, *D. celastrina*, *D. eres*, *D. helicis* and *D. pulla*. Modern descriptions and illustrations are provided for these species. Newly designed primers are introduced to amplify and sequence the Apn2 (DNA-lyase) gene in *Diaporthe*. Based on phylogenetic informativeness profiles, EF1- α , Apn2 and HIS genes are recognised as the best markers for defining species in the *D. eres* complex.

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Introduction

In the last two decades much progress has been made in the ability to define fungal species through the use of molecular data (Hibbett and Taylor 2013; Hyde et al. 2013). Circumscribing species within cryptic species complexes that have complicated life histories is essential for determining patterns of speciation and potential hyperdiversity within a genus (Bickford et al. 2007; Silva et al. 2012a; Fekete et al. 2012; O'Donnell et al. 2013). Genealogical Concordance Phylogenetic Species Recognition (GCPSR) as an approach

for defining fungal species was proposed by Taylor et al. (2000), based on Avise and Ball's (1990) genealogical concordance species concept requiring the analysis of several unlinked genes. This approach is often used as an alternative to morphological and biological species recognition (Dettman et al. 2003a). However, there have been relatively a few evaluations of the utility of genes to delineate closely related species in genera with broad host ranges and wide geographic distributions (Giraud et al. 2008; Dupis et al. 2012; Groenewald et al. 2013; Wikee et al. 2013; Salgado-Salazar et al. 2013).

The principles of GCPSR are based on the assumption that recombination within a lineage is likely to be the reason for conflict within gene trees, with the transition from conflict to congruence representing the species boundaries (Taylor et al. 2000). Selecting multiple genes with strong phylogenetic signals, the absence of significant incongruence and the application of standard criteria to determine the species boundaries are important factors in the best practices of resolving species complexes (O'Donnell et al. 2000; Bischoff et al. 2009; Watanabe et al. 2011; Salichos and Rokas 2013; Damm et al. 2013; Quaedvlieg et al. 2014). Dettman et al. (2003a) further upgraded the operational criteria of GCPSR with the implementation of a two-step process to resolve complex species level phylogenies in fungi. Independent evolutionary lineages are recognised by genealogical concordance and non-discordance, and subsequently these lineages are subjected to the ranking based on genetic differentiation and exhaustive subdivision process to determine the species limits (Dettman et al. 2003a, b). These methods have been implemented in species complexes including the model ascomycete *Neurospora* (Dettman et al. 2003b, 2006) and some important plant pathogenic fungal genera (O'Donnell et al. 2004; Taylor et al. 2006; Cai et al. 2011; Laurence et al. 2014). The genus *Diaporthe* comprises pathogenic, endophytic and saprobic species with both temperate and tropical geographic distributions (Rehner and Uecker 1994; Rossman et al. 2007; Udayanga et al. 2011; Huang et al. 2013). Species recognition criteria in *Diaporthe* have evolved from morphology and host associations (Wehmeyer 1933) to the recent use of phylogenetic species recognition (Castlebury et al. 2003; Santos and Phillips 2009; Santos et al. 2011; Udayanga et al. 2012a, b; Gomes et al. 2013; Tan et al. 2013).

Diaporthe eres Nitschke, the type species of the genus, was originally described by Nitschke (1870), from *Ulmus* sp. collected in Germany. Wehmeyer (1933) listed a number of synonyms under *D. eres* with approximately 70 host associations belonging to a wide range of plant families based on morphological characters. Despite Wehmeyer's (1933) broad concept of *D. eres*, a comprehensive study of this species has not been attempted (Udayanga et al. 2011; Gomes et al. 2013). Few of the synonyms listed in Wehmeyer's taxonomic treatment have been accepted by later studies or re-examined using molecular data. The oldest name associated with *D. eres* is *Phomopsis*

velata (Sacc.) Traverso and the editors of *Index Fungorum* have recently listed *D. eres* as a synonym of *P. velata* along with many other synonyms including names belonging to *Chorostate*, *Cucurbitaria*, *Diatrype*, *Phoma*, *Phomopsis*, *Sclerophoma*, *Sclerophomella*, and *Valsa* (Index Fungorum 2014). Considering its status as the generic type and its wide use in the literature, Rossman et al. (2014) proposed to conserve the name *Diaporthe eres* over all potential synonyms.

Wehmeyer (1933) based his species concept on morphology rather than host association and observed that *Diaporthe eres* might be regarded as a species complex. Barr (1978) recognised three sections of *Diaporthe* based on ascospore morphology including *Diaporthe* section *Diaporthe* typified by *D. eres*. Although a broad species concept has historically been associated with *D. eres*, the lack of an ex-type or epi-type culture for this generic type species has been a major issue. The incorporation of DNA-sequence-based comparative methods to accurately define the type species is essential to provide a stable platform for the taxonomy of a genus (Crous et al. 2014). The available identifications of *D. eres* in disease reports and other recent phylogenetic studies have been based solely on morphology or more recently on comparison with reference sequences in GenBank. Despite the previous taxonomic definitions based on morphology and host association and recently vouchered sequences, the phylogenetic limits of the *D. eres* species complex are still unknown.

Diaporthe eres has also been regarded as a minor pathogen causing leaf spots, stem cankers and diseases of woody plants in diverse families including the *Ericaceae*, *Juglandaceae*, *Rosaceae*, *Sapindaceae*, *Ulmaceae*, *Vitaceae* and others, mostly in temperate regions worldwide (Vrandečić et al. 2010; Anagnostakis 2007; Thomidis and Michailides 2009; Baumgartner et al. 2013). In addition, it is considered a pathogen with plant health inspection and quarantine significance (Cline and Farr 2006). Several recent disease reports of *D. eres* include cane blight on blackberry in Croatia (Vrandečić et al. 2010), pathogen of butternut (*Juglans cinerea*) in Connecticut (Anagnostakis 2007), shoot blight and canker disease of peach in Greece (Thomidis and Michailides 2009), stem canker of *Salsola tragus* in Russia (Kolomiets et al. 2009), on *Vaccinium* species in Europe (Lombard et al. 2014) and association with wood cankers of grapevines in Croatia (Kaliterna et al. 2012) and in the USA (Baumgartner et al. 2013). It is reported less frequently on herbaceous plants such as the *Cucurbitaceae* (Garibaldi et al. 2011; Gomes et al. 2013).

The aims of this study are as follows: 1) to define the species limits of *D. eres* and closely related species based on multi-gene genealogies; 2) to designate epi-type specimens for *D. eres* and related species including *D. alnea*, *D. bicincta*, *D. celsastrina*, *D. helcis* and *D. pulla* and provide modern descriptions and illustrations with synonyms; and 3) to critically evaluate phylogenetic species concepts in *Diaporthe*,

providing insights into the usefulness of various genes within this species complex.

Materials and methods

Sampling and morphology

Sources of isolates, additional fresh specimens and cultures obtained from contributors are listed in Table 1. Specimens of *D. eres* were initially collected from *Ulmus* in Germany and subsequent collections were made from the same host to identify both the sexual and asexual morphs. Morphological descriptions are based on type or epitype specimens and cultures including pycnidia developing on water agar with sterilized alfalfa stems. Digital images were captured and cultural characteristics were observed as described in Udayanga et al. (2014).

DNA extraction, PCR and sequencing

DNA was extracted and the ITS, EF1- α , CAL, TUB and ACT genes were amplified following the protocols outlined by Udayanga et al. (2014). The FG1093 (60s ribosomal protein L37) was amplified using the universal primers for Ascomycota, E1F1 and E3R1 (Walker et al. 2012) following the touch-down PCR protocol outlined by the same study. HIS (Histone-3) genes were amplified as described in Gomes et al. (2013) using the primer pair CYLH3F (Crous et al. 2004b) and H3-1b (Glass and Donaldson 1995). Apn2 primers for *Diaporthe* were designed and the conditions were optimised as described in this study and amplified under conditions of 95 °C for 1 min, (95 °C : 30 s, 54 °C:50 s, 72 °C:1 min) \times 39 cycles, 72 °C for 10 min extension in PCR mixtures used for the other genes in Udayanga et al. (2014). PCR products were visualised in 1 % agarose gel electrophoresis (Udayanga et al. 2014) and then purified with ExoSAP-IT (USB Corp., Cleveland, Ohio) according to the manufacturer's instructions and sequenced with the BigDye Terminator 3.1 cycle sequencing kit (Applied Biosystems, Foster City, California) on an Applied Biosystems 3130xl Genetic Analyser using the same amplification primers for each of the gene regions.

Apn2 (DNA lyase) primer design and assessment of utility within *Diaporthe*

An alignment of the complete sequences of Apn2-Mat genes of *Diaporthe* W and G types in Kanematsu et al. (2007) (AB199324-27) with a selected set of homologous Apn2 genes available in GenBank including *Colletotrichum caudatum* (JX076930-32), *C. cereale* (EU365102, 365045, 365117), *C. fragariae* (FR719119), *C. fructicola* (FR719124), *C. gloeosporioides* (FR719121-22,

FR719126), *C. siamense* (FR719125), and *Thielavia terrestris* chromosome A (XM003651303), *Myceliophthora thermophila* Chromosome 1 (CP003002), and the mating type A locus from *Neurospora terricola* (HE600070), *N. pannonica* (HE600067) and *N. africana* (HE600066) were used to design the forward and reverse primers to amplify the Apn2 gene. The target fragment contained the DNA-(apurinic or apyrimidinic site) lyase (Apn2) gene approximately 800 bp including an intron region of 70–100 bp. The forward primer (apn2fw2: GCMATGTTYGAMATYCTGGAG) and the reverse primer (apn2rw2: CTT GGTCTCCCAGCAGGTG AAC) were designed based on the proximal end of first exon and the distal end of the second exon region relatively conserved across the alignment. The selected primers were then evaluated for thermal properties, GC content, 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).

Gradient PCR and reagent optimisations were used to develop the standard protocols for amplification. Twelve reactions across an annealing temperature gradient of 65–50 °C for each of the test isolates were performed in three replicates. The optimal annealing temperature was determined by the intensity of the amplicons visualised by agarose gel electrophoresis. Primers were initially tested against a panel of 20 species selected from a broad range of *Diaporthe* species and including the representative isolates of *Ophiodiaporthe cyatheae* (AR5192) and *Mazzantia galii* (AR4658). PCR products were purified and sequenced using the protocols detailed above.

Sequence alignment and phylogenetic analysis

Raw sequences were assembled with Sequencher 4.9 for Windows (Gene Codes Corp., Ann Arbor, Michigan). The consensus sequences were then initially aligned using MAFFTv.7 (Katoh and Standley 2013) (<http://mafft.cbrc.jp/alignment/server/>) and optimised in the SATEv.2.2.7 (Simultaneous Alignment and Tree Estimation) high throughput alignment platform (<http://phylo.bio.ku.edu/software/sate/sate.html>) (Liu et al. 2012). Newly generated ITS and EF1- α sequences were analysed with all available type-derived sequences listed in Udayanga et al. (2011, 2012a) and Gomes et al. (2013) to determine initial identities of the isolates.

ML gene-trees were estimated using the software RAXML 7.4.2 Black Box (Stamatakis 2006; Stamatakis et al. 2008) in the CIPRES Science Gateway platform (Miller et al. 2010). For the concatenated dataset all free modal parameters estimated by RAXML with ML estimate of 25 per site rate categories. The RAXML software accommodated the GTR model of nucleotide substitution with the additional options

Table 1 Isolates and sequences used in this study

Species	Isolate/culture collection*	Host	Host family	Location	Collector	GenBank accessions									
						ACT	Apm2	CAL	EFL- α	FGI093	HIS	ITS	TUB		
<i>D. alleghaniensis</i>	CBS 495.72	<i>Betula alleghaniensis</i>	Betulaceae	Canada	R.H. Arnold	JQ807299	KJ380963	KC343249	GQ250298	KJ381045	KC343491	FJ889444	KC843228		
<i>D. abaea</i>	CBS 146.46	<i>Alnus</i> sp.	Betulaceae	Netherlands	S. Truter	KJ420774	KJ380969	KC343250	KC343734	KJ381037	KC343492	KC343008	KC343976		
	CBS 159.47	<i>Alnus</i> sp.	Betulaceae	Netherlands	S. Truter	KJ420775	KJ380970	KC343251	KC343735	KJ381038	KC343493	KC343009	KC343977		
	LCM22b.02a	<i>Alnus</i> sp.	Betulaceae	USA	L.C. Mejia	KJ420776	KJ380971	KJ435020	KJ210557	KJ381039	KJ420883	KJ210535	KJ420825		
	LCM22b.02b	<i>Alnus</i> sp.	Betulaceae	USA	L.C. Mejia	KJ420777	KJ380972	KJ435021	KJ210558	KJ381040	KJ420884	KJ210536	KJ420826		
	DP0659=CBS 121004	<i>Juglans</i> sp.	Juglandaceae	USA	A.Y. Rossman	KJ420771	KJ380976	KC343376	KC343860	KJ381042	KC343618	KC343134	KC344102		
<i>D. bicipita</i>															
<i>D. celastrina</i>	CBS 139.27	<i>Celastrus</i> sp.	Celastraceae	USA	L.E. Wehmeyer	KJ420769	KJ380974	KC343289	KC343773	KJ381041	KC343531	KC343047	KC344015		
<i>D. citri</i>	AR3405	<i>Citrus</i> sp.	Rutaceae	USA	L. W. Timmer	KC843234	KJ380981	KC843157	KC843071	KJ381049	KJ420881	KC843311	KC843187		
<i>D. citricrinensis</i> <i>eres</i>	ZJUD034A=CBS 134242	<i>Citrus</i> sp.	Rutaceae	China	F. Huang	KJ420779	KJ380980	KC843234	KC843071	KJ381048	KJ420880	KC843311	KC843187		
	ZJUD034B=M1040	<i>Citrus</i> sp.	Rutaceae	China	F. Huang	KJ420778	KJ380979	KJ435042	KJ210562	KJ381047	KJ420879	KJ210539	KJ420829		
	AR5193=CBS 138594	<i>Ulmus laevis</i>	Ulmaceae	Germany	R. Schumacher	KJ420760	KJ380958	KJ434999	KJ210550	KJ381003	KJ420850	KJ210529	KJ420799		
	AR5196=CBS 138595	<i>Ulmus laevis</i>	Ulmaceae	Germany	R. Schumacher	KJ420766	KJ380932	KJ435006	KJ210554	KJ381021	KJ420866	KJ210533	KJ420817		
	DP0438	<i>Ulmus minor</i>	Ulmaceae	Austria	W. Jaklitsch	KJ420765	KJ380935	KJ435016	KJ210553	KJ381020	KJ420886	KJ210532	KJ420816		
	LCM114.01a=CBS 138598	<i>Ulmus</i> sp.	Ulmaceae	USA	L.C. Mejia	KJ420754	KJ380919	KJ435027	KJ210545	KJ380988	KJ420837	KJ210521	KJ420787		
	LCM114.01b	<i>Ulmus</i> sp.	Ulmaceae	USA	L.C. Mejia	KJ420754	KJ380918	KJ435026	KJ210544	KJ380987	KJ420836	KJ210520	KJ420786		
	FAU483	<i>Malus</i> sp.	Rosaceae	Netherlands	F.A. Uecker	JQ807326	KJ380933	KJ435022	JQ807422	KJ381031	KJ420874	KJ210537	KJ420827		
	DAN001A=M1115	<i>Daphne laureola</i>	Thymelaeaceae	France	unknown	KJ420750	KJ380914	KJ434994	KJ210540	KJ380982	KJ420831	KJ210516	KJ420781		
	DAN001B=M1116	<i>Daphne laureola</i>	Thymelaeaceae	France	unknown	KJ420751	KJ380915	KJ434995	KJ210541	KJ380983	KJ420832	KJ210517	KJ420782		
	AR5197	<i>Rhododendron</i> sp.	Ericaceae	Germany	R. Schumacher	KJ420764	KJ380931	KJ435014	KJ210552	KJ381016	KJ420863	KJ210531	KJ420812		
	CBS 439.82	<i>Cotoneaster</i> sp.	Rosaceae	UK	H. Butin	KC843231	KJ380920	JX197429	GQ250341	KJ380989	KC343574	FJ889450	JX275437		
	AR3519	<i>Corylus avellana</i>	Betulaceae	Austria	W. Jaklitsch	KJ420758	KJ380922	KJ435008	KJ210547	KJ380991	KJ420839	KJ210523	KJ420789		
	FAU506	<i>Cornus florida</i>	Cornaceae	USA	F.A. Uecker	JQ807328	KJ380925	KJ435012	JQ807403	KJ380994	KJ420842	KJ210526	KJ420792		
	FAU570	<i>Oxydendrum arboreum</i>	Ericaceae	USA	F.A. Uecker	JQ807335	KJ380926	KJ435025	JQ807410	KJ380996	KJ420844	KJ210527	KJ420794		
	AR3723	<i>Rubus fruticosus</i>	Rosaceae	Austria	W. Jaklitsch	JQ807273	KJ380941	KJ435024	JQ807354	KJ380995	KJ420843	JQ807428	KJ420793		
	FAU522	<i>Sassafras albidum</i>	Lauraceae	USA	F.A. Uecker	JQ807331	KJ380924	KJ435010	JQ807406	KJ380993	KJ420841	KJ210525	KJ420791		
	DP0666	<i>Juglans cinerea</i>	Juglandaceae	USA	S. Anagnostakis	KJ420756	KJ380921	KJ435007	KJ210546	KJ380990	KJ420838	KJ210522	KJ420788		
	DP0667=CBS 135428	<i>Juglans cinerea</i>	Juglandaceae	USA	S. Anagnostakis	KC843232	KJ380923	KC843155	KC843121	KJ380992	KJ420840	KC843328	KC843229		
	AR3560	<i>Irbidnum</i> sp.	Adoxaceae	Austria	W. Jaklitsch	JQ807270	KJ380939	KJ435011	JQ807351	KJ380998	KJ420846	JQ807425	KJ420795		
	AR5224	<i>Hedera helix</i>	Araliaceae	Germany	R. Schumacher	KJ420763	KJ380961	KJ435036	KJ210551	KJ381006	KJ420853	KJ210530	KJ420802		
	AR5231	<i>Hedera helix</i>	Araliaceae	Germany	R. Schumacher	KJ420767	KJ380936	KJ435038	KJ210555	KJ381022	KJ420867	KJ210534	KJ420818		
	AR5223=CBS 138599	<i>Acer nigrum</i>	Sapindaceae	Germany	R. Schumacher	KJ420759	KJ380938	KJ435000	KJ210549	KJ380997	KJ420845	KJ210528	KJ420830		
	CBS 109767=AR3538	<i>Acer</i> sp.	Sapindaceae	Austria	W. Jaklitsch	JQ807294	KJ380940	KC343317	KC343801	JF319006	KC343559	DQ491514	KC344043		
	DLR12A=M1117=CBS 138597	<i>Vitis vinifera</i>	Vitaceae	France	L. Philippe	KJ420752	KJ380916	KJ434996	KJ210542	KJ380984	KJ420833	KJ210518	KJ420783		
	DLR12B=M1118	<i>Vitis vinifera</i>	Vitaceae	France	L. Philippe	KJ420753	KJ380917	KJ434997	KJ210543	KJ380985	KJ420834	KJ210519	KJ420784		
	AR4347	<i>Vitis vinifera</i>	Vitaceae	Korea	S.K. Hong	JQ807275	KJ380929	KJ435030	JQ807356	KJ381009	KJ420856	JQ807430	KJ420805		
	Dt-C005/1	<i>Hydrangea macrophylla</i>	Hydrangeaceae	Portugal	J.M. Santos	–	–	–	GQ250334	–	–	GQ250203	–		
	Dt-C005/2	<i>Hydrangea macrophylla</i>	Hydrangeaceae	Portugal	J.M. Santos	–	–	–	GQ250335	–	–	GQ250204	–		

Table 1 (continued)

Species	Isolate/culture collection*	Host	Host family	Location	Collector	GenBank accessions									
						ACT	Apr2	CAL	EFL-α	FGI093	HIS	ITS	TUB		
<i>Hydrangea macrophylla</i>	DI-C005/3		<i>Hydrangeaceae</i>	Portugal	J.M. Santos	–	–	–	GQ250336	–	–	–	GQ250205	–	–
<i>Hydrangea macrophylla</i>	DI-C005/4		<i>Hydrangeaceae</i>	Portugal	J.M. Santos	–	–	–	GQ250342	–	–	–	GQ250208	–	–
<i>Hydrangea macrophylla</i>	DI-C005/5		<i>Hydrangeaceae</i>	Portugal	J.M. Santos	–	–	–	GQ250343	–	–	–	GQ250209	–	–
<i>Hydrangea macrophylla</i>	DI-C005/6		<i>Hydrangeaceae</i>	Portugal	J.M. Santos	–	–	–	GQ250344	–	–	–	GQ250210	–	–
<i>Hydrangea macrophylla</i>	DI-C005/7		<i>Hydrangeaceae</i>	Portugal	J.M. Santos	–	–	–	GQ250345	–	–	–	GQ250211	–	–
<i>Hydrangea macrophylla</i>	DI-C005/8		<i>Hydrangeaceae</i>	Portugal	J.M. Santos	–	–	–	GQ250337	–	–	–	GQ250206	–	–
<i>Hydrangea macrophylla</i>	DI-C005/9		<i>Hydrangeaceae</i>	Portugal	J.M. Santos	–	–	–	GQ250346	–	–	–	GQ250212	–	–
<i>Hydrangea macrophylla</i>	DI-C005/10		<i>Hydrangeaceae</i>	Portugal	J.M. Santos	–	–	–	GQ250347	–	–	–	GQ250213	–	–
<i>Prunus</i> sp.	AR4355		<i>Rosaceae</i>	Korea	S.K. Hong	JQ807278	KJ380942	KJ435035	JQ807359	KJ381001	KJ420848	JQ807433	KJ420797		
<i>Prunus</i> sp.	AR4367		<i>Rosaceae</i>	Korea	S.K. Hong	JQ807283	KJ380962	KJ435019	JQ807364	KJ381029	KJ420873	JQ807438	KJ420824		
<i>Prunus mume</i>	AR4346		<i>Rosaceae</i>	Korea	S.K. Hong	JQ807274	KJ380955	KJ435003	JQ807355	KJ381027	KJ420872	JQ807429	KJ420823		
<i>Prunus persici</i>	AR4348		<i>Rosaceae</i>	Korea	S.K. Hong	JQ807276	KJ380952	KJ435004	JQ807357	KJ381015	KJ420862	JQ807431	KJ420808		
<i>Pyrus pyrifolia</i>	AR3669=MAFF 625029		<i>Rosaceae</i>	Japan	S. Kanematsu	JQ807340	KJ380930	KJ435002	JQ807415	KJ381012	KJ420859	JQ807466	KJ420807		
<i>Pyrus pyrifolia</i>	AR3670=MAFF 625030		<i>Rosaceae</i>	Japan	S. Kanematsu	JQ807341	KJ380950	KJ435001	JQ807416	KJ381011	KJ420858	JQ807467	KJ420807		
<i>Pyrus pyrifolia</i>	AR3671=MAFF 625033		<i>Rosaceae</i>	Japan	S. Kanematsu	JQ807342	KJ380954	KJ435017	JQ807417	KJ381018	KJ420865	JQ807468	KJ420814		
<i>Pyrus pyrifolia</i>	AR3672=MAFF 625034		<i>Rosaceae</i>	Japan	S. Kanematsu	JQ807343	KJ380937	KJ435023	JQ807418	KJ381023	KJ420868	JQ807469	KJ420819		
<i>Pyrus pyrifolia</i>	DP0177		<i>Rosaceae</i>	New Zealand	W. Kandula	JQ807304	KJ380945	KJ435041	JQ807381	KJ381024	KJ420869	JQ807450	KJ420820		
<i>Pyrus pyrifolia</i>	DP0591		<i>Rosaceae</i>	New Zealand	W. Kandula	JQ807319	KJ380946	KJ435018	JQ807395	KJ381025	KJ420870	JQ807465	KJ420821		
<i>Pyrus pyrifolia</i>	AR4369		<i>Rosaceae</i>	Korea	S. K. Hong	JQ807285	KJ380953	KJ435005	JQ807366	KJ381017	KJ420864	JQ807440	KJ420813		
<i>Pyrus pyrifolia</i>	DP0180		<i>Rosaceae</i>	New Zealand	W. Kandula	JQ807307	KJ380928	KJ435029	JQ807384	KJ381008	KJ420855	JQ807453	KJ420804		
<i>Pyrus pyrifolia</i>	DP0179		<i>Rosaceae</i>	New Zealand	W. Kandula	JQ807306	KJ380944	KJ435028	JQ807383	KJ381007	KJ420854	JQ807452	KJ420803		
<i>Pyrus pyrifolia</i>	DP0590		<i>Rosaceae</i>	New Zealand	W. Kandula	JQ807318	KJ380951	KJ435037	JQ807394	KJ381014	KJ420861	JQ807464	KJ420810		
AR4373			<i>Rhamnaceae</i>	Korea	S.K. Hong	JQ807287	KJ380957	KJ435013	JQ807368	KJ381002	KJ420849	JQ807442	KJ420798		
AR4374			<i>Rhamnaceae</i>	Korea	S.K. Hong	JQ807288	KJ380943	KJ434998	JQ807369	KJ380986	KJ420835	JQ807443	KJ420785		
AR4357			<i>Rhamnaceae</i>	Korea	S.K. Hong	JQ807279	KJ380949	KJ435031	JQ807360	KJ381010	KJ420857	JQ807434	KJ420806		
AR4371			<i>Rosaceae</i>	Korea	S.K. Hong	JQ807286	KJ380927	KJ435034	JQ807367	KJ381000	KJ420847	JQ807441	KJ420796		
FAU532			<i>Cupressaceae</i>	USA	F.A. Uecker	JQ807333	KJ380934	KJ435015	JQ807408	KJ381019	KJ420885	JQ807333	KJ420815		
CBS113470			<i>Fagaceae</i>	Australia	K.A. Seifert	KJ420768	KJ380956	KC343388	KC343372	KJ381028	KC343630	KC343146	KC344114		
AR4349			<i>Urticaceae</i>	Korea	S.K. Hong	JQ807277	KJ380947	KJ435032	JQ807358	KJ381026	KJ420871	JQ807432	KJ420822		
AR4363			<i>Rosaceae</i>	Korea	S.K. Hong	JQ807281	KJ380948	KJ435033	JQ807362	KJ381013	KJ420860	JQ807436	KJ420809		
DNPI28 (=BYDI,M1119)			<i>Fagaceae</i>	China	S.X. Jiang	KJ420762	KJ380960	KJ435040	KJ210561	KJ381005	KJ420852	JF957786	KJ420801		
DNPI29 (=BYD2, M1120)			<i>Fagaceae</i>	China	S.X. Jiang	KJ420761	KJ380959	KJ435039	KJ210560	KJ381004	KJ420851	JQ619886	KJ420800		
CBS 587.79			<i>Pinaceae</i>	Japan	G. H. Boerema	KJ420770	KJ380975	KC343395	KC343879	KJ381030	KC343637	KC343153	KC344121		

Table 1 (continued)

Species	Isolate/culture collection*	Host	Host family	Location	Collector	GenBank accessions							
						ACT	Apn2	CAL	EFI- α	FG1093	HIS	ITS	TUB
<i>D. helveticus</i>	AR5211=CBS 138596	<i>Hedera helix</i>	Araliaceae	France	A. Gardienet	KJ420772	KJ380977	KJ435043	KJ210559	KJ381043	KJ420875	KJ210538	KJ420828
<i>D. neilliae</i>	CBS 144.27	<i>Spiraea</i> sp.	Rosaceae	USA	L.E. Wehmeyer	KJ420780	KJ380973	KC343386	KC343870	KJ381046	KC343628	KC343144	KC344112
<i>D. pulla</i>	CBS 338.89	<i>Hedera helix</i>	Araliaceae	Yugoslavia	M. Cevetovic	KJ420773	KJ380978	KC343394	KC343878	KJ381044	KC343636	KC343152	KC344120
<i>D. vaccinii</i>	CBS 135436=DF5032	<i>Vaccinium corymbosum</i>	Ericaceae	USA	D.F. Farr	JQ807303	KJ380964	KC849457	JQ807380	KJ381032	KJ420877	AF317570	KC843225
	FAU633	<i>Vaccinium macrocarpon</i>	Ericaceae	USA	F.A. Uecker	JQ807338	KJ380966	KC849456	JQ807413	KJ381034	KJ420878	U11360,U11414	KC843226
	FAU446	<i>Vaccinium macrocarpon</i>	Ericaceae	USA	F. Caruso	JQ807322	KJ380967	KC849455	JQ807398	KJ381035	KJ420882	U11317,U11367	KC843224
	CBS 160.32	<i>Vaccinium macrocarpon</i>	Ericaceae	USA	C.L. Shear	JQ807297	KJ380968	KC343470	GQ250326	KJ381036	KC343712	AF317578	JX270436
	FAU 468	<i>Vaccinium macrocarpon</i>	Ericaceae	USA	F.A. Uecker	JQ807323	KJ380965	KC849458	JQ807399	KJ381033	KJ420876	U113327,U11377	KC843227

*AR, DAN, DNP, FAU, DLR, DF, DP, LCM, M: isolates in SMMML culture collection, USDA-ARS, Beltsville, MD, USA; CBS: CBS-KNAW Fungal Biodiversity Centre, Utrecht, The Netherlands; Di-005/1-10: obtained from Santos et al. 2010; MAFF: NIAS Genebank Project, Ministry of Agriculture, Forestry and Fisheries, Japan

of modeling rate heterogeneity (Γ) and proportion invariable sites (I). These analyses utilised the rapid bootstrapping algorithm in RAxML. All isolates were subjected to a multi-gene analysis of seven genes including Apn2, EF1- α , CAL, HIS, FG1093, ACT and TUB regions, excluding the ITS region from the combined analysis. Individual datasets were tested for congruency using the 70 % reciprocal bootstrap (BS) threshold method as described by Gueidan et al. (2007).

Evolutionary models for phylogenetic analyses were selected independently for each locus using MrModeltest 2.3 (Nylander 2004) under the Akaike Information Criterion (AIC) implemented in both PAUP v.4. and MrBayes v3. Phylogenetic reconstructions of concatenated and individual gene-trees were performed using both Bayesian (BI) Markov Chain Monte Carlo and Maximum Likelihood (ML) criteria. Bayesian reconstructions were performed using MrBayes 3.1.2 (Huelsenbeck and Ronquist 2001; Ronquist et al. 2005). Six simultaneous Markov chains were run for 100000 generations with increments of additional generations when needed until the standard deviation of split frequencies are reached to 0.01 and trees are converged and trees were sampled every 100th generation resulting in 10000 total trees. The first 25 % of the trees, representing the burn-in phase of the analyses, were discarded and the remaining trees used for calculating posterior probabilities (PP) in the majority rule consensus tree. PAUPv 4.0b10 was used to conduct maximum parsimony analyses. Trees were inferred using the heuristic search option with 1000 random sequence additions. The Maxtrees option was unlimited, branches of zero length were collapsed and all equally parsimonious trees were saved. Maximum parsimony trees generated were compared with BI and ML trees, with bootstrap support values indicated on the trees shown. Phylogenetic trees and data files were viewed in MEGA 5 (Tamura et al. 2011), Treeview (Page 1996) and Fig tree v1.4 (Rambaut and Drummond 2008). All the sequences generated were deposited in GenBank (Table 1) and alignments and trees in TreeBASE (Study 16003) and typifications (MBT178529–178541) in MycoBank (Crous et al. 2004a).

Phylogenetic species recognition

In order to determine the species boundaries, we applied the criteria previously described by Dettman et al. (2003a). Clades were genealogically concordant if they were present in at least some of the gene trees and genealogically non-discordant if they were strongly supported ($MP \geq 70\%$; $ML \geq 70\%$) in a single gene and not contradicted at or above this level of support in any other single gene tree. This criterion prohibited poorly supported non-monophyly at one locus from undermining well-supported monophyly at another locus. In addition, species limits were determined conclusively if resolved with strong support ($PP \geq .95$; $ML \geq 70\%$; $MP \geq 75\%$) in all analyses of the combined seven gene dataset (excluding

ITS). Since the variability of ITS sequences within the *D. eres* clade resulted in confusion, also confirmed by Santos et al. (2010), we opted to use the combined seven gene alignment to reconstruct the evolutionary relationships.

When deciding which independent evolutionary lineages should be ranked as phylogenetic species, genetic differentiation and exhaustive subdivision criteria were applied (Dettman et al. 2003a, 2006). Genetic differentiation required that lineages be well-differentiated, preventing minor terminal lineages from being recognised as phylogenetic species. Exhaustive subdivision required that all individuals be classified into phylogenetic species and no individuals be left unclassified. The technique involved tracing from the terminal nodes of the tree, collapsing all lineages that were not subtended by an independent evolutionary lineage (Dettman et al. 2006; Laurence et al. 2014).

Testing phylogenetic informativeness

To determine loci most suitable for species level phylogenetic inference in closely related species within *Diaporthe*, we employed the phylogenetic informativeness profiling method (Townsend 2007) implemented in PhyDesign (Lopez-Giraldez and Townsend 2011, <http://phydesign.townsend.yale.edu/>). Phylogenetic informativeness (PI) was measured from a partitioned combined dataset of ten ex-types and taxonomically authenticated species for the ITS, EF1- α , TUB, CAL, ACT, HIS, FG1093 and Apn2 genes. The maximum likelihood tree from RAxML analysis of the concatenated data set was ultrametricised using Mesquite (Maddison and Maddison 2011). Per gene and per site informativeness for all partitions were determined using PhyDesign and the rates of change for each site determined under the HyPhy criteria (Pond et al. 2005).

Results

DNA Sequencing, Apn2 new primers and phylogenetic analyses

Four hundred new sequences were generated in this study (Table 1) from 68 living cultures of *Diaporthe* for eight genes (ACT, Apn2, CAL, EF1- α , HIS, FG1093, ITS and TUB). Additional sequences were obtained from GenBank. Evaluation of the newly designed Apn2 primers (apnfw2/apanrw2) determined that the melting temperatures (T_m) of apn2fw2=49–56 °C and apn2rw2=58.6 °C with GC content of apn2fw2=38–57 % and apn2rw2=59 %. No hairpin formation or self-complementarities were found. The optimal annealing temperature for the primer pair was determined to be 54 °C by the by gradient PCR using amplification conditions

outlined in materials and methods. Amplification and sequencing of 20 different isolates of *Diaporthe* outside of the *D. eres* species complex (GenBank accessions KM016673–KM016694) including additional isolates of *Ophiodiaporthe cyatheae* (AR5192, KM016693) and *Mazzantia galii* (AR4658, KM016692) were successful (Supplementary material 1/ESM 1).

Eight different alignments corresponding to each individual gene, a combined alignment of all eight genes, and a combined alignment of the seven genes excluding the ITS were analysed. Comparison of the alignment properties and nucleotide substitution models are provided in Table 2. Phylogenetic trees inferred from EF1- α and ITS sequences for all isolates, a summary of the results of GCPSR in RAxML cladogram and a phylogram of combined analysis of seven genes are presented with annotations for species, host and geographic origin (Figs. 1, 2, 3).

Single gene analyses and comparison

The ITS and EF1- α sequence alignment consisted of 548 and 369 characters respectively, with 78 isolates including the outgroup taxa. Phylogenetic trees obtained from maximum likelihood (ML), parsimony (MP), and Bayesian (BI) analysis were compared for the placement of each isolate, topology of the tree and clade stability. The topology of the ML tree inferred from RAxML was identical to BI and MP trees with reference to the major subclades and is presented as Fig. 1. Alignment properties and model selections are shown in Table 2.

The ITS phylogeny has limited resolution within the species complex often resulting in an inconclusive branching order and lack of bootstrap support at the internodes, resulting in two major clusters. Analysis of each region of the ITS sequences of *Diaporthe eres* with the reference annotated sequence (KC343073) revealed an approximately 176 bp span for ITS1 and 161 bp for ITS2 region with the intermediate 5.8 s rDNA partition spanning approximately 157 bp. The differences within two ITS1 clusters were consistent although the two clusters were not completely congruent with the ITS2 region.

We obtained two different isolates from a single ascospore and conidium (AR5193, AR5196) derived from two twigs of *Ulmus* collected at the same time from the same individual tree in Germany, where the field collections were made. Both of these isolates were determined to be *D. eres* based on morphology of the asexual and sexual morphs. However, the single ascospore-derived isolate (AR5193) and the single conidium-derived isolate (AR5196) had different ITS sequences and were placed in different major groups in the ITS phylogenetic tree (Fig. 1). However, they were determined to be the same species based on EF1- α and all other genes.

Table 2 Alignment properties and nucleotide substitution models

Genes/loci	ITS	EF1- α	CAL	TUB	ACT	FG1093	HIS	Apn2	combined 7 genes
Characters included in analysis (with gaps)	548	369	429	517	259	379	501	769	3193
Characters excluded in analysis	–	–	101	–	–	–	–	–	101
Invariable characters	479	266	309	434	186	263	381	645	2563
Parsimony informative characters (%)	43 (8 %)	62 (16 %)	45(10 %)	69(13 %)	40(15 %)	44(11 %)	69(13 %)	78(10 %)	427(13 %)
Uninformative variable characters	26	41	75	14	33	72	51	46	203
Nucleotide substitution models	GTR+I+G	HKY+G	GTR+I+G	HKY+G	GTR+I+G	HKY+G	GTR+I+G	HKY+G	GTR+I+G

Inspection of the ITS alignment also revealed that isolates can share similarity in the ITS1 and ITS2 regions both within and between species in this complex. The ITS1 region of *Diaporthe vaccinii* is identical to most of the isolates identified as *D. eres*. In contrast, the ITS2 region is different between the two species. *Diaporthe citri* and *D. citrichinensis* share ITS similarities with the other species in the complex. However, the two species are clearly diverged when analyses using the other genes are performed and therefore regarded as outgroup taxa in the analyses.

As opposed to the ITS, the EF1- α phylogenetic tree clearly distinguishes species boundaries except in a few closely related species that could only be distinguished in the combined analyses. The EF1- α phylogenetic tree was used as an initial guide to determine the species limits and tested with all other genes and in various combinations. Nodes that were supported ($\geq 70\%$) in the EF1- α phylogeny were initially recognised as species to be later confirmed by the strict application of GCPSR criteria. Comparison of each single gene phylogeny revealed that the isolates recognised as *D. eres* in the EF1- α phylogeny grouped together with significant bootstrap support with the other genes; however, minor genetic variation was always present in the species recognised in combined tree. Also according to the genealogical non-discordance, the distinct ITS groups could only be recognised as poorly supported clades contradicted by the other gene trees and therefore were not supported as distinct phylogenetic species (Fig. 1).

Genealogical concordance phylogenetic species recognition

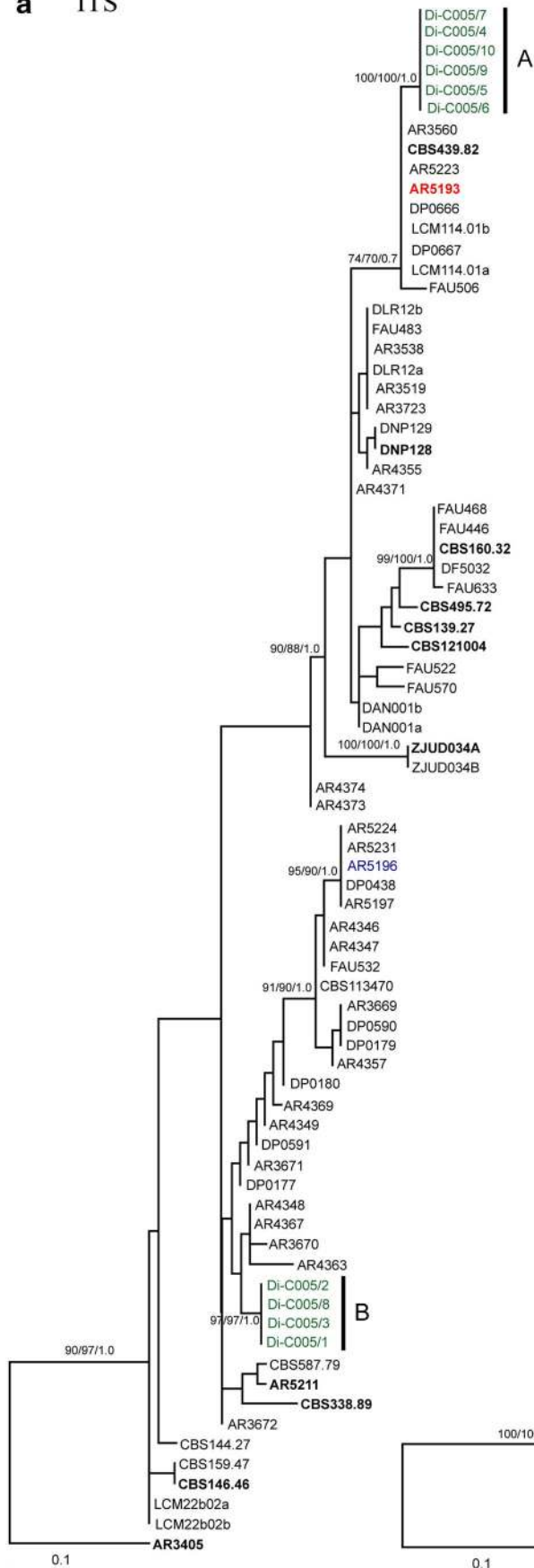
The combined sequence alignment of seven genes comprised 3293 total characters for 68 isolates. An ambiguously aligned region of 100 bp in the CAL gene (2677–2777) in the combined alignment, was excluded from the analysis. The phylogenetic tree inferred from ML analysis was identical to the Bayesian and parsimony trees in terms of major clades and branching order. A total of 25

independent evolutionary lineages were recognised based on given criteria of the ML/MP $\geq 70\%$ bootstrap support in single genes and are summarised on the combined cladogram (Fig. 2). Lineage 11 was only supported by the tubulin gene tree and contradicted by all seven other gene trees including ITS and lineage 13 was poorly supported by the combined tree and contradicted in all single gene trees. Therefore the two lineages were excluded under genealogical non-discordance criterion. The other lineages were supported by more than one gene at the same level as in the EF1- α tree (Fig. 1) and when not supported in a gene tree, they were not contradicted. Therefore these lineages were selected under genealogical concordance criterion for further analysis to determine the species limits.

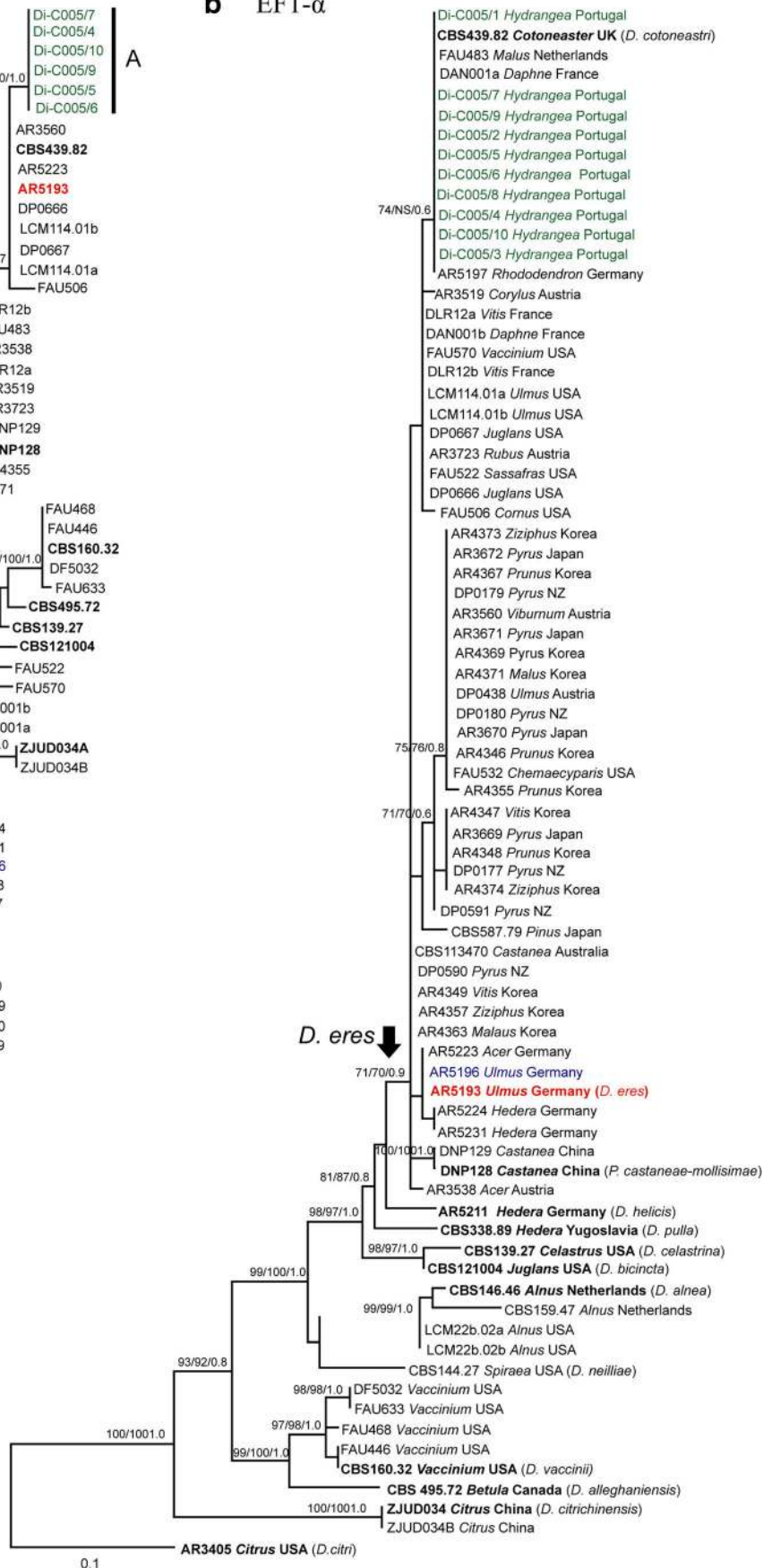
To fulfill the genetic differentiation criterion, the terminal lineages 1, 2, 3, 6, 9, 10, 11, 12, 15, 17, 20, 22 and 24 (blue numbers) in the combined analysis were excluded from the exhaustive subdivision process (Fig. 2). The remaining 11 lineages were used in the exhaustive subdivision process, which involved tracing from the terminal nodes of the tree. All lineages not subtended by an independent evolutionary lineage were collapsed, to satisfy that all individuals should be classified and none remained unclassified. To satisfy the exhaustive subdivision criterion, poorly supported lineage numbers 4, 5, 8 were collapsed under lineage number 7, which is supported by all seven genes and combined analysis, to recognise phylogenetic species 1 (PS 1). The PS 2 and PS 3 were recognised based on the support of each single gene trees as distinct sister taxa

Fig. 1 Comparison of the ITS and the EF1- α phylogenetic trees: The phylograms resulted from RAxML analysis of a) ITS and b) EF1- α regions. The ML, MP bootstrap values $\geq 70\%$, bayesian PP ≥ 0.75 are indicated above the branches. The trees are rooted with *Diaporthe citri* (AR3405). The sequences of Di-C005/1-10 (green) were obtained from Santos et al. 2010. Ex-type and ex-epitype cultures are in bold

a ITS



b EF1- α



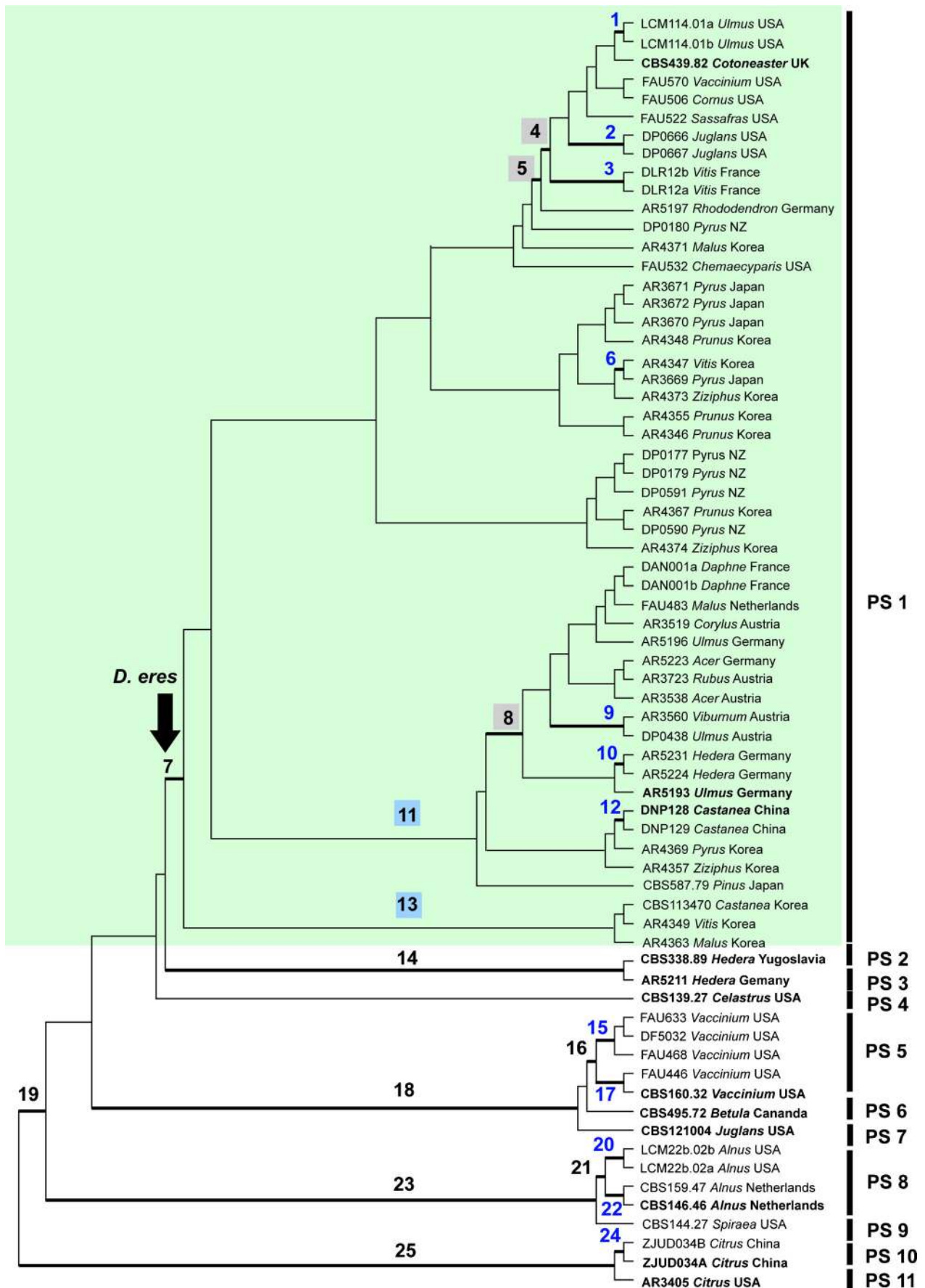


Fig. 2 The summary of independent evolutionary lineages recognised based on genealogical concordance, genealogical non-discordance criteria and ranking according to genetic differentiation and exhaustive subdivision indicated on the RAxML cladogram based on combined analysis of 7 genes (ACT, Apn2, CAL, EF1- α , HIS, FG1093 and TUB). Taxon labels indicate strain number, host and country. Labels to the right the branch labels indicate phylogenetic species identified by the analyses. Bold branches numbered in blue and black were supported by the majority of the loci or supported by at least one locus but not contradicted by any other locus. The non bold branches numbered with blue fill squares (11 and 13) indicate branches that were poorly supported in combined analysis and contradicted in single gene trees. The terminal branch numbers (blue) were excluded from the ranking process under the genetic differentiation criterion. The bold branches numbered with grey fill squares (4, 5 and 8) are collapsed under branch 7 in the exhaustive subdivision process. PS 1- PS 11 indicates the phylogenetic species recognised by genealogical non-discordance and exhaustive subdivision. The limit of PS 1 is indicated by a down arrow at number 7 selected through exhaustive subdivision; with green shade indicates all the isolates belong to *D. eres*

represented by singletons. PS 4-PS 11 were recognised based on exhaustive subdivision of the rest of the lineages later assigned to distinct species based on placement of ex-type and ex-epitype isolates.

The tree generated from the RAxML analysis was used to represent the phylogeny annotated with host and geographic origin of the each isolate and determination of species (Fig. 3). The phylogenetic species recognised in the above analyses (PS 1-PS 11) (Fig. 2) were assigned to named species based on ex-type and ex-epitype isolates and supported with morphological studies of all available isolates. The species determination was highly similar. The EF1- α phylogenetic tree and the clade credibility values of each of the methods increased when compared to the EF1- α phylogenetic tree with a relatively stable tree topology. The limit of *D. eres* was determined based on the well-supported node at lineage number 7 assigned as PS 1 in the combined phylogenetic tree with application of GCPSR criteria. Therefore, a total of nine phylogenetic species were recognised within the species complex, as follows: PS 1 as *D. eres*, PS 2 as *D. pulla*, PS 3 as *D. helicis*, PS 4 as *D. celsastrina*, PS 5 as *D. vaccinii*, PS 6 as *D. alleghaniensis*, PS 7 as *D. bicincta*, PS 8 as *D. alnea*, PS 9 as *D. neilliae* when using two closely related species, *D. citri* (PS 11) and *D. citrichinensis* (PS 10) as out-group taxa in the combined analysis (Fig. 2). Therefore, the limit of the *D. eres* species complex was determined to correspond to node 19 in Fig. 2, with nine accepted species, and *D. citri* and *D. citrichinensis* as basal lineages. *Diaporthe pulla* (PS 2) and *D. helicis* (PS 3) appeared to be closely related sister taxa and were closely related to *D. eres* (PS 1). However, based on the comparison of each single gene tree, these two species diverged from *D. eres* and each should be recognised as distinct phylogenetic species.

Phylogenetic informativeness of each locus

The informativeness profiles indicated that the EF1- α , Apn2 and HIS genes are the best markers to resolve the phylogenetic species included in this analysis (Fig. 4). The EF1- α and ACT genes performed the best in terms of phylogenetic informativeness per site. In comparison with the percentage parsimony informative characters of each gene (Table 2), EF1- α (16 %) and ACT (15 %) regions show a congruent result with the phylogenetic informativeness per site.

Taxonomy

Based on the phylogenetic analyses, the type species of *Diaporthe*, *D. eres*, is circumscribed along with eleven closely related but phylogenetically distinct lineages, each of which is briefly described and illustrated. If a modern description already exists, a reference is given and the species is provided with host association, distribution and notes on taxonomy and phylogeny. As listed after the descriptions, type and additional specimens were observed for each species. Epitype specimens were designated for six species. In addition, ex-type, ex-epitype, and additional cultures were observed, if available.

Diaporthe eres Nitschke, Pyrenomycetes Germanici 2: 245 (1870), nom. cons. prop. Fig. 5

= *Phoma oblonga* Desm., Annl. Sci. Nat., Bot., sér. 3, 22: 218 (1853)

= *Phomopsis oblonga* (Desm.) Traverso, Fl. ital. crypt., Pars 1: Fungi. Pyrenomycetae. Xylariaceae, Valsaceae, Ceratostomataceae: 248 (1906)

= *Phomopsis cotoneastri* Punith., Trans. Br. mycol. Soc. 60: 157 (1973)

= *Diaporthe cotoneastri* (Punith.) Udayanga, Crous & K.D. Hyde, Fungal Diversity 56: 166 (2012)

= *Phomopsis castaneae-mollisimae* S.X. Jiang & H.B. Ma, Mycosystema 29: 467 (2010)

= *Diaporthe castaneae-mollisimae* (S.X. Jiang & H.B. Ma) Udayanga, Crous & K.D. Hyde Fungal Diversity 56: 166 (2012)

= *Phomopsis fukushii* Tanaka & S. Endô, in Endô, J. Pl. Prot. Japan 13: [1] (1927)

Perithecia on dead twigs 200–300 μm diam, black, globose, subglobose or irregular, densely clustered in groups, deeply immersed in host tissue with tapering necks, 300–700 μm long protruding through substrata. *Asci* (39–) 48.5–58.5(–61) μm \times (6.5–)7–9 (–11) μm (\bar{x} \pm SD = 53 \pm 5 \times 8.0 \pm 0.7, n = 30), unitunicate, 8-spored, sessile, elongate to clavate. *Ascospores* (11–)12.5–14.5(–15.5) \times 3–4 μm (\pm SD = 13.5 \pm 1 \times 3.5 \pm 0.3, n = 30), hyaline, two-celled, often 4-guttulate, with larger guttules at centre and smaller ones at the ends, elongated to elliptical.

Pycnidia on alfalfa twigs on WA, 200–250 µm diam, globose, embedded in tissue, erumpent at maturity, with a 200–300 µm long, black, elongated neck, often with yellowish, conidial cirrus extruding from ostiole, walls parenchymatous, consisting of 3–4 layers of medium brown *textura angularis*. *Conidiophores* 10–15×2–3 µm, hyaline, smooth, unbranched, ampulliform, straight to sinuous. *Conidiogenous cells* 0.5–1 µm diam, phialidic, cylindrical, terminal, slightly tapering towards the apex. *Paraphyses* absent. *Alpha conidia* (6–)6.5–8.5(–9)×3–4 µm ($\bar{x}\pm\text{SD}=7.5\pm0.5\times2.5\pm0.5$, $n=30$), abundant in culture and on alfalfa twigs, aseptate, hyaline, smooth, ovate to ellipsoidal, often biguttulate, base subtruncate. *Beta conidia* (18–)22–28(29)×1–1.5 µm ($\text{SD}=25\pm2\times1.3\pm0.3$, $n=30$), formed in culture and alfalfa stems in some isolates, aseptate, hyaline, smooth, fusiform to hooked, base sub-truncate.

Cultural characteristics: In dark at 25 °C for 1 wk, colonies on PDA fast growing, 5.5±0.2 mm/day ($n=8$), white, aerial, fluffy mycelium, reverse centre dark pigmentation developing in centre; producing abundant, black stromata at maturity.

Host range: *Abutilon* (*Malvaceae*), *Acer* (*Sapindaceae*), *Alliaria* (*Brassicaceae*), *Allium* (*Amaryllidaceae*), *Arctium* (*Asteraceae*), *Castanea* (*Fagaceae*), *Chamaecyparis*, *Juniperus*, (*Cupressaceae*), *Corylus* (*Betulaceae*), *Cornus* (*Cornaceae*), *Cotoneaster*, *Malus*, *Prunus*, *Pyrus*, *Rubus*, *Sorbus* (*Rosaceae*), *Cucumis* (*Cucurbitaceae*), *Daphne* (*Thymelaeaceae*), *Fraxinus*, *Osmanthus* (*Oleaceae*), *Hedera* (*Araliaceae*), *Hordeum* (*Poaceae*), *Ilex* (*Aquifoliaceae*), *Juglans* (*Juglandaceae*), *Laburnum*, *Phaseolus*, *Wisteria* (*Fabaceae*), *Laurus*, *Sassafras* (*Lauraceae*), *Magnolia* (*Magnoliaceae*), *Opuntia* (*Cactaceae*), *Picea*, *Pinus* (*Pinaceae*), *Rhododendron*, *Vaccinium* (*Ericaceae*), *Rumex* (*Polygonaceae*), *Skimmia* (*Rutaceae*), *Ulmus* (*Ulmaceae*), *Viburnum* (*Adoxaceae*), *Vitis* (*Vitaceae*), *Ziziphus* (*Rhamnaceae*) (including the host association confirmed with molecular data in Gomes et al. 2013)

Geographic distribution: Austria, China, France, Korea, Germany, Italy, Japan, Latvia, Netherlands, New Zealand, UK, USA

Type material of Diaporthe eres — GERMANY, Nordrhein-Westfalen, Munsterland, Munster Botanical Gardens, on twigs of *Ulmus* sp., June 1865, T. Nitschke, (B 70 0009145, lectotype designated here; MBT178528, isolectotypes ex herb. Munster; B 70 0009146, B 70 0009147); Carpinion forest, on dead, attached, corticated twigs of *Ulmus laevis*, 5 January 2013, R. Jarling, comm. R. Schumacher (BPI 892912, epitype designated here, ex-epitype culture AR5193=CBS 138594; MBT178527).

Phoma oblonga — FRANCE, on twigs of *Ulmus campestris*, unknown collector (bound specimen of Desmazieres, *Plantes Cryptogames du Nord de la France*, Ed. 2, ser. 2. No. 60 in BPI, lectotype designated here; MBT178529). GERMANY, Carpinion forest, on dead,

attached, corticated twigs of *Ulmus laevis*, 5 January 2013, R. Jarling, comm. R. Schumacher (BPI 892913, epitype designated here, ex-epitype culture AR5196=CBS 138595; MBT178530).

Phomopsis castaneae-mollissima — CHINA, Taian, Shangdong, leaf of *Castanea mollissima*, April 2006, S.X. Jiang (CLS 0612, holotype not seen, ex-type culture BYD1=DNP128 observed), ex-isotype culture BYD4=DNP129.

Diaporthe cotoneastri — UK, Scotland, Ayr, on *Cotoneaster* sp., May 1982, H. Butin (isotype CBS-H 7633 not seen, ex-isotype culture CBS 439.82 observed).

Phomopsis fukushii JAPAN, Ibaraki, on *Pyrus pyrifolia*, August 1994, S. Kanematsu, (BPI 892933, neotype designated here, ex-neotype culture MAFF625034=AR3672; MBT178531).

Additional material examined: AUSTRALIA, New South Wales, on *Castanea sativa* (chestnuts in store), 5 July 1999, K.A. Seifert 932 (culture CBS 113470=DAOM 226800); AUSTRIA, Vienna, 21st District, Marchfeldkanalweg, grid square 7764/2, on dead twigs of *Ulmus minor*, 17 November 2002, W. Jaklitsch WJ 2021 (BPI 843626, culture DP0438); Vienna, 22nd District. Lobau (Oelhafen), grid square 7865/1, on dead stems of *Acer campestre*, 21 October 2000, W. Jaklitsch WJ 1643 (BPI 748435, culture AR3538); Niederoesterreich, Buschberg, grid square 7464/1, on *Rubus fruticosus*, 11 August 2001. W. Jaklitsch WJ 1771 (BPI 843611, culture AR3723); Niederoesterreich, Losenheim, Laerchkogel, on *Corylus avellana*, 30 September 2000, W. Jaklitsch WJ 1605 (BPI 747936, culture AR3519=CBS 109497); Wograda, St. Margareten, Kaernten, grid square 9452/3, on *Viburnum lantana*, 27 October 2000, W. Jaklitsch WJ 1662, (BPI 749133, culture AR3560); FRANCE, on *Daphne laurifolia* (culture DAN001a=M1115), *ibid*, DAN001b=M1116; on *Vitis vinifera*, Larignon Phillipe (culture DLR12A=M1117= CBS 138597, DLR12B=M1118); GERMANY, mixed forest, on dead twigs of *Acer nugundo*, 13 April 2013, R. Jarling, comm. R. Schumacher (culture AR5223= CBS 138599); on dead attached twigs of *Hedera helix*, 26 March 2013, R. Jarling, comm. R. Schumacher (culture AR5224); Planar forest, on attached bud of *Rhododendron* sp., 3 January 2013, comm. R. Schumacher (culture AR5197); JAPAN, Ibaraki, on *Pyrus pyrifolia*, S. Kanamatsu, August 1994 (culture AR3670=MAFF625030, AR3671=MAFF625033, AR3669=MAFF625929); on *Pinus pantepella*, G.H. Boerema, May 1979 (CBS-H 16732, alfalfa stem in culture BPI 892918, culture CBS587.79); KOREA, Eumsnus, on *Prunus persica*, S.K. Hong, Pho 0348 (culture AR4355); Punggi-eup, on *Malus pumila* var. *dulcissima*, S.K. Hong, BD 102 (culture AR4371); Anseong-si, on *Ziziphus jujube*, S.K. Hong, Pho 0345 (culture AR4373), KOREA: Geumsan-gun, on *Ziziphus jujube*, S.K. Hong, Pho 0330 (AR4374); Bubal-eup, on *Prunus mume*, S.K. Hong, BD 173 (culture AR4346); on *Vitis vinifera*, S.K. Hong (culture AR4347); on *Chamaecyparis thyoides*, F.A. Uecker (culture FAU 532); on *Ziziphus jujuba* (culture AR4357); on *Pyrus*

pyrifolia, S.K. Hong (culture AR4369); on *Vitis* sp., S.K. Hong (culture AR4349); on *Prunus persici*, S.K. Hong (culture AR4348); on *Prunus* sp. (culture AR4367); on *Malus* sp., S.K. Hong (culture AR4363); NETHERLANDS, on branches of *Malus* sp. (culture FAU483); NEW ZEALAND, Waikato region, on *Pyrus pyrifolia* (Cultivar - Nashi Asian Pear) (culture DP0179, DP0177, DP0180); on *Pyrus pyrifolia*, W. Kandula WK-NP204 (culture DP0590); on *Pyrus pyrifolia*, W. Kandula WK-NP-104 (culture DP0591); USA, New York, Adirondack Mountains, Buttermilk Falls, on twigs of *Ulmus* sp., 7 June 2007, L.C. Mejia (culture LCM114.01a=CBS 138598, LCM114.01b); New Jersey, on *Sassafras albida* (culture FAU522); Virginia: on *Oxydendrum arboreum* (culture FAU570); Maryland, on *Cornus florida* (culture FAU506); North Carolina, Old Fort, on bark from canker on *Juglans cinerea*, June 2002, S. Anagnostakis (cultures DP0666, DP0667).

Notes: *Diaporthe eres* was designated as the type species by Nitschke (1870) and this has been widely accepted in the literature (Wehmeyer 1933; Barr 1978; Brayford 1990; Rossman et al. 2007). The asexual morph of *D. eres* has been known as *Phomopsis oblonga* (basionym: *Phoma oblonga* (Wehmeyer 1933; Udayanga et al. 2011). Considering the obscurity of the older names listed as synonyms in Wehmeyer (1933) and the difficulty of determining their identity within the genus *Diaporthe*, Rossman et al. (2014) proposed to conserve the name *D. eres* over these older synonyms.

Originating from the same host and country as the lectotype, an epitype of *D. eres* is here designated. Many recent collections and isolates included in the phylogenetic analysis were from the same and different hosts in Germany and throughout the temperate regions of the world. The diagnostic morphological features of *D. eres* are the black stroma, perithecia generally immersed in the host tissue with necks protruding through ruptured host tissue with large asci (48.5–58.5 $\mu\text{m} \times 7\text{--}9 \mu\text{m}$) and ascospores (12.4–14.4 $\times 3\text{--}4 \mu\text{m}$) compared to other species of *Diaporthe*. Among the cultures used in this study, the majority sporulated on PDA or WA + alfalfa stems producing abundant black pycnidia and conidial masses. Only alpha conidia were observed in some cultures while both alpha and beta conidia were abundant in other cultures. The sexual morph was not observed in culture. Significant morphological differences were not observed in cultures of different ITS types or cultures derived from different hosts. The geo-ecological data for isolates identified here as *D. eres* suggest that this species has a widespread distribution and a broad host range as a pathogen, endophyte or saprobe (Toti et al. 1993; Sieber and Dorworth 1994; Vajna 2002; Sieber 2007; Casieri et al. 2009).

Diaporthe alleghaniensis R.H. Arnold, Can. J. Bot. 45: 787 (1967). Fig. 6a–c

Pycnidia on alfalfa twigs on WA 100–200 μm diam, globose, embedded in tissue, erumpent at maturity, with a slightly

elongated neck 100–180 μm long, black, often with yellowish, conidial cirrus extruding from ostiole, walls parenchymatous, consisting of 3–4 layers of medium brown *textura angularis*. **Conidiophores** 9–15 $\times 1\text{--}2 \mu\text{m}$, hyaline, smooth, unbranched, ampulliform, cylindrical to sub-cylindrical. **Conidiogenous cells** 0.5–1 μm diam, phialidic, cylindrical, terminal, slightly tapering towards apex. **Paraphyses** absent. **Alpha conidia** 7–9 $\times 3\text{--}4 \mu\text{m}$ ($\bar{x} \pm \text{SD} = 8 \pm 0.5 \times 3.5 \pm 0.3$, $n = 30$), abundant in culture and on alfalfa twigs, aseptate, hyaline, smooth, ovate to ellipsoidal, biguttulate or multiguttulate, base sub-truncate. **Beta conidia** not observed.

Cultural characteristics: In dark at 25 °C for 1 wk, colonies on PDA fast growing, 5.8 ± 0.2 mm/day ($n = 8$), white, aerial mycelium with concentric rings, reverse grey pigmentation developing in centre; stroma not produced in 1 wk old cultures.

Type material: CANADA, Ontario, Abinger Township, Lennox and Addington Co., Vennacher, P.S.P. 10, on branch of *Betula lenta*, 16 September 1953, R. Horner, J. Newman, A.W. Hill (DAOM 45776, holotype not seen, ex-type culture CBS 495.72 observed).

Host range: On dead branches of *Betula alleghaniensis* (*Betulaceae*).

Geographic distribution: Canada (Ontario), also reported from New Brunswick, Quebec, USA (NH, NY, VT) by Arnold (1967).

Notes: Based on phylogenetic analyses, *Diaporthe alleghaniensis* is clearly distinguished from closely related cryptic taxa. It was recognised as a facultative parasite of yellow birch (*Betula alleghaniensis*) on which it causes an annual bark canker and foliage disease (Arnold 1967). According to the protologue, it is morphologically distinguished from *Diaporthe eres* based on the narrow cylindrical asci each with a truncate apex and the narrow cylindrical-ellipsoid ascospores with a variable position of the single septum. However, conidia in culture could not be distinguished from those of *D. eres*.

Diaporthe alnea Fuckel, Jahrb. nassau. Ver. Naturk. 23–24: 207 (1870) Fig. 6d–n

= *Phomopsis alnea* Höhn., Sber. Akad. Wiss. Wien, Math.-naturw. Kl., Abt. 1 115: 681 (1906)

Perithecia on dead twigs 200–300 μm diam, black, globose to conical, scattered evenly on dead twigs, immersed in host tissue with elongated, 300–400 μm long necks, protruding through substrata in clusters. **Asci** 36–46 $\mu\text{m} \times 6\text{--}7 \mu\text{m}$ ($\bar{x} \pm \text{SD} = 40 \pm 5 \times 6.5 \pm 0.7$, $n = 30$), unitunicate, 8-spored, sessile, elongate to clavate. **Ascospores** (11–)12.5–13.5(–14) $\times 2.5\text{--}3 \mu\text{m}$ ($\bar{x} \pm \text{SD} = 12.7 \pm 0.8 \times 2.8 \pm 0.3$, $n = 30$), hyaline, two-celled, often 4-guttulate, with larger guttules at centre and smaller ones at ends, elongated to elliptical.

Pycnidia on alfalfa twigs on WA 100–200 μm diam, globose to subglobose, embedded in tissue, erumpent at maturity, with black, 100–200 μm long necks, cream, conidial cirrus

extruding from ostiole; walls parenchymatous, consisting of 3–4 layers of medium brown *textura angularis*. *Conidiophores* 9–16×1–2 µm, hyaline, smooth, unbranched, ampulliform, cylindrical to sub-cylindrical, with larger basal cell. *Conidiogenous cells* 0.5–1 µm diam, phialidic, cylindrical, terminal, slightly tapering towards apex. *Paraphyses* absent. *Alpha conidia* 8–10×2–3 µm ($\bar{x}\pm\text{SD}=9\pm0.5\times2.5\pm0.2$, $n=30$), abundant in culture and on alfalfa twigs, aseptate, hyaline, smooth, ellipsoidal, biguttulate or multiguttulate, base subtruncate. *Beta conidia* not observed.

Cultural characteristics: In dark at 25 °C for 1 wk, colonies on PDA fast growing, 6±0.2 mm/day ($n=8$), white, aerial mycelium turning grey at edges of plate, reverse yellowish pigmentation developing in centre; stroma not produced in 1 wk old culture.

Host range: On species of *Alnus* including *A. glutinosa*, *A. rugosa* and *A. sinuata* (*Betulaceae*)

Geographic distribution: Europe (Germany, Netherlands), USA

Type material: GERMANY, on twigs of *Alnus glutinosa*, 1894, L. Fuckel (FH, Fungi rhenani 1988, lectotype designated here; MBT178532); Hesse, Oestrich, *Alnus glutinosa*, 1894, L. Fuckel (BPI 615718, Isolectotype); NETHERLANDS, on *Alnus* sp., June 1946, S. Truter 605 (BPI 892917, epitype designated here, ex-epitype culture CBS 146.46; MBT178534).

Additional material examined: CZECH REPUBLIC, Moravia, Sternberg, on *Alnus glutinosa*, February 1922, J. Piskor (BPI 615717); USA, Idaho, Moscow Mtns., on dead stem of *Alnus sinuata*, 2 July 1898, C.V. Piper (BPI 616606); Maine, North New Portland, on twigs of *Alnus rugosa*, 3 August 2006, L.C. Mejia (culture LCM22b.02a); Maryland, Takoma Park, on *Alnus* sp., 1 July 1918, C.H. Kauffman (BPI 615716); Michigan, Isle Royale, Rock Harbor, on *Alnus* sp., 15 July 1904, E.T. Harper, Susan A. Harper (BPI 616605); New York, Tripoli, Ft. Ann, on *Alnus* sp., 28 June 1914, S.H. Burnham 104 (BPI 615284).

Notes: *Diaporthe alnea* is represented here by isolates on *Alnus glutinosa* from Europe and *A. rugosa* in the USA. The geographic origins of CBS isolates of *D. alnea* were previously uncertain although the collector's name is known as S. Truter (Gomes et al. 2013). Truter's (1947) doctoral dissertation concerned the die-back of European alder and presumably the collections originated in the Netherlands or close by in Europe. Herein, *D. alnea* is epitypified with one of Truter's isolates based on the historical authenticity and the morphological similarity of this isolate to the type specimen.

The name *Diaporthe nivosa* Ellis & Everh. has been applied to an ascomycete from *Alnus* in the USA. However, observation of the type specimen of *Diaporthe nivosa* revealed that it is a *Melanconis* sp., having a well-developed ectostromata and ascospores characteristic of that genus, thus *D. nivosa* is not similar with *D. alnea*.

Type material of *Diaporthe nivosa* examined: USA, Michigan, Isle Royale, Lake Superior, on dead wood of *Alnus* sp., July 1889, E.W.D. Holway, Ellis & Everhart, North American Fungi Second Series 2535 (BPI 616604, lectotype designated here; MBT178535).

Diaporthe bicincta (Cooke & Peck) Sacc., Syll. fung. (Abellini) 1: 622 (1882). Fig. 7a–c

Basionym. *Valsa bicincta* Cooke & Peck, in Peck, Ann. Rep. N.Y. St. Mus. nat. Hist. 29: 64 (1878) [1876]

Pycnidia on alfalfa twigs on WA 200–300 µm diam, globose, embedded in tissue, erumpent at maturity, well-developed, black stroma with a slightly elongated, 50–150 µm long necks, often with off-white, conidial cirrus extruding from ostiole; walls parenchymatous, consisting of 3–4 layers of medium brown *textura angularis*. *Conidiophores* 7–12×1–2 µm, hyaline, smooth, unbranched, ampulliform, cylindrical to sub-cylindrical. *Conidiogenous cells* 0.5–1 µm diam, phialidic, cylindrical, terminal, slightly tapering towards apex. *Paraphyses* 80–100×1–2 µm, hyaline, smooth, unbranched, elongated with acute apex. *Alpha conidia* 9–12×2–3.5 µm ($\bar{x}\text{SD}=10\pm1\times3\pm0.3$, $n=30$), abundant on alfalfa twigs, aseptate, hyaline, smooth, ovate to ellipsoidal, biguttulate or multiguttulate, base subtruncate. *Beta conidia* not observed.

Cultural characteristics: In dark at 25 °C for 1 wk, colonies on PDA moderate growth rate, 3.8±0.2 mm/day ($n=8$), white, aerial mycelium turning to grey at edges of plate, reverse white in centre; stroma produced in 1 wk old culture with abundant conidia.

Host range: On *Juglans cinerea* and *Juglans* sp. (*Juglandaceae*)

Geographic distribution: Canada (Ontario); USA (Iowa, New York, Pennsylvania, Tennessee).

Type material: USA, New York, Greenbush, on branch of *Juglans cinerea*, (NYS F 468, holotype); Tennessee, Great Smoky Mts National Park, dead wood of *Juglans* sp., 8 May 2006, L. Vasilyeva (BPI 878472, epitype designated here, ex-epitype culture DP0659=CBS 121004; MBT178536).

Additional material examined: CANADA, Ontario, Granton, on dead branches of *Juglans* sp., July 1898, J. Dearness (BPI 615762, 615766); USA, Iowa, Decorah, on dead branches of *Juglans* sp., June 1892, E.W.D. Holway (BPI 615761, BPI 615765); Pennsylvania, Bethlehem, on twigs of *Juglans cinerea*, 9 June 1922, C.L. Shear 4043, det. F. Petrak (BPI 615764).

Notes: *Diaporthe bicincta* has long paraphyses and larger conidia (9–12×2–3.5 µm) than *D. juglandina* on *Juglans* in Europe. The isolate CBS 121004 was deposited as *D. juglandina* (Gomes et al. 2013); however, this isolate was originally from the USA (Tennessee) and is here confirmed as *D. bicincta* based on a morphological comparison with the type and non-type specimens.

Diaporthe celastrina Ellis & Barthol., J. Mycol. 8: 173 (1902). Fig. 7d–f

Pycnidia on host and alfalfa twigs on WA 200–300 µm diam, globose, embedded in tissue, erumpent at maturity, well developed, black stroma with a 50–150 µm long necks, often with an off-white, conidial cirrus extruding from ostiole; walls parenchymatous, consisting of 3–4 layers of medium brown *textura angularis*. *Conidiophores* 7–21 × 1–2 µm, hyaline, smooth, unbranched, ampulliform, cylindrical. *Conidiogenous cells* 0.5–1 µm diam, phialidic, cylindrical, terminal, slightly tapering towards apex. *Paraphyses* absent. *Alpha conidia* 9–12 × 2–3.5 µm ($\bar{x} \pm \text{SD} = 10 \pm 0.8 \times 2.7 \pm 0.3$, $n=30$) abundant on alfalfa twigs, aseptate, hyaline, smooth, ellipsoidal, biguttulate, multiguttulate, or eguttulate, base subtruncate. *Beta conidia* not observed.

Cultural characteristics: In dark at 25 °C for 1 wk, colonies on PDA fast growing, 5.8 ± 0.2 mm/day ($n=8$), white aerial mycelium, reverse white in centre; stroma produced in 1 wk old culture.

Host range: On *Celastrus scandens* (Celastraceae).

Geographic distribution: USA (KS, VA).

Type material/USA, Kansas, Clyde, *Celastrus scandens*, 18 May 1901, E. Bartholomew 2856 (BPI 615293, holotype). USA, on *Celastrus scandens*, September 1927, L.E. Wehmeyer (BPI 892915, epitype designated here, ex-epitype culture CBS 139.27; MBT178537).

Additional material examined USA, Virginia, Blacksburg, on *Celastrus scandens*. 13 October 1936, C.L. Shear (BPI 615294).

Notes: *Diaporthe celastrina* was originally described from *Celastrus scandens* in the USA (Kansas) and the epitype designated here is collected from the USA on the same host and also identified by L.E. Wehmeyer. The host *Celastrus scandens* (American Bittersweet, Celastraceae) is native to central and northeastern North America.

Diaporthe helicis Niessl, Verh. Naturforsch. Ver., Brünn 16: 50 (1876). Fig. 7g–i

[=*Diaporthe nitschkei* J. Kunze, Fungi Selecti Exs. 124. (1877), nom. nud.]

Pycnidia on host and alfalfa twigs on WA 200–300 µm diam, globose, embedded in tissue, erumpent at maturity, well developed, black stroma with a black, 50–150 µm long neck, often with an off white, conidial cirrus extruding from ostiole; walls parenchymatous, consisting of 3–4 layers of medium brown *textura angularis*. *Conidiophores* (6–) 8–15 (16.5) × 1–2 µm, hyaline, smooth, unbranched, ampulliform, cylindrical to clavate. *Conidiogenous cells* 0.5–1 µm diam, phialidic, cylindrical, terminal, tapering slightly towards apex. *Paraphyses* absent. *Alpha conidia* (5.5–) 6–8 (9.5) × 2.5–3.5 µm ($\bar{x} \pm \text{SD} = 7 \pm 0.5 \times 3 \pm 0.2$, $n=30$), abundant on alfalfa twigs, aseptate, hyaline, smooth, cylindrical to ellipsoidal, biguttulate or multiguttulate, base subtruncate. *Beta conidia* not observed.

Cultural characteristics: In dark at 25 °C for 1 wk, colonies on PDA fast growing, 5.6 ± 0.2 mm/day ($n=8$), white, aerial mycelium turning to grey, reverse white, turning to grey in centre; stroma produced in 1 wk old culture with abundant conidia.

Host range: On vines and leaves of *Hedera helix* (Araliaceae)

Geographic distribution: Europe (France, Germany)

Type material: GERMANY, Saxony, Islebiam, on vines of *Hedera helix*, June 1875, J. Kunze (bound collection in BPI Joannes Kunze, Fungi Selecti Exsiccata 124, lectotype designated here; MBT178538, isolectotypes BPI 1108439; BPI 1108445); FRANCE, Veronnes, on vines of *Hedera helix*, 10 March 2011, A. Gardiennet (BPI 892919, epitype designated here, ex-epitype culture AR5211=CBS; MBT178539).

Notes: When Niessl (1876) described *Diaporthe helicis*, he referred to the J. Kunze specimen that was distributed as J. Kunze, Fungi Sel. Exsiccata 124 labeled *Diaporthe nitschkei*. Although that exsiccata number was issued in 1875, the label does not include a description and thus that name was not published. The name *D. helicis* published 1 year later is typified by that same exsiccata number.

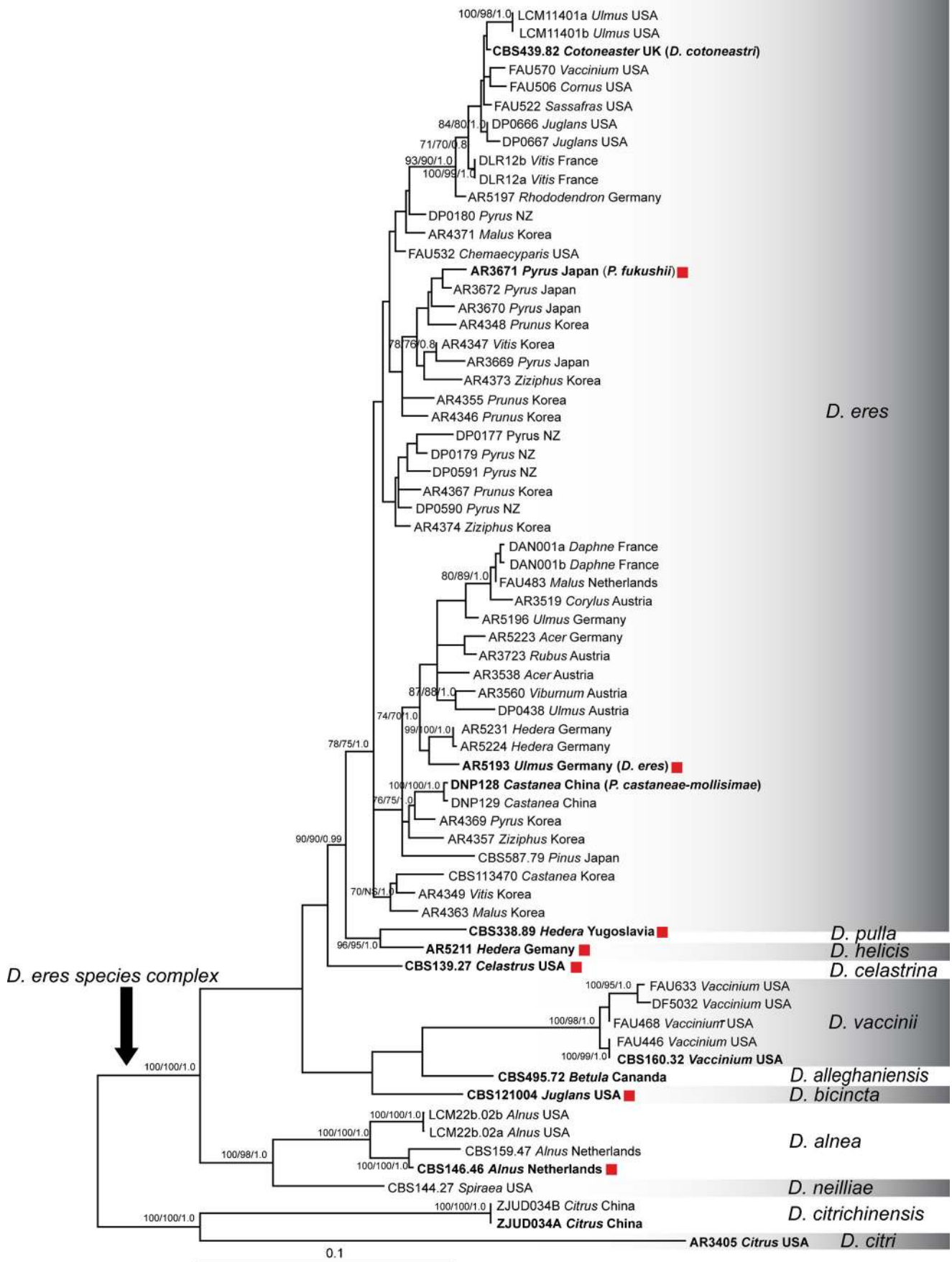
Observations of the type specimens and additional material from *Hedera* confirmed that the fresh collection from France is *D. helicis* and belongs in the same species complex as does *D. pulla* described below. A comparison of representatives of *D. helicis* and *D. pulla* based on eight gene alignments and combined analysis revealed genetic differences suggesting that these two species are distinct.

The third species on *Hedera*, *D. hederiae*, was described from the UK. Observations of a recent collection from *Hedera* in the UK confirmed that it is morphologically differ from *D. helicis* and *D. pulla*. The asexual morph produced by the isolate (M1078, in SMML culture collection, specimen BPI892914), from the UK has longer conidiophores (20–45 × 2–2.4 µm) and the paraphyses are abundant, while *D. helicis* and *D. pulla* have shorter conidiophores (8–15 × 1–2 µm) and paraphyses are absent. The ITS (KM111543) sequence similarity of the above referenced isolate from the UK confirmed that *D. hederiae* can be a synonym of *D. rudis* (see Udayanga et al. (2014) for description and illustration).

Type material of *Diaporthe hederiae* examine UK, Boxhill, on vines of *Hedera helix*, July 1930, E.W. Mason Detr. L.E. Wehmeyer (BPI 1108438).

Diaporthe neilliae Peck, Ann. Rep. N.Y. St. Mus. nat. Hist. 39: 52 (1887) [1886]. Fig. 8a–d

Perithecia on dead twigs, 200–300 µm diam, black, globose to conical, scattered irregularly, immersed in host tissue with elongated, 300–400 µm long necks protruding through substrata. *Asci* 36–50 µm × 7–10 µm ($\bar{x} \pm \text{SD} = 45 \pm 5 \times 8.5 \pm 0.7$, $n=30$), unitunicate, 8-spored, sessile, elongate to clavate. *Ascospores* (11–) 12–13.5 (–14.5) × 3.5–4 µm ($\bar{x} \pm \text{SD} = 13 \pm 0.8 \times 3.8 \pm 0.3$, $n=30$), hyaline, two-celled, often 4-guttulate,



◀ **Fig. 3** The RAxML phylogram based on combined alignment of 7 genes (ACT, Apn2, CAL, EF1- α , HIS, FG1093 and TUB) of *Diaporthe eres* species complex. The ML, MP bootstrap values ≥ 70 %, bayesian PP ≥ 0.75 are indicated above the branches. The tree is rooted with *Diaporthe citri* (AR3405) and *D. citrichinensis* (ZJUD034A, B). Ex-type and ex-epitype cultures are in bold. Epitypes and neotypes designated in this study are indicated with a red squares

with larger guttules at centre and smaller one at ends, elongated to elliptical.

Cultural characteristics: In dark at 25 °C for 1 wk, colonies on PDA slow growing, 2.6 ± 0.2 mm/day ($n=8$), white, aerial mycelium, reverse white, turning to grey in centre; no conidia produced.

Host range: On *Physocarpus opulifolius* (*Rosaceae*).

Geographic distribution: USA (New York).

Type material: USA, New York, West Albany, on stems of *Physocarpus opulifolius*, C.H. Peck (NYS, holotype not examined, BPI 616581, isotype observed).

Additional material examined USA, on *Spiraea* sp., September 1927, L.E. Wehmeyer (BPI 892921, CBS 144.27).

Notes: *Diaporthe neilliae* is known only from the host species *Physocarpus opulifolius*; however, this host has been placed in various genera and has been reported as being on *Neillia opulifolia*, *Opulaster opulifolus* and *Spiraea opulifolia*, all names for the same species. This rosaceous host is native to North America, thus the isolate identified by L.E. Wehmeyer is used to represent this taxon; however, due to lack of information about its origin, it is not designated as the epitype.

Diaporthe pulla Nitschke, Pyrenomycetes Germanici 2: 249 (1870) Fig. 8e–g

= *Phoma pulla* Sacc., *Michelia* 2: 96 (1880)

≡ *Phomopsis pulla* (Sacc.) Traverso, Fl. ital. crypt., Pyrenomycetae (Florence) 2: 244 (1906)

Pycnidia on host and alfalfa twigs on WA 200–300 μ m diam, globose, embedded in tissue, erumpent at maturity, well developed, black stromata, 50–300 μ m long necks, often with bright yellow, conidial cirrus extruding from ostiole, walls parenchymatous, consisting of 3–4 layers of medium brown *textura angularis*. *Conidiophores* (10–) 12–20 (–25) \times 1–2 μ m, hyaline, smooth, unbranched, ampulliform, cylindrical to clavate. *Conidiogenous cells* 0.5–1 μ m diam, phialidic, cylindrical, terminal, slightly tapering towards the apex. *Paraphyses* absent. *Alpha conidia* (6–) 6.5–7.5 (8) \times (2–) 2.5–3.5 (–4) μ m ($\bar{x} \pm SD = 7 \pm 0.5 \times 3 \pm 0.5$, $n=30$), abundant on alfalfa twigs, aseptate, hyaline, smooth, cylindrical to ellipsoidal, biguttulate or multi-guttulate, base subtruncate. *Beta conidia* not observed.

Cultural characteristics: In dark at 25 °C for 1 wk, colonies on PDA fast growing, 5.6 ± 0.2 mm/day ($n=8$), white aerial mycelium, reverse white, turning to grey in centre; black stromata produced in 1 wk with abundant conidia.

Host range: On dead and dying vines and leaves of *Hedera helix* (*Araliaceae*).

Geographic distribution: Europe (Czech Republic, France, Germany, Italy, Serbia)

Type material: GERMANY, on vines of *Hedera helix*, (Fries Scleromyceti Sueciae No. 307 (BPI Sbarbaro Collection, Bound, Centuries III (part) to V. in BPI as *Sphaeria spiculosa*, lectotype designated here; MBT178540); SERBIA, Belgrade, on vines of *Hedera helix*, July 1989, M. Muntanola-Cvetkovic (BPI 892920, epitype designated here, ex-epitype culture, CBS 338.89; MBT178541).

Additional material examined: CZECH REPUBLIC (as Czechoslovakia), Maehren, Sternberg, in garden, stems of *Hedera helix*, October 1934, J. Piskor (BPI 801639); GERMANY, Schmilka, on stems of *Hedera helix*, September 1903, W. Krieger (BPI 1108429); Hesse, Oestrich, on stems of *Hedera* sp., L. Fuckel (BPI 1108479); ITALY, Castel Gandolfo, Rome, on stems of *Hedera helix*, July 1904, D. Saccardo (BPI 1108428).

Notes: *Diaporthe pulla* is distinguished from *D. heliciis* based primarily on molecular phylogenetic differences. The combined alignment of eight genes that includes the two isolates from *Hedera* as well as the single gene analysis support the distinction of *D. pulla* from *D. heliciis*. The other isolates from *Hedera* in Europe were identified as *D. eres* and *D. rudis*. A number of specimens are listed by Nitschke (1870) under the description of *Diaporthe pulla*. The specimens selected here as lectotype was among them and is not the type of *Sphaeria spiculosa* Batsch.

Diaporthe vaccinii Shear, United States Department of Agriculture Technical Bulletin 258: 7(1931)

= *Phomopsis vaccinii* Shear, N.E. Stevens & H.F. Bain, United States Department of Agriculture Technical Bulletin 258:7 (1931)

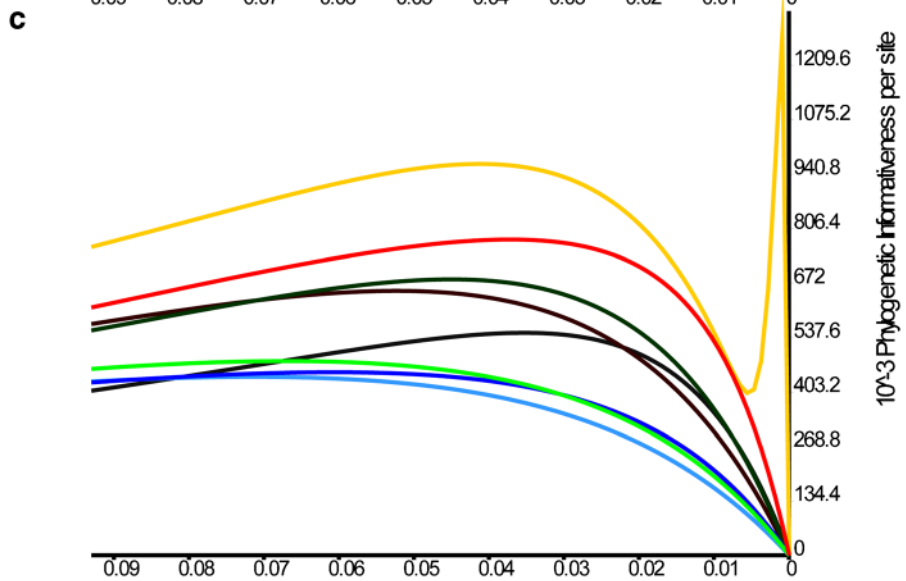
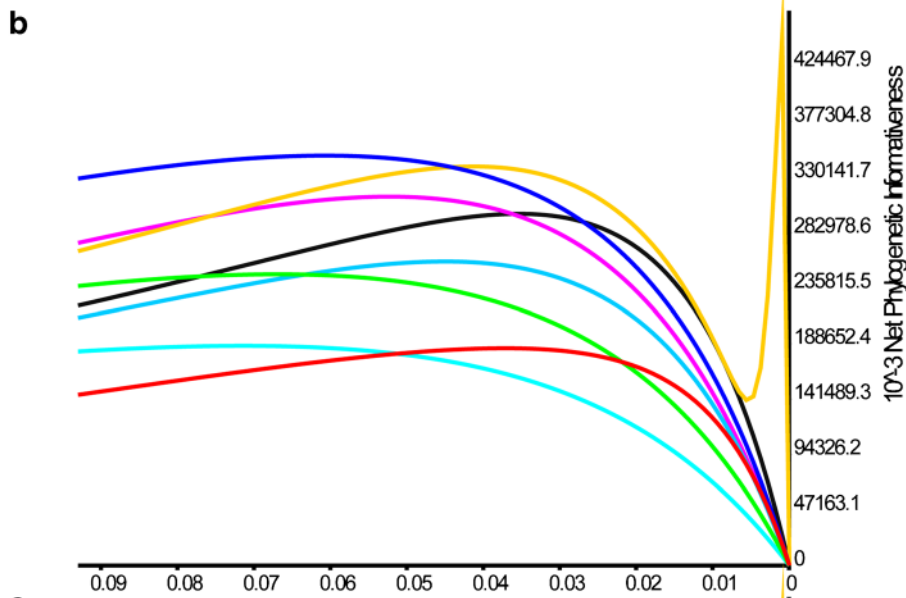
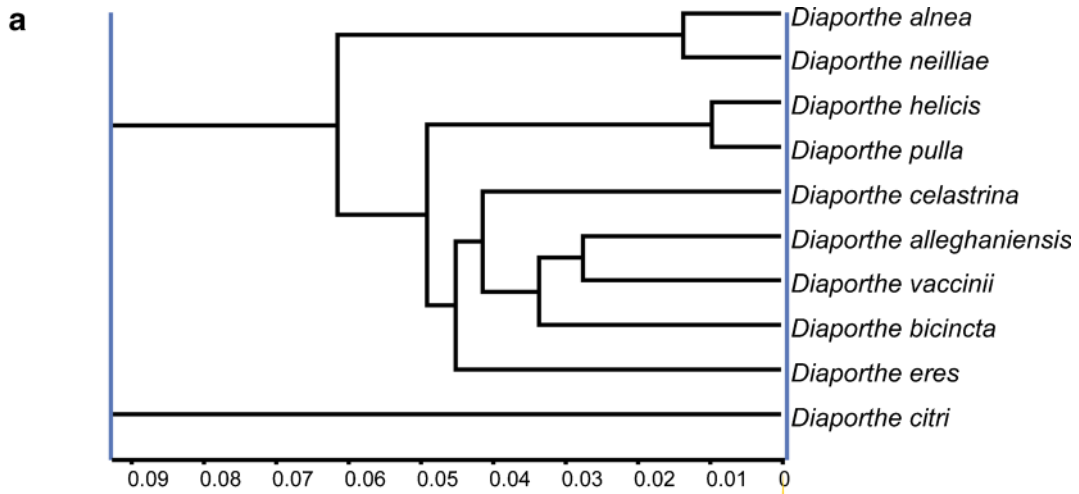
For description and illustrations, see Farr et al. (2002).

Host range: *Vaccinium corymbosum*, *V. macrocarpon*, *V. oxycoccous* (*Ericaceae*) (including the host association confirmed with molecular data in Lombard et al. 2014).

Geographic distribution: Latvia, Lithuania, Netherlands and USA

Type material examined: USA, Oregon, Clatsop, on *Vaccinium macrocarpon*, H.F. Bain, 1924 (BPI 617410, holotype).

Additional material examined USA, Massachusetts, on *Vaccinium macrocarpon*, C.L. Shear (authentic culture CBS 160.32); Oregon, Seaside, *Vaccinium macrocarpon*, 1923, H.F. Bain, (BPI 617405), *ibid*, 2 September 1924, C.L. Shear (BPI 617411); Oregon, Carnahan, *Vaccinium macrocarpon*, 20 September 1924, H.F. Bain, det. C.L. Shear (BPI 617406); Oregon, Intercepted Seattle Washington #009527, *Vaccinium macrocarpon*, 3 May 1972, coll. W.H. Taussig, det. F.G. Pollack (BPI 617407); Oregon, Seaside, *Vaccinium macrocarpon*, 1923, H.F. Bain (BPI 617408); Unknown, fruit



◀ **Fig. 4** Profiles of phylogenetic informativeness for the 10 cryptic species compared within *D. eres* species complex (based on types, epitypes or taxonomically authenticated isolates) and 8 genes included in the study. a) Ultrametric tree generated from the combined analysis of Apn2, ACT, ITS, EF1- α , TUB, CAL, FG1093 and HIS genes b) Net Phylogenetic informativeness c) Phylogenetic informativeness per site. d) key

of *Vaccinium macrocarpon*, 1 March 1929, H.F. Bain (BPI 617409).

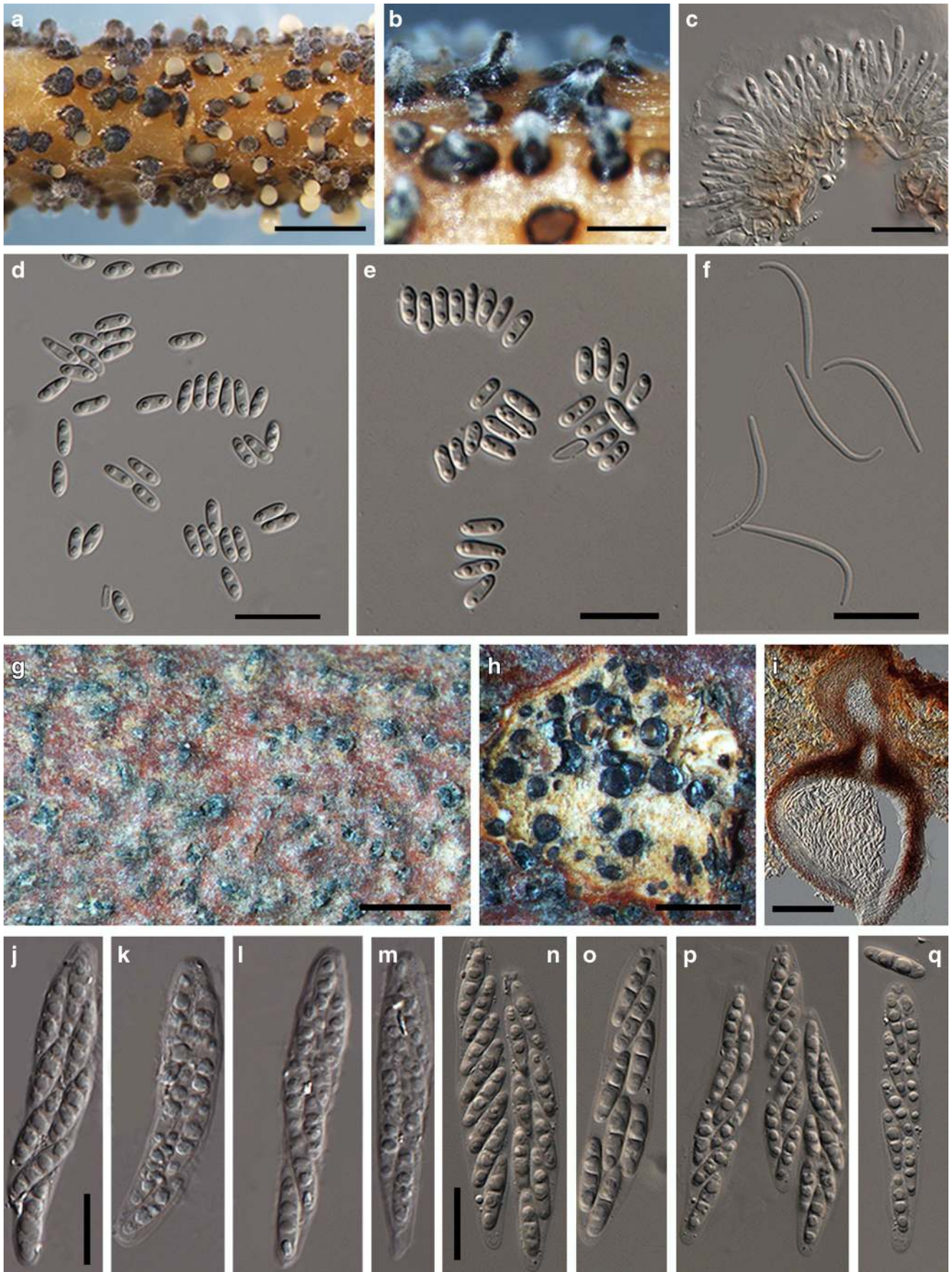
Notes: The type specimen of *Diaporthe vaccinii* was examined but no useful structures remain as had been noted previously by Wehmeyer (1933) and Farr et al. (2002). The authentic specimen listed in Farr et al. (2002) serves here as the reference material including sequences used in that study. Additional authentic material examined included the asexual morph with pycnidial structures and alpha conidia. *Diaporthe vaccinii* is known to cause twig blight and fruit rot of *Vaccinium* species and is primarily reported from the USA and it is reported on *Vaccinium* in Europe along with several other common taxa including *D. eres* (Lombard et al. 2014). However, this is one of relatively host specific pathogens within *Diaporthe* infecting on *Vaccinium* spp.

Discussion

Fungi are excellent models for studying eukaryotic evolution with many examples of highly diverse species complexes with multiple recently diverged sibling species (Dettman et al. 2003b, 2006; Kohn 2005; Pringle et al. 2005; Giraud et al. 2008). The genus *Diaporthe* is composed of species varying from relatively host-specific to species with broad host ranges. For instance *D. alnea* (on *Alnus* spp.), *D. citri* (on *Citrus* spp.), *D. vaccinii* (on *Vaccinium* spp.) and *D. ampelina* (formerly known as *Phomopsis viticola* on *Vitis* spp.) are known to be relatively host specific species, are often pathogenic, and show less infraspecific variability (Udayanga et al. 2014). The majority of the host-specific species are generally pathogens causing mild to serious diseases on their respective host plants. The occurrence of these host-specific pathogens supports the hypothesis of host switching and specialization in the speciation within diaporthelean genera (Sogonov et al. 2008; Mejia et al. 2008, 2011; Crous et al. 2012; Voglmayr et al. 2012; Walker et al. 2014). In contrast, species occurring on a wide range of hosts are mostly opportunistic pathogens or secondary invaders on saprobic host substrata. These species often show high genetic diversity and are sometimes regarded as species complexes (Gomes et al. 2013). Udayanga et al. (2014) recognised *D. foeniculina* and *D. rudis* as species occurring on an extensive range of hosts similar to *D. eres* as resolved in this study.

It is essential to define the generic type species *Diaporthe eres* for a meaningful phylogenetic reappraisal of *Diaporthe*, as well as to reveal its biology, ecology and host associations (Udayanga et al. 2011; Gomes et al. 2013; Rossman et al. 2014). *Diaporthe eres* has been reported as a weak to moderate pathogen of woody plants. Kaliterna et al. (2012) reported the association of *D. eres* with grapevine trunk disease in Croatia having moderate pathogenicity. They suggest that this plurivorous species could play an important role in the aetiology of grapevine trunk disease. Baumgartner et al. (2013) characterised the isolates of *Diaporthe* from North American vineyards and recognised the wide occurrence of *D. eres* in their collection. Interestingly, they recovered both ITS types of *Diaporthe eres*, one of which was named *Phomopsis fukushii* because of the high similarity with authentic isolates from Japan included in their analysis. However, they did not notice any morphological variability or differences in virulence and pathogenicity within the two groups. The weak pathogenic *D. eres* has been widely reported associated with ericaceous, rosaceous fruit trees and grapevines from Asia, Europe and USA (Kanematsu et al. 1999, 2000, 2007; Kaliterna et al. 2012; Lombard et al. 2014). Additionally *Phomopsis* sp. 6, reported from South Africa (van Niekerk et al. 2005), was confirmed as *D. eres* based on the sequence comparison, which also supports the association of this species as a weak pathogen or opportunistic saprobe of grape in different geographic regions. Gomes et al. (2013), observed an unresolved sub-clade, which they referred to as the *Diaporthe nobilis* species complex, represented by CBS 587.79, CBS 113470 and some of the isolates used in our analysis. Many of the isolates in that clade clustered within *Diaporthe eres* based on the application of GCPSR in our analysis except for CBS 338.89, which is identified herein as *D. pulla*. We confirm that this poorly supported non-monophyletic grouping can be observed when ITS sequences are included in the combined analysis. Therefore, the recognition of the *Diaporthe nobilis* species complex (*sensu* Gomes) is redundant. As large numbers of sequences from *Diaporthe* species have accumulated, subsequent rigorous analyses have shown that the interpretation of phylogenetic trees at species level is subject to much confusion, especially in taxa associated with broad host ranges (Udayanga et al. 2014). These issues are not only significant in biodiversity and evolutionary contexts, but also in situations in which the accurate identification of plant pathogenic species is required for quarantine or other purposes.

The nuclear ribosomal internal transcribed spacer (ITS) region has been proposed as the standard fungal barcode (Schoch et al. 2012) and is also being used for sequence-based species delimitation in environmental surveys of fungi (Horton and Bruns 2001; Begerow et al. 2010; Peršoh 2013; Schoch et al. 2014). For a majority of groups of fungi, ITS is the predominantly available sequence in public databases



◀ **Fig. 5** Morphology of *Diaporthe eres* a. Pycnidia on alfalfa stem on WA b. pycnidial necks protruding on alfalfa stem c. conidiophores d. α -conidia f. β -conidia g. Ectostroma on the dead twigs of *Ulmus* sp. h. Perithecia i. Ascomata in section j–q. Asci and ascospores, *Specimens*: a–e. AR5193 epitype culture g–m. B 70 0009145 lectotype specimen, n–q. epitype specimen (BPI 892912), *Scale bars*: a=1000 μ m, b=500 μ m, c=10 μ m, d,e=15 μ m f=10 μ m g=1000 μ m, h=500 μ m, i=100 μ m, j–q=15 μ m

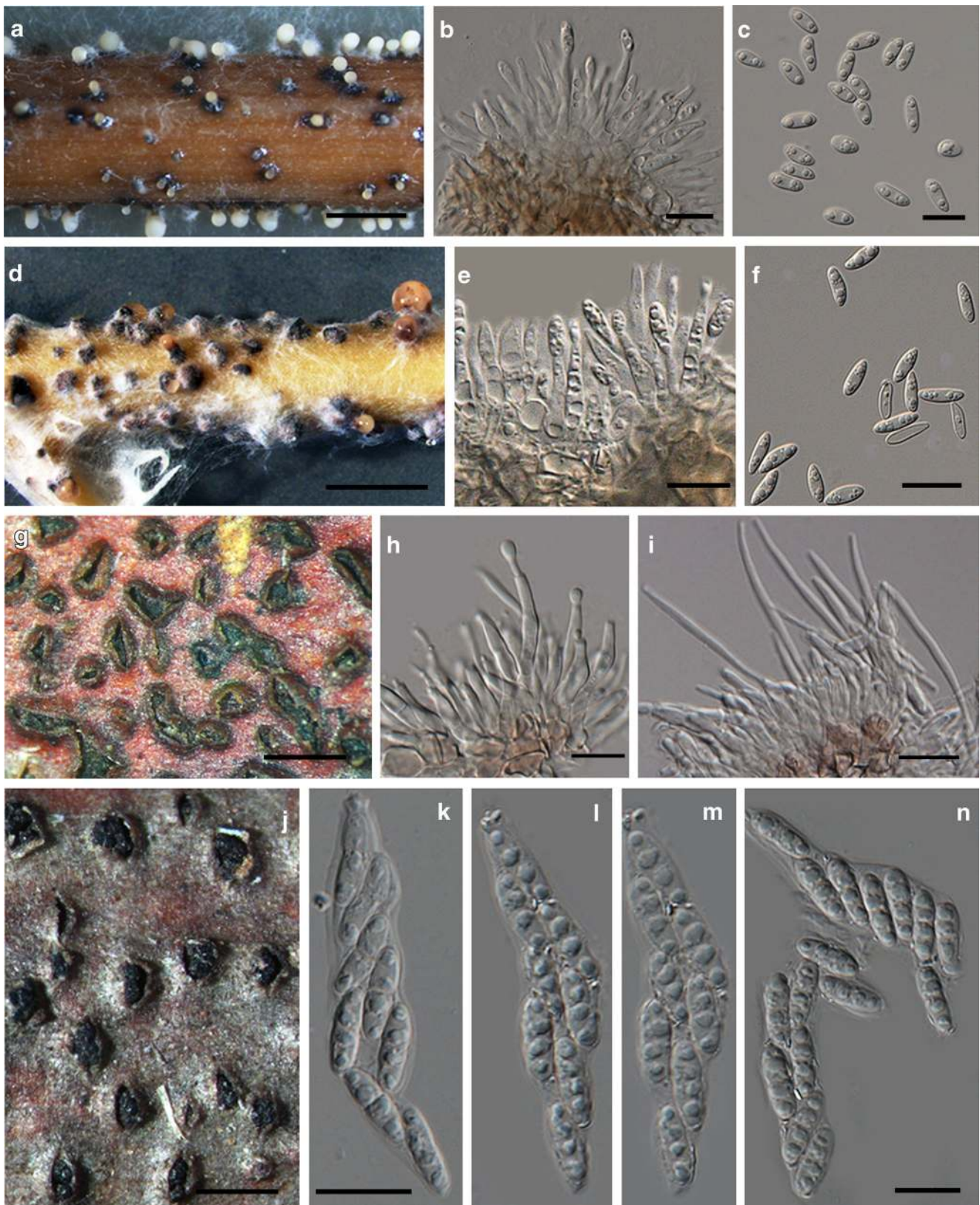
(Nilsson et al. 2008, 2014; Kõljalg et al. 2013). Although ITS has been widely used in fungal systematics to delimit species and to understand evolutionary relationships, there are several known issues with the effectiveness of this region including the overestimating and underestimating fungal diversity (Schoch et al. 2012, 2014). On average the variability of the ITS1 exceeds that of ITS2, while the 5.8S fragment embedded between these two regions is highly conserved, and results of phylogenetic analysis of the complete sequence may differ from the analysis of the individual sub-loci (Nilsson et al. 2008; Monard et al. 2013).

The ITS region in the nuclear ribosomal cistron has undergone non-concerted patterns of evolution leading to paralogous ITS types within species in some important plant pathogenic genera (O'Donnell and Cigelnik 1997; Nilsson et al. 2008; Santos et al. 2010) and is considered by some authors to be uninformative due to the lack of interspecific variation or even misleading in some fungi (Crouch et al. 2009; Gaziz et al. 2011; Maharachchikumbura et al. 2012; Weir et al. 2012). Although complications resulting from ITS sequence data in *Diaporthe* have been recognised by several previous authors, they have not been thoroughly examined (Farr et al. 2002; Murali et al. 2006; Udayanga et al. 2014). In Santos et al. (2010) two ITS types tentatively named as A and B recovered from the isolates Di-C005/1-10 from *Hydrangea* in Portugal, derived from 10 individual sibling ascospores from the same perithecium were similar to the two large groups observed in our analysis (Fig. 1-a). However, our study reveals that the unidentified isolates Di-C005/1-10 belong to *Diaporthe eres* and cluster together as one species in the EF1- α phylogenetic tree. These differences were confined to the ITS1 region and are more extensive than the minor differences often noted among isolates of a single species. Sequence heterogeneity was not noted in the EF1- α and mating type genes for these same sibling isolates and the isolates were fully reproductively compatible (Santos et al. 2010). The same study further noted that both ITS types were not found in the genome of the same isolate, indicating that the different ITS types are independently segregated in meiotic events in this species. Comparison of the geographic origins and host associations of the isolates of *D. eres* used in this study with respect to the occurrence of two ITS types revealed that the different ITS sequences can be observed even within the same geographic region and the same host. We detected no evidence of sympatric patterns or host specialisation related to these ITS

populations. The discordance of ITS versus other gene trees in combination with a lack of informative morphological characters to delineate taxa have led to a confused taxonomic situation within this species complex. In this study, ITS analysis resulted in an unresolved phylogenetic tree without definitive bootstrap support at the internodes, highly discordant to the trees resulting from the other seven genes. Therefore ITS region was not used in the combined analysis. The conflict among gene trees can be reasonably explained by recombination among individuals within a species (Milgroom 1996; Geiser et al. 1998; Matute et al. 2006). However, in each of the species within *D. eres* complex, either the genealogical nondiscordance rule (Dettman et al. 2003a) or the genealogical concordance criterion has been fulfilled, revealing that there are significant barriers to gene flow among these species defined.

The seven gene analysis excluding the discordant ITS data resulted in a robust tree congruent with the EF1- α and other single genes. The species boundaries within the *D. eres* species complex were resolved in this study by application of criteria of phylogenetic species recognition (Taylor et al. 2000; Dettman et al. 2003a) revealing cryptic diversity that may be obscured by biological species recognition, morphology and discordance of genes. Several similar conclusions have been made in other fungal groups with cryptic species diversity, which also display little or no morphological variation (Dettman et al. 2003a, 2006; Walker et al. 2012; Weir et al. 2012; Manamgoda et al. 2013; Laurence et al. 2014).

The structure of the mating type genes and the association with *Apn2* genes in *Diaporthe* were illustrated by Kanematsu et al. (2007). DNA-lyase genes have not traditionally been used as molecular markers in fungi; however, the association with mating type genes of fungi is known in relation to their structure. The *Apn2* region has recently been used in conflicting genera like *Colletotrichum* (Crouch and Tomaso-Peterson 2012; Silva et al. 2012b; Doyle et al. 2013; Sharma et al. 2013) and the *Apn2* and *Apn2*/MAT-IGS (intergenic spacer between 3' end of the DNA lyase and mating type locus MAT1-2) genetic markers recommended as a better marker in disentangling the *C. gloeosporioides* species complex (Silva et al. 2012a, b). Mating type genes of *Diaporthe* were amplified in several previous studies and utilised in phylogenetic analyses (Santos et al. 2010, 2011). Portions of the α -1 box in MAT 1-1-1 gene (141 bp) and a portion of HMG domain of MAT 1-2-1 (229 bp) regions were shown to have less utility as phylogenetic markers than for screening mating types of isolates (Santos et al. 2010). The MAT phylogenetic trees were strongly correlated with EF1- α phylogenetic tree. However, MAT genes were less informative for more closely related species that could potentially be regarded as one biological species. At least some of taxa in species complexes might be regarded as reproductively compatible, but are distinct phylogenetic species. In our analyses of the available



mating type sequences of the *D. eres* species complex with those generated by Santos et al. (2010) (as *Diaporthe*

cotoneastri and *D. vaccinii*), species could not be distinguished based on MAT1-1-1 or MAT1-2-1 gene trees (trees

Fig. 6 Morphology of *Diaporthe alleghaniensis* (a–c), *D. alnea* (d–n). Pycnidia on alfalfa stem on WA, **b**. Conidiophores **c**. α - conidia **d**. Pycnidia on alfalfa stem **e**. conidiophores **f**. α - conidia **g**. infected stem of *Alnus* sp. with ruptures on bark and pycnidia **h**. α - conidiophores and conidiogenous cells **i**. β - conidiophores and conidia **j**. Ectostroma on twigs of *Alnus* sp. **k–m**. Asci **n**. Ascospores, *Specimens*: a–c. ex-type culture CBS 495.72, d–f. culture LCM22b.02a, g–h. lectotype specimen Fungi rhenani 1988 in FH, i–n. isolectotype specimen BPI 615718, *Scale bars*: a=800 μ m, b,c=10 μ m, d=3000 μ m, e,f=12 μ m, g=500 μ m, h,i=12 μ m, j=1000 μ m, k–n=15 μ m

not shown). However, in heterothallic species mating type genes may not always be appropriate as phylogenetic markers due to their absence in different strains. To our knowledge, this study is the first ever utility of *Apn2* gene as a phylogenetic marker within the genus *Diaporthe*. The comparison of

phylogenetic informativeness revealed that it is a competing marker for *EF1- α* and *HIS* genes. The *Apn2* region has the advantage of being highly informative and bearing a shorter hypervariable intron region allowing a more accurate global alignment that is sometimes impossible with *EF1- α* in this genus.

The phylogenetic informativeness profiles generated based on PhyDesign were used to compare each locus with respect to the species hypothesis inferred based on the multi-gene phylogenetic analysis. *Apn2*, *EF1- α* and *HIS* genes showed the highest net phylogenetic informativeness, with *EF1- α* showing the highest informativeness per site. The phylogenetic informativeness per site is useful in comparing the relative power of genes regardless of gene length. These profiles are useful in determining the most informative genes for facilitating

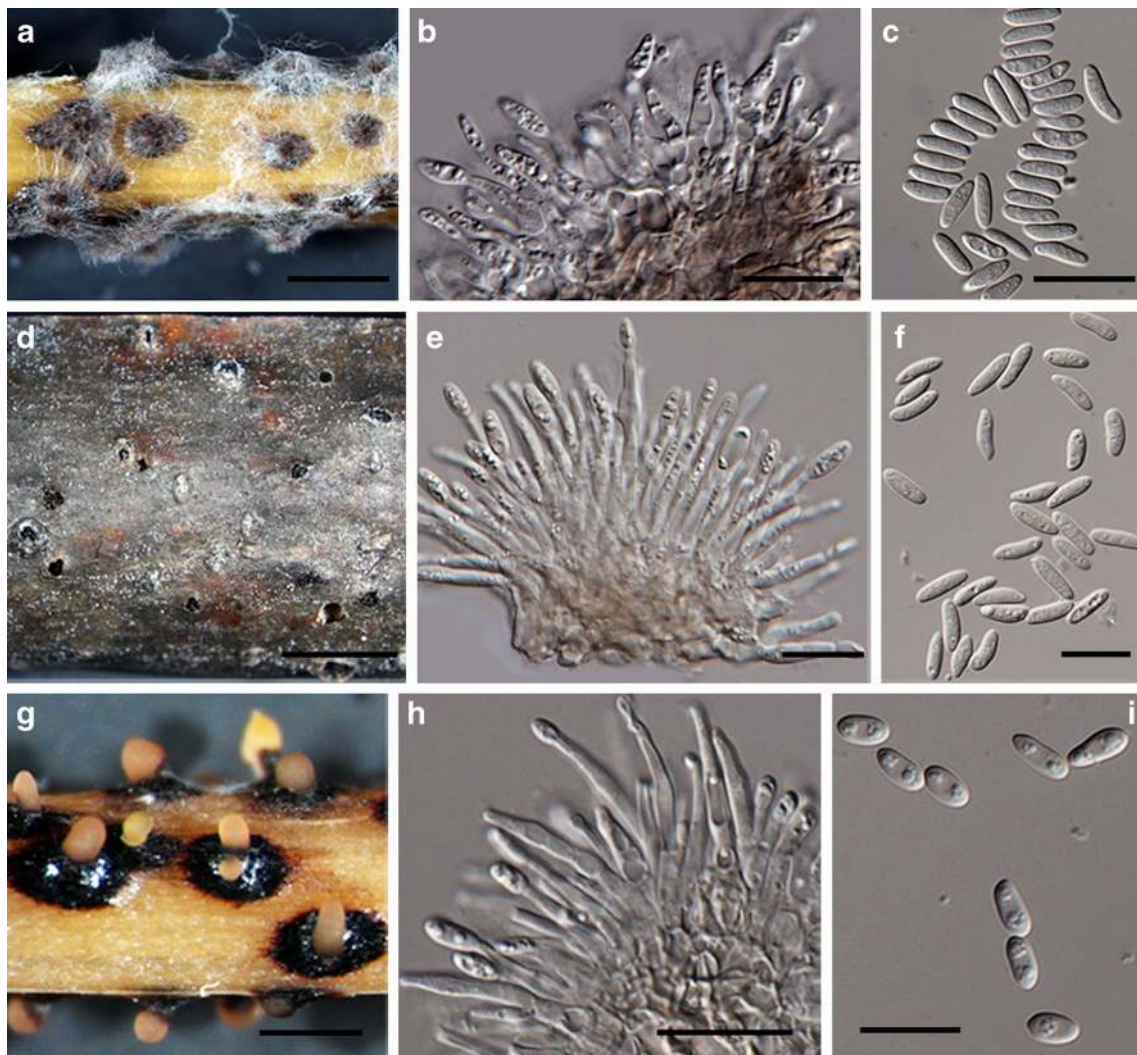


Fig. 7 Morphology of *Diaporthe bicincta* (a–c), *D. celastrina* (d–f), *D. helicis* (g–i) **a**. Pycnidia on alfalfa stem on WA **b**. Conidiophores **c**. α -conidia **d**. Surface view of infected stem of *Celastrus scandens* with pycnidia **e**. conidiophores **f**. α - conidia **g**. Pycnidia on alfalfa stem on

WA **h**. conidiophores **i**. α -conidia. *Specimens*: a–c. ex-epitype culture CBS 121004, d–f. Holotype BPI 615293 g–i. ex-epitype culture (AR5211), *Scale bars*: a=1000 μ m, b,c=15 μ m, d=2000 μ m, e,f=12 μ m, g=, 1000 μ m, h,i=10 μ m

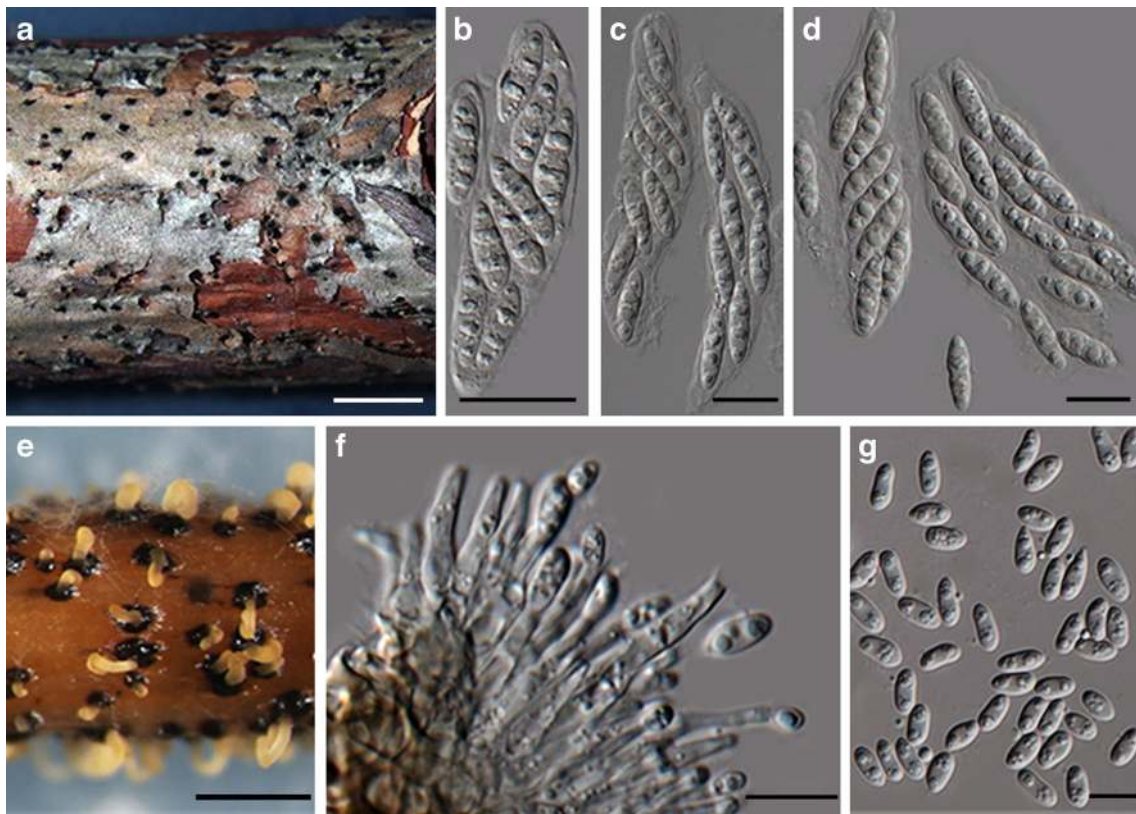


Fig. 8 Morphology of *Diaporthe neilliae* (a–d) and *D. pulla* (e–g) a. Ectostoma on dead stem of *Physocarpus opulifolius* b–c. Asci d. Asci and ascospores e. Pycnidia on alfalfa stem on WA f. conidiophores g. α -

conidia, *Specimens*: a–d. Holotype of *D. neilliae* BPI 616581, e–g. ex-type culture CBS 338.89, *Scale bars*: a=2000 μ m, b=15 μ m, c,d=12 μ m e=1800 μ m, f=12 μ m, g=8 μ m

locus prioritisation and increasing the efficiency of sequencing for phylogenetic purposes (Townsend 2007). The relatively recent “phantom” spikes in EF1- α phylogenetic informativeness plots arise because the maximum likelihood estimate for the rate of a few sites has its peak at infinity, which has little biological meaning (<http://phydesign.townsend.yale.edu/faq.html>). The EF1- α gene was used initially to provide an estimate of the species boundaries with six additional genes including ACT, Apn2, CAL, FG1093, HIS and TUB genes compared individually and in combinations. The approximately 300 bp complete intron sequence of the translation elongation factor1- α has previously been recognised as a powerful marker within *Diaporthe* to define cryptic species (Castlebury et al. 2001; Santos et al. 2010; Udayanga et al. 2012a, b, 2014)

The infraspecific variability of the highly informative genes as well as the less informative genes is a factor to be considered in the large scale evolutionary reconstruction of the genus. However, it is important to increase sampling of each species from a wide range of hosts using additional genes to clarify the topological conflicts of single gene analyses. Novel species may be encountered in unexplored ecological

niches in which these fungi occur as endophytes, pathogens or saprobes.

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