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Diaporthe juglandicola sp. nov. (Diaporthales, Ascomycetes), evidenced by morphological characters and phylogenetic analysis

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Abstract

Diaporthe juglandicola sp. nov, collected from diseased branches of *Juglans mandshurica* in Beijing, China, is described and illustrated in this paper. Evidence for this new species is provided by its holomorphic morphology and phylogenetic analysis. Morphologically, the asexual morph produces hyaline, aseptate, ellipsoidal, alpha conidia (8.1–8.7 × 2.3–2.9 µm), while the sexual morph produces 8-spored, unitunicate, clavate to cylindric asci and fusoid, 0–1-septate ascospores. The phylogeny inferred from combined multi-locus sequences (CAL, HIS, ITS, TEF1-α, TUB) grouped the isolates of the new species into a distinct lineage.

Key words – dieback – molecular phylogeny – new species – taxonomy

Introduction

The genus *Diaporthe* (syn. *Phomopsis*) was established by Nitschke (1870). Species of *Diaporthe* occur widely in natural ecosystems, comprising endophytes and saprobes, as well as plant pathogens (Uecker 1988, Rehner & Uecker 1994, Rossman & Palm-Hernández 2008, Udayanga et al. 2011, 2012a, b). According to Index Fungorum, there are 977 names in *Diaporthe* and 980 names in *Phomopsis*, although the relationships between the asexual and sexual taxa are mostly unclear. The current International Code of Nomenclature for algae, fungi, and plants (ICN) requires a single-name for pleomorphic fungi (McNeill et al. 2012). *Diaporthe* (1870) is older than the asexual morph name *Phomopsis* (1905) and is recommended for use (Rossman et al. 2015).

Species in *Diaporthe* were originally identified based on host association and morphological characters (van der Aa et al. 1990). However, the utility of morphology has generally been shown to be of little use for species identification as there is considerable plasticity of the characters within a species (Rehner & Uecker 1994, Mostert et al. 2001, Santos & Phillips 2009, Santos et al. 2010, Udayanga et al. 2011, 2012a). Host association has also been shown to bear little significance when referring to phylogenetic relationships (Brayford 1990, Rehner & Uecker 1994, Mostert et al. 2001, Santos & Phillips 2009, Udayanga et al. 2011, 2012a, b, 2014b, 2015). More than one species of *Diaporthe* can often be recovered from a single host, while one species can be associated with many different hosts (Santos & Phillips 2009, Diogo et al. 2010, Santos et al. 2011, Gomes et al. 2013). Multi-locus phylogenetic analyses have become the most effective tool for taxonomic studies to identify cryptic fungal species in *Diaporthe* (Cai et al. 2011a, b, Udayanga et al. 2012a, b, Gomes et al. 2013, Huang et al. 2013, 2015, Udayanga et al. 2014a, b, 2015, Fan et al. 2015, Du et

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al. 2016, Gao et al. 2016).

In China, several *Diaporthe* species have been characterized and illustrated based on morphological characteristics and multi-locus phylogeny (Huang et al. 2013, 2015, Gao et al. 2014, 2015, 2016, Fan et al. 2015, Du et al. 2016). During investigations of forest pathogens in Beijing, fresh specimens from symptomatic branches of *Juglans mandshurica* were collected. From these, one additional *Diaporthe* species is newly described and characterized based on morphological characters and multi-locus phylogeny (CAL, HIS, ITS, TEF1-α, TUB). Both morphology and sequence data confirmed that the new collection represents a new species in *Diaporthe* following the guidelines for new species in Jeewon & Hyde (2016).

Materials & Methods

Isolation

Fresh collections of *Diaporthe* were made from infected branches of *Juglans mandshurica* collected in Beijing, China. Strains were isolated from fresh diseased branches and grown from single ascospores or conidia plated on potato dextrose agar (PDA) following method by Chomnunti et al. (2014). After incubation at 25°C for up to 24h, single germinating conidia were removed and plated onto fresh PDA plates. Two strains (one from the asexual morphs and the other from the sexual morphs) were used in the phylogenetic analysis (Table 1). Specimens and isolates of the new species are deposited in the Museum of Beijing Forestry University (BJFC). Axenic cultures are maintained in the China Forestry Culture Collection Center (CFCC).

Morphology

Morphological observations were based on features of the fruiting bodies produced on infected plant tissues and micromorphology, supplemented by cultural characteristics. Morphological characteristics of the fruiting bodies were recorded with a Leica stereomicroscope (M205 FA). Micromorphological observations were determined with a Leica compound microscope (DM 2500). More than 20 fruiting bodies were sectioned, both vertically and horizontally, and 50 spores were selected randomly for measurement. For observation of culture characteristics, two strains were selected for the species, and three cultures were replicated for each strain. All cultures incubated on PDA in the dark at 25°C were observed and recorded. This included colony colour, texture and arrangement of the conidiomata in culture, at 3, 7, and 30-days in darkness.

DNA extraction, PCR amplification, and sequencing

Genomic DNA was extracted from colonies grown on PDA overlain with cellophane using a modified CTAB method (Doyle & Doyle 1990) and then estimated by electrophoresis in 1% agarose gels, and the quality was measured by NanoDrop™ 2000 (Thermo, USA) following the user manual (Desjardins et al. 2009). PCR amplifications were performed in DNA Engine (PTC-200) Peltier Thermal Cycler (Bio-Rad Laboratories, CA, USA). The ITS region was amplified using primers ITS1 and ITS4 (White et al. 1990). The CAL region was amplified using primers CAL-228F and CAL-737R (Carbone & Kohn 1999). The HIS region was amplified using primers CYLH4F (Crous et al. 2004a) and H3-1b (Glass & Donaldson 1995). The partial translation elongation factor 1-alpha (TEF1-α) gene region was amplified using primers EF1-728F and EF1-986R (Carbone & Kohn 1999) and TUB was amplified using primers Bt2a and Bt2b (Glass & Donaldson 1995). The PCR amplification products were estimated visually by electrophoresis in 2% agarose gels. DNA sequencing was performed using an ABI PRISM® 3730XL DNA Analyzer with BigDye® Terminater Kit v.3.1 (Invitrogen) at the Shanghai Invitrogen Biological Technology Company Limited (Beijing, China).

DNA sequence analysis

Sequences from this study, along with reference sequences from GenBank (Table 1) were

aligned with MAFFT v.6 (Katoh & Toh 2010) and edited manually with MEGA6 (Tamura et al. 2013). Phylogenetic analysis was carried out with PAUP v.4.0b10 for maximum parsimony (MP) analysis (Swofford 2003), MrBayes v.3.1.2 for Bayesian analysis (Ronquist & Huelsenbeck 2003), and PhyML v.7.2.8 for maximum likelihood (ML) analysis (Guindon et al. 2010). The analyses were performed on the combined multi-gene dataset (CAL, HIS, ITS, TEF1-α, TUB) to compare *Diaporthe* species from other ex-type reference in recent studies (Udayanga et al. 2011, 2012a, b, 2014a, b, 2015, Gomes et al. 2013, Gao et al. 2014, 2015, Fan et al. 2015). *Diaporthella corylina* (CBS 121124) was selected as outgroup in this analysis (Gomes et al. 2013). Trees were visualised with FigTree v.1.3.1 (Rambaut & Drummond 2010).

Table 1 Isolates and GenBank accession numbers used in this study.

Species	Isolates	GenBank accession numbers				
		ITS	CAL	HIS	TEF1-α	TUB
D. alleghaniensis^{T}	CBS 495.72	KC343007	KC343249	KC343491	KC343733	KC343975
D. alnea^{T}	CBS 146.46	KC343008	KC343250	KC343492	KC343734	KC343976
D. ampelina^{T}	STE-U 2660	AF230751	AY745026	–	AY745056	JX275452
D. amygdali^{T}	CBS 126679	KC343022	KC343264	KC343506	KC343748	KC343990
D. angelicae^{T}	CBS 111592	KC343027	KC343269	KC343511	KC343753	KC343995
D. apiculatum^{T}	LC3418	KP267896	–	–	KP267970	KP293476
D. arecae^{T}	CBS 161.64	KC343032	KC343274	KC343516	KC343758	KC344000
D. arengae^{T}	CBS 114979	KC343034	KC343276	KC343518	KC343760	KC344002
D. aspalathi^{T}	CBS 117169	KC343036	KC343278	KC343520	KC343762	KC344004
D. australafricana^{T}	CBS 111886	KC343038	KC343280	KC343522	KC343764	KC344006
D. biconispora^{T}	ICMP20654	KJ490597	–	KJ490539	KJ490476	KJ490418
D. bicincta^{T}	CBS 121004	KC343134	KC343376	KC343618	KC343860	KC344102
D. biguttulata^{T}	ICMP20657	KJ490582	–	KJ490524	KJ490461	KJ490403
D. biguttusis^{T}	CGMCC 3.17081	KF576282	–	–	KF576257	KF576306
D. castaneae-mollisimae^{T}	DNP 128	JF957786	JX197430	–	JX275401	JX275438
D. celastrina	CBS 139.27	KC343047	KC343289	KC343531	KC343773	KC344015
D. citri^{T}	CBS 135422	KC843311	KC843157	–	KC843071	KC843187
D. citrichinensis^{T}	ZJUD34	JQ954648	KC357494	–	JQ954666	–
D. cotoneastri^{T}	CBS 439.82	KC343090	KC343332	KC343574	KC343816	KC344058
D. crotalariae^{T}	CBS 162.33	KC343056	KC343298	KC343540	KC343782	KC344024
D. discoidispora^{T}	ICMP20662	KJ490624	–	KJ490566	KJ490503	KJ490445
D. eres^{T}	AR5193	KJ210529	KJ434999	KJ420850	KJ210550	KJ420799
D. eugeniae	CBS 444.82	KC343098	KC343340	KC343582	KC343824	KC344066
D. fraxini-angustifoliae^{T}	BRIP 54781	JX862528	–	–	JX862534	KF170920
D. fusicola^{T}	CGMCC 3.17087	KF576281	KF576233	–	KF576256	KF576305
D. gardeniae	CBS 288.56	KC343113	KC343355	KC343597	KC343839	KC344081
D. gulyae	BRIP 54025	JF431299	JN645803	–	–	–
D. helicis^{T}	AR5211	KJ210538	KJ435043	KJ420875	KJ210559	KJ420828
D. hickoriae^{T}	CBS 145.26	KC343118	KC343360	KC343602	KC343844	KC344086
D. hongkongensis^{T}	CBS 115448	KC343119	KC343361	KC343603	KC343845	KC344087
D. infecunda^{T}	CBS 133812	KC343126	KC343368	KC343610	KC343852	KC344094
***D. juglandicola*^{T}**	**CFCC 51134**	**KU985101**	**KX024616**	**KX024622**	**KX024628**	**KX024634**
D. juglandicola	**CFCC 51135**	**KU985102**	**KX024617**	**KX024623**	**KX024629**	**KX024635**
D. litchicola^{T}	BRIP 54900	JX862533	–	–	JX862539	KF170925
D. longicicola^{T}	CGMCC 3.17089	KF576267	–	–	KF576242	KF576291
D. lusitanicae^{T}	CBS 123212	KC343136	KC343378	KC343620	KC343862	KC344104
D. mahothocarpus^{T}	CGMCC 3.15181	KC153096	–	–	KC153087	KF576312
D. melonis^{T}	CBS 507.78	KC343142	KC343384	KC343626	KC343868	KC344110
D. multigutullata^{T}	ICMP20656	KJ490633	–	KJ490575	KJ490512	KJ490454
D. musigena^{T}	CBS 129519	KC343143	KC343385	KC343627	KC343869	KC344111
D. neilliae^{T}	CBS 144.27	KC343144	KC343386	KC343628	KC343870	KC344112
D. neoarctii^{T}	CBS 109490	KC343145	KC343387	KC343629	KC343871	KC344113

D. nobilis	CBS 113470	KC343146	KC343388	KC343630	KC343872	KC344114
D. nomurai	CBS 157.29	KC343154	KC343396	KC343638	KC343880	KC344122
D. nothofagi[T]	BRIP 54801	JX862530	–	–	JX862536	KF170922
D. novem[T]	CBS 127270	KC343156	KC343398	KC343640	KC343882	KC344124
D. oraccinii[T]	LC3166	KP267863	–	KP293517	KP267937	KP293443
D. ovalispora[T]	ICMP20659	KJ490628	–	KJ490570	KJ490507	KJ490449
D. ovoicicola[T]	CGMCC 3.17092	KF576264	KF576222	–	KF576239	KF576288
D. pascoei[T]	BRIP 54847	JX862532	–	–	JX862538	KF170924
D. penetriteum[T]	LC3353	KP714505	–	KP714493	KP714517	KP714529
D. pseudomangiferae[T]	CBS 101339	KC343181	KC343423	KC343665	KC343907	KC344149
D. pseudophoenicicola[T]	CBS 462.69	KC343184	KC343426	KC343668	KC343910	KC344152
D. pterocarpicola[T]	MFLUCC 100580	JQ619887	JX197433	–	JX275403	JX275441
D. pulla[T]	CBS 338.89	KC343152	KC343394	KC343636	KC343878	KC344120
D. rostrata[T]	CFCC 50062	KP208847	KP208849	KP208851	KP208853	KP208855
D. rostrata	CFCC 50063	KP208848	KP208850	KP208852	KP208854	KP208856
D. rudis[T]	AR3422	KC843331	KC843146	–	KC843090	KC843177
D. schini[T]	CBS 133181	KC343191	KC343433	KC343675	KC343917	KC344159
D. subclavata[T]	ICMP20663	KJ490587	–	KJ490529	KJ490466	KJ490408
D. terebinthifolii[T]	CBS 133180	KC343216	KC343458	KC343700	KC343942	KC344184
D. unshiuensis[T]	CGMCC 3.17569	KJ490587	–	KJ490529	KJ490466	KJ490408
D. vaccinii[T]	CBS 160.32	KC343228	KC343470	KC343712	KC343954	KC344196
D. virgiliae[T]	CMW 40755	KP247573	–	–	–	KP247582
D. viticola[T]	CBS 113201	KC343234	KC343476	KC343718	KC343960	KC344202
D. woodii	CBS 558.93	KC343244	KC343486	KC343728	KC343970	KC344212
Diaporthella corylina	CBS 121124	KC343004	KC343246	KC343488	KC343730	KC343972

[T]Ex-type/Ex-epitype isolates. New species is bold.

For MP analyses, trees were inferred using the heuristic search option, TBR branch swapping and 1000 random sequence additions. Maxtrees were set to 5000, branches of zero length were collapsed and all equally parsimonious trees were saved. Clade stability was assessed with a bootstrap analysis of 1000 replicates (Hillis & Bull 1993). Other calculated parsimony scores were tree length (TL), consistency index (CI), retention index (RI) and rescaled consistency (RC). ML analysis was performed with a GTR site substitution model (Guindon et al. 2010). Branch support was evaluated by a bootstrapping (BS) method with 1000 replicates (Hillis & Bull 1993).

MrModeltest v. 2.3 was used to estimate the best nucleotide substitution model settings for each gene ((TrN + I + G) for ITS; (HKY + I + G) for CAL and TEF1-α; (GTR + I + G) for HIS) (Posada & Crandall 1998). The best fit model (GTR + I + G) was selected for CAL, HIS, ITS, TEF1-α and TUB sequence datasets. Bayesian inference (BI) analysis employing a Markov Chain Monte Carlo (MCMC) algorithum was performed to confirm the topology of the tree (Rannala & Yang 1996). Suitable nucleotide substitution models were determined using MrModeltest v.2.3 (Posada & Crandall 1998). Sequences data were deposited in GenBank (Table 1). The multilocus file was deposited inTreeBASE (www.treebase.org) as accession S20403. The taxonomic novelty was deposited in MycoBank (Crous et al. 2004b).

Results

Phylogeny

A total of 67 combined CAL, HIS, ITS, TEF1-α, TUB sequences (including one outgroup) were aligned, comprising 2854 characters after alignment. Of these, 1342 characters were constant, 368 variable characters were parsimony-uninformative and 1144 characters were parsimony informative. The MP analysis resulted in 4 equally parsimonious trees and the first tree (TL = 5645, CI = 0.461, RI = 0.727, RC = 0.335) is shown in Fig. 1. The phylogenetic tree obtained from ML and BI with the MCMC algorithm was consistent with the MP tree. The branches with significant Bayesian posterior probability (≥ 0.90) in BI are thickened in the phylogenetic tree. Isolates in the current study clustered in a distinct clade with high support (MP/ML/BI=100/100/1). It is

recognized as a novel species and this is supported by morphological traits.

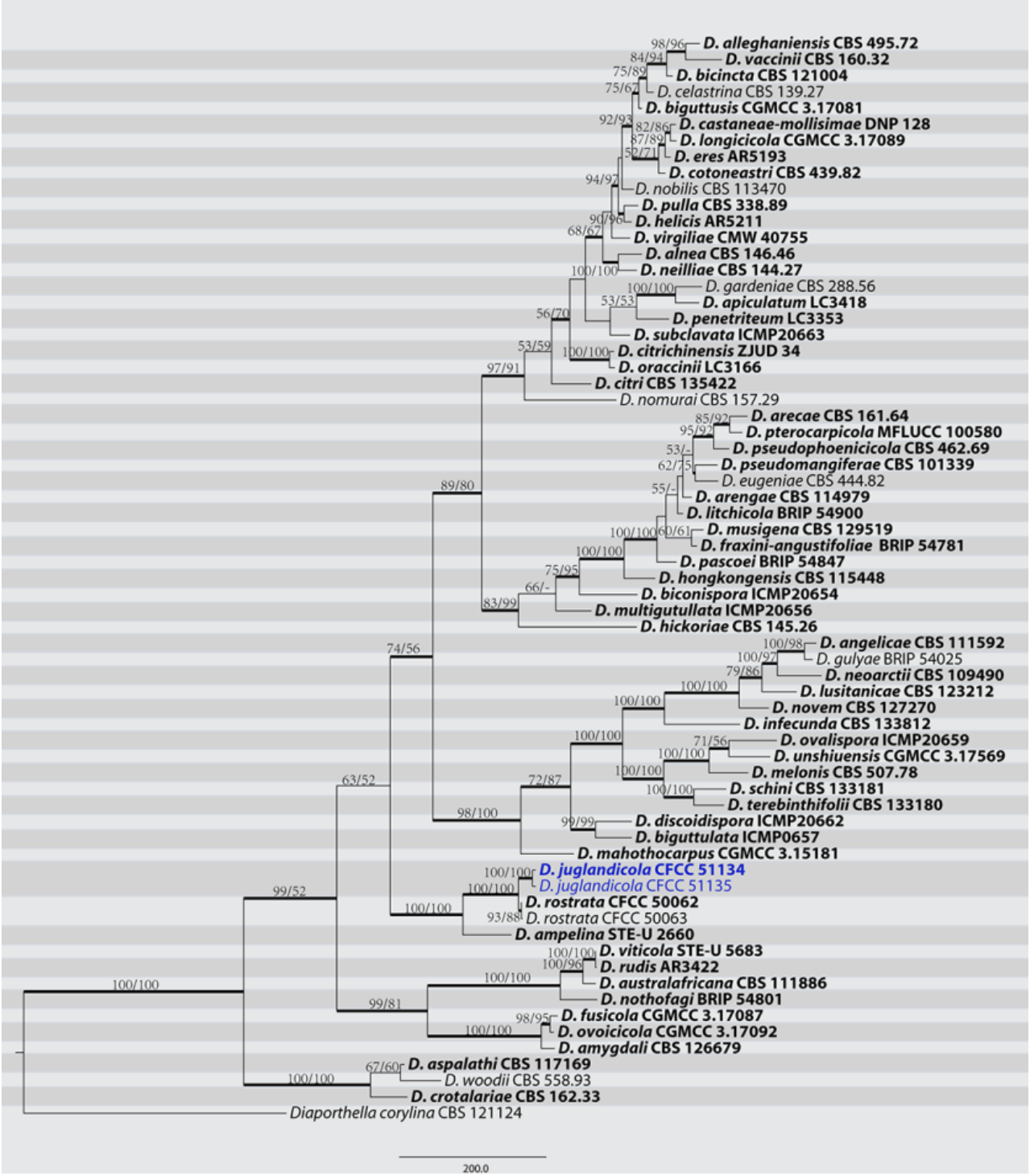

Figure 1 – Phylogram of CAL, HIS, ITS, TEF1-α and TUB regions based on MP, ML and Bayesian analysis. Values above the branches indicate maximum parsimony bootstrap (MP BP ≥ 50 %) and maximum likelihood bootstrap (ML BP ≥ 50%). Thickened branches represent posterior probabilities (BI PP ≥ 0.90) from Bayesian inference. Scale bar = 200 nucleotide substitutions. The new sequences resulting from the current study are in blue. Ex-type strains are in bold.

Taxonomy

Diaporthe juglandicola C.M. Tian & Q. Yang, sp. nov. Figs 2–3

Index Fungorum number: 552939; Faces of fungi number: FoF03111

Etymology – *juglandicola* (Lat.): named after the host genus, *Juglans*.

Holotype – BJFC-S1342.

Host/Distribution – from branches of *Juglans mandshurica* in China.

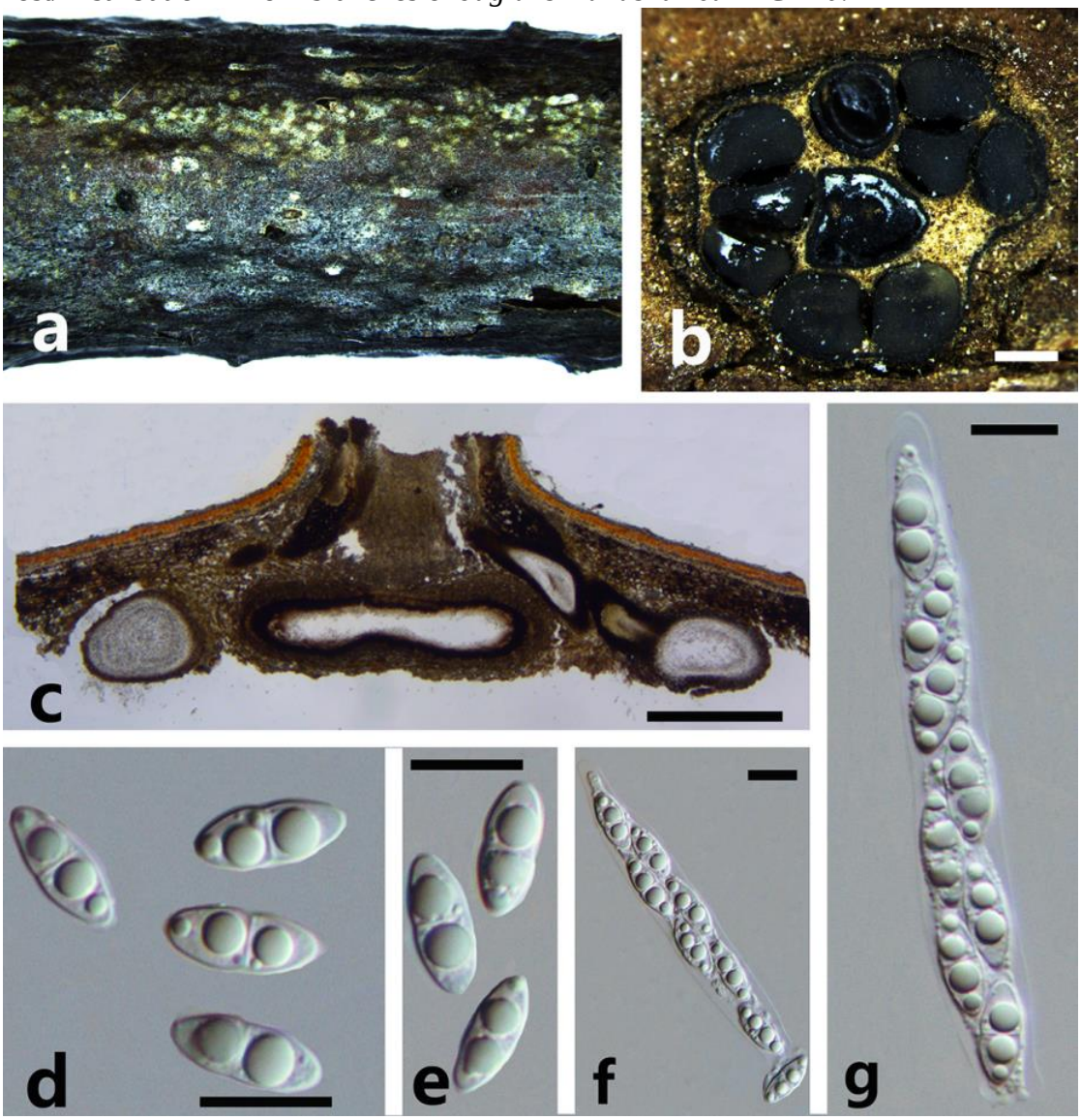

Figure 2 – The sexual morph of *Diaporthe juglandicola* from *Juglans mandshurica* (BJFC-S1342). **a** Habit of ascostroma on a twig. **b** Transverse sections through ascostroma. **c** Longitudinal sections through ascostroma. **d**–**e** Ascospores. **f** Asci with ascospores. **g** Asci. Scale bars: b–c = 500 μm, d–g = 10 μm.

Pathogens on branches and twigs of *Juglans mandshurica*. **Sexual morph**: *Ascostromata* (1300–)1550–2200(−2350) μm (av. = 2000 μm, n = 20) diameter, immersed in bark, erumpent, with 5–10 perithecia in black entostromata, extending to a large circular area with a black conceptacle, sometimes with central conidiomatal stromata surrounded by perithecium. *Perithecia* (350–)545–775(−850) μm (av. = 700 μm, n = 20) diameter, black, scattered, arranged circularly, ovoid to spherical. *Asci* (68–)70–79(−82.5) × (7–)7.5–9.5(−10.5) μm (av. = 73 × 8.5 μm, n = 20) diameter, 8-spored, unitunicate, clavate to cylindric, sessile. *Ascospores* (8.5–)10–13.5(−15) × (3–)3.5–5.5(−6) μm (av. = 12 × 5 μm, n = 50) diameter, biseriate to partially biseriate in the ascus, fusoid, hyaline, 0–1-septate, 2–4-guttulate, smooth-walled. **Asexual morph**: *Conidiomatal stromata* immersed, erumpent through the bark surface, separate, globose, with a single locule. *Ectostromatic* disc black, with one ostiole per disc, (300–)445–550(–600) μm (av. = 500 μm, n = 20) diam. *Locule* undivided, (600–)650–780(−900) μm (av. = 750 μm, n = 20) diam. *Conidiophores* reduced to *conidiogenous cells. Conidiogenous cells* (13.5–)15.5–18(−20) × 1–1.2 μm (av. = 16 × 1 μm, n = 50) diam, hyaline, branched, phialides, cylindrical, straight or slightly curved. *Alpha conidia* (7.5–)8–9(−9.5) × 2.5–3.0(−3.2) μm (av. = 8.5 × 2.7 μm, n = 50) diameter, hyaline, aseptate,

smooth, abundant in culture and on twigs, ellipsoidal, biguttulate. Beta conidia not present.

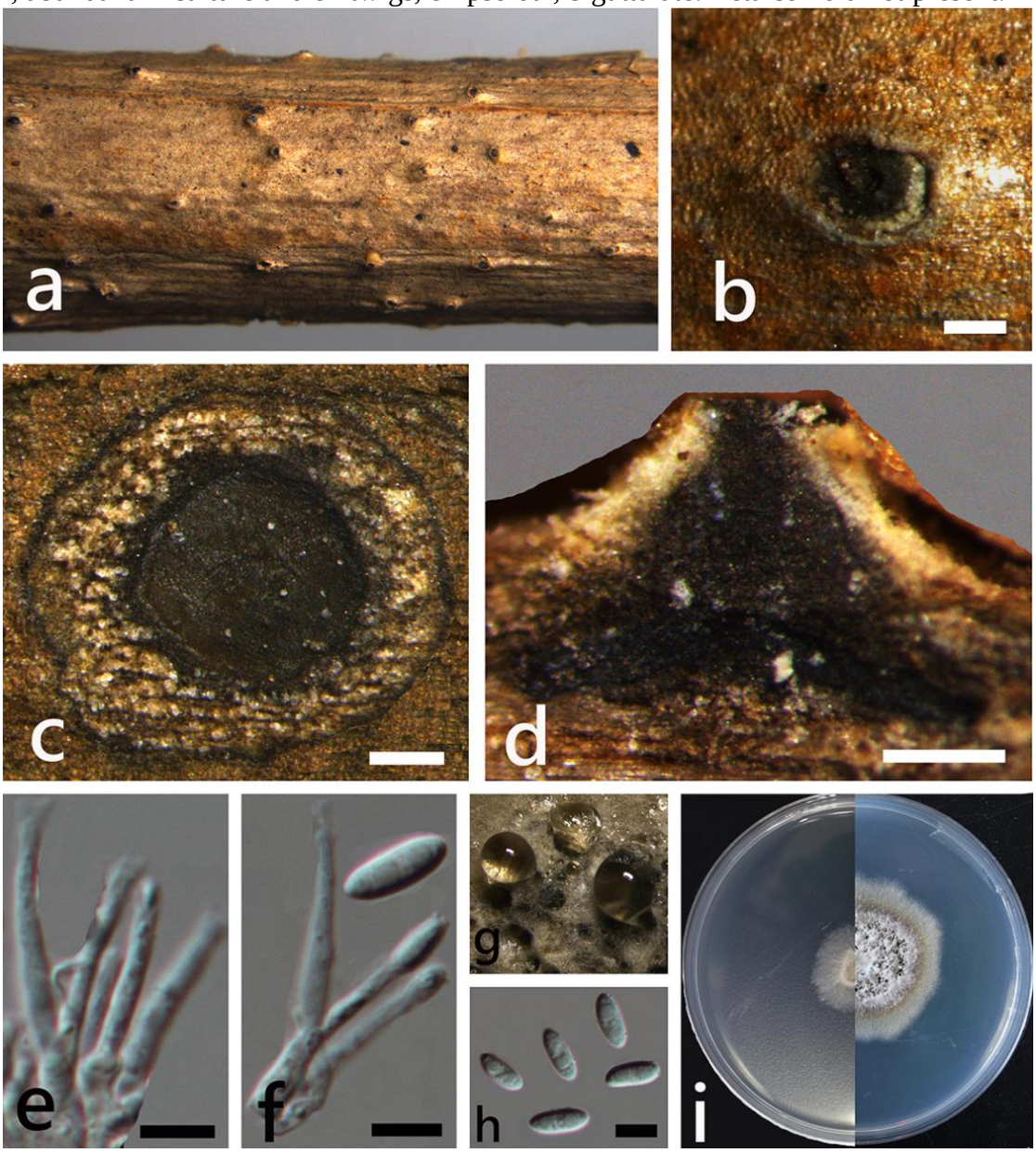

Figure 3 – The asexual morph of *Diaporthe juglandicola* from *Juglans mandshurica* (BJFC-S1341). **a**–**b** Habit of conidiomata on a twig. **c** Transverse sections through conidiomata. **d** Longitudinal sections through conidiomata. **e**–**f** Conidiophores. **g** conidiomata in culture. **h** alpha conidia. **i** Colonies on PDA at 3 days (left) and 30 days (right). Scale bars: b–d = 200 μm, e–f, h = 5 μm.

Culture characters – Culture incubated on PDA at first white, becoming slightly brown. Aerial mycelium white, cottony, with irregular margin. Conidiomata distributed over agar surface.

Material examined – CHINA, Beijing City, Yanqing County, Songshan Nature Reserve, 40°30'26.30"N, 115°47'44.45"E, 810 m asl, on twigs and branches of *Juglans mandshurica*, coll. S.S. Hao, 9 May 2015 (BJFC-S1342, **holotype**), ex-type culture, CFCC 51134. Beijing City: Mentougou District, 39°57'35.21"N, 115°26'06.50"E, 1157 m asl, on twigs and branches of *Juglans mandshurica*, coll. Q. Yang, 17 August 2015 (BJFC-S1341, paratype), living culture, CFCC 51135.

Notes – This new species is introduced as molecular data showed it to be distinct, and this is also supported by morphological traits. The phylogram clustered in 67 clades with 57 ex-type *Diaporthe* strains distinguished the new species with high support (MP/ML/BI=100/100/1) (Fig. 1). *Diaporthe juglandicola* appears closely related to *D. rostrata* (identity in ITS: 464/464; in CAL: 482/485; in HIS: 441/451; in TEF1-α: 326/330; in TUB: 693/695). Morphologically, it is

characterized by ellipsoidal, aseptate, biguttulate alpha conidia and unitunicate, clavate to cylindric asci. However, *Diaporthe juglandicola* can be distinguished by its bigger perithecia (545–775 vs. 280–370 μm) and smaller asci (70–79 × 7.5–9.5 vs. 72–86 × 10.5–13.5μm), ascospores (10–13.5 × 3.5–5.5 vs. 13–16.5 × 5.5–6.5 μm) (Fan et al. 2015).

Discussion

In this study, *Diaporthe juglandicola* was found on twigs and branches of *Juglans mandshurica* in China with both sexual and asexual morphs. Two representative strains, one from an ascospore and one from a conidium, were used in the phylogenetic analysis. Two specimens, one with both sexual and asexual morphs, and the other with asexual morph only are deposited as the holotype and paratype. We describe and illustrate the holomorph of this species, the asexual morph occasionally occurred around or in the centre of the sexual perithecia (Fig. 3c). And it is clearly distinguished from *D. rostrata*, which also reported from *Juglans mandshurica* in China. *Diaporthe juglandicola* can be distinguished from *D. rostrata* by the smaller asci, ascospores and bigger perithecia. Besides, *D. juglandicola* has a small group of ascomata (5–10 vs. 13–32) and can be separated by the size of smaller alpha coidia (8.0–9.0 × 2.5–3.0 vs. 8.5–11.5 × 4–5 μm) (Fan et al. 2015).

The morphological characteristics usually used for species delimitation in *Diaporthe* include cultural appearance, shape and size of conidiomata, conidiophores and alpha and beta conidia (Rehner & Uecker 1994, Santos et al. 2011, Thompson et al. 2011, Udayanga et al. 2011, 2012a, b, 2014a, b, 2015, Gomes et al. 2013, Gao et al. 2014, 2015, 2016). However, species delimitation in *Diaporthe* based on morphological characters is challenging, as most taxa in culture do not produce all spore states of the asexual (alpha, beta and gamma conidia) or the sexual morph (Udayanga et al. 2014a, b, 2015). By means of molecular sequence data, much progress has been made towards identifying and characterizing in the genus *Diaporthe* (Gao et al. 2014, 2015, 2016, Udayanga et al. 2014a, b, 2015, Dissanayake et al. 2015, Fan et al. 2015, Du et al. 2016). Taylor et al. (2000) proposed the Genealogical Concordance Phylogenetic Species Recognition (GCPSR) concept to recognize the limits of fungi species, using the phylogenetic concordance of multiple unlinked genes. The adoption of genealogical concordance for species recognition in *Diaporthe* enabled us to distinguish species that were otherwise not possible to identify due to either sterility, or the lack or loss of specific character states (Udayanga et al. 2014a, b, 2015). In this study, phylogenetic analysis based on sequences of multiple loci (CAL, HIS, ITS, TEF1-α and TUB) demonstrated one distinctive new species in *Diaporthe* with highly supported clades and supported by the holomorphic morphology

Acknowledgements

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