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RESEARCH ARTICLE

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Dark septate endophytic pleosporalean genera from semiarid areas

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Key words

Dothideomycetes
endophytes
Massarineae
mating
phylogeny
sexual state
taxonomy

Abstract Dark septate endophytes (DSE) are distributed worldwide as root-colonising fungi, and frequent in environments with strong abiotic stress. DSE is not a taxon, but constitutes numerous fungal taxa belonging to several orders of *Ascomycota*. In this study we investigate three unidentified DSE lineages belonging to *Pleosporales* that were found previously in semiarid sandy grasslands. For molecular phylogenetic studies seven loci (ITS, partial 18S nrRNA, 28S nrRNA, actin, calmodulin, transcription-elongation factor 1-α and ß-tubulin genes) were amplified and sequenced. Based on morphology and the resulting molecular phylogeny these isolates were found to represent three novel genera within the *Pleosporales*, namely *Aquilomyces*, *Flavomyces* and *Darksidea*, with eight novel species. Molecular data revealed that monotypic *Aquilomyces* belongs to *Morosphaeriaceae*, monotypic *Flavomyces* represents an *incertae sedis* lineage related to *Massarinaceae*, and *Darksidea*, with six new species, is allied to the *Lentitheciaceae*. During this study we tested numerous conditions to induce sporulation, and managed for the first time to induce several DSE to form their sexual morphs.

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INTRODUCTION

Dark septate endophytes (DSE) compose a form-group of root associated fungi (RAF) (Jumpponen & Trappe 1998a), which generally have melanised hyphae, colonise the root epidermis and the cortex inter- and intracellularly and form densely septated intracellular structures, called microsclerotia (Jumpponen & Trappe 1998a, Barrow & Aaltonen 2001, Yu et al. 2001, Addy et al. 2005, Mandyam & Jumponnen 2008, Peterson et al. 2008). DSE fungi occur in all climate regions and major biome types (Mandyam & Jumponnen 2005, Rodriguez et al. 2009, Porras-Alfaro & Bayman 2011) and are relatively frequent in extreme and nutrient-limited environments such as arid and semiarid areas (Kovács & Szigetvári 2002, Rodriguez et al. 2009, Khidir et al. 2010). Although there is an increasing research interest in DSE, our knowledge on the diversity and function of these fungi is limited, especially compared to mycorrhizal fungi.

No sexual morph of DSE is known and their sexual relations are not completely understood (Jumpponen & Trappe 1998a, Grünig et al. 2008, Rodriguez et al. 2009). Even conidiogenesis is infrequent and conidiogenous processes have only been induced in a minority of isolates after specific treatments (Jumpponen & Trappe 1998a, Sieber & Grünig 2006).

Dark septate endophytes represent a group of fungal endophytes from different ascomycete lineages forming similar structures (Jumpponen & Trappe 1998a). They generally belong to numerous orders of phylum *Ascomycota* (e.g. *Capnodiales*, *Chaetothyriales*, *Eurotiales*, *Helotiales*, *Hypocreales*, *Microascales*, *Pleosporales*, *Sordariales*, *Xylariales* – see e.g. Jumpponen & Trappe 1998a, Addy et al. 2005, Newsham 2011, Knapp et al. 2012, Andrade-Linares & Franken 2013). The majority of our knowledge on DSE fungi has thus far been gained through studies of fungi in the *Helotiales* (class *Leotiomycetes*) focusing on several DSE fungi e.g. *Cadophora finlandica* (≡ *Phialophora finlandica*) and the widely studied *Phialocephala fortinii* s.l. – *Acephala applanata* species complex (PAC) (e.g. Fernando & Currah 1996, Jumpponen & Trappe 1998b, Caldwell et al. 2000, Tellenbach et al. 2011, Reininger et al. 2012). *Pleosporales*, which is the largest order of *Dothideomycetes* (Schoch et al. 2009, Zhang et al. 2012), is one of the most represented orders in DSE communities of semiarid areas (Porras-Alfaro et al. 2008, Knapp et al. 2012).

In a previous study, the compositional diversity of DSE fungi colonising native and invasive plants of semiarid sandy areas on the Great Hungarian Plain was investigated (Knapp et al. 2012). Based on an in vitro resynthesis assay, isolates of 14 lineages were considered as DSE fungi, several groups of which could not be identified. Three of these groups (DSE-4, DSE-8 and DSE-7 sensu Knapp et al. 2012) clustered in the *Pleosporales*. In case of DSE-4, no similar sequences of either cultured or uncultured fungi were found in public databases. Although group DSE-7 was found to be the third most frequent DSE clade (Knapp et al. 2012), and similar findings were obtained in other studies (Porras-Alfaro et al. 2008, Khidir et al. 2010, Herrera et al. 2010), the identity and phylogenetic placement of this taxon and other DSE fungi in the *Pleosporales* remained unclear.

The main aim of our study was therefore to conduct a taxonomic study of the pleosporalean DSE groups originating from semiarid sandy areas (Knapp et al. 2012). We further aimed to collect more isolates of group DSE-7 to study the intragroup heterogeneity, and conduct a multi-locus molecular phylogenetic and morphological comparison of isolates.

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Table 1 Collection details, culture collection numbers, GenBank accession numbers (ITS, LSU, SSU, ACT, TUB, CAL, TEF) and other details of strains included in this study.

Species	Strain no.[1]	Isolate no.	Host plant	Collection date	Strains published[2]	GenBank Accession no.[3]						
						ITS	LSU	SSU	ACT	TUB	CAL	TEF
Aquilomyces patris	CBS 135661 = CPC 22895 (ex–type)	DSE-4 / 099	*Populus alba*	July 2005	REF099	KP184002	KP184041	KP184077	KP184119	–	KP184154	–
	CBS 135760 = CPC 22896	DSE-4 / 100	*P.alba*	July 2005	REF100	KP184004	KP184042	KP184078	KP184120	–	KP184155	–
	CBS 135662 = CPC 22897	DSE-4 / 101	*P. alba*	July 2005	REF101	KP184003	KP184043	KP184079	KP184121	–	KP184156	–
Darksidea alpha	CBS 135627 = CPC 22861	DSE-7 / 1	*Bromus tectorum*	June 2012	–	KP183966	KP184005	KP184044	KP184093	KP184192	KP184122	KP184160
	CBS 135628 = CPC 22862	DSE-7 / 2	*Festuca vaginata*	June 2012	–	KP183965	KP184035	KP184054	KP184083	KP184193	KP184123	KP184161
	CBS 135630 = CPC 22864	DSE-7 / 4	*Stipa borysthenica*	July 2012	–	KP183987	KP184032	KP184058	KP184090	KP184195	KP184126	KP184162
	CBS 135631 = CPC 22865	DSE-7 / 5	*F. vaginata*	September 2010	–	KP183968	KP184033	KP184051	KP184101	KP184196	KP184142	KP184180
	CBS 135632 = CPC 22866	DSE-7 / 6	*F. vaginata*	September 2010	–	KP183969	KP184034	KP184052	KP184103	KP184197	KP184143	KP184181
	CBS 135641 = CPC 22875	DSE-7 / 15	*Ailanthus altissima*	July 2008	REF132	KP183970	KP184037	KP184055	KP184086	KP184205	KP184124	KP184163
	CBS 135642 = CPC 22876	DSE-7 / 16	*F. vaginata*	July 2005	REF133	KP183967	KP184026	KP184053	KP184097	KP184206	KP184141	KP184182
	CBS 135643 = CPC 22877	DSE-7 / 17	*S. borysthenica*	May 2005	REF135	KP183988	KP184022	KP184048	KP184091	KP184207	KP184139	KP184171
	CBS 135644 = CPC 22878	DSE-7 / 18	*F. vaginata*	July 2005	REF136	KP183989	KP184030	KP184059	KP184100	KP184208	KP184127	KP184179
	CBS 135645 = CPC 22879	DSE-7 / 19	*S. borysthenica*	May 2005	REF137	KP183990	KP184036	KP184060	KP184092	KP184209	KP184140	KP184172
	CBS 135646 = CPC 22880	DSE-7 / 20	*F. vaginata*	July 2005	REF138	KP183994	KP184015	KP184066	KP184084	KP184210	KP184128	KP184164
	CBS 135647 = CPC 22881	DSE-7 / 21	*F. vaginata*	July 2005	REF139	KP183997	KP184018	KP184061	KP184094	KP184211	KP184125	KP184175
	CBS 135648 = CPC 22882	DSE-7 / 22	*F. vaginata*	July 2012	–	KP183993	KP184016	KP184062	KP184104	KP184212	KP184129	KP184165
	CBS 135649 = CPC 22883	DSE-7 / 23	*F. vaginata*	July 2012	–	KP183991	KP184025	KP184063	KP184105	KP184213	KP184130	KP184170
	CBS 135650 = CPC 22884 (ex–type)	DSE-7 / 24	*F. vaginata*	July 2012	–	KP183998	KP184019	KP184049	KP184102	KP184214	KP184131	KP184166
	CBS 135651 = CPC 22885	DSE-7 / 25	*F. vaginata*	July 2012	–	KP183996	KP184017	KP184064	KP184087	KP184215	KP184132	KP184167
	CBS 135652 = CPC 22886	DSE-7 / 26	*F. vaginata*	July 2012	–	KP183992	KP184020	KP184050	KP184089	KP184216	KP184133	KP184168
	CBS 135653 = CPC 22887	DSE-7 / 27	*F. vaginata*	July 2012	–	KP183995	KP184021	KP184065	KP184085	KP184217	KP184134	KP184169
	CBS 135654 = CPC 22888	DSE-7 / 28	*F. vaginata*	July 2012	–	KP183972	KP184010	KP184045	KP184095	KP184218	KP184135	KP184176
	CBS 135655 = CPC 22889	DSE-7 / 29	*F. vaginata*	July 2012	–	KP183975	KP184011	KP184046	KP184096	KP184219	–	KP184177
	CBS 135656 = CPC 22890	DSE-7 / 30	*F. vaginata*	July 2012	–	KP183971	KP184007	KP184047	KP184088	KP184220	KP184136	KP184178
	CBS 135659 = CPC 22893	DSE-7 / 33	*S. borysthenica*	July 2012	–	KP183973	KP184012	KP184056	KP184098	KP184223	KP184137	KP184173
	CBS 135660 = CPC 22894	DSE-7 / 34	*S. borysthenica*	July 2012	–	KP183974	KP184014	KP184057	KP184099	KP184224	KP184138	KP184174
D. beta	CBS 135636 = CPC 22870	DSE-7 / 10	*F. vaginata*	July 2005	REF127	KP183976	KP184008	KP184076	KP184114	KP184200	KP184152	–
	CBS 135637 = CPC 22871 (ex–type)	DSE-7 / 11	*F. vaginata*	July 2005	REF128	KP183978	KP184023	KP184074	KP184112	KP184201	KP184153	KP184189
	CBS 135657 = CPC 22891	DSE-7 / 31	*F. vaginata*	July 2012	–	KP183977	KP184009	KP184075	KP184113	KP184221	KP184150	KP184190
D. gamma	CBS 135633 = CPC 22867	DSE-7 / 7	*F. vaginata*	July 2005	REF123	KP183984	KP184031	KP184072	KP184110	KP184198	KP184149	KP184187
	CBS 135634 = CPC 22868 (ex–type)	DSE-7 / 8	*F. vaginata*	July 2005	REF124	KP183985	KP184028	KP184073	KP184111	KP184199	KP184151	KP184188
	CBS 135635 = CPC 22869	DSE-7 / 9	*F. vaginata*	July 2005	REF125	KP183986	–	–	–	–	–	–
D. delta	CBS 135629 = CPC 22863	DSE-7 / 3	*F. vaginata*	June 2012	–	KP183980	KP184006	KP184067	KP184106	KP184194	KP184144	KP184183
	CBS 135638 = CPC 22872 (ex–type)	DSE-7 / 12	*F. vaginata*	July 2005	REF129	KP183981	KP184024	KP184069	KP184107	KP184202	KP184145	KP184184
	CBS 135639 = CPC 22873	DSE-7 / 13	*Fumana procumbens*	July 2008	REF130	KP183982	KP184027	KP184068	KP184108	KP184203	KP184146	KP184185
D. epsilon	CBS 135658 = CPC 22892 (ex–type)	DSE-7 / 32	*S. borysthenica*	July 2012	–	KP183983	KP184029	KP184070	KP184109	KP184222	KP184147	KP184186
D. zeta	CBS 135640 = CPC 22874 (ex–type)	DSE-7 / 14	*F. vaginata*	July 2005	REF131	KP183979	KP184013	KP184071	KP184115	KP184204	KP184148	KP184191
Flavomyces fulophazii	CBS 135664 = CPC 22899	DSE-8 / 143	*F. vaginata*	July 2005	REF143	KP184000	KP184039	KP184081	KP184116	–	KP184159	–
	CBS 135761 = CPC 22900 (ex–type)	DSE-8 / S	*F. vaginata*	July 2012	–	KP184001	KP184040	KP184082	KP184118	–	KP184158	–
Periconia macrospinosa	CBS 135663 = CPC 22898	DSE-8 / B	*F. vaginata*	June 2012	–	KP183999	KP184038	KP184080	KP184117	–	KP184157	–

[1] CBS: CBS Fungal Biodiversity Centre, Utrecht, The Netherlands; CPC: Collection Pedro Crous, housed at CBS.

[2] Strains published by Knapp et al. 2012. PLoS ONE 7: e32570.

[3] ITS: internal transcribed spacer regions of the nrDNA and intervening 5.8S nrDNA; LSU: partial 28S large subunit of the nrRNA gene; SSU: partial 18S small subunit of the nrRNA gene; ACT: partial actin gene; TUB: partial beta-tubulin gene; CAL: partial calmodulin gene; TEF: partial translation elongation factor 1-alpha gene. All of the strains were isolated from surface sterilized healthy root segments of healthy-looking host plants. Isolates were collected by Dániel G. Knapp Alexandra Pintye, Erik Zajta and Gábor M. Kovács at Fülöpháza, Hungary (N46°52' E19°25') except the strain CBS 135641, which was collected near Tatárszentgyörgy, Hungary (N47°03.5' E19°24.3').

MATERIAL AND METHODS

Isolates and locus selection

Roots of three native grass species – sand feather grass (*Stipa borysthenica)*, *Festuca vaginata* and cheat grass (*Bromus tectorum)* – were collected from semiarid grasslands near Fülöpháza (N46°52' E19°25'), Hungary, during the summer of 2012 (for detailed description of the site see Kovács & Szigetvári 2002 and Knapp et al. 2012). Isolates were collected from surface-sterilised healthy roots as described in Knapp et al. (2012), and cultivated on a Modified Melin-Norkrans agar (MMN, Marx 1969). As the colony morphology of isolates with similar or even identical nrDNA ITS sequences can be completely different in the clade DSE-7, we designed a diagnostic PCR primer pair for rapid identification of isolates belonging to this clade. Based on the internal transcribed spacer regions ITS alignment of our sequences and sequences from GenBank we designed specific forward (DSE7F: GTTGTTTCCTCGGCAGGTC) and reverse (DSE7R: ACGACCGCTGCCAATACC) primers targeting the ITS1 and ITS2 regions, and amplifying an c. 300-bp long segment. Nineteen isolates obtained from roots of the three grass species (Table 1) with positive reaction to the designed primers, were kept for further study.

Two additional isolates obtained during the present survey were included in this study. Both belonged to group DSE-8. One of the isolates (CBS 135761) had colony characteristics similar to isolate REF143 (CBS 135664) and was considered a member of group DSE-8 (Knapp et al. 2012). The other isolate (CBS 135663) belonged to another subclade of DSE-8 representing *Periconia macrospinosa*, and was used in the phylogenetic analyses (see below) (Knapp et al. 2012).

Other than the isolates collected during the present study, 19 isolates obtained previously (Knapp et al. 2012) were also included: 15 isolates of group DSE-7, three isolates of group DSE-4 and one isolate (REF143) from group DSE-8 (Table 1), representing 40 isolates in total. All isolates investigated in this study are deposited in the CBS culture collection (CBS 135627–135664, 135760, 135761), and nomenclatural novelties and descriptions deposited in MycoBank (www.MycoBank.org, Crous et al. 2004).

Sporulation

To induce sporulation, isolates were cultured onto different media in 9-cm Petri dishes; potato-dextrose agar (PDA), synthetic nutrient-poor agar (SNA), malt extract agar (MEA), oatmeal agar (OA) (see Crous et al. 2009), MMN (Marx 1969) and Murashige-Skoog agar (MS, Murashige & Skoog 1962, M5524 Sigma-Aldrich Co. LLC., USA) were used. All cultures were incubated at room temperature in the dark, while cultures on MMN and MS were also incubated in the dark at 10 °C.

Furthermore, isolates were also cultured onto the following autoclaved plant parts laid on media to promote sporulation: barley shoots, pine needles, stinging nettle stems and rye grass roots on SNA, and white elm stems on MS. Two parallel replicates of each representative isolate studied were incubated at room temperature and at 10 °C in the dark.

Three parallel replicates of each representative isolate of the groups were cultured onto MEA and MMN and three different treatments applied: i) colonies were burned with a red-hot needle and incubated for 4 wk at room temperature; ii) colonies were exposed daily to near-ultraviolet light for 3 × 10 min for 3 d and subsequently incubated for 4 wk at room temperature, and afterwards stored in dark at 4 °C for several months; and iii) colonies were allowed to dry out on the laboratory bench over a period of 3 mo at room temperature.

Morphology

Observations were made with a Zeiss V20 Discovery stereomicroscope, and with a Zeiss Axio Imager 2 light microscope using differential interference contrast (DIC) illumination and an AxioCam MRc5 camera and ZEN software. Measurements and photographs were made from structures mounted in clear lactic acid. Colony morphology was assessed on MMN and MEA media inoculated with 4-mm-diam fungal plugs in 5-cm Petri dishes at room temperature after 4 wk. Colony colours (surface and reverse) were established using the colour charts of Rayner (1970).

DNA extraction, amplification and phylogeny

DNA was isolated from all isolates, amplified, and sequenced using both forward and reverse primers for partial ITS, partial 18S nrRNA (SSU), 28S nrRNA (LSU), actin (ACT) and calmodulin (CAL) genes; for the DSE-7 isolates, partial transcription-elongation factor 1-α (TEF) and ß-tubulin (TUB) gene sequences were also determined. For rapid identification of DSE-7 isolates, a small amount of mycelium was disrupted in 30 µL TE, incubated on 96 °C for 10 min, and used in the diagnostic PCR performed in a Biometra Gradient Thermocycler (Biometra) with an initial denaturing at 95 °C for 5 min, followed by 35 cycles of denaturation at 95 °C for 30 s, annealing at 55 °C for 30 s, extension 72 °C for 40 s and 5 min final extension at 72 °C. The PCR products visualised under UV light after electrophoresis on a 1.5 % agarose gel at 100 V for 20 min.

Genomic DNA was extracted from fungal mycelia using the UltraClean™ Microbial DNA Isolation Kit (Mo Bio Laboratories, Inc., Solana Beach, CA, USA) according to the manufacturer's instructions.

The primers V9G (de Hoog & Gerrits van den Ende 1998) and LR5 (Vilgalys & Hester 1990) were used to amplify the nrDNA operon region containing the 3' end of the 18S nrRNA gene (SSU), the ITS region and the partial 28S nrRNA gene (LSU). The ITS region and partial 28S nrRNA gene were sequenced using internal primers ITS4 and ITS5 (White et al. 1990) and LR0R (Rehner & Samuels 1994) and LR5 (Vilgalys & Hester 1990) primers. The primers NS1 and NS4 (White et al. 1990) were used to amplify and sequence part of the 18S nrRNA gene. The partial actin gene (ACT) was amplified using the primers ACT-512F (Carbone & Kohn 1999) and ACT-2Rd (Quaedvlieg et al. 2011) and part of the translation elongation factor 1-α gene (TEF) using EF1-728F (Carbone & Kohn 1999) and EF-2Rd (Groenewald et al. 2013) primers. The primers CAL-228F (Carbone & Kohn 1999) and CAL-2Rd (Groenewald et al. 2013) were used to amplify part of the calmodulin gene (CAL) while the primers CYLTUB1F (Groenewald et al. 2013) and Bt-2b (Glass & Donaldson 1995) were used to amplify part of the ß-tubulin gene (TUB). The protocols outlined by Groenewald et al. (2013) were followed for the amplification of these loci. The PCR products were sequenced in both directions using the primers listed above and the BigDye Terminator v. 3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA), according to the manufacturer's recommendations, and analysed with an ABI Prism 3730XL DNA Analyzer (Applied Biosystems). The sequences were compiled from electrophoregrams using the Pregap4 and Gap4 software packages (Staden et al. 2000) and deposited in GenBank (KP183965–KP184224). The sequences obtained were compared to sequences in public databases using a blast search (http://blast.ncbi.nlm.nih.gov/Blast.cgi) (Altschul et al. 1990).

Phylogenetic analyses

Three datasets were used in the phylogenetic analyses. The order-level dataset was used to gain information about the phylogenetic position of groups DSE-4, DSE-7 and DSE-8 in *Pleo-*

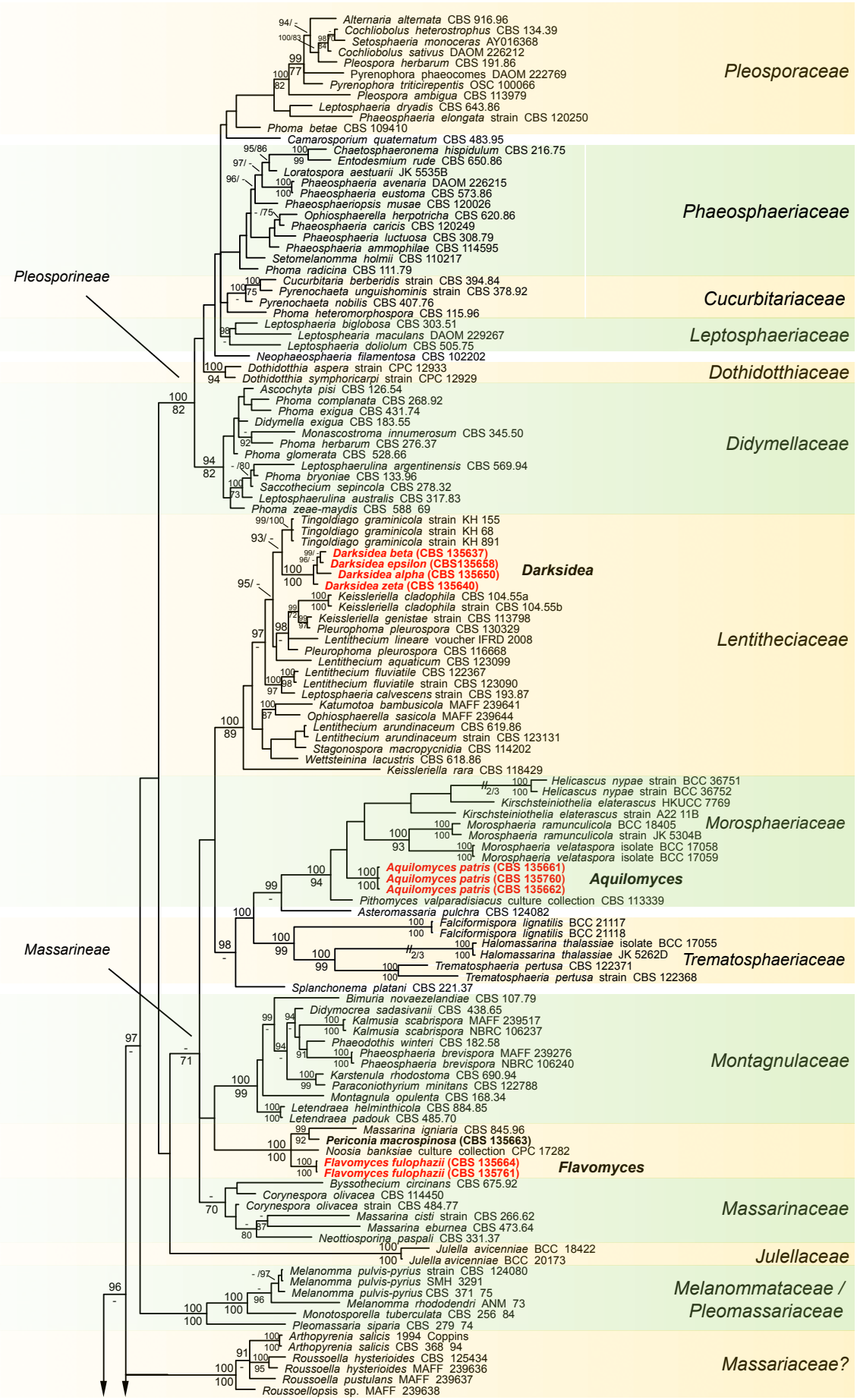


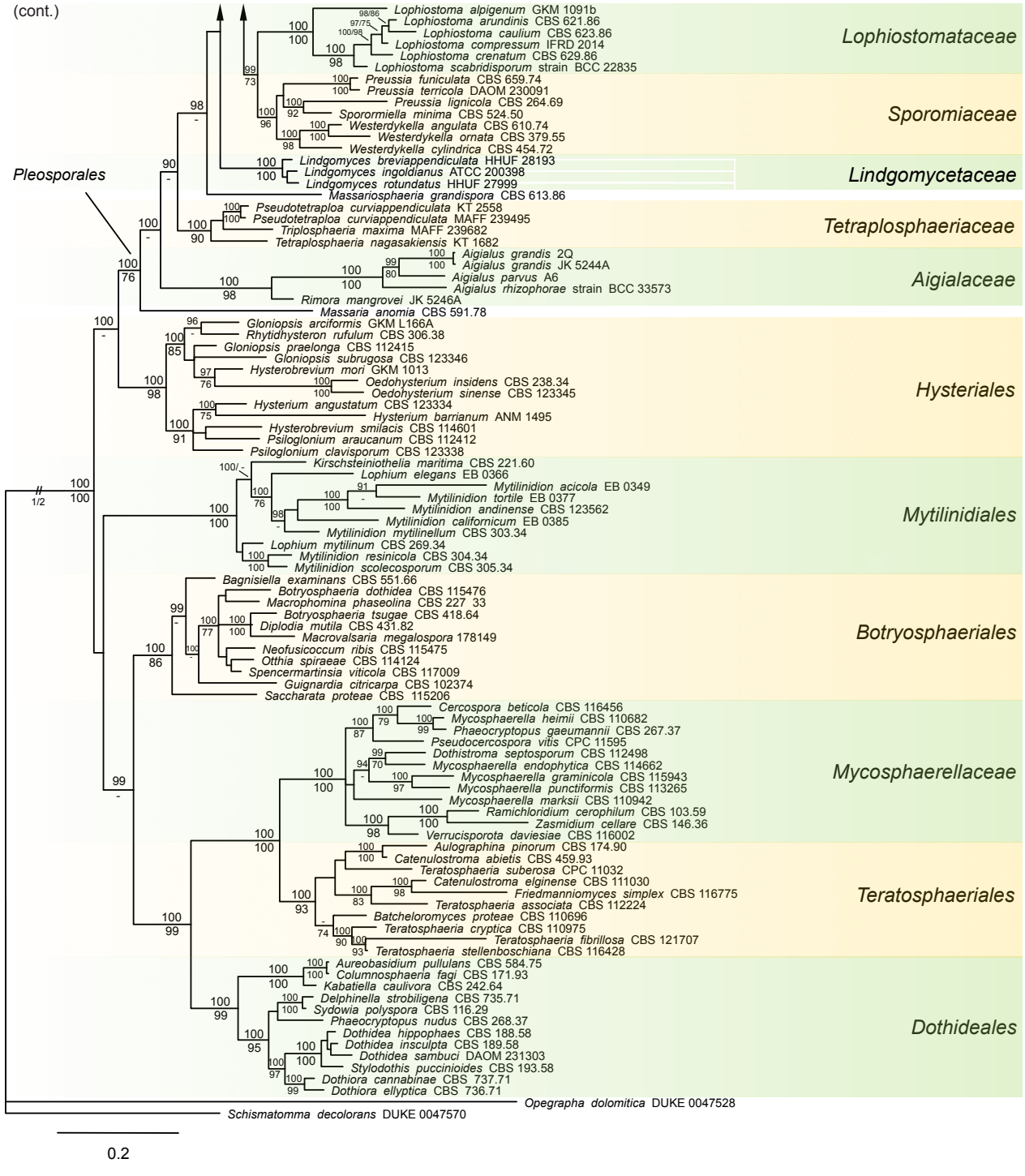
Fig. 1 Phylogenetic tree of representative sequences from *Dothydeomycetes* and from the dark septate endophyte genera *Darksidea*, *Aquilomyces* and *Flavomyces* (labelled with red). The 50 % majority rule consensus phylogram inferred from the Bayesian analysis of the combined dataset of three loci (LSU, SSU, TEF). Bayesian posterior probabilities (≥ 90) are shown as percentages before slashes or above branches, ML bootstrap support values (≥ 70) are shown after slashes or below branches. *Schismatomma decolorans* (DUKE 0047570) served as outgroup. Highlighted sections indicate affiliations to families or orders. The scale bar indicates 0.2 expected changes per site per branch.

sporales (Fig. 1). This dataset also contains taxa belonging to other dothideomycete orders, but only *Pleosporales* was represented by all its known families. The family-level dataset was used to study the position of group DSE-7 within the family *Lentitheciaceae* (Fig. 2). The third dataset was established to study the intra-group phylogenetic relation of DSE-7 isolates (Fig. 3).

Alignments of our sequences supplemented with sequences from GenBank of respective loci were made using the online version of MAFFT v. 6 (Katoh & Toh 2008). The alignments were checked and edited with MEGA v. 5 (Tamura et al. 2011) and deposited in TreeBASE (study S16626; www.treebase.org). The best-fit nucleotide substitution model was selected using the program jModelTest (Posada 2008) considering the selection of the Akaike Information Criterion (AIC) and the model was implemented in the Bayesian analyses performed with MrBayes v. 3.1.2 (see details below) (Huelsenbeck & Ronquist 2001, Ronquist & Huelsenbeck 2003). Topological convergence was checked by AWTY (Nylander et al. 2008). RAxML phylogenetic analyses were carried out with raxmlGUI v. 1.3 (Silvestro & Michalak 2012) and GTR+G nucleotide substitution model was used with ML estimation of base frequencies and a ML bootstrap analysis with 1 000 replicates was used to test the support of the branches. The phylogenetic trees were visualised and edited by FigTree v. 1.4.0 (http://tree.bio.ed.ac.uk/software/figtree) and MEGA v. 5 (Tamura et al. 2011).

The order-level multi-loci phylogenetic analyses (Fig. 1) were carried out using the partial LSU, SSU and TEF sequences. The following priors were set in MrBayes for the different partitions: all partitions had dirichlet base frequencies and GTR+I+G model with inverse gamma-distributed rates were implemented for SSU and TEF, and the SYM+I+G model with inverse gamma-distributed rates for LSU. Four Markov chains were run for 10 000 000 generations sampled every 500 generations with a burn-in value set at 7 500 sampled trees.

Fig. 1 (cont.)



For the family-level phylogeny (Fig. 2) the partial LSU, SSU and TEF sequences of representative strains of DSE-7 and sequences of taxa reported to belong to family *Lentithecia*ceae were examined. In the Bayesian analysis the substitution models GTR+I+G were implemented for SSU and TEF, and HKY+I+G for LSU. Four Markov chains were run for 15 000 000 generations and sampled every 1 500 generations with a burn-in value set at 3 000 sampled trees.

For the intra-group phylogenetic analysis of isolates of DSE-7 (Fig. 3) the more variable sequences, namely ITS, partial ACT, TUB, CAL and TEF, were used. The GTR+I+G model with inverse gamma-distributed rates was implemented for ITS and partial ACT, TUB and TEF, while CAL was analysed using the K2P model. To gain phylogenetic information of indel regions of the ITS (Nagy et al. 2012), indel coding was accomplished with FastGap v. 1.1 (Borchsenius 2007). The two-parameter Markov (Mk2 Lewis) model was used for the analysis of the binary matrix of indel characters appended to the alignments in the Bayesian analyses. Four Markov chains were run for 10 000 000 generations sampled every 1 000 generations with a burn-in value set at 3 000 sampled trees.

RESULTS

Molecular phylogeny

Targeted DNA sequences of isolates could be used for the phylogenetic analyses excluding the following that failed to be amplified: TEF of strain CBS 135636, CAL of strain CBS 135655 and all regions except ITS of strain CBS 135635 (Table 1). The TUB sequence of strain CBS 135646 failed to align properly with other TUB sequences and was therefore excluded from further analysis.

According to the results of the order-level phylogenetic analyses (Fig. 1), all three DSE groups unambiguously belong to the order *Pleosporales* and are nested in different families. The group DSE-4 represents a basal lineage in *Morosphaeriaceae*. The distinct isolates of group DSE-8 form a well-supported *incertae sedis* clade together with *Massarina igniaria* (CBS 845.96), *Periconia macrospinosa* (CBS 135663) and *Noosia banksiae* (CPC 17282) in the suborder *Massarineae*. There was almost no sequence heterogeneity of the loci among the studied isolates in the group DSE-4 and DSE-8 (data not shown). Representative isolates of group DSE-7 formed a well-supported clade in the family *Lentithecia*ceae in a sister group position with *Tingoldiago graminicola*.

In the family-level phylogenetic analyses group DSE-7 also formed a well-supported distinct clade as a sister group of *Tingoldiago graminicola* (Fig. 2). The type species of the family, *Lentithecium fluviatile*, grouped with *L. calvescens* while *L. arundinaceum* formed a distant lineage with *Stagonospora macropycnidia*. Interestingly, both species isolated from bamboo, *Katumotoa bambusicola* and *Ophiosphaerella sasicola*, formed a common clade (Fig. 2).

The intra-group phylogenetic analyses of group DSE-7 (Fig. 3) resulted in well-supported distinct clades. One clade comprised the majority of isolates (23 isolates) with a moderate intra-clade heterogeneity at all loci studied (data not shown). The second and third clades formed monophyletic groups with 3 and 2 isolates, respectively. The fourth and fifth clades formed monophyletic groups with 3 and 1 isolates, respectively. The sixth clade was a basal distinct lineage consisting of a single isolate (Fig. 3). Results of the molecular phylogenetic analyses reinforce our hypothesis that these lineages represent three novel genera within the *Massarineae*.

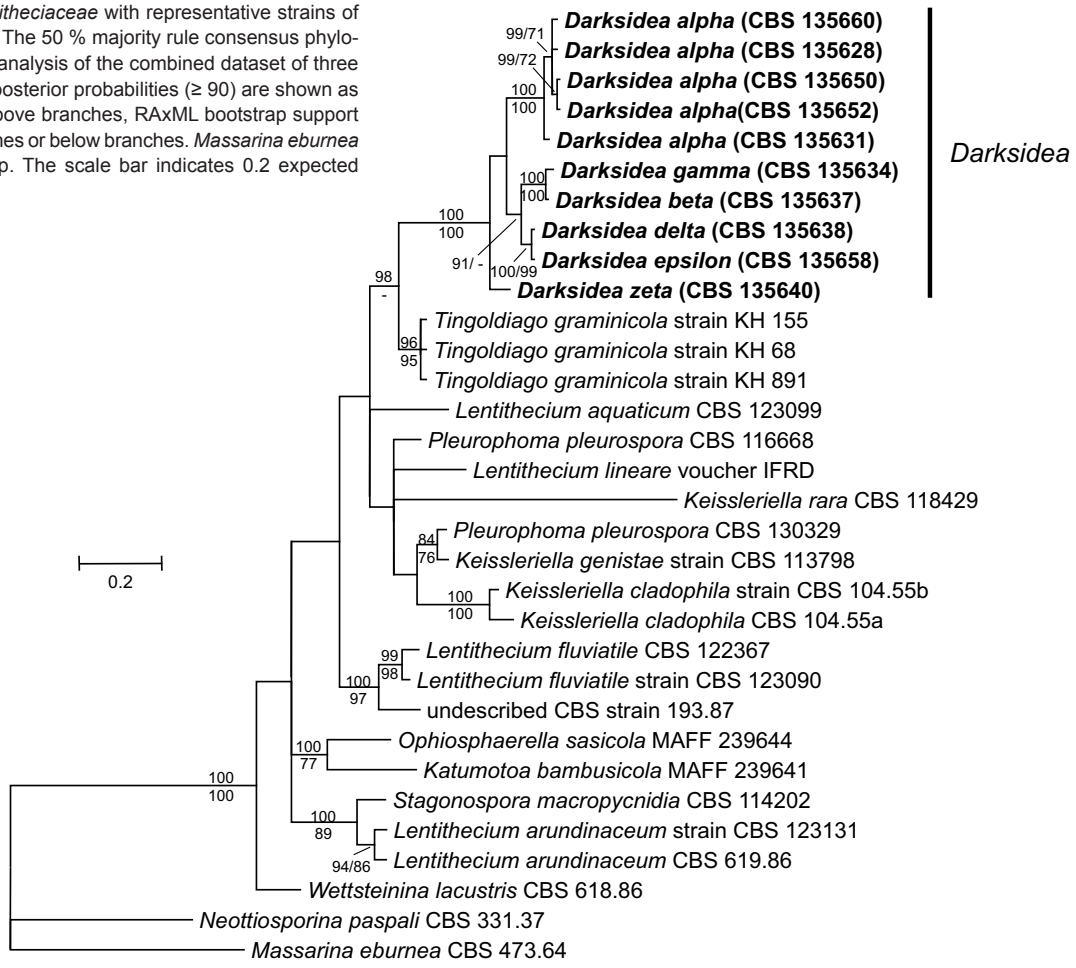


Fig. 2 Phylogenetic tree of *Lentithecia*ceae with representative strains of the new genus *Darksidea* (**bold**). The 50 % majority rule consensus phylogram inferred from the Bayesian analysis of the combined dataset of three loci (LSU, SSU, TEF). Bayesian posterior probabilities (≥ 90) are shown as percentages before slashes or above branches, RAxML bootstrap support values (≥ 70) are shown after slashes or below branches. *Massarina eburnea* (CBS 47364) served as outgroup. The scale bar indicates 0.2 expected changes per site per branch.

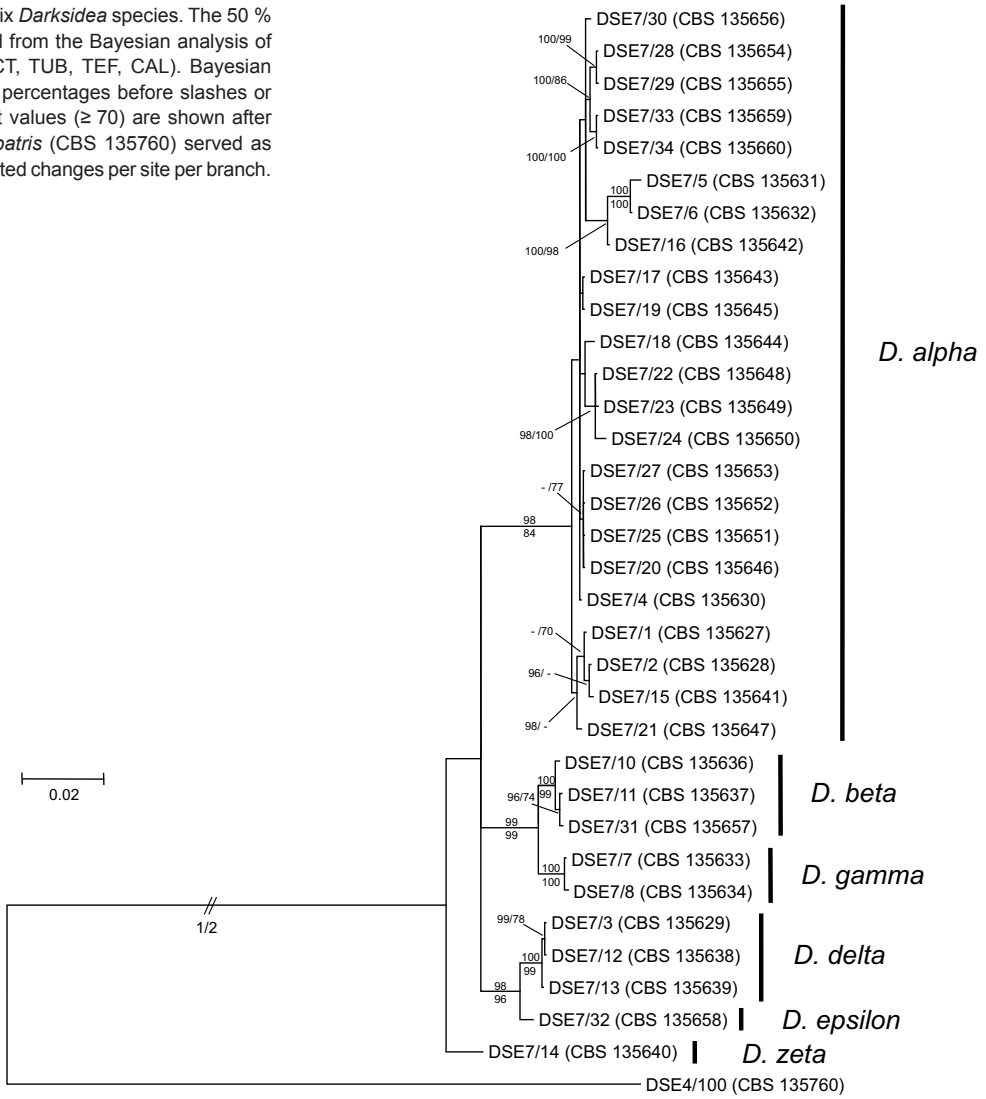


Fig. 3 Phylogenetic tree of the isolates of six *Darksidea* species. The 50 % majority rule consensus phylogram inferred from the Bayesian analysis of the combined dataset of five loci (ITS, ACT, TUB, TEF, CAL). Bayesian posterior probabilities (≥ 90) are shown as percentages before slashes or above branches, RAxML bootstrap support values (≥ 70) are shown after slashes or below branches. *Aquilomyces patris* (CBS 135760) served as outgroup. The scale bar indicates 0.02 expected changes per site per branch.

Taxonomy

Although numerous media and culture conditions were set and tested to induce sporulation, sporocarp-like structures could be observed only under one specific circumstance. These structures were observed on the surface of stinging nettle stem kept on SNA at room temperature 3–4 wk after inoculation. Sporocarps were produced by several isolates of DSE-7, but not of DSE-4 or DSE-8. Fertile isolates of the DSE-7 group formed dark brown, globose sporocarps with asci and ascospores. Although we tried to reproduce the sporulation using the same circumstances several times, subsequent attempts proved unsuccessful.

Aquilomyces patris D.G. Knapp, Kovács, J.Z. Groenew. & Crous, *gen. & sp. nov*. — MycoBank MB810756 (genus); MB810757 (species); Fig. 1, 4

Etymology. Named after the dark hyphae and colony (aquilus = dark coloured), and in honour and memory of DGK's father (= patris).

Aquilomyces patris differs from its closest phylogenetic neighbour, *Morosphaeria ramunculicola* (BCC 18405) by unique fixed alleles in the LSU and SSU loci based on alignments of the separate loci deposited in TreeBASE as study S16626: LSU positions: 129 (T), 139 (A), 145 (C), 232 (T), 335 (C), 349 (C), 355 (A), 357 (T), 369 (A), 382 (T), 422 (G), 478 (G), 550 (T), 661 (C), 662 (T), 669 (T); SSU positions: 24 (C), 70 (T), 71 (C), 72 (C), 79 (C), 80 (T), 295 (G), 1002 (C), 1094 (C), 1123 (C).

Culture characteristics — Colonies covering the Petri dish after 4 wk at 24 °C. On MMN colonies fluffy, spreading with abundant aerial mycelium, olivaceous-grey to pale-brown with smoke-grey marginal zone. Colonies on MEA smoke-grey to olivaceous-grey, somewhat flat, less fluffy with pale grey marginal zone.

Specimens examined. Hungary, Fülöpháza, N46°52' E19°25', in root of *Populus alba*, July 2005, *G.M. Kovács & A. Pintye* (holotype permanently preserved in a metabolically inactive state CBS 135661; other numbers CPC 22895 = DSE-4/099 = REF099); ibid., (CPC 22896 = CBS 135760 = DSE-4/100 = REF100); ibid., (CPC 22897 = CBS 135662 = DSE-4/101 = REF101).

Notes — Isolates belonging to the genus *Aquilomyces* are root endophytes associated with white poplar (*Populus alba*) in Fülöpháza, Hungary.

Flavomyces fulophazii D.G. Knapp, Kovács, J.Z. Groenew. & Crous, *gen. & sp. nov*. — MycoBank MB810758 (genus); MB810759 (species); Fig. 1, 4

Etymology. Named after the characteristic pale yellow-eosine pigment diffusing into the medium, and after the collection site Fülöpháza in Hungary.

Flavomyces fulophazii is a fungal root endophyte. *Flavomyces fulophazii* (CBS 135761) differs from its closest phylogenetic neighbour, *Massarina igniaria* (CBS 84596) (Fig. 1) by unique fixed alleles in the LSU and SSU loci based on alignments of the separate loci deposited in TreeBASE as study S16626: LSU

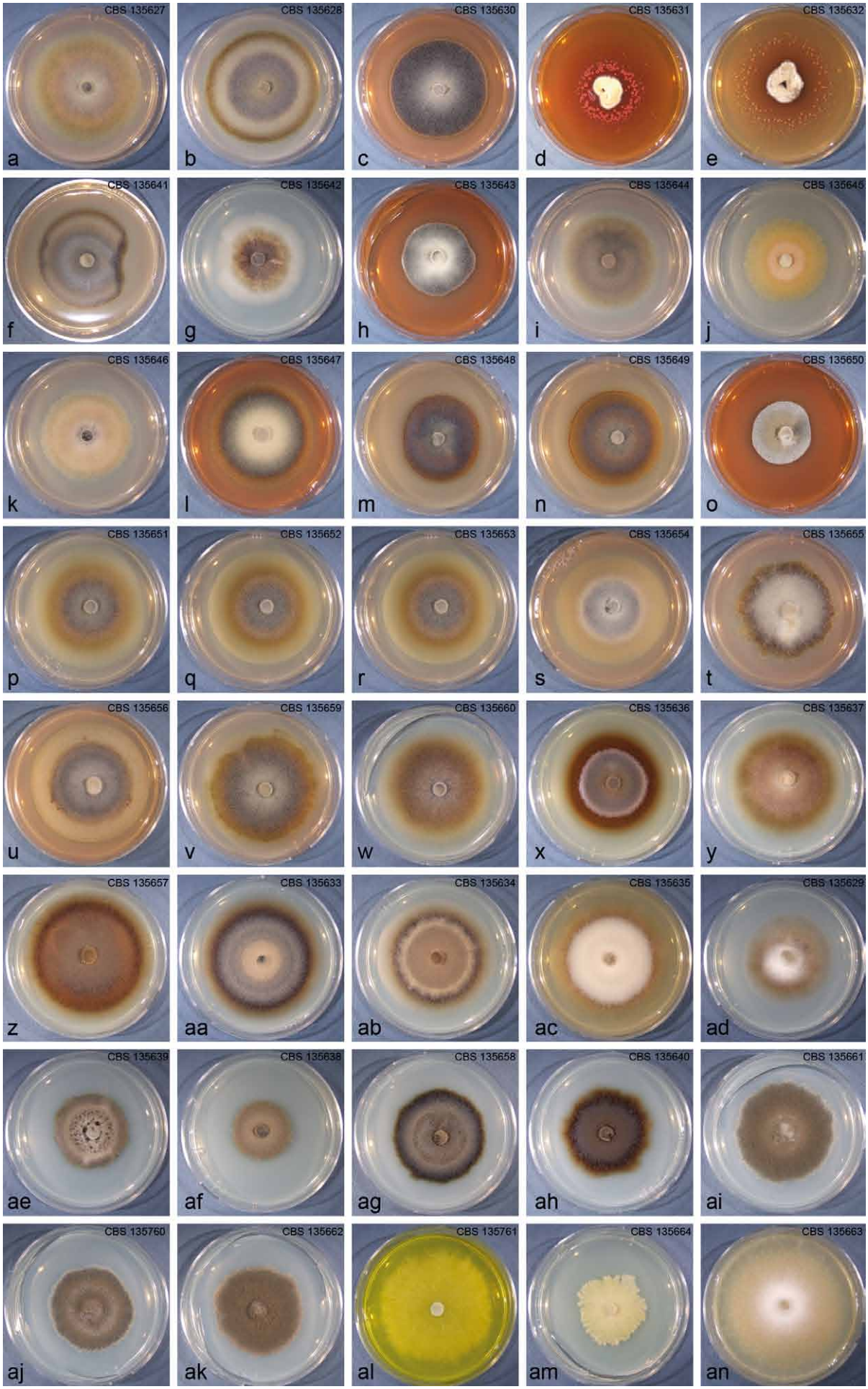


Fig. 4 Colonies of the strains of the three novel genera and *P. macrospinosa* on MMN media. a–w. *Darksidea alpha*; x–z. *Darksidea beta*; aa–ac. *Darksidea gamma*; ad–af. *Darksidea delta*; ag. *Darksidea epsilon*; ah. *Darksidea zeta*; ai–ak. *Aquilomyces patris*; al–am. *Flavomyces fulophazii*; an. *Periconia macrospinosa*.

positions: 38 (C), 48 (A), 65 (T), 74 (C), 78 (G), 84 (C), 146 (deletion), 147 (T), 174 (C), 435 (C), 456–481 (insertion), 490 (C), 624 (G), 630 (C), 658–672 (insertion), 860 (C), 862 (G), 864 (A); SSU positions: 22 (C), 26 (G), 34 (G), 39 (G), 71 (T), 294 (C), 408–834 (insertion), 890 (A), 971 (G), 1011 (C), 1028 (A), 1050 (G), 1081(A), 1082 (T), 1083 (A), 1084 (G), 1090 (G).

Culture characteristics — Colonies covering the Petri dish after 2 wk at 24 °C. On MMN colonies submerged, white to pale yellow with sparse hyaline aerial mycelium, with diffuse pale yellow-eosine pigment forming in media. After years of storage the ability to stain the medium can be lost, while colonies become flat with lobate marginal zone. On MEA colonies submerged, white with more aerial hyaline mycelium.

Specimens examined. Hungary, Fülöpháza, N46°52' E19°25', in root of *Festuca vaginata*, July 2012, *E. Zajta & D.G. Knapp* (holotype permanently preserved in a metabolically inactive state CBS 135761; other numbers CPC 22900 = DSE-8/S); Fülöpháza, N46°52' E19°25', in root of *Festuca vaginata*, July 2005, *G.M. Kovács & A. Pintye* (CPC 22899 = CBS 135644 = DSE-8/143 = REF143).

Notes — Isolates belonging to the genus *Flavomyces* are root endophytes associated with *Festuca vaginata* in semiarid grasslands near Fülöpháza, Hungary.

Darksidea D.G. Knapp, Kovács, J.Z. Groenew. & Crous, *gen. nov*. — MycoBank MB810760; Fig. 1–6

Etymology. The name of the genus alludes to the fact that these fungi belong to dark septate endophytes, the enigmatic root colonising fungal group 'on the dark side'.

Type species. *Darksidea alpha* D.G. Knapp, Kovács, J.Z. Groenew. & Crous, *sp. nov.*

Ascomata globose, brown; ostiole not seen; wall of 3–4 layers of brown *textura angularis*, surface of *textura epidermoidea*. *Pseudoparaphyses* intermingled among asci, hyaline, septate, hyphal, anastomosing. *Asci* bitunicate, clavate to ellipsoid, with weakly developed apical chamber, stipitate, 4–6-spored. *Ascospores* multiseriate in asci, hyaline, guttulate, aseptate, thick-walled, ellipsoid. The genus *Darksidea* contains root endophytic fungi associated almost exclusively with grasses in arid and semiarid areas. *Darksidea* isolates can be collected from surface-sterilised roots and can be cultured and maintained on general media. Using the primer pairs DSE7F / DSE7R (this study), a c. 300-bp-long partial ITS region of fungi belonging to the genus *Darksidea* can be amplified by PCR.

Notes — Colony morphology is variable among *Darksidea* spp. Shape, growth characteristics, colour, presence of exudates and diffusion can even vary among isolates of the same species (Fig. 6). Cultures are generally sterile. Isolates were collected from surface-sterilised roots of *Festuca vaginata*, sand feather grass (*Stipa borysthenica*), cheat grass (*Bromus tectorum*) and two dicots, namely sprawling needle sunrose (*Fumana procumbens*) and tree of heaven (*Ailanthus altissima*) (Knapp et al. 2012). Sequence data of uncultured *Darksidea* spp. were gained from below-ground tissues of indian ricegrass (*Stipa hymenoides*) (Hawkes et al. 2006), blue grama grass (*Bouteloua gracilis*) (e.g. Green et al. 2008), sand dropseed (*Sporobolus cryptandrus*) (e.g. Herrera et al. 2011b), *Stipa grandis* (Su et al. 2010), European beachgrass (*Ammophila arenaria*) (Sánchez-Márquez et al. 2008) and soil of semiarid grasslands (e.g. Porras-Alfaro et al. 2011). Sequence data of *Darksidea* spp. have been obtained from roots of dominant

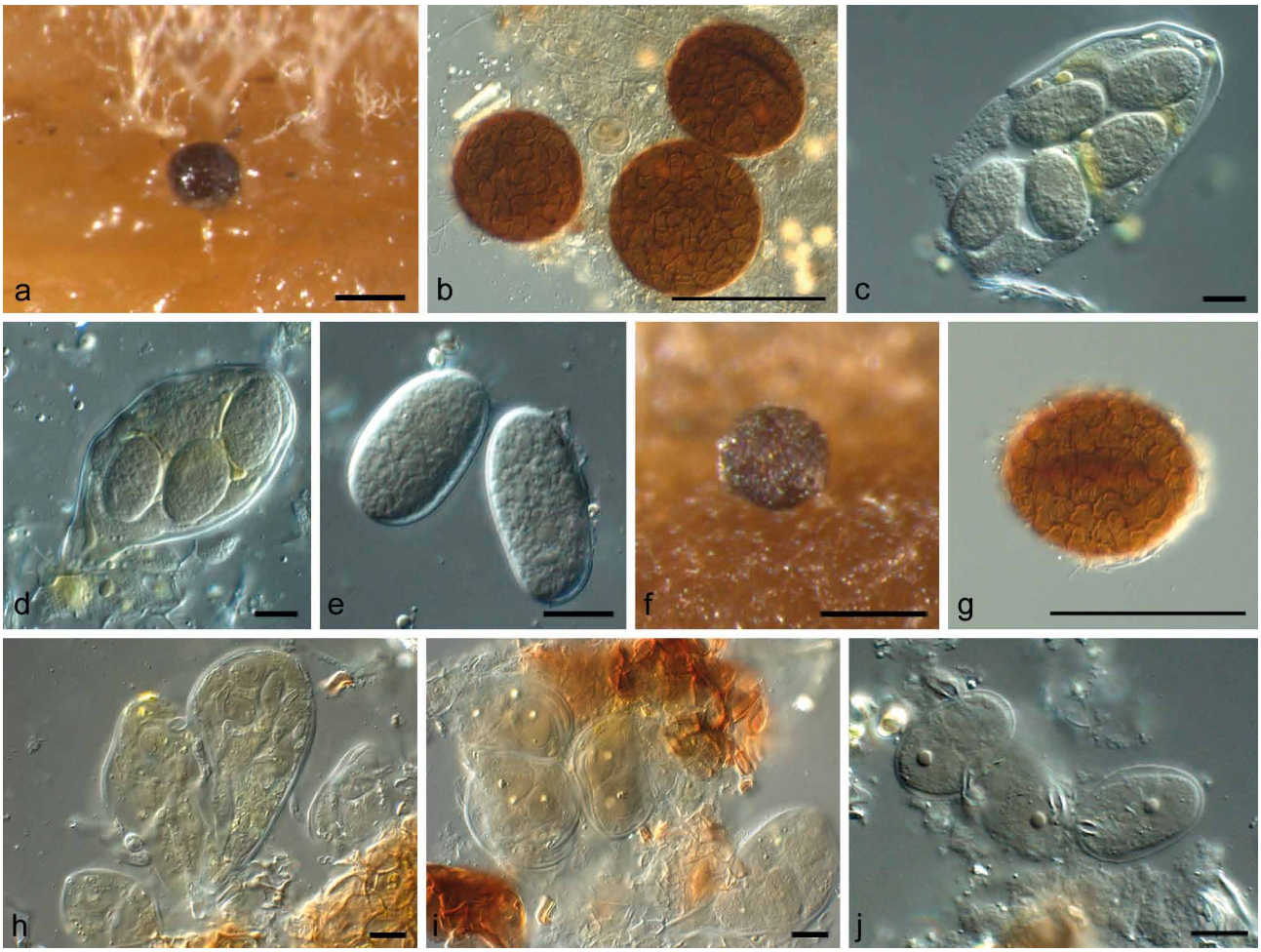


Fig. 5 Ascus and ascospores of *Darksidea* spp. — a–e. *Darksidea zeta* (CBS 135640). a, b. Ascomata; c, d. asci; e. ascospores. — f–j. *Darksidea alpha* (CBS 135650). f, g. Ascomata; h, i. asci; j. ascospores. — Scale bars: a, b = 200 µm, f, g = 180 um, all others = 10 µm.

grasses of arid-semiarid regions in at least three different continents (Porras-Alfaro et al. 2008, Su et al. 2010, Knapp et al. 2012).

Darksidea alpha D.G. Knapp, Kovács, J.Z. Groenew. & Crous, *sp. nov*. — MycoBank MB810761; Fig. 3–5

Etymology. Referring to the Greek alphabet.

Ascomata globose, brown, up to 180 µm diam; ostiole not seen; wall of 3–4 layers of *textura angularis*, 5–10 µm diam, surface of *textura epidermoidea*. *Pseudoparaphyses* intermingled among asci, hyaline, septate, hyphal, anastomosing, 2–3 µm diam. *Asci* bitunicate, clavate, with weakly developed apical chamber, stipitate, 60–80 × 40–45 µm, 4–6-spored. *Ascospores* multiseriate in asci, hyaline, guttulate, aseptate, thick-walled, ellipsoid, 18–30 × 12–17 µm. *Darksidea alpha* is a fungal root endophyte. *Darksidea alpha* (CBS 135650) differs from *Tingoldiago graminicola* (KH 68) by unique fixed alleles in the LSU and SSU loci based on alignments of the separate loci deposited in TreeBASE as study S16626: LSU positions: 30 (A); SSU positions: 442 (C), 467 (C), 555 (C), 649 (C), 675 (C), 742 (G), 743 (A), 815 (T), 816 (T).

Culture characteristics — On both MMN and MEA colonies can be white-yellow (e.g. CBS 135645 or CBS 135646), dark-grey (e.g. CBS 135630 or CBS 135643) or pale brown (e.g. CBS 135653). Colonies can be slow-growing (e.g. CBS 135631 and CBS 135632 do not reach the edge of the 5-cm Petri dish in 12 wk at 24 °C) or fast-growing (e.g. CBS 135627 and CBS 135628 covering the 5-cm Petri dish after 2 wk at 24 °C), flat with an entire edge without sparse aerial mycelium (e.g. CBS 135643 and CBS 135650), or fluffy with a submerged marginal zone (e.g. CBS 135655). The majority of strains stain the agar from pale orange-brown to deep red (e.g. CBS 135631, CBS 135643, CBS 135647 and CBS 135650). Two strains also produced red crystals in the agar (CBS 135631 and CBS 135632), while some strains secreted sparse exudate droplets (e.g. CBS 135654 and CBS 135655).

Specimens examined. Hungary, Fülöpháza, N46°52' E19°25', in root of *Festuca vaginata*, July 2012, *D.G. Knapp* & *E. Zajta* (holotype permanently preserved in a metabolically inactive state CBS 135650; other numbers CPC 22884 = DSE-7/24); ibid., (CPC 22862 = CBS 135628 = DSE-7/2); ibid., (CPC 22882 = CBS 135648 = DSE-7/22); ibid., (CPC 22883 = CBS 135649 = DSE-7/23); ibid., (CPC 22885 = CBS 135651 = DSE-7/25); ibid., (CPC 22886 = CBS 135652 = DSE-7/26); ibid., (CPC 22887 = CBS 135653 = DSE-7/27); ibid., (CPC 22888 = CBS 135654 = DSE-7/28); ibid., (CPC 22889 = CBS 135655 = DSE-7/29); ibid., (CPC 22890 = CBS 135656 = DSE-7/30); Sept. 2010, *D.G. Knapp* (CPC 22865 = CBS 135631 = DSE-7/5); ibid., (CPC 22866 = CBS 135632 = DSE-7/6); July 2005, *G.M. Kovács & A. Pintye* (CPC 22876 = CBS 135642 = DSE-7/16 = REF133); ibid., (CPC 22878 = CBS 135644 = DSE-7/18 = REF136); ibid., (CPC 22880 = CBS 135646 = DSE-7/20 = REF138); ibid., (CPC 22881 = CBS 135647 = DSE-7/21 = REF139); in root of *Stipa borysthenica*, July 2012, *D.G. Knapp* & *E. Zajta* (CPC 22864 = CBS 135630 = DSE-7/4); May 2005, *G.M. Kovács & A. Pintye* (CPC 22877 = CBS 135643 = DSE-7/17 = REF135); ibid., (CPC

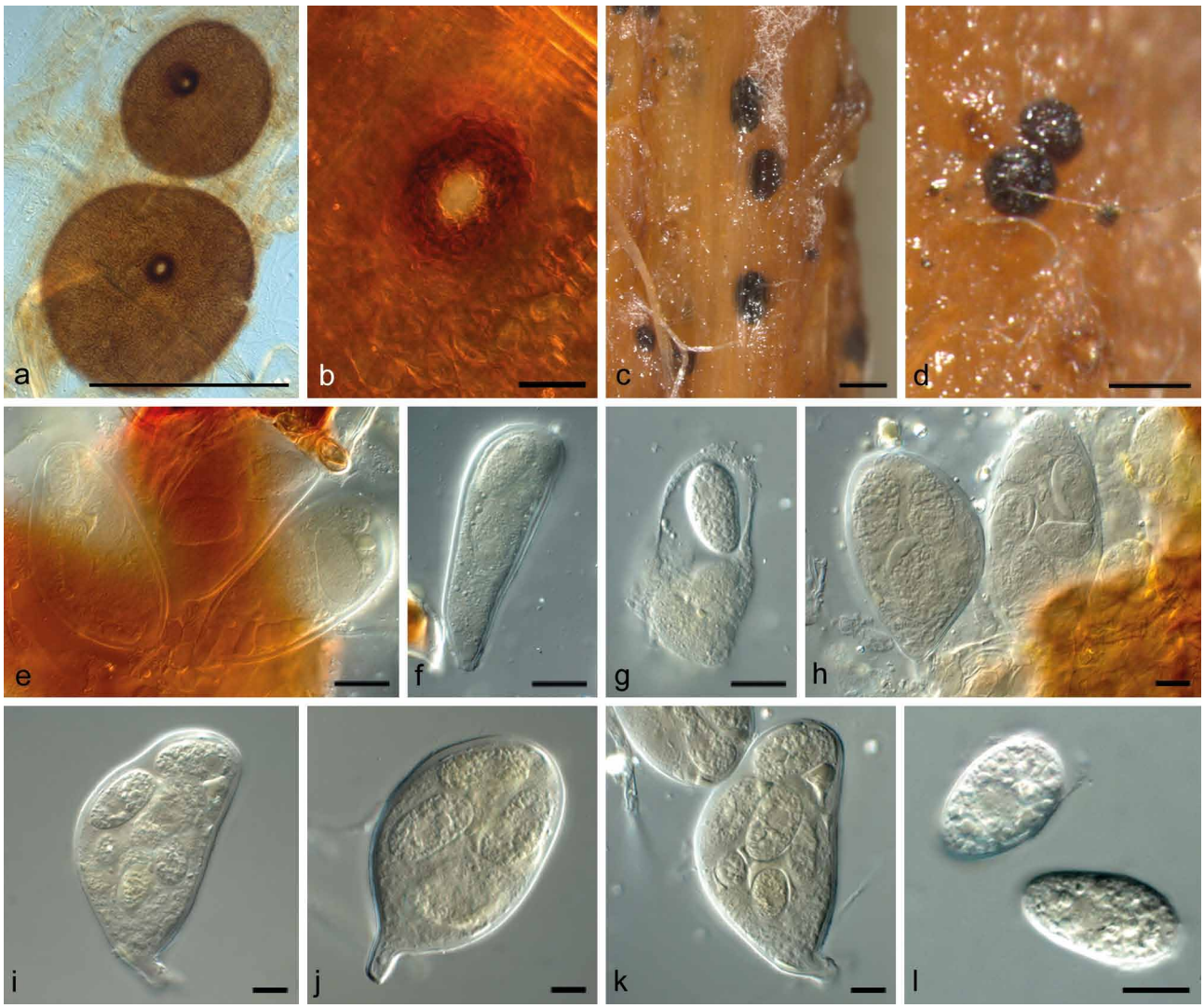


Fig. 6 Ascus and ascospores of *Darksidea* spp. — a–e. *Darksidea gamma* (CBS 135634). a–c. Conidiomata showing ostioles; d. ascomata; e–g. asci. — h–l. *Darksidea beta* (CBS 135637). h–k. Asci; l. ascospores. — Scale bars: a, c = 300 µm, d = 200 um, all others = 10 µm.

22879 = CBS 135645 = DSE-7/19 = REF137); July 2012, *D.G. Knapp* & *E. Zajta* (CPC 22893 = CBS 135659 = DSE-7/33); ibid., (CPC 22894 = CBS 135660 = DSE-7/34); in root of *Bromus tectorum*, June 2012, (CPC 22861 = CBS 135627 = DSE-7/1); Tatárszentgyörgy, N47°03.5' E19°24.3', in root of *Ailanthus altissima*, July 2008, *D.G. Knapp* (CPC 22875 = CBS 135641 = DSE-7/15 = REF132).

Notes — Sporocarp-like structures of several isolates were observed once on the surface of autoclaved stinging nettle stem kept on SNA at room temperature 3–4 wk after the inoculation. The colony morphology of *D. alpha* isolates is highly diverse.

Darksidea beta D.G. Knapp, Kovács, J.Z. Groenew. & Crous, *sp. nov.* — MycoBank MB810762; Fig. 3, 4, 6

Etymology. Referring to the Greek alphabet.

Ascomata globose, brown, up to 250 µm diam; ostiole not seen; wall of 3–4 layers of *textura angularis*, 5–10 µm diam, surface of *textura epidermoidea*. *Pseudoparaphyses* intermingled among asci, hyaline, septate, hyphal, anastomosing, 4–5 µm diam. *Asci* bitunicate, ellipsoid, with weakly developed apical chamber, stipitate, 50–90 × 35–50 µm, 4–6-spored. *Ascospores* multiseriate in asci, hyaline, guttulate, aseptate, thick-walled, ellipsoid, 23–30 × 14–19 µm. *Darksidea beta* is a fungal root endophyte. *Darksidea beta* (CBS 135637) differs from *D. alpha* (CBS 135650) by unique fixed alleles in the ITS, ACT and TEF loci based on alignments of the separate loci deposited in TreeBASE as study S16626: ITS positions: 102 (T), 263 (T), 607 (A), 614 (deletion); ACT positions: 292 (T), 400 (C), 553 (T); TEF positions: 201 (T), 414 (A).

Culture characteristics — Colonies covering the Petri dish after 2–3 wk at 24 °C. On MMN colonies are smoke-grey to pale brown with a pale brown marginal zone, and sparse aerial mycelium, and a submerged pale brown marginal zone. On MEA colonies are smoke-grey to olivaceous-grey, with grey to pale brown aerial mycelium, often with exudates.

Specimens examined. Hungary, Fülöpháza, N46°52' E19°25', in root of *Festuca vaginata*, July 2005, *G.M. Kovács & A. Pintye* (holotype permanently preserved in a metabolically inactive state CBS 135637; other numbers CPC 22871 = DSE-7/11 = REF128); ibid., (CPC 22870 = CBS 135636 = DSE-7/10 = REF127); July 2012, *D.G. Knapp & E. Zajta* (CPC 22891 = CBS 135657 = DSE-7/31).

Notes — Sporocarp-like structures of strain CBS 135637 were observed on the surface of autoclaved stinging nettle stem kept on SNA at room temperature 3–4 wk after inoculation.

Darksidea gamma D.G. Knapp, Kovács, J.Z. Groenew. & Crous, *sp. nov*. — MycoBank MB810763; Fig. 3, 4, 6

Etymology. Referring to the Greek alphabet.

Forming sterile, erumpent, subglobose, brown pycnidial-like sporocarps with a central ostiole. *Ascomata* globose, brown, up to 200 µm diam; ostiole not seen; wall of 3–4 layers of *textura angularis*, 5–10 µm diam, surface of *textura epidermoidea. Pseudoparaphyses* intermingled among asci, hyaline, septate, hyphal, anastomosing, 2–3 µm diam. *Asci* bitunicate, clavate, with weakly developed apical chamber, stipitate, 60–80 × 20–30 µm, 4–6-spored. *Ascospores* multiseriate in asci, hyaline, granular to guttulate, aseptate, thick-walled, ellipsoid, 14–25 × 9–14 µm. *Darksidea gamma* is a fungal root endophyte. *Darksidea gamma* (CBS 135634) differs from *D. alpha* (CBS 135650) by unique fixed alleles in the ITS, ACT and TEF loci based on alignments of the separate loci deposited in TreeBASE as study S16626: ITS positions: 104 (T), 224 (T), 250 (G), 251 (C), 252 (A), 262 (T), 484 (T), 498 (T), 595 (G), 601 (deletion); ACT positions: 72 (G), 134 (T), 140 (T), 161 (deletion), 164 (C), 175 (C), 616 (T); TEF positions: 8 (C), 113 (A), 150 (T), 619 (T), 688 (T).

Culture characteristics — Colonies covering the Petri dish after 2–3 wk at 24 °C. On MMN colonies smoke-grey to olivaceous-grey, with sparse aerial mycelium, and a submerged pale brown marginal zone. On MEA colonies smoke-grey to olivaceous-grey, with abundant white aerial mycelium; exudates often observed in concentric rings.

Specimens examined. Hungary, Fülöpháza, N46°52' E19°25', in root of *Festuca vaginata*, July 2005, *G.M. Kovács & A. Pintye* (holotype permanently preserved in a metabolically inactive state CBS 135634; other numbers CPC 22868 = DSE-7/8 = REF124); ibid., CPC 22867 = CBS 135633 = DSE-7/7 = REF123); ibid., CPC 22869 = CBS 135635 = DSE-7/9 = REF125.

Notes — Ascomata of CBS 135634 were observed on the surface of autoclaved stinging nettle stem kept on SNA at room temperature 3–4 wk after inoculation.

Darksidea delta D.G. Knapp, Kovács, J.Z. Groenew. & Crous, *sp. nov*. — MycoBank MB810764; Fig. 3, 4

Etymology. Referring to the Greek alphabet.

Darksidea delta is a fungal root endophyte. *Darksidea delta* (CBS 135638) differs from *D. alpha* (CBS 135650) by unique fixed alleles in the ITS, ACT, TEF and CAL loci based on alignments of the separate loci deposited in TreeBASE as study S16626: ITS positions: 238 (T), 281 (A), 465 (C); ACT positions: 200 (T), 337 (C), 547 (T); TEF positions: 38 (C), 39 (G), 40 (A), 230 (T), 404 (T); CAL positions: 142 (A), 256 (T), 633 (T).

Culture characteristics — Colonies covering the 5-cm Petri dish after 3–5 wk at 24 °C on MMN. Colonies smoke-fluffy, olivaceous-grey to pale brown, spreading with abundant aerial mycelium, exudates often observed in concentric rings. Colonies covering the dish after 6 wk at 24 °C on MEA. Colonies smoke-grey to yellow or white with an entire edge and sparse aerial mycelium, exudates generally observed.

Specimens examined. Hungary, Fülöpháza, N46°52' E19°25', in root of *Festuca vaginata*, July 2005, *G.M. Kovács & A. Pintye* (holotype permanently preserved in a metabolically inactive state CBS 135638; other numbers CPC 22872 = DSE-7/12 = REF129); June 2012, *D.G. Knapp & E. Zajta* (CPC 22863 = CBS 135629 = DSE-7/3); in root of *Fumana procumbens*, June 2008, *D.G. Knapp* (CPC 22873 = CBS 135639 = DSE-7/13 = REF130).

Notes — Sporocarp-like structures of isolate CBS 135629 were observed when colonies were inoculated onto the surface of autoclaved stinging nettle stem on SNA at room temperature for 3–4 weeks. These structures remained sterile.

Darksidea epsilon D.G. Knapp, Kovács, J.Z. Groenew. & Crous, *sp. nov*. — MycoBank MB810765; Fig. 3, 4

Etymology. Referring to the Greek alphabet.

Darksidea epsilon is a fungal root endophyte. *Darksidea epsilon* (CBS 135658) differs from *D. alpha* (CBS 135650) by unique fixed alleles in the ITS, ACT, TUB and CAL loci based on alignments of the separate loci deposited in TreeBASE as study S16626: ITS positions: 86 (T), 289 (T); ACT positions: 108 (A), 189 (T), 322 (T), 406 (G); TUB positions: 37 (T), 402 (G); CAL positions: 56–57 (deletion), 461 (A), 579 (T).

Culture characteristics — Colony covering the Petri dish after 3 wk at 24 °C on MMN. Colony olivaceous-grey to brown with pale-brown marginal zone, spreading with abundant aerial mycelium; colony secretes exudates in the middle. On MEA colonies cover the Petri dish after 6 wk at 24 °C; surface yellow to grey with sparse aerial mycelium, secreting exudates.

Specimens examined. Hungary, Fülöpháza, N46°52' E19°25', in root of *Stipa borysthenica*, July 2012, *E. Zajta & D.G. Knapp* (holotype permanently preserved in a metabolically inactive state CBS 135658; other numbers CPC 22892 = DSE-7/32).

Notes — Isolate CBS 135658 remained sterile.

Darksidea zeta D.G. Knapp, Kovács, J.Z. Groenew. & Crous, *sp. nov*. — MycoBank MB810766; Fig. 3–5

Etymology. Referring to the Greek alphabet.

Ascomata globose, brown, erumpent, up to 200 µm diam; ostiole not seen; wall of 3–4 layers of *textura angularis*, 5–10 µm diam, surface of *textura epidermoidea*. *Pseudoparaphyses* intermingled among asci, hyaline, septate, hyphal, anastomosing, 2–3 µm diam. *Asci* bitunicate, ellipsoid, with weakly developed apical chamber, stipitate, 60–80 × 40–50 µm, 4–6-spored. *Ascospores* multiseriate in asci, hyaline, guttulate, aseptate, thick-walled, ellipsoid, 19–30 × 12–15 µm. *Darksidea zeta* is a fungal root endophyte. *Darksidea zeta* (CBS 135640) differs from *D. alpha* (CBS 135650) by unique fixed alleles in the ITS, ACT, TUB, TEF and CAL loci based on alignments of the separate loci deposited in TreeBASE as study S16626: ITS positions: 173 (G), 220 (C), 246 (deletion), 256 (T), 604 (A); ACT positions: 42 (T), 94 (C), 156 (C), 161 (C), 166–168 (deletion), 173 (T), 183 (G), 185 (T), 186 (C), 250 (T), 253 (A), 525 (T), 577 (T); TUB positions: 63 (T), 81 (T), 246 (C), 276 (T), 282 (T); TEF positions: 10 (C), 153 (G), 194 (T), 202 (G), 230 (G), 269 (C), 362 (T), 409 (G), 622 (T), 835 (T), 877 (C); CAL positions: 107 (T), 167 (G), 258 (G), 307 (C), 431 (G).

Culture characteristics — Colony covering the Petri dish after 4–5 wk at 24 °C on MMN. Colony on MMN brown-grey with sparse aerial mycelium, with pale brown submerged marginal zone, colony secretes exudates in the middle. On MEA colonies cover the Petri dish after 2 wk at 24 °C; colony white to smoke-grey with moderate aerial mycelium, secreting exudates in concentric rings.

Specimens examined. Hungary, Fülöpháza, N46°52' E19°25', in root of *Festuca vaginata*, July 2005, *G.M. Kovács & A. Pintye* (holotype permanently preserved in a metabolically inactive state CBS 135640; other numbers CPC 22874 = DSE-7/14 = REF131).

Notes — Ascomata of isolate CBS 135640 were observed on the surface of autoclaved stinging nettle on SNA incubated at room temperature for 3–4 wk.

DISCUSSION

In spite of the increasing general interest in DSE, our knowledge on the diversity and distribution of these fungi is still limited. Only c. 30 DSE species have been described to date (Wang & Wilcox 1985, Jumpponen & Trappe 1998a, Knapp et al. 2012, Andrade-Linares & Franken 2013, Walsh et al. 2014). Furthermore, only a fraction of these DSE species have been well-defined and tested to determine if they really fulfil the definition of DSE.

To the best of our knowledge the *Pleosporales* includes several DSE fungi: *Rhizopycnis vagum* (Andrade-Linares et al. 2011, DSE-3 in Knapp et al. 2012), '*Phoma'* sp. (Junker et al. 2012), *Periconia macrospinosa* (Mandyam et al. 2010, Knapp et al. 2012), 'Unknown 1[d]' and 'Unknown 2[e]' isolates (sensu Jumpponen & Trappe 1998a), *Alternaria* sp. (DSE-5), *Setophoma sacchari* (DSE-6), *Embellisia* sp. (DSE-9), *Curvularia* sp. (DSE-10) and the eight new species of *Aquilomyces*, *Flavomyces* and *Darksidea* described in the present study. Although finding discriminating morphological features would have been preferred, this was not possible for all taxa, and thus we employed genealogical concordance of several loci to aid us in the description of these taxa.

The three new genera described here nested in the suborder *Massarineae* in *Pleosporales*, which comprises mostly saprobic species of terrestrial or aquatic environments (Zhang et al. 2012).

The new genus *Darksidea* belongs to the *Lentitheciaceae* (Zhang et al. 2009), although this family is still poorly resolved. The morphological features of *Darksidea* make the *Lentitheciaceae* even more diverse, suggesting that there is a lack of reliable, unique morphological features for the family (Zhang et al. 2012). *Lentitheciaceae* comprises saprobic fungi living in freshwater and wet habitats, occurring on angiosperm debris (Zhang et al. 2009). However, species belonging to *Darksidea* originated from roots of grasses and soils from arid and semiarid environments. Zhang et al. (2009) reported that the *Lentitheciaceae* was split into two subclades based on molecular phylogenetic data, with species in subclade 'V-A' occurring exclusively on monocotyledons, while species in subclade 'V-B' were associated with dicotyledonous woody substrates in freshwater environments. However, the growing number of sampled taxa changed and masked these differences (Zhang et al. 2012), and it was further supported by our findings. Sexual morphs in *Lentitheciaceae* have lenticular ascomata, trabeculate to broadly cellular pseudoparaphyses, cylindrical to clavate asci with short pedicels, uni-, tri- to multiseptate, fusiform or filiform ascospores (Zhang et al. 2009). *Tingoldiago graminicola*, the sister group of genus *Darksidea*, is a freshwater ascomycete characterised by flattened, globose, immersed to erumpent ascomata, and numerous cellular pseudoparaphyses (Hirayama et al. 2010). The ascomata, asci and pseudoparaphyses of *Darksidea* support it as pleosporalean (Zhang et al. 2009, 2012), although morphologically it is clearly distinct from the *Lentitheciaceae*, a family in which it clusters.

Aquilomyces clusters in the *Morosphaeriaceae*, representing a new basal genus among the genera *Heliascus*, *Kirschsteiniothelia*, *Morosphaeria*, *Pithomyces* and *Asteromassaria* (*A. pulchra*), albeit the latter is just 'tentatively assigned' in *Morosphaeriaceae* (Zhang et al. 2012). The *Morosphaeriaceae* was introduced by Suetrong et al. (2009) as one of the current five families of *Massarineae* (Zhang et al. 2012), and represents a well-supported clade including the genera *Morosphaeria*, *Helicascus* and *Kirschsteiniothelia* (Suetrong et al. 2009). This family includes mostly marine species with subglobose ascomata and 8-septate ascospores in thick-walled, fissitunicate asci.

Flavomyces fulophazii formed a well-supported clade with *Massarina igniaria*, *Periconia macrospinosa* and *Noosia banksiae*, but this clade did not form a monophyletic group with the other members of family *Massarinaceae* including the type species *Massarina eburnea*. *Massarina igniaria* nested as a basal lineage to the family in previous phylogenetic analyses encompassing more loci and a smaller set of related taxa (Zhang et al. 2009, 2012). After the addition of *Flavomyces fulophazii*, *Noosia banksiae* and *P. macrospinosa*, these taxa clustered separately, representing an apparently unknown family.

The genus *Darksidea* described in this study has been reported from several countries according to previous publications and GenBank entries. The first representative of *Darksidea* was detected from a root of *Stipa hymenoides* originating from a semiarid grassland in Utah, USA (GenBank AY929107, Hawkes et al. 2006). Sánchez-Márquez et al. (2008) also isolated a strain (Unidentified *Pleosporales* B, GenBank AM921730) from a rhizome of *Ammophila arenaria* in sand dunes of the northern coast of Galicia (Spain). Green et al. (2008) gained ITS sequences of *Darksidea* from roots of a dominant grass species, *Bouteloua gracilis*, from rhizosphere soil and from biological soil crusts from semiarid grassland in central New Mexico, USA. In their study on soil fungal communities in the same semiarid grassland, Porras-Alfaro et al. (2011) obtained further *Darksidea* sequences from biological soil crusts. In a previous study focusing on the root-associated fungal community of *B. gracilis*, Porras-Alfaro et al. (2008) found that the RAF community was dominated by a novel group of dark

septate fungi within the order *Pleosporales*, named 'clade A', including 'subclade B' and 'subclade C'. Analyses of those ITS sequences with those of our isolates show that subclade B unambiguously grouped into *Darksidea* and is most probably grouped with the four species described here. Khidir et al. (2010) also obtained several sequences belonging to the aforementioned 'subclade B', and hypothesised that the clade is a *Paraphaeosphaeria* species (sensu Câmara et al. 2001). This taxonomic hypothesis – later used in Herrera et al. (2010), too – cannot be supported, as *Paraphaeosphaeria* is unambiguously nested in *Montagnulaceae* (Verkley et al. 2014). Herrera et al. (2010) compared the RAF communities of *B. gracilis* along a latitudinal gradient and concluded that the most consistent and common members of the RAF community belonged to this clade. Sequences of *Darksidea* were also gained from the dung of mammalian herbivores from two distinct grasslands (Herrera et al. 2011a), and from a root of *S. cryptandrus* in a rainfall manipulation experiment study (Herrera et al. 2011b). The genus was also found in the Eurasian Steppe Belt in both the western (Knapp et al. 2012, see above) and the eastern region (Su et al. 2010). Su et al. (2010) investigated fungal endophytes of the grass *Stipa grandis* in the semiarid steppe zone of the Inner Mongolia Plateau, and one of the isolates obtained from a root of *S. grandis* (named *Pleosporales* sp. 3 (GenBank HM007086)) was conspecific with *D. alpha*. Based on this finding we assume that *Darksidea* is one of the common members of the core DSE community hypothesised to be shared by the semiarid grassland areas worldwide (Knapp et al. 2012).

Although our trials to induce sporulation were not consistent and reproducible, we could detect ascomata, and in several cases asci and ascospores. Although asexual sporulation is also rarely observed among many of the DSE fungi (Jumpponen & Trappe 1998a), it could be induced in culture (e.g. Sieber & Grünig 2006), e.g. extreme long incubation times at low temperatures (Wang & Wilcox 1985, Grünig et al. 2009). Previous studies hypothesised that the sexual stage of some DSE exists and/or existed (e.g. Grünig et al. 2004). Zaffarano et al. (2011) studied the MAT locus structure of thousands of strains of 19 PAC species from various hosts, continents and ecosystems and hypothesised that cryptic sexual reproduction regularly occurs in the PAC. Although data from population genetic studies, genome analysis and attribution of MAT genes could provide evidence of a possible sexual state (e.g. Zaffarano et al. 2011), inducing the sexual morph for DSE fungi has to date been unsuccessful. Although sterile ascocarpium-like structures with no ascospores were observed in studies investigating other DSE species in *Acephala* sp. (UAMH 6816, Currah et al. 1993), to our best knowledge, the present study is the first in which sexual morphs formed by DSE fungi were observed. This demonstrated capability for ascospore production in DSE fungi might help us to better understand the widespread and common occurrence of these root colonizing fungi.

Conclusions

DSE fungi constitute a polyphyletic form-group of fungi representing several orders of the *Pezizomycotina*. Not only are their functional contributions to ecosystems 'elusive' (Mandyam & Jumpponen 2005), but also, their taxonomic diversity is far from known. The three genera described from a semiarid sandy region in the present study illustrate that even distinct new lineages of DSE can still be identified. These well-defined and formally described DSE lineages from distantly related families can be useful in future comparative studies focusing on whether these endophytes have functional similarities, or whether the eponymous morphological characteristics are the only similarities. Since root-associated pleosporalean fungi, including *Darksidea* species, seem to be common in arid and semiarid regions of different continents (e.g. Porras-Alfaro et al. 2008, Su et al. 2010, Knapp et al. 2012), they can be used in experiments aimed at broadening our understanding the function of DSE fungi in arid and semiarid environments.

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