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ORIGINAL ARTICLE

Two pleosporalean root-colonizing fungi, *Fuscosphaeria hungarica* gen. et sp. nov. and *Delitschia chaetomioides*, from a semiarid grassland in Hungary

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Abstract
In this study, we investigated two unidentified lineages of root-colonizing fungi belonging to the order Pleosporales (Dothideomycetes), which were isolated from *Festuca vaginata* (*Poaceae*), a dominant grass species in the semiarid sandy grasslands of Hungary. For molecular phylogenetic studies, seven loci (internal transcribed spacer, partial large subunit and small subunit region of nrRNA, partial transcription elongation factor 1-α, RNA polymerase II largest subunit, RNA polymerase II second largest subunit, and ß-tubulin genes) were amplified and sequenced. Based on morphology and multilocus phylogenetic analyses, we found that one lineage belonged to *Delitschia chaetomioides* P. Karst. (*Delitschiaceae*), and the isolates of the other lineage represented a novel monotypic genus in the family *Trematosphaeriaceae* (suborder Massarineae). For this lineage, we proposed a new genus, *Fuscosphaeria,* represented by a single species, *F. hungarica*. In both lineages, only immature and degenerated sporocarps could be induced. These were sterile, black, globose, or depressed globose structures with numerous mycelioid appendages submerged in culture media or on the surface of autoclaved plant materials. Both species are first reported here as root-colonizing fungi.

Keywords Ascomycetes · Dark septate endophytes · Endophytic fungi · Root-associated fungi · Systematics · Taxonomy

Introduction

Fungal root endophytes are common non-pathogenic colonizers of various plant species, including gramineous hosts and woody species (Rodriguez et al. 2009; Sieber and Grünig 2013; Lukešová et al. 2015). This artificial group of fungi also comprises pathogens and saprobes, which, at some point in their life cycle, colonize plant tissues without causing symptoms of tissue damage (Wilson 1995); and it seems that in this group, species commonly considered as endophytes are rather pathogenic (Schlegel et al. 2016) or possess an expanded repertoire of genes linked with saprobic abilities (Knapp et al. 2018). These root colonizers are relatively frequent in arid and semiarid grasslands worldwide (Mandyam and Jumpponen 2005), where they inhabit healthy roots of different grass species (Porras-Alfaro et al. 2008; Knapp et al. 2012, 2019). They are generally ascomycetes and usually have pigmented hyphae, which is why they are commonly called dark septate endophytes (DSE) (Jumpponen and Trappe 1998; Porras-Alfaro and Bayman 2011). The root-associated fungal communities of gramineous plants in grassland ecosystems are diverse (e.g., Khidir et al. 2010; Li et al. 2018). *Festuca vaginata* Waldst. et Kit. ex Willd. is one of the dominant grass species in sandy areas, such as the semiarid open sandy grasslands of the interfluves of the Danube and Tiscia (Szabó et al. 2017), which are also inhabited by a wide spectrum of root-colonizing non-mycorrhizal fungi (Knapp et al. 2012, 2015) as well as arbuscular mycorrhizae (Endresz et al. 2013). Similar to other grasses dominating certain ecosystems across the temperate region, such as *Andropogon*, *Bouteloua*, *Festuca*, and *Stipa* species (e.g., Porras-Alfaro et al. 2008; Mandyam et al. 2010; Knapp et al. 2012, 2019) *F. vaginata* accommodates a core community of root-associated fungi, which was concluded using isolation-based techniques (Khidir et al. 2010; Knapp et al. 2012, 2019).

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The order Pleosporales is one of the most common orders in grassland ecosystems, comprising a plethora of grass root endophytes (Zhang et al. 2012; Jumpponen et al. 2017). Several common pleosporalean DSE fungi have been studied to date, including *Darksidea* species (Knapp et al. 2015, 2019) and the relatively well-studied *Periconia macrospinosa* (see Mandyam et al. 2010; Knapp et al. 2018). Pleosporales includes an increasing number of root endophytic species and genera; for example, in the last year only, several novel DSE lineages were investigated and formally described, such as *Alfoldia vorosii* (Crous et al. 2019), *Laburnicola rhizohalophila* (Yuan et al. 2019), *Kiskunsagia ubrizsyi* (Crous et al. 2019), and *Posidoniomyces atricolor* (Vohník et al. 2019). Pleosporalean root endophytes belong to various families, some within the suborders Pleosporineae and Massarineae. To date, root-colonizing fungi have not been reported in the families *Delitschiaceae* and *Trematosphaeriaceae*.

The family *Delitschiaceae* is a basal family (along with *Massariaceae*) in the order Pleosporales (Zhang et al. 2012; Hyde et al. 2013), comprising mainly terrestrial and saprobic species occurring on herbivore dung (e.g., Kruys et al. 2006; Doveri 2011), aged wood, and plants (Hyde et al. 2013) or submerged wood (Hyde and Steinke 1996; Rivera-Chávez et al. 2019); one species was identified from decaying fruits of *Nypa fruticans* (Jayasiri et al. 2019). The family is monogeneric, including only the genus *Delitschia*, with relatively few species (Jayasiri et al. 2019). Similarly to *Delitschia* species, species in the family *Trematosphaeriaceae* (suborder Massarineae) are usually saprobic fungi living on decomposed woody materials submerged in mangroves, and they were also recorded on the oil palm, which is a terrestrial plant species (Suetrong et al. 2011). In addition, pathogenic species from the family *Trematosphaeriaceae* were also reported as the causal agents of human mycetoma (Ahmed et al. 2014). This family includes three genera, *Falciformispora*, *Halomassarina*, and *Trematosphaeria* (Suetrong et al. 2011).

During our investigations of root-colonizing fungi of dominant grass species in the semiarid sandy open grasslands of Kiskunság in the Great Hungarian Plain, we isolated some fungi from healthy roots of *F. vaginata*, and found that they represented two distinct taxa. The present study aimed to identify these taxa based on multilocus molecular phylogeny and morphological comparison of the obtained isolates.

Materials and methods

Plant material sampling and fungi isolation

The roots of *Festuca vaginata* were sampled in the semiarid grassland of the Great Hungarian Plain near Fülöpháza, Hungary (N46° 52′, E19° 25′) during the spring of 2014 (for detailed description of the study site, see Kovács and Szigetvári 2002 and Knapp et al. 2012). Isolates were collected from surface-sterilized healthy roots as described in Knapp et al. (2012). We selected ten isolates representing two different lineages (DSE-87 and DSE-88 groups, with five isolates per group) (Table 1). The holotype specimen of the novel taxon was dried, and in form of a biologically inert agar culture, it was deposited in the herbarium of the Hungarian Natural History Museum, Budapest (BP) under the accession number 111139BP. Ex-type and other cultures investigated in this study were deposited in the culture collection of the Westerdijk Fungal Biodiversity Institute (CBS 147250–147251), and nomenclatural novelties and descriptions were deposited in MycoBank (www.MycoBank.org, Crous et al. 2004).

Fungal morphology and sporulation

The five isolates of each taxon were subcultured onto PDA and MEA media inoculated with fungal plugs (5 mm diameter) in Petri dishes (5 cm diameter). Growth rate and colony characteristics were recorded in cultures grown for 2 or 5 weeks at 22 °C. Morphological characteristics of the fungal structures were examined by bright-field and phase-contrast microscopy using a ZEISS AxioScope2 microscope equipped with an AxioCam ICc5 camera (Zeiss, Germany) along with the Zeiss ZEN 2011 software. Measurements and photographs were made using structures mounted in clear lactic acid. Colors were designated according to Rayner (1970).

To induce sporulation, the isolates were cultured on autoclaved pine needles and stinging nettle stems laid on water agar (WA) media in Petri dishes (9 cm diameter) at 22 °C for 3 months. The isolates were also cultured on WA media supplemented with minced vegetables (carrot, turnip, celery, and kohlrabi) at a pH of 3.5.

DNA extraction and amplification

Genomic DNA was extracted from fungal mycelia using the DNeasy Plant Mini Kit (Qiagen) following the manufacturer's instructions. Seven loci were amplified and sequenced: internal transcribed spacer (ITS), partial 18S small subunit (SSU) and partial 28S large subunit (LSU) of the nrDNA, partial beta-tubulin gene (*TUB*), translation elongation factor 1-alpha gene (*TEF*), partial RNA Polymerase II largest subunit gene (*RPB1*), and partial RNA polymerase II second largest subunit gene (*RPB2*). The following primers were used for the amplification and sequencing: for ITS, ITS1F/ITS4 (White et al. 1990; Gardes and Bruns 1993); for SSU, NS1/NS4 (White et al. 1990); for LSU, LROR/LR6 (Rehner and Samuels 1994; Vilgalys and Hester 1990); for *TUB*, Bt2a/Bt2b (Glass and Donaldson 1995); for *TEF*, EF1-983/EF1-2218R (Rehner and Buckley 2005); for *RPB1*, RPB1-A_f/RPB1-C_r (Stiller and Hall 1997; Matheny et al. 2002); and for *RPB2*, RPB2-6F/RPB2-7R (Liu et al. 1999). Additional

Table 1 Designations and GenBank accession numbers of *Delitschia chaetomioides* and *Fuscosphaeria hungarica* strains isolated in this study

Species	Strains[a]	GenBank Accession numbers[b]						
		ITS	LSU	SSU	*TEF*	*RPB1*	*RPB2*	*TUB*
Delitschia chaetomioides	DSE871 = CBS 147251	MW209042	MW209067	MW209047	MW238837	–	MW238833	MW238828
D. chaetomioides	DSE872	MW209043	MW209068	MW209048	MW238838	–	–	MW238829
D. chaetomioides	DSE873	MW209044	MW209069	MW209049	MW238839	–	MW238834	MW238830
D. chaetomioides	DSE874	MW209045	MW209070	MW209050	–	–	MW238835	MW238831
D. chaetomioides	DSE875	MW209046	MW209071	MW209051	MW238840	–	MW238836	MW238832
Fuscosphaeria hungarica	DSE881	MW209052	MW209057	MW209063	MW238841	–	–	MW238824
F. hungarica	DSE882	MW209053	MW209058	MW209064	MW238842	–	–	–
F. hungarica	DSE883 = CBS 147250 (ex-type)[c]	MW209054	MW209059	MW209065	MW238843	MW238822	–	MW238825
F. hungarica	DSE884	MW209055	MW209060	MW209066	MW238844	MW238823	–	MW238826
F. hungarica	DSE885	MW209056	MW209061	MW209062	MW238845	–	–	MW238827

[a] All the strains were isolated by Dániel G. Knapp from surface-sterilized healthy root segments of healthy-looking *Festuca vaginata* plants at semiarid grasslands of Fülöpháza, Hungary (N46° 52′ E19° 25′) in April 22, 2014

[b] *ITS* internal transcribed spacer region of nrDNA, *LSU* partial 28S large subunit of the nrDNA, *SSU* partial 18S small subunit of the nrDNA, *TUB* partial beta-tubulin gene, *TEF* translation elongation factor 1-alpha gene, *RPB1* partial RNA polymerase II largest subunit gene, *RPB2* partial RNA polymerase II second largest subunit gene

[c] Holotype material is deposited in the herbarium of the Hungarian Natural History Museum, Budapest under the barcode HNHM-MYC-009694

primers, D2for (ATAAAACGGCCGTGACTGTC) and D2rev (GAGGGGATTAAGAGATCC), were used for sequencing the ITS region of DSE-87 isolates. The *RPB1* and *TUB* and regions of some isolates were sequenced but were not used in the phylogenetic analyses (Table 1).

All PCR amplifications were performed in a final volume of 20 μL. Reaction components included 1 μL of 10 μM forward and reverse primers (Sigma-Aldrich, Germany), 2 μL of the DNA template, and 10 μL of the Dream Taq Green PCR Master Mix (Thermo Fisher Scientific, USA), or Phusion Green Hot Start II High-Fidelity PCR Master Mix (Thermo Fisher Scientific, USA) for the ITS of isolates of DSE-87 and *RPB1* of the DSE-88 group.

The cycling times and temperatures were as follows: an initial denaturation step for 10 min at 95 °C; 35 cycles at 95 °C for 45 s (for ITS and *RPB2*), 30 s (for LSU and *TEF*), or 15 s (for SSU and *RPB2*); 1 min (for SSU, LSU, and *TEF*), 45 s (for ITS), or 30 s (*RPB2*) at an annealing temperature of 55 °C (for ITS and *TEF*), 52 °C (for SSU and *RPB2*), or 48 °C (foe LSU); 3 min (for *TEF*), 90 s (SSU and LSU), or 1 min (for *RPB2* and ITS) at 72 °C; and a final extension step of 10 min (for ITS) or 7 min (for SSU, LSU, *TEF*, and *RPB2*). Additionally, *RPB1* amplifications were performed with the following parameters: 98° °C for 2 min, followed by 15 cycles of 10 s at 98 °C, 20 s at 67 °C and 13 s at 72 °C, and 30 cycles of 10 s at 98 °C, 20 s at 52 °C, and 15 s at 72 °C, with a final extension step of 2 min at 72 °C. Additionally, for the ITS of the DSE-87 group, cycling times and temperatures were as follows: 98 °C for 2 min, followed by 36 cycles of 5 s at 98 °C, 5 s at 60 °C, and 15 s at 72 °C, and a final extension step of 5 min at 72 °C. The PCR products were separated on 1.5% agarose gel containing GelRed (Biotium Inc., CA, USA) in 0.5 × TBE buffer and visualized under UV light.

The sequences were compiled from electropherograms using the Pregap4 and Gap4 software packages (Staden et al. 2000) and deposited in GenBank (Table 1). The obtained sequences were compared with the accessions in the National Center for Biotechnology Information database (NCBI, http://www.ncbi.nlm.nih.gov/Blast.cgi) using the BLAST search (http://blast.ncbi.nlm.nih.gov/Blast.cgi) (Altschul et al. 1990).

Phylogenetic analyses

We combined and aligned our sequences with those from representative taxa in GenBank using the online version of MAFFT 7 (Katoh and Standley 2013) and the E-INS-i method. The alignments were examined and edited using MEGA 7 (Kumar et al. 2016). In the case of the DSE-87 group, a family level dataset was used to obtain information about the phylogenetic position of our sequences among all sequences and taxa of *Delitschiaceae* and representative sequences from *Massariaceae* and other related families (Supplementary Table 1). In the phylogenetic analysis, *Hysterium angustatum* (CBS 123334) and *Psiloglonium araucanum* (CBS 112412) served as multiple outgroups. In this dataset, for multilocus analysis, we used ITS, LSU, SSU, *TEF*, and *RPB2* as well as the indels coded from the ITS, LSU, and SSU regions (Nagy et al. 2012) using a simple indel coding algorithm (Simmons et al. 2001; Young and Healy 2003) with the program FASTGAP (Borchsenius 2009). Therefore, in the first dataset, eight partitions were used. The second dataset contained sequences of DSE-88 group and taxa representing lineages of the pleosporalean suborder Massarineae (Supplementary Table 2), in which they grouped. In this analysis, ITS, LSU, SSU, and *TEF* were used as four partitions and *Leptosphaeria doliolum* var. *doliolum* (CBS 505.75) served as outgroup. Bayesian inference (BI) analyses were performed with MRBAYES 3.1.2 (Ronquist and Huelsenbeck 2003) using a GTR + G substitution model for the nucleotide partitions and the two-parameter Markov (Mk2 Lewis) model for the indel partition. Four Markov chains were run for 10,000,000 generations sampling every 1000 generations with a burn-in value set at 4000 sampled trees. Maximum likelihood (ML) phylogenetic analysis was carried out with the RAXMLGUI 1.3 implementation (Silvestro and Michalak 2012; Stamatakis 2014). A GTR + G nucleotide substitution model was used for nucleotide partitions with ML estimation of base frequencies, and the indel data were treated as binary data. ML bootstrap (BS) analysis with 1000 replicates was used to test the support of the branches. Phylogenetic trees were visualized and edited in MEGA 7 (Kumar et al. 2016) and deposited in TreeBASE (www.treebase.org) as study S26261.

Results

Morphology

The colonies of DSE-87 isolates on MEA were brownish grey, fluffy with an abundant aerial mycelium, and with pale brown or white marginal zone. PDA colonies were olivaceous-grey or greenish white, fluffy with a sparse aerial mycelium, and with brownish white marginal zone. Terminal and intercalary chlamydospores were formed (Fig. 1), and sporocarp-like structures could be produced. These were sterile, black, and globose morphs with numerous mycelioid appendages, which were observed when the isolates were cultured on the WA media supplemented with minced vegetables or pine needles and kept at 20 °C 3 months after inoculation (Fig. 1). Discoloration (color change from orange to white) of the WA media with minced vegetables was noticed.

The colonies of DSE-88 isolates on the three different media were yellowish to dark grey, flat, and they changed the agar color from orange or light brown to dark brown (Fig. 2). In addition to chlamydospores (Fig. 2), sterile, depressed, and globose sporocarp-like structures were observed on the

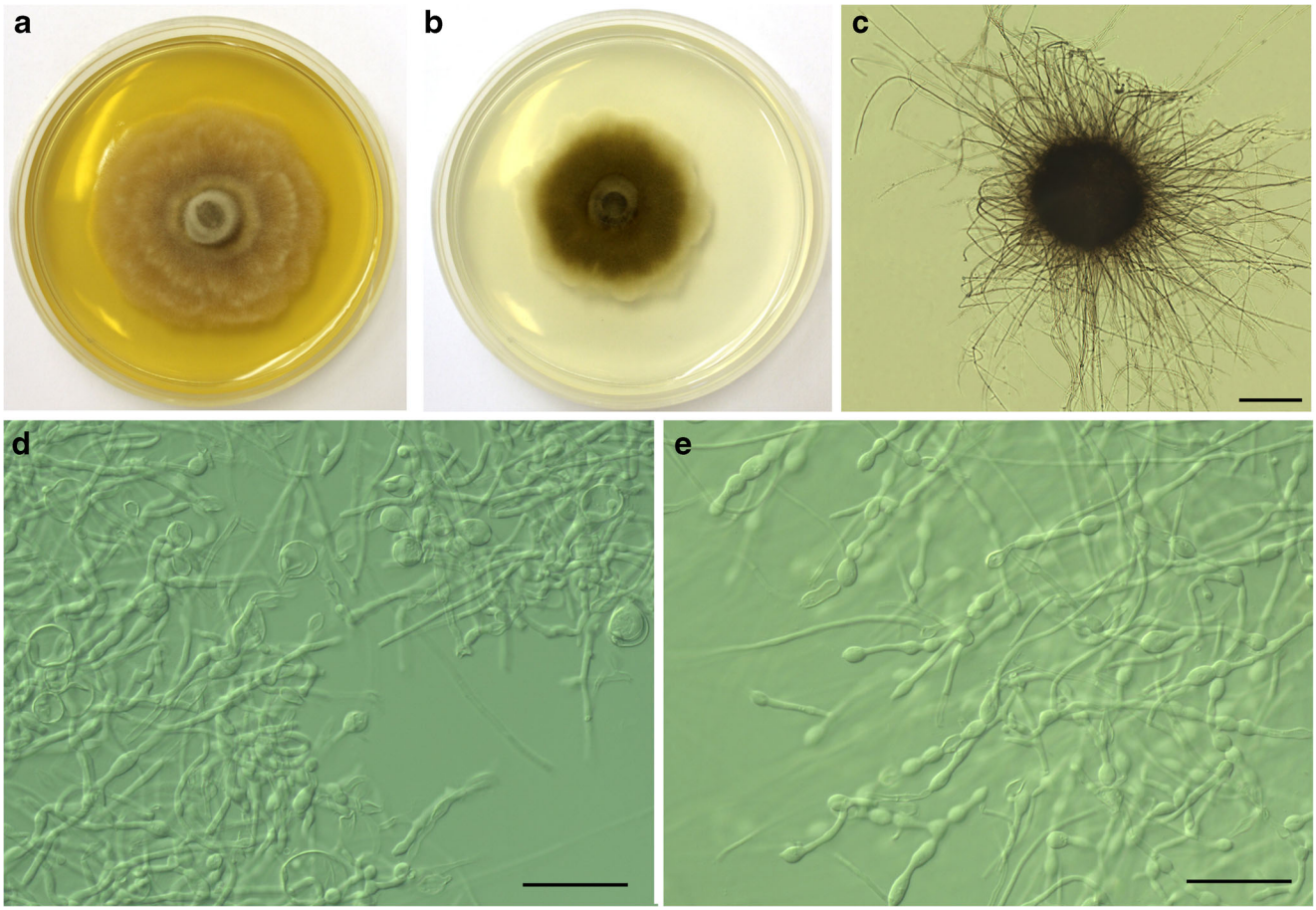


Fig. 1 *Delitschia chaetomioides* (CBS 147251). **a** Colony on MEA. **b** Colony on PDA. **c** Sterile sporocarp-like structure produced submerged in WA media supplemented with minced vegetables. **d** Terminal chlamydospores produced on MEA. **e** Intercalary chlamydospores produced on MEA. Scale bars: 50 μm

surface of autoclaved stinging nettles and on isolates cultured on the WA media supplemented with minced vegetables or pine needles (for detailed colony description, see the taxonomy section).

Neither mature sexual or asexual morphs nor ascospore or conidium production by the strains was observed in any of the media or conditions tested, or in the laboratory where the strains were maintained and kept for years before the present study.

Molecular phylogeny

The targeted DNA sequences of the isolates could be used for the phylogenetic analyses (ITS, LSU, SSU, and *TEF* for both groups, and *RPB2* for the DSE-87 group), excluding the *TEF* region of strain DSE874 and *RPB2* region of strain DSE872, which failed to be amplified (Table 1). Sequencing of the ITS region of the DSE-87 isolates was problematic because amplification of this region resulted in an unusually large (~ 2200 bp) amplicon/product. Thus, for sequencing the whole ITS region, two additional primers (D2for and D2rev) were needed.

Based on the BLAST search of the NCBI's GenBank nucleotide database, the closest ITS hits of the DSE-87 isolates showed no unambiguous relations to a certain species; only weak similarities with sequences of certain pleosporalean species were observed. Using the LSU, SSU, and *TEF* sequences, relatively clear similarities to a *Delitschia* species were found (Supplementary Table 3). Results of BLAST analyses of different DSE-88 isolate sequences showed no very similar hits (Supplementary Table 4).

For the DSE-87 group, the combined dataset consisted of 32 taxa and 7965 characters, and the group was positioned together with the strains of *Delitschia chaetomioides* P. Karst. within the family *Delitschiaceae* (Fig. 3). The five isolates formed a well-supported (PP = 1, ML BS = 99) distinct clade besides four *D. chaetomioides* strains. These nine strains together formed strongly supported (PP = 1, ML BS = 90) clades with strong internal clades, showing the unambiguous position of the isolates presented here as *D. chaetomioides* (Fig. 3).

For the DSE-88 group, the analyses of the combined dataset consisting of 93 taxa and 5403 characters showed that the group supported an independent monophyletic clade in

Trematosphaeriaceae (Massarineae) as a basal lineage (Fig. 4). The families *Trematosphaeriaceae* and *Morosphaeriaceae* together formed a monophyletic group, and the DSE-88 clade was a sister group (PP = 1, ML BS = 83) with species from the genera *Halomassarina*, *Falciformispora*, and *Trematosphaeria*, as well as with the species from the three known genera of *Trematosphaeriaceae* (Fig. 4). The results of molecular phylogenetic analyses indicated that the DSE-88 isolates collected in the semiarid grasslands represented a novel monospecific genus.

Taxonomy

Fuscosphaeria hungarica *D.G. Knapp & Pintye*, gen. et sp. nov. (Figs. 2, 4)

MycoBank: MB 835597, MB 835598

Typification: HUNGARY. KISKUNSÁG: semiarid sandy open grassland near Fülöpháza, N46° 52′ E19° 25′, in root of *Festuca vaginata*, 22 Apr 2014, *D.G. Knapp DSE883* (*CBS 147250*) (**holotype** 111139BP). GenBank: ITS = MW209054; LSU = MW209059; SSU = MW209065; *TEF* = MW238843; *RPB1* = MW238822; *TUB* = MW238825.

Etymology: *Fusco* (from the Latin word *fuscus*, meaning dark, swarthy) + *sphaeria* (from the Greek word *sphairion*, diminutive of *sphaira*, meaning ball, sphere, referring to its pigmented ascomata-like structures). The species was named "hungarica" because its occurrence solely in one semiarid Hungarian grassland.

Fuscosphaeria hungarica differs from one of its closest phylogenetic neighbors *Trematosphaeria pertusa* CBS 122368, the ex-type strain of the type species of *Trematosphaeria* (*Treamatosphaeriaceae*), by its unique fixed alleles in the LSU, SSU, and *TEF* loci, which was found based on the alignments of separate loci deposited in TreeBASE as study S26261: LSU positions: 54 (T), 61 (C), 62 (C), 63 (G), 64 (T), 67–70 (deletion), 172 (A), 181 (T), 197 (T), 204 (C), 216 (A), 235 (T), 237 (T), 337 (G), 383 (T), 386 (C), 387 (T), 389 (C), 391 (T), 392 (A), 401 (T), 408 (deletion), 412 (T), 414–415 (deletion), 429 (C), 430 (G), 446 (T), 449 (G), 468 (C), 474 (G), 476 (C), 479 (deletion), 481 (T),

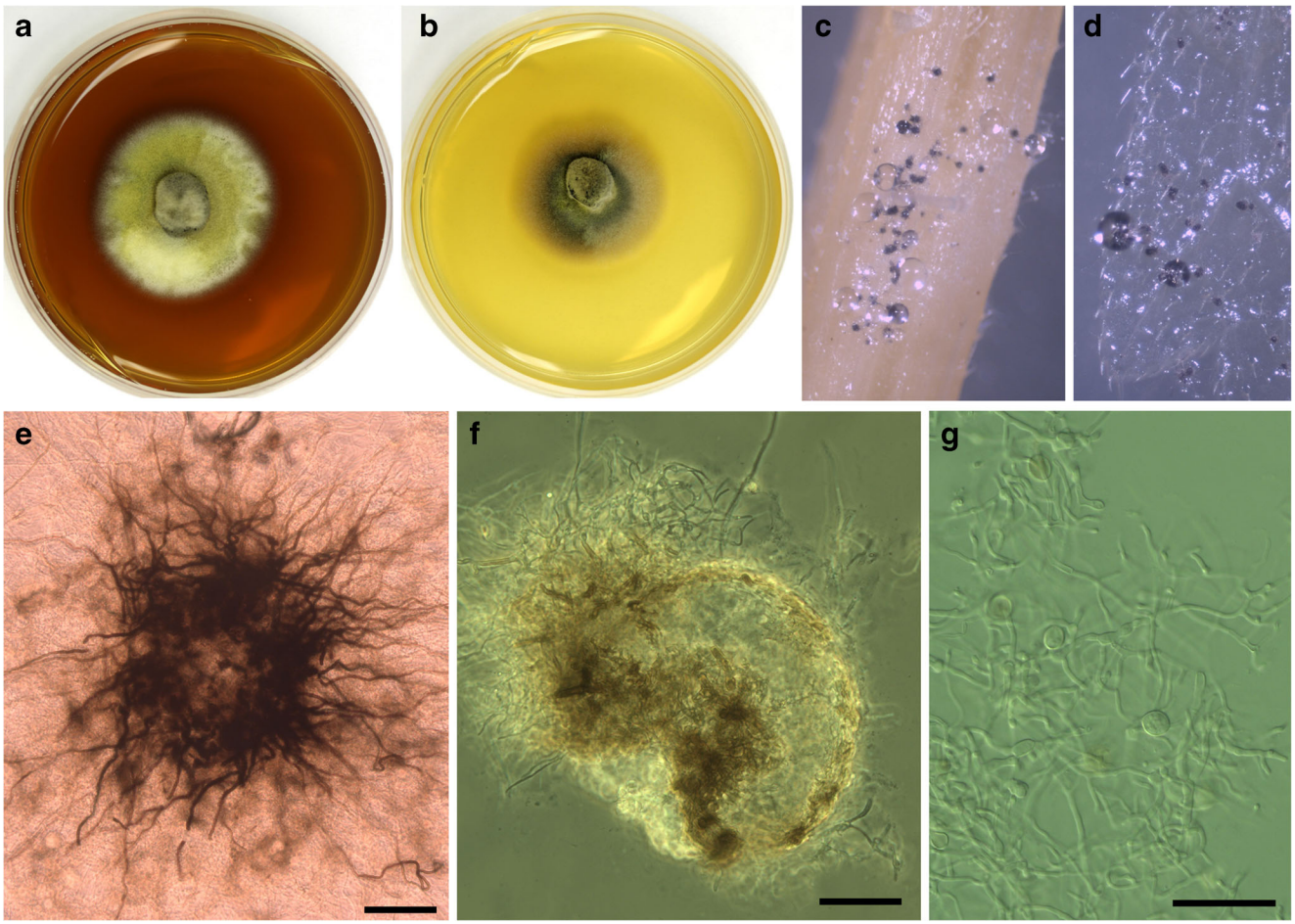


Fig. 2 *Fuscosphaeria hungarica* (ex-holotype, CBS 147250). **a** Colony on MEA. **b** Colony on PDA. **c**, **d** Sporocarp-like structure produced on the surface of stinging nettle. **e** Developing sporocarp-like structure. **f** Cross section of a sporocarp-like structure formed submerged in WA media supplemented with pine needles. **g** Terminal chlamydospores produced on PDA. Scale bars: 50 μm

484 (T), 486 (G), 497 (A), 537 (C), 545 (G), 547 (T), 553 (C), 561 (deletion), 564 (T), 571 (A), 578 (C), 582 (C), 663 (A), 669 (A), 674 (A), 690 (G), 708 (T), 709 (C), 710–720 (deletion), 724 (T); SSU positions: 116 (C), 120 (A), 128 (G), 133 (G), 159 (G), 160 (C), 160 (C), 162 (C), 164 (T), 165 (T), 166 (C), 167 (G), 168 (G), 169 (G), 187 (G), 198 (G), 206 (T), 263 (A), 273 (T), 293 (G), 296 (G), 313 (G), 317 (C), 324 (G), 335 (A), 343 (C), 378 (C), 383 (C), 395 (G), 398 (G), 506 (G), 507 (A), 508 (C), 509 (A), 510 (T), 511 (T), 512 (C), 513 (A), 515 (C), 517 (C), 523 (A), 524 (T), 539 (A), 540 (G), 545 (T), 553 (C), 554 (C), 555 (T), 556 (C), 557 (G), 558 (C), 560 (A), 562 (C), 563 (G), 564 (C), 566 (G), 568 (G), 572 (T), 573 (T), 575 (C), 583 (A), 597 (C), 598 (T), 601 (G), 623 (A), 641 (G), 642 (A), 653 (A), 654 (T), 655 (C), 656 (G), 660 (A), 662 (G), 663 (A), 664 (T), 673 (A), 675 (T), 689 (G), 691 (C), 692 (A), 693 (C), 705 (T), 711 (A), 728 (G), 729 (T), 740 (C), 748 (C), 779 (A), 780 (T), 793 (C), 801 (A), 817 (C), 831 (G), 834 (C), 839 (G), 841 (deletion), 912 (deletion), 918 (deletion), 920 (T), 923 (T), 924 (T), 939 (G), 941 (G), 990 (G), 991 (G), 992 (A), 993 (G), 994 (T), 995 (C), 996 (G), 997 (C), 998 (G), 999 (C), 1000 (T), 1001 (T), 1002 (C), 1003 (G), 1004 (C), 1005 (A), 1009 (G), 1012 (C), 1023 (G), 1024 (A), 1025 (A), 1034 (T), 1035 (T), 1037 (T), 1038 (C), 1040 (C), 1043 (G), 1044–1045 (deletion), 1051 (C), 1052 (C), 1055 (A), 1056 (A), 1057 (A), 1059 (A), 1196 (T), 1197 (C), 1222 (deletion), 1223 (G), 1316 (G), 1326 (T), 1329 (G), 1332 (C), 1333 (T), 1388 (A), 1424 (T), 1425 (T), 1454 (T); TEF positions: 24 (C), 30 (G), 37 (C), 55 (T), 91 (C), 94 (C), 115 (G), 116 (A), 118 (G), 127 (C), 133 (T), 152 (A), 169 (G), 172 (C), 174 (T), 176 (C), 178 (C), 184 (C), 190 (A), 196 (G), 208 (T), 217 (T), 220 (A), 226 (C), 241 (A), 250 (C), 256 (C), 263 (A), 283 (G), 284 (C), 289 (G), 290 (A), 331 (G), 334 (C), 338 (T), 339 (C), 341 (T), 343 (T), 352 (T), 355 (G), 358 (C), 361 (A), 364 (C), 367 (T), 372 (G), 386 (A), 394 (C), 397 (T), 409 (C), 415 (A), 418 (C), 421 (T), 439 (C), 448 (C), 454 (G), 483 (C), 491 (T), 493 (C), 511 (C), 517 (T), 538 (A), 544 (C), 562 (G), 569 (A), 570 (C), 571 (T), 572 (G), 578 (C), 580 (C), 589 (T), 607 (T), 619 (C), 634 (T), 637 (C), 643 (T), 649 (T), 652 (T), 655 (T), 673 (T), 685 (C), 688 (A), 712 (T), 715 (C), 733 (G), 745 (T), 769 (T), 778 (T), 781 (T), 793 (T), 802 (G), 832 (C), 835 (G), 865 (C), 868 (T), 895 (C), 898 (A), 916 (C), 922 (C).

Culture characteristics: Colonies on MEA are yellowish grey and flat with sparse aerial mycelium. Strains stain the agar dark brown. On PDA, colonies are dark grey and flat with a pale brown marginal zone, and they stain the agar light brown. Chlamydospores were formed.

Additional material examined: HUNGARY. KISKUNSÁG: semiarid sandy open grassland near

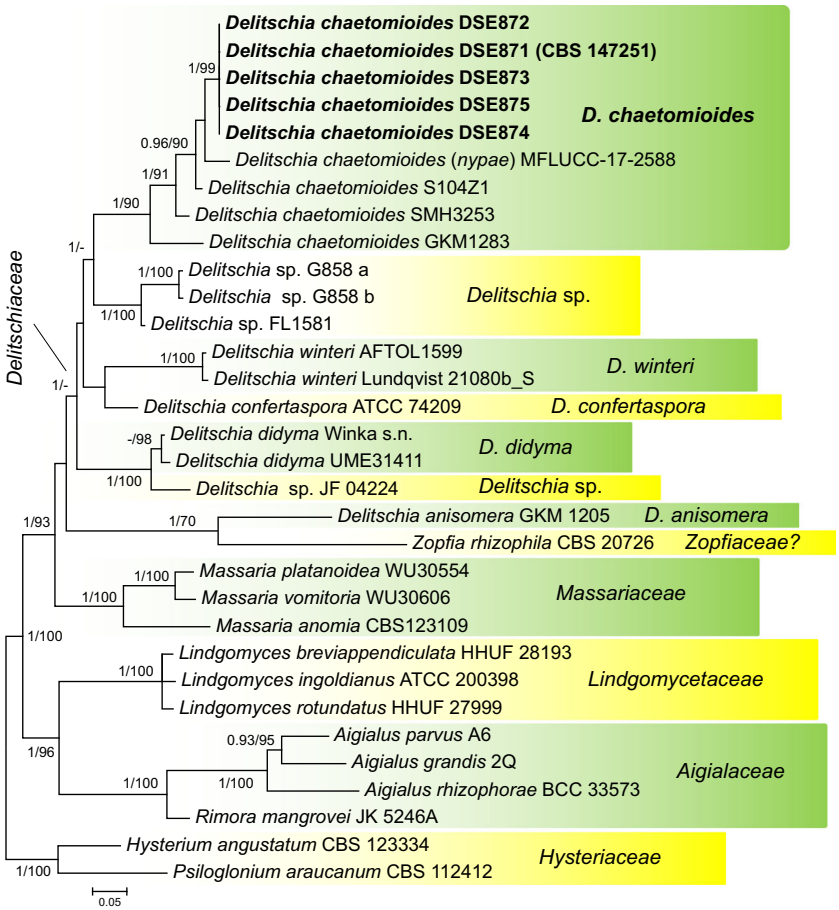
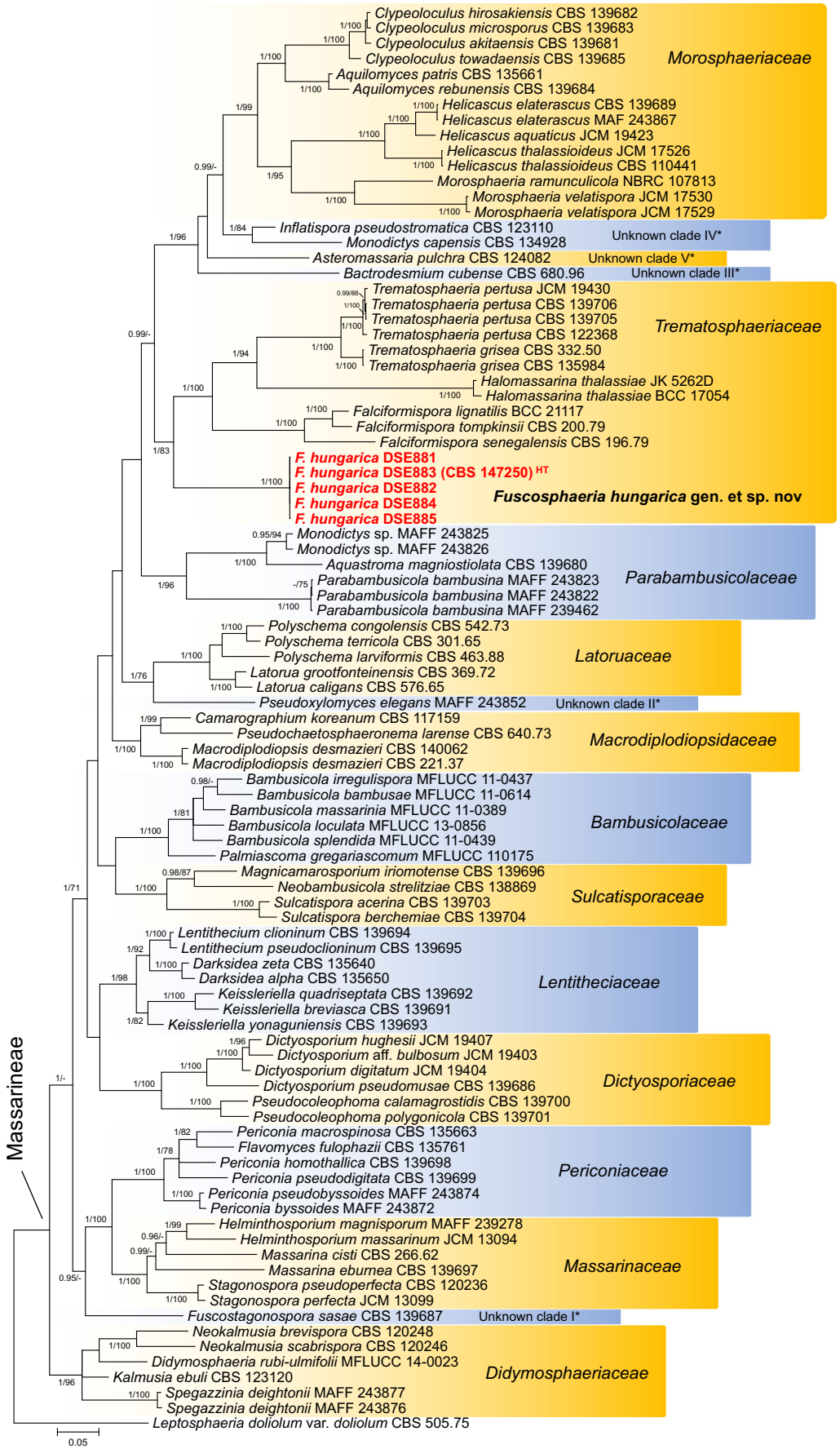


Fig. 3 Phylogenetic tree of *Delitschia* species and related representative taxa from basal groups of Pleosporales. Strains presented in this study are shown in bold. The 50% majority rule consensus phylogram inferred from the Bayesian analysis of the combined dataset of five loci (ITS, LSU, SSU, *TEF*, *RPB2*) and coded indels from ITS, LSU, and SSU as three additional partitions. Bayesian posterior probabilities (≥ 90) are shown before slashes, ML bootstrap support values (≥ 70) are shown after slashes. *Hysterium angustatum* (CBS 123334) and *Psiloglonium araucanum* (CBS 112412) served as multiple outgroups. Highlighted sections indicate affiliations to species or families. The scale bar indicates 0.5 expected changes per site per branch


Clypeoloculus hirosakiensis CBS 139682
Clypeoloculus microsporus CBS 139683
Clypeoloculus akitaensis CBS 139681
Clypeoloculus towadaensis CBS 139685
Morosphaeriaceae
Aquilomyces patris CBS 135661
Aquilomyces rebunensis CBS 139684
Helicascus elaterascus CBS 139689
Helicascus elaterascus MAF 243867
Helicascus aquaticus JCM 19423
Helicascus thalassioideus JCM 17526
Helicascus thalassioideus CBS 110441
Morosphaeria ramunculicola NBRC 107813
Morosphaeria velatispora JCM 17530
Morosphaeria velatispora JCM 17529
Inflatispora pseudostromatica CBS 123110
Monodictys capensis CBS 134928
Unknown clade IV*
Asteromassaria pulchra CBS 124082
Unknown clade V*
Bactrodesmium cubense CBS 680.96
Unknown clade III*
Trematosphaeria pertusa JCM 19430
Trematosphaeria pertusa CBS 139706
Trematosphaeria pertusa CBS 139705
Trematosphaeria pertusa CBS 122368
Trematosphaeriaceae
Trematosphaeria grisea CBS 332.50
Trematosphaeria grisea CBS 135984
Halomassarina thalassiae JK 5262D
Halomassarina thalassiae BCC 17054
Falciformispora lignatilis BCC 21117
Falciformispora tompkinsii CBS 200.79
Falciformispora senegalensis CBS 196.79
F. hungarica DSE881
F. hungarica DSE883 (CBS 147250) HT
F. hungarica DSE882
F. hungarica DSE884
F. hungarica DSE885
Fuscosphaeria hungarica gen. et sp. nov
Monodictys sp. MAFF 243825
Monodictys sp. MAFF 243826
Aquastroma magniostiolata CBS 139680
Parabambusicola bambusina MAFF 243823
Parabambusicola bambusina MAFF 243822
Parabambusicola bambusina MAFF 239462
Parabambusicolaceae
Polyschema congolensis CBS 542.73
Polyschema terricola CBS 301.65
Polyschema larviformis CBS 463.88
Latorua grootfonteinensis CBS 369.72
Latorua caligans CBS 576.65
Latoruaceae
Pseudoxylomyces elegans MAFF 243852
Unknown clade II*
Camarographium koreanum CBS 117159
Pseudochaetosphaeronema larense CBS 640.73
Macrodiplodiopsis desmazieri CBS 140062
Macrodiplodiopsis desmazieri CBS 221.37
Macrodiplodiopsidaceae
Bambusicola irregulispora MFLUCC 11-0437
Bambusicola bambusae MFLUCC 11-0614
Bambusicola massarinia MFLUCC 11-0389
Bambusicola loculata MFLUCC 13-0856
Bambusicola splendida MFLUCC 11-0439
Palmiascoma gregariascomum MFLUCC 110175
Bambusicolaceae
Magnicamarosporium iriomotense CBS 139696
Neobambusicola strelitziae CBS 138869
Sulcatispora acerina CBS 139703
Sulcatispora berchemiae CBS 139704
Sulcatisporaceae
Lentithecium clioninum CBS 139694
Lentithecium pseudoclioninum CBS 139695
Darksidea zeta CBS 135640
Darksidea alpha CBS 135650
Keissleriella quadriseptata CBS 139692
Keissleriella breviasca CBS 139691
Keissleriella yonaguniensis CBS 139693
Lentitheciaceae
Dictyosporium hughesii JCM 19407
Dictyosporium aff. bulbosum JCM 19403
Dictyosporium digitatum JCM 19404
Dictyosporium pseudomusae CBS 139686
Pseudocoleophoma calamagrostidis CBS 139700
Pseudocoleophoma polygonicola CBS 139701
Dictyosporiaceae
Periconia macrospinosa CBS 135663
Flavomyces fulophazii CBS 135761
Periconia homothallica CBS 139698
Periconia pseudodigitata CBS 139699
Periconia pseudobyssoides MAFF 243874
Periconia byssoides MAFF 243872
Periconiaceae
Helminthosporium magnisporum MAFF 239278
Helminthosporium massarinum JCM 13094
Massarina cisti CBS 266.62
Massarina eburnea CBS 139697
Stagonospora pseudoperfecta CBS 120236
Stagonospora perfecta JCM 13099
Massarinaceae
Fuscostagonospora sasae CBS 139687
Unknown clade I*
Neokalmusia brevispora CBS 120248
Neokalmusia scabrispora CBS 120246
Didymosphaeria rubi-ulmifolii MFLUCC 14-0023
Kalmusia ebuli CBS 123120
Spegazzinia deightonii MAFF 243877
Spegazzinia deightonii MAFF 243876
Didymosphaeriaceae
Leptosphaeria doliolum var. doliolum CBS 505.75
Massarineae
0.05

◀ **Fig. 4** Phylogenetic tree of *Fuscosphaeria hungarica* isolates and representative species of families in the suborder Massarineae (Pleosporales). Strains presented in this study are shown in bold. The 50% majority rule consensus phylogram inferred from the Bayesian analysis of the combined dataset of four loci (ITS, LSU, SSU, *TEF*). Bayesian posterior probabilities (≥90) are shown before slashes, ML bootstrap support values (≥70) are shown after slashes. *Leptosphaeria doliolum* var. *doliolum* (CBS 505.75) served as outgroup. Highlighted sections indicate affiliations to families and asterisks indicate unknown clades sensu Tanaka et al. (2015). The scale bar indicates 0.5 expected changes per site per branch

Fülöpháza, N46° 52′ E19° 25′, on the roots of *Festuca vaginata*, 22 Apr 2014, *D.G. Knapp DSE881*; ibid., *DSE882*; ibid., *DSE884*; ibid., *DSE885*.

Notes: Sterile, depressed, globose, sporocarp-like structures were formed on the surface of autoclaved stinging nettle and submerged in WA media supplemented with minced vegetables or pine needles kept at 20 °C 3 months after inoculation. The change in the color of the WA media with minced vegetables from orange to dark brown was observed.

Ecology and distribution: Sandy grasslands of the Kiskunság region of the Great Hungarian Plain, in the areas dominated by *Festuca vaginata* and *Stipa borysthenica* as dominant grasses. Isolates belonging to the genus *Fuscosphaeria* are root-colonizing fungi associated with *F. vaginata* in Fülöpháza, Hungary. There is neither an isolate/morph-based nor an uncultured sequence-based report of this species from any other geographical region.

Discussion

In the present study, the DSE-87 isolates representing *Delitschia chaetomioides* (*Delitschiaceae*) were first reported as root-colonizing fungi, and a novel species *Fuscosphaeria hungarica* belonging to a monotypic genus was introduced based on phylogenetic analyses and morphological characters. The species was nested as a basal lineage in the family *Trematosphaeriaceae.* Although in vitro resynthesis experiments and microscopical investigation of root colonization were not carried out to prove the true endophytic nature of fungi (Grunewaldt-Stöcker and von Alten 2016), in the present study, we considered them to be root-colonizing fungi as they were isolated from healthy tissues of the grass *F. vaginata*, indicating that they might be considered as fungal root endophytes.

The monogeneric family *Delitschiaceae* comprises mainly saprobic species inhabiting herbivore dung, wood, or fruits (Kruys et al. 2006; Doveri 2011; Hyde et al. 2013; Jayasiri et al. 2019). Based mainly on the LSU and SSU sequences, *Delitschiaceae* forms a monophyletic basal lineage of Pleosporales (Hyde et al. 2013; Jayasiri et al. 2019; Haridas et al. 2020). ITS sequencing may be complicated in all species belonging to this family, which was indicated by the low number of sequences deposited online; however, because of the great length and potential variability of this region, ITS sequencing could provide crucial information for species delimitation in this group. The ITS region of these species could also provide misleading information for phylogenetic reconstructions, because the nrDNA sequences can be a primary source of artifacts (Kolařík and Vohník 2018). Nevertheless, obtaining the ITS sequences in combination with the sequences from other loci (e.g., *TEF* and *RPB2*) for more *Delitschia* species could be used to better characterize their lineages and may lead to the separation of the clade comprising the DSE-87 isolates from *D. chaetomioides*.

Delitschiaceae species are described by sexual morphs, and asexual characters cannot be found in their descriptions. Our DSE-87 isolates belonged to the species *Delitschia chaetomioides*, which produces embedded, scattered, or clustered perithecia. These ascomata can be black, opaque, globose, or pyriform, and are surrounded by dark hairs. These hairs are brown, septate, flexuous, branched or unbranched with blunt apices, and have thickened walls, up to 1000 μm in length. The asci of *D. chaetomioides* are eight-spored and cylindrical, and their ascospores are uniseriate, oblong-ellipsoid, at maturity almost black and opaque, each surrounded by a gelatinous layer (Luck-Allen and Cain 1975). In the present study, we also observed dark haired sporocarp-like structures after which the species was named (referring to the typical structures of *Chaetomium* species). The observed structures were sterile, without asexual or sexual spores, and were almost five times smaller than the usual mature ascomata of *D. chaetomioides* containing ascospores; however, the morphological characteristics of these morphs as well as the globose shape and abundant appendages or hairs are in accordance with the original description of this species (Luck-Allen and Cain 1975).

D. chaetomioides has been generally reported as a saprobic fungus living on herbivore dung, but it has also been isolated from the shoots of healthy plants, in which it was considered as an endophyte. Khoyratty et al. (2015) isolated *D. chaetomioides* from the ovaries of *Vanilla planifolia* and identified it by the LSU sequence. Based on the BLASTN search, one of the closest ITS hits to our *D. chaetomioides* isolates was an endophyte isolated from the leaves and stems of *Trifolium subterraneum* from semiarid dehesa ecosystems (grasslands with scattered trees and a herbaceous understory) in Spain (Lledó et al. 2016). The isolates were considered as unidentified fungi, and their sequence was deposited as Dothideomycete sp1 (GenBank accession number KP698360). The same sequences were obtained from the endophytes of another legume species (*Ornithopus compressus*) during the investigation of the same dehesa sites (Santamaria et al. 2018). Doust et al. (2017) obtained more ITS sequences of *D. chaetomioides* (GenBank accession number KX611078)

from the endophytes of the persian oak (*Quercus brantii*) originating from a xerophytic, cold-resistant deciduous oak forest. Based on the collection area of our *D. chaetomioides* isolates and similar sequences from GenBank, we hypothesized that this species can be more strongly associated with the plants from semiarid ecosystems than previously assumed.

Trematosphaeriaceae species are usually saprobes or pathogens (Suetrong et al. 2011; Ahmed et al. 2014). Recently, a couple of taxa were excluded, and based on the LSU, SSU, *TEF,* and *RPB2* regions it was found that this family comprises three genera: *Falciformispora*, *Halomassarina*, and *Trematosphaeria* (Suetrong et al. 2011; Tanaka et al. 2015). Their ascomata are solitary, scattered or produced in groups, initially immersed, becoming erumpent, or semiimmersed, subglobose, black, and have an apex with a short papilla. Their asci are eight-spored, bitunicate, fissitunicate, cylindro-clavate, pedicellate, and possess an ocular chamber. Their ascospores are biseriate to uniseriate, fusiform, hyaline or dark brown, trans-septate, and variously ornamented (Suetrong et al. 2011; Hyde et al. 2013). Only hyphopodia-like structures were reported as anamorphs produced after 6 months in cultures (Suetrong et al. 2011). In the present study, the colonies grown on different media and plant materials were checked periodically for 9 months; no sexual morphs were observed, but only immature or degenerated sporocarp-like structures were found (Fig. 2). Although *Fuscosphaeria* might represent an *incertae sedis* clade in a sister position with *Trematosphaeriaceae*, we considered *F. hungarica* to be a representative of the most basal group of the family due to its close phylogenetic position and similar morphological characteristics of its ascomata-like structures to the ascomata described in other species from the same family (Suetrong et al. 2011).

Based on a BLAST search of ITS sequences, even if the best hit was below 90% of similarity, the closest hits of *F. hungarica* were designated as endophytic fungi from different semiarid and arid sites according to GenBank entries and previous publications. During a survey on the effects of grazing on root-associated fungi in a semiarid grassland, Chen et al. (2018) obtained sequences from unidentified fungi (GenBank accession number KX823409) similar to the ITS sequence of *F. hungarica*. Khidir et al. (2010) detected uncultured root-associated fungi with similar ITS sequences (GenBank accession number FJ362211) from the roots of dominant grasses in semiarid grasslands. Another similar ITS sequence was that of *Trematosphaeria* sp. (GenBank accession number KY114922) associated with the stem and roots of halophytic species colonizing desert ecosystems (Li et al. 2020). One of the closest hits was another fungal endophyte (GenBank accession number KF887091) originating from *Ferula sinkiangensis* (Sun et al. 2014).

Root-colonizing endophytic fungi are considered to be asexual (Jumpponen and Trappe 1998); however, sterile, immature, and relatively small ascomata-like structures can rarely be observed under experimental conditions. Currah et al. (1993) observed sterile ascomata-like structures of a DSE species, but without ascospores. Recently, in the case of the worldwide distributed root endophytic pleosporalean genus *Darksidea*, Knapp et al. (2015) reported induced ascomata production, and some of these structures contained asci and ascospores. Similar to the taxonomical description presented in the present study, novel root endophytic species were generally described without sexual morphs (e.g., Knapp et al. 2015; Ashrafi et al. 2018; Crous et al. 2019; Vohník et al. 2019).

We are not aware of any previously published isolates related to *Fuscosphaeria*, and *D. chaetomioides* has also been rarely isolated in previous studies (Khoyratty et al. 2015; Lledó et al. 2016; Santamaria et al. 2018). The reports on the presence of these species are probably biased because of faster growing fungi, which are capable of growing rapidly on media and suppressing other fungal species (Hyde and Soytong 2008). Although based on the results of isolation techniques, these two species are not among the dominant lineages of root-associated fungi (e.g., Knapp et al. 2012, 2015), sequences of both species were found in our databases of the ITS-based metabarcoding study of the soil fungal community in different sites of the grassland near Fülöpháza (Vajna, Knapp, and Kovács, unpublished results), strengthening the idea of their common presence in this semiarid area. Further investigations of non-pathogenic root colonizers of gramineous plants may reveal their broader occurrence on semiarid grasslands, which was found for several root endophytes (Knapp et al. 2019).

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Compliance with ethical standards

The authors declare that they have no conflict of interest. All the data and materials used in the publication are deposited in public databases and culture collections. All authors contributed to the study conception and design. Material preparation, data collection, and analyses were performed and the manuscript was written by Alexandra Pintye and Dániel G. Knapp. All authors commented on previous versions of the manuscript, then read and approved the final manuscript.

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