



Confusion surrounding *Didymosphaeria*—phylogenetic and morphological evidence suggest Didymosphaeriaceae is not a distinct family

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Abstract

Didymosphaeriaceae is a ubiquitous fungal family that is reported to include saprobic, endophytic and pathogenic species associated with a wide variety of substrates. The family is characterized by 1-septate ascospores and trabeculate pseudoparaphyses, mainly anastomosing above the asci. In recent treatments *Appendispora*, *Didymosphaeria*, *Roussoella*, *Phaeodothis* and *Verruculina* were placed in the family. The aim of the present study is to delineate phylogenetic lineages within Didymosphaeriaceae and allied genera. A new species, *Didymosphaeria rubi-ulmifolii*, was isolated and identified based on morphological characters and phylogenetic analyses of partial 18S nrDNA and 28S nrDNA nucleotide sequence data. *Didymosphaeria rubi-ulmifolii* clustered with Montagnulaceae as a separate genus, while two putative strains (HKUCC 5834 and CMW 22186) of *D. futilis* from GenBank clustered with Cucurbitariaceae and Didymellaceae, respectively. The new species is characterized by immersed to slightly erumpent ascomata immersed under a clypeus, a peridium with compressed cells of *textura intricata*, long trabeculate pseudoparaphyses, anastomosing mostly above the asci and brown, 1-septate ascospores with granulate ornamentation. Phylogenetic analysis in combination with morphology and a review of literature show that *Appendispora*, *Phaeodothis*, *Roussoella* and *Verruculina* should be excluded from the family. *Phaeodothis* belongs in Montagnulaceae, *Verruculina* in Testudinaceae, while *Appendispora* and *Roussoella* belong in Roussoellaceae. The position of Didymosphaeriaceae as a distinct family, based on 1-septate ascospores and trabeculate pseudoparaphyses, mainly anastomosing above the asci is doubtful. Fresh collections of more *Didymosphaeria* strains are needed for epitypification and to obtain sequence data to establish if this family can be maintained.

Keywords: Didymosphaeriaceae, epitypification, Montagnulaceae, new species

Introduction

Munk (1953) introduced the family Didymosphaeriaceae typified by the genus *Didymosphaeria*. The family was characterized by 1-septate ascospores and trabeculate pseudoparaphyses which anastomosed mostly above the asci (Aptroot 1995). Species can be found mainly in terrestrial habitats as saprobes or parasites on woody branches and herbaceous stems and leaves, and are also parasitic on other fungi (Hyde *et al.* 2013). Based on its trabeculate

pseudoparaphyses, Barr (1990) referred Didymosphaeriaceae to the order Melanommatales, however, the importance of trabeculate pseudoparaphyses was later disputed (Liew *et al.* 2000). Due to the uncertainty of the placement, several authors have referred the family to different higher taxa. von Arx & Müller (1975) treated Didymosphaeriaceae as a synonym of the Pleosporaceae. Didymosphaeriaceae was maintained as a separate family within Pleosporales by Aptroot (1995) based on the 1-septate ascospores and trabeculate pseudoparaphyses. Lumbsch & Huhndorf (2007) assigned Didymosphaeriaceae to Montagnulaceae, while Zhang *et al.* (2012) tentatively included Didymosphaeriaceae as a separate family in the Pleosporales under the suborder Pleosporineae. In the same study it was shown that two strains of *Didymosphaeria fulilis* (HKUCC 5834 and CMW 22186), clustered separately in Cucurbitariaceae and Didymellaceae (Zhang *et al.* 2012). The understanding of the type species, *Didymosphaeria fulilis*, and Didymosphaeriaceae is therefore confused.

Based on trabeculate pseudoparaphyses embedded within a gel matrix and brown ascospores, *Appendispora* was assigned to Didymosphaeriaceae (Barr 1987, Hyde 1994). Barr (1990) also included *Neotestudina* in this family based on the morphology of the ascospores. Kohlmeyer & Volkmann-Kohlmeyer (1990) also assigned *Coronopapilla* to Didymosphaeriaceae, while *Verruculina enalia* (Kohlm.) Kohlm. & Volkm.-Kohlm. (= *Lojkania enalia* (Kohlm.) M.E. Barr = *Didymosphaeria enalia* Kohlm.) was also included in the family, based on its characteristic ascospores. Lumbsch & Huhndorf (2010) included five genera in Didymosphaeriaceae, *i.e.*, *Appendispora*, *Didymosphaeria*, *Phaeodothis*, *Rousoella* and *Verruculina*, but Zhang *et al.* (2012) accepted only three genera for the family, namely *Appendispora*, *Didymosphaeria* and *Phaeodothis*.

Several asexual morphs have been reported for Didymosphaeriaceae. Sivanesan (1984) stated that *Didymosphaeria* has *Ascochyta* and *Periconia* asexual states, while *Keissleriella* also included in Didymosphaeriaceae by Sivanesan (1984), produces *Dendrophoma* asexual states. Phylogenetic studies based on multigene analysis have shown that *Keissleriella* clusters within Lentitheciaceae, while *Dendrophoma cytisporoides* Sacc., the generic type of *Dendrophoma* clusters with *Chaetosphaeria hebetiseta* Réblová & W. Gams, and *C. callimorpha* (Mont.) Sacc., thus *Dendrophoma* belongs in Lentitheciaceae (Zhang *et al.* 2012, Schoch *et al.* 2009, Crous *et al.* 2012). Ellis (1971) reported *Periconia* asexual states for *Didymosphaeria*, but this was due to the misidentification of *Lophiostoma* as *Didymosphaeria* (Seifert *et al.* 2011, Hyde *et al.* 2013). The generic type of *Ascochyta*, *A. pisi* Lib., clustered within Didymellaceae and, therefore, the placement of *Ascochyta* in Didymosphaeriaceae is doubtful (Gruyter *et al.* 2009, Hyde *et al.* 2013). *Rousoella* produces a *Cytoplea* asexual state in culture (Hyde *et al.* 1996). Liu *et al.* (2014) have introduced a new family Rousoellaceae in Pleosporales to accommodate *Rousoella* species. Thus, the placement of the asexual states of Didymosphaeriaceae is also confused.

We have been studying the families of Pleosporales in order to provide a natural classification of this large order (Ariyawansa *et al.* 2013a,b,c, Ariyawansa *et al.* 2014, Zhang *et al.* 2009, 2012). Some studies have been based only on morphological characterization, while others have used molecular analysis (Ariyawansa *et al.* 2013a,b,c, Ariyawansa *et al.* 2014, Zhang *et al.* 2011, 2012). Given the considerable taxonomic confusion, the present study, based on phylogenetic analyses of nrDNA sequence data, was undertaken to (i) validate the familial placement of *Didymosphaeria* and assess whether it signifies natural groupings, (ii) verify which morphological characters are phylogenetically significant by observing the type species of the genera *Didymosphaeria* and *Phaeodothis*, and (iii) establish characters useful for generic delineation.

Materials and methods

Specimen examination

Fresh material of a *Didymosphaeria* species was collected on dead branches of *Rubus ulmifolius* in Forlì-Cesena, Ravaldino, Italy during 2012. Specimens were brought to the laboratory in Zip lock plastic bags®. Ascomata were removed directly from natural substrates without incubation. Herbarium specimens were obtained on loan from the Swedish Museum of Natural History (S) and the New York Botanical Garden (NY). The samples were processed and examined following the method described in Ariyawansa *et al.* (2013a,b). The fresh and herbarium materials were examined for fungal fruiting bodies using a Motic SMZ 168 dissecting microscope. Hand sections of the fruiting structures were mounted in water, examined with a Nikon ECLIPSE 80i compound microscope and photographed using a Canon 450D digital camera fitted to the microscope. Measurements were made with the Tarosoft (R) Image Frame Work program and images used for figures were processed with Adobe

Photoshop CS3 Extended version 10.0 software (Adobe Systems, The United States). Isolations were made from single ascospores following a modified method of Chomnunti *et al.* (2011, 2014). Contents of the sectioned fruiting body were transferred to a drop of sterile water on a flame-sterilized slide. Drops of the spore suspension were pipetted and spread on a Petri dish containing 2% water agar (WA) and incubated at 25°C overnight. Germinated ascospores were transferred singly to malt extract agar (MEA) media (Alves *et al.* 2006, Liu *et al.* 2011). Voucher specimens are deposited in the herbarium of Mae Fah Luang University (MFLU), Chiang Rai, Thailand and cultures are deposited at the Mae Fah Luang University Culture Collection (MFLUCC), Biodiversity and Climate Research Centre (BiK-F), International Collection of Microorganisms from Plants (ICMP) and BIOTEC Culture Collection (BCC), the latter under Material Transfer Agreement No. 4/2010 (MTA).

DNA extraction, PCR amplification and sequencing

Fungal isolates were grown on MEA for 28 days at 25°C in the dark. Genomic DNA was recovered from the growing mycelium using the Biospin Fungus Genomic DNA Extraction Kit (BioFlux®), but using sterile white quartz sand and a Kontes® battery-powered pestle grinder in 1.5 mL microfuge tubes.

Polymerase chain reaction (PCR) was carried out using known primer pairs. NS1 and NS4 were used to amplify a region spanning the small subunit rDNA (White *et al.* 1990). LROR and LR5 primer pairs were used to amplify a segment of the large subunit rDNA (Vilgalys & Hester 1990) and internal transcribed spacers was amplified by primer pairs ITS1 and ITS4 (White *et al.* 1990). The amplification procedure was performed in a 50 µl reaction volume containing 5–10 ng DNA, 0.8 units Taq polymerase, 1X PCR buffer, 0.2 mM d’NTP, 0.3 µM of each primer with 1.5 mM MgCl₂ (Cai *et al.* 2009). Amplification conditions were setup for initial denaturation of 5 min at 95°C, followed by 35 cycles of 45 s at 94°C, 45 s at 48°C and 90 s at 72°C, and a final extension period of 10 min at 72°C (Phillips *et al.* 2008). The PCR products were observed on 1% agarose electrophoresis gels stained with ethidium bromide. Purification and sequencing of PCR products were carried at Shanghai Sangon Biological Engineering Technology & Services Co. (China).

Phylogenetic analysis

The large and small subunits of the nuclear ribosomal RNA genes (LSU, SSU) were included in the analysis. All sequences obtained from GenBank and those used in Hyde *et al.* (2013), Schoch *et al.* (2009) and Zhang *et al.* (2012) and are listed in Table 2. Multiple sequence alignments were generated with MAFFT v. 6.864b (<http://mafft.cbrc.jp/alignment/server/index.html>). The alignments were checked visually and improved manually where necessary. Concordance of the SSU and LSU genes datasets was estimated with the partition-homogeneity test implemented with PAUP v. 4.0b10 (Swofford 2002). *Dothidea sambuci* was selected as out group taxon. New sequences are deposited in GenBank (Table 1).

TABLE 1. Synopsis of the characteristics of three species of *Didymosphaeria*.

Species	Ascomata (µm)	Peridium (µm)	Pseudoparaphyses (µm)	Asci (µm)	Ascospores (µm) and ornamentation
<i>D. decolorans</i>	150–300 × 200–320	30–40	0.8–1.5	55–80 × 5–8	8–13 × 4–6, smooth
<i>D. futilis</i>	100–150 × 120–160	10–20	0.5–1	75–85 × 4–6	7–10 × 3–5, spinulose
<i>D. rubi-ulmifolii</i>	140–250 × 150–280	7–15	0.5–1	60–90 × 4–8	6–10 × 3–6, granulate

Maximum likelihood analyses including 1,000 bootstrap replicates were run using RAxML v. 7.2.6 (Stamatakis & Alachiotis 2006, 2008). The online tool Findmodel (<http://www.hiv.lanl.gov/content/sequence/findmodel/findmodel.html>) was used to determine the best nucleotide substitution model for each partition. The best scoring tree was selected with a final likelihood value of -21860.18890. The resulting replicates were plotted on to the best scoring tree obtained previously. Maximum likelihood bootstrap values (ML) equal or greater than 50% are given below or above each node in red (Fig. 1).

MEGA 5.0 (Tamura *et al.* 2011) was used for minimum evolution (ME) inference, with default settings except for assuming pairwise deletion, and using the Tamura-Nei nucleotide substitution model. For inferring tree robustness, 1,000 bootstrap replicates were carried out and bootstrap values (ME) equal or greater than 50% are given below or above each node in green (Fig. 1).

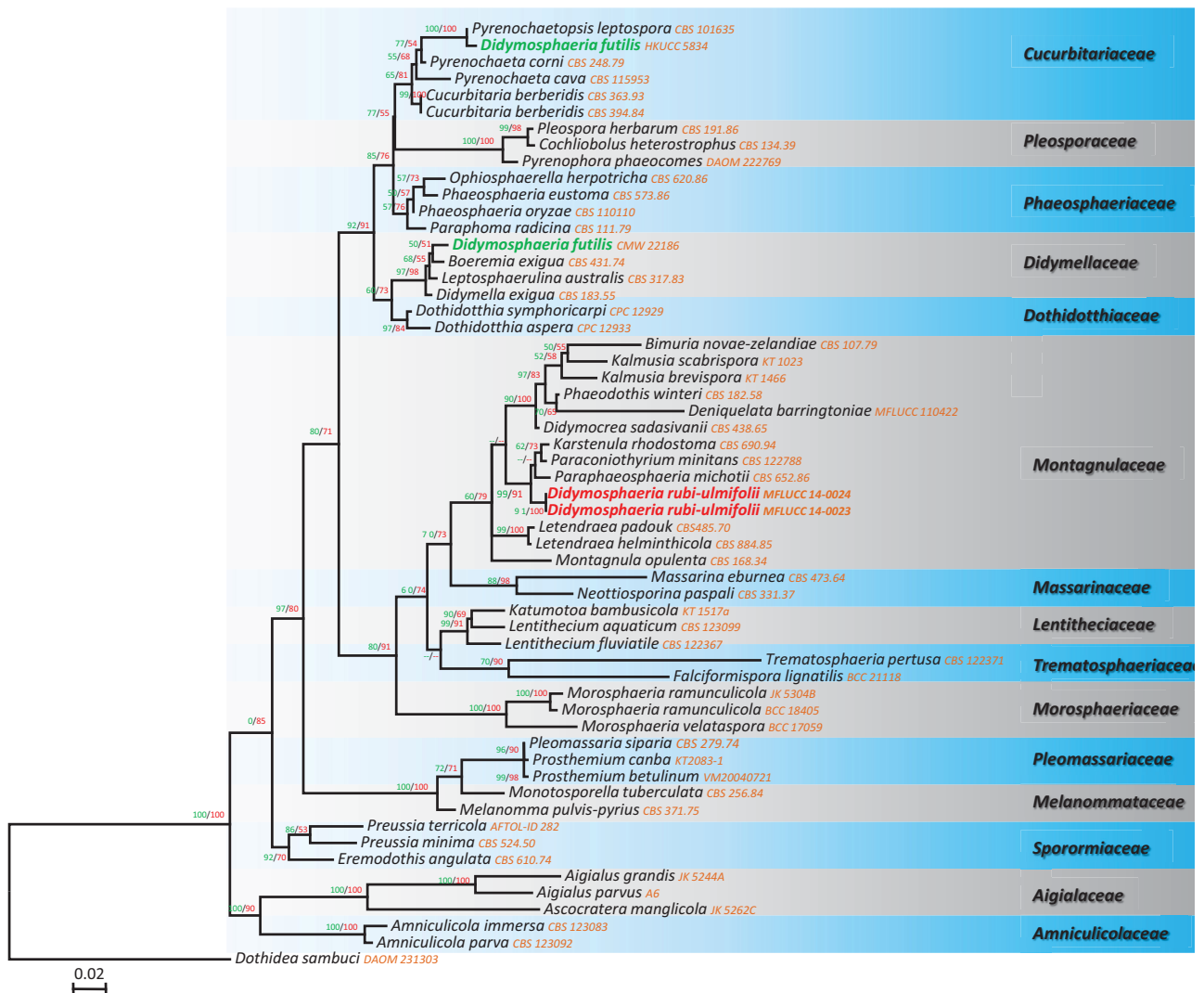


FIGURE 1. Best scoring RAxML tree based on a combined dataset of SSU and LSU with bootstrap support values for maximum likelihood (red) and minimum evolution (green) greater than 50% given below and above the nodes. *Dothidea sambuci* is the out group taxon. The original isolate numbers are noted after the species names.

TABLE 2. Isolates used in this study and their GenBank accession numbers. Newly deposited sequences are shown in bold.

Taxon	Voucher/culture	LSU	SSU
<i>Aigialus grandis</i>	JK 5244A	GU301793	GU296131
<i>Aigialus parvus</i>	A6	GU301795	GU296133
<i>Amniculicola immersa</i>	CBS 123083	FJ795498	GU456295
<i>Amniculicola parva</i>	CBS 123092	FJ795497	GU296134
<i>Ascocratera manglicola</i>	JK 5262C	GU301799	GU296136
<i>Bimuria novaezelandiae</i>	CBS 107.79	AY016356	AY016338
<i>Boeremia exigua</i>	CBS 431.74	EU754183	EU754084
<i>Cochliobolus heterostrophus</i>	CBS 134.39	AY544645	AY544727
<i>Cucurbitaria berberidis</i>	CBS 363.93	GQ387605	GQ387544
<i>Cucurbitaria berberidis</i>	CBS 394.84	GQ387606	GQ387545
<i>Deniquelata barringtoniae</i>	MFLUCC 110422	JX254655	JX254656

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TABLE 2 (continued)

Taxon	Voucher/culture	LSU	SSU
<i>Didymella exigua</i>	CBS 183.55	EU754155	EU754056
<i>Didymocrea sadasivani</i>	CBS 438.65	DQ384103	DQ384066
<i>Didymosphaeria futilis</i>	HKUCC 5834	GU205219	GU205236
<i>Didymosphaeria futilis</i>	CMW22816	EU552123	
<i>Didymosphaeria rubi-ulmifolii</i>	MFLUCC 14-0024	KJ436585	KJ436587
<i>Didymosphaeria rubi-ulmifolii</i>	MFLUCC 14-0023	KJ436586	KJ436588
<i>Dothidea sambuci</i>	DAOM 231303	AY544681	AY544722
<i>Dothidotthia aspera</i>	CPC 12933	EU673276	EU673228
<i>Dothidotthia symphoricarpi</i>	CPC 12929	EU673273	EU673224
<i>Eremodothis angulata</i>	CBS 610.74	DQ384105	DQ384067
<i>Falciformispora lignatilis</i>	BCC 21118	GU371827	GU371835
<i>Kalmusia scabriscpora</i>	KT 1023	AB524593	AB524452
<i>Karstenula rhodostoma</i>	CBS 690.94	GU301821	GU296154
<i>Katumotoa bambusicola</i>	KT 1517a	AB524595	AB524454
<i>Lentithecium aquaticum</i>	CBS 123099	GU301823	GU296156
<i>Lentithecium fluviatile</i>	CBS 122367	GU301825	GU296158
<i>Leptosphaerulina australis</i>	CBS 317.83	GU301830	GU296160
<i>Letendraea helminthicola</i>	CBS 884.85	AY016362	AY016345
<i>Letendraea padouk</i>	CBS485.70	AY849951	GU296162
<i>Massarina eburnea</i>	CBS 473.64	GU301840	GU296170
<i>Melanomma pulvis-pyrius</i>	CBS 371.75	GU301845	
<i>Monotosporella tuberculata</i>	CBS 256.84	GU301851	
<i>Montagnula opulenta</i>	CBS 168.34	DQ678086	AF164370
<i>Morosphaeria ramunculicola</i>	JK 5304B	GQ925854	GQ925839
<i>Morosphaeria ramunculicola</i>	BCC 18405	GU479794	GU479760
<i>Morosphaeria velataspora</i>	BCC 17059	GQ925852	GQ925841
<i>Neottiosporina paspali</i>	CBS 331.37	EU754172	EU754073
<i>Ophiosphaerella herpotricha</i>	CBS 620.86	DQ678062	DQ678010
<i>Paraconiothyrium minitans</i>	CBS 122788	EU754173	EU754074
<i>Paraphaeosphaeria michotii</i>	CBS 652.86	GQ387581	GQ387520
<i>Paraphoma radicina</i>	CBS 111.79	EU754191	EU754092
<i>Phaeodothis winteri</i>	CBS 182.58	GU301857	
<i>Phaeosphaeria eustoma</i>	CBS 573.86	DQ678063	DQ678011
<i>Phaeosphaeria oryzae</i>	CBS 110110	GQ387591	
<i>Pleomassaria siparia</i>	CBS 279.74	DQ678078	
<i>Pleospora herbarum</i>	CBS 191.86	DQ247804	DQ247812
<i>Preussia minima</i>	CBS 524.50	DQ678056	DQ678003
<i>Preussia terricola</i>	AFTOL-ID 282	AY544686	AY544726
<i>Prosthemia betulinum</i>	VM20040721	AB553754	
<i>Prosthemia canba</i>	KT2083-1	AB553760	

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TABLE 2 (continued)

Taxon	Voucher/culture	LSU	SSU
<i>Pyrenochaeta cava</i>	CBS 115953	GQ387607	GQ387546
<i>Pyrenochaeta corni</i>	CBS 248.79	GQ387608	GQ387547
<i>Pyrenochaetopsis leptospora</i>	CBS 101635	GQ387627	GQ387566
<i>Pyrenophora phaeocomes</i>	DAOM 222769	DQ499596	DQ499595
<i>Trematosphaeria pertusa</i>	CBS 122371	GU301876	GU348999

Results

Phylogeny of combined 18S and 28S nrDNA gene datasets

The combined SSU and LSU dataset comprised 57 taxa, including two new strains of *Didymosphaeria rubaei*. Results of the partition-homogeneity test ($P = 0.107$) indicated that the SSU and LSU gene trees reflect the same underlying phylogeny. Therefore, these datasets were combined and analyzed by using several tree-building programs and the resulting trees were compared.

Phylogenetic analysis

The combined SSU and LSU gene dataset of 15 families in the Pleosporales is shown (Fig. 1). All trees (ML and ME) were similar in topology and not significantly different (data not shown). A best scoring RAxML tree is shown in Fig. 1 with the value of -21860.18890. The strains of *Didymosphaeria rubi-ulmifolii* (MFLUCC 14-0023 and 14-0024) clustered in the family Montagnulaceae, but were separated from other genera of the family and sister to *Paraphaeosphaeria* clade. The two putative strains of *Didymosphaeria futilis* (HKUCC 5834 and CMW 22186) from GenBank clustered in Cucurbitariaceae and Didymellaceae, respectively.

Taxonomy

Didymosphaeriaceae Munk, Dansk bot. Ark. 15(2): 128 (1953) MycoBank: MB 80702

Notes:—The familial status of the Didymosphaeriaceae is questionable. Lumbsch & Huhndorf (2007) assigned it to the Montagnulaceae, while von Arx & Müller (1975) treated it as a synonym of the Pleosporaceae. Eriksson & Hawksworth (1993) listed *Didymosphaeria*, *Montagnula*, *Verruculina* and, doubtfully, *Roussoella* in this family. *Neotestudina* was subsequently referred to the Testudinaceae based on molecular data (Schoch *et al.* 2009, Seutrong *et al.* 2009). Aptroot (1995a) suggested that Didymosphaeriaceae species are probably close to other families in the Melanommatales with 1-septate ascospores (often recognized at ordinal level and as the Pyrenulales), viz Reuquenellaceae, Pyrenulaceae, Trypetheliaceae and Massariaceae and many taxa were excluded from the Didymosphaeriaceae, *i.e.* *Aaosphaeria*, *Amphisphaeria*, *Astrosphaeriella*, *Dothidotthia*, *Flagellosphaeria*, *Kirschsteiniotelia*, *Megalotremis*, *Montagnula*, *Munkovalsaria*, *Mycomicrothelia*, *Parapyrenis*, *Phaeodothis*, and *Verruculina*. Huhndorf & Lumbsch (2010) treated Didymosphaeriaceae as a separate family and assigned to Pleosporales and included five genera, viz *Appendispora*, *Didymosphaeria*, *Roussoella*, *Phaeodothis* and *Verruculina*. When considering morphological data only (peridium comprising flattened or irregular cells, 1-septate ascospores and trabeculate pseudoparaphyses mostly anastomosing above the asci), the family Didymosphaeriaceae appears to be a distinct family of Pleosporales. However, based on the molecular data the status of Didymosphaeriaceae as a distinct family is doubtful (Fig. 1). Collections of authentic *Didymosphaeria futilis* are needed for epitypification and to obtain sequence data to confirm that Didymosphaeriaceae is a synonym of Montagnulaceae.

Saprobic on woody branches and herbaceous stems and leaves. Sexual state: *Ascomata* solitary, scattered, or in small groups, immersed to erumpent, globose to ovoid, ostiolate. *Ostiole* papillate with a pore-like opening, ostiolar canal filled with hyaline cells (periphyses). *Peridium* 1-layered, thin, composed of brown pseudoparenchymatous cells of *textura intricata*. *Hamathecium* of dense, trabeculate, pseudoparaphyses, anastomosing mostly above the asci. *Asci* 8-spored, bitunicate, fissitunicate, cylindrical, with a furcated pedicel, apically rounded with an indistinct ocular chamber. *Ascospores* uniseriate, ellipsoid, brown, 1-septate, wall with different ornamentations. Asexual state: see notes below.

Notes:—The genus *Didymosphaeria* was introduced by Fuckel (1870) for three species of ascomycetes with two-celled ascospores. This genus *sensu lato*, comprised species having a wide distribution and a broad host range. Saccardo (1882) restricted the genus to only those species with brown ascospores (Aptroot 1995a). More than 100 species have been excluded from *Didymosphaeria* by various authors (Barr 1989a,b, 1990, 1992a,b, Hawksworth 1985a,b, Hawksworth & Boise 1985, Hawksworth & Diederich 1988, Scheinpflug 1958). Aptroot (1995a) included over 400 epithets of *Didymosphaeria* in his monograph of the genus, after examining over 3,000 species, and accepted only seven species.

The placement of *Didymosphaeria* is confused. Initially, the genus comprised three species and most authors accepted *Didymosphaeria epidermidis* (Fries) Fuckel as the type but, the type material comprises a coelomycete (Aptroot 1995a). The type of the only original species, *D. rubi* Fuckel, could not be found in G or in any other herbarium (Aptroot 1995a). Considering these facts Hawksworth & David (1989) proposed to conserve the genus with a lectotype specimen, and selected the widespread and common *D. fulvis* (Fungi Rhenani 1770) as the type for the Didymosphaeriaceae (Aptroot 1995a). This proposal was accepted by the Yokohama Botanical Congress (1993), in agreement with the Committee for Fungi of the International Association of Plant Taxonomists. Aptroot (1995a) listed seven species *i.e.*, *D. bisphaerica*, *D. conoidea*, *D. dimastospora*, *D. fulvis*, *D. massarioides*, *D. oblitescens*, and *D. spinosa*, which are closely related with the generic type of *Didymosphaeria* without considering differences of host or country of origin. Aptroot (1995a) proposed that *Didymosphaeria* belonged in the family Didymosphaeriaceae, order Pleosporales based on a peridium containing pseudoparenchymatous cells, a hamathecium consisting of narrow, trabeculate pseudoparaphyses, mainly anastomosing above the asci and brown, 1-septate ascospores (Kirk *et al.* 2008).

Asexual stages of *Didymosphaeria* are unclear. Sivanesan (1984) reported that *Didymosphaeria* has *Ascochyta* and *Periconia* asexual states, while Kirk *et al.* (2008) linked *Fusicladiella*-like and *Phoma*-like species to the genus. Linking *Didymosphaeria* to asexual states should be treated with caution until a lectotype of *D. fulvis* has been sequenced.

Type species: *Didymosphaeria fulvis* (Berk. & Broome) Rehm, Hedwigia 18: 167 (1879) MycoBank MB 223613 (Fig. 2a–o)

≡ *Sphaeria epidermidis* Berk. & Broome, Ann. Mag. nat. Hist., Ser. 2 9: 326 (1852)

Saprobic on dead wood. Sexual state: *Ascomata* 110–140 × 120–160 µm (\bar{x} = 130 × 140 µm, n = 10), scattered, or in small groups, immersed to slightly erumpent, rarely nearly superficial, under a clypeus, globose to subglobose, membranous, papillate. *Papilla* black, with a pore-like ostiole, ostiolar canal filled with periphyses. *Peridium* 10–20 (\bar{x} = 15 µm, n = 10) µm wide, 1-layered, composed of hyaline pseudoparenchymatous compressed cells of *textura intricata*, fusing at the outside with the host. *Hamathecium* of dense, 0.5–1 µm (\bar{x} = 0.8 µm, n = 20) broad, long, trabeculate pseudoparaphyses, anastomosing frequently above the asci, embedded in mucilage. *Asci* 75–85 × 4–6 µm (\bar{x} = 78 × 6 µm, n = 20), 8-spored, bitunicate, fissitunicate, cylindrical, pedicellate, rounded apex with an indistinct ocular chamber. *Ascospores* 7–10 × 3–5 µm (\bar{x} = 9 × 5 µm, n = 40), uniseriate, slightly overlapping, ellipsoid with obtuse ends, brown, 1-septate, slightly to not constricted at the septum, with distinctly spinulose ornamentation. Asexual state: unknown.

Material examined: UNITED KINGDOM. England: Norfolk, on dead stem of *Rosa* sp., March 1850, M.J. Berkeley (K 147683!, holotype of *Sphaeria epidermidis*).

Notes:—The type species of *Didymosphaeria*, *Didymosphaeria futilis* (Berk. & Broome) Rehm was initially described as *Sphaeria futilis* by Berk. & Broome (1852). Aptroot (1995a) synonymized more than 40 species under *Didymosphaeria futilis*. The placement of *D. futilis* is confused because several molecular studies (e.g., Zhang *et al.* 2012) and the present study have shown that the putatively named strains of *D. futilis* obtained from GenBank (HKUCC 5834 and CMW 22186) clustered in different families. Therefore, fresh collections of *Didymosphaeria futilis* are needed so that molecular data can be used to validate the natural taxonomic affinities of this genus.



FIGURE 2. *Didymosphaeria futilis* (holotype) a–b. Herbarium packet and specimen. c. Close-up of ascomata. d. Section through ascoma. e. Close-up of peridium. f. Arrangement of asci and pseudoparaphyses in hamathecium. g. Broad, long trabeculate pseudoparaphyses, anastomosing mostly above the asci. h–k. Cylindrical asci with an indistinct ocular chamber. l–o. Ascospores with distinct spinulose ornamentation. Scale bars: c = 100 μ m, d–g = 10 μ m, h–k = 20 μ m, l–o = 5 μ m.



FIGURE 3. *Didymosphaeria decolorans* (holotype). a. Herbarium packet and specimen. b–c. Close up of the ascomata. d. Section through ascoma. e. Close-up of the ostiole. f. Long trabeculate pseudoparaphyses. g. Long trabeculate pseudoparaphyses. h–l. Immature and mature asci. m–r. Immature and mature ascospores with smooth wall. Scale bars: b = 500 μ m, c = 200 μ m, d = 50 μ m, h–l = 20 μ m, e, f = 10 μ m, g, m–r = 5 μ m.

Didymosphaeria decolorans Rehm, Hedwigia 37(Beibl.): (143) (1898) MycoBank: MB 224571 (Fig. 3a–r)

Fungicolous or saprobic on other fungi or dead wood. Sexual state: *Ascomata* 150–300 × 200–320 µm (\bar{x} = 280 × 300 µm, n = 10), solitary to aggregated, immersed, subsequently erumpent through host periderm, globose to subglobose, membranous, dark brown to black, papillate. *Papilla* black, with a pore-like ostiole, ostiolar canal filled with periphyses. *Peridium* 30–40 (\bar{x} = 35 µm, n = 10) µm wide, 1-layered, composed of brown pseudoparenchymatous cells of *textura angularis*. *Hamathecium* of dense, 0.8–1.5 µm (\bar{x} = 1 µm, n = 20) broad, long trabeculate pseudoparaphyses, anastomosing mostly above the asci, embedded in mucilage. *Asci* 55–80 × 5–8 µm (\bar{x} = 75 × 7 µm, n = 20), 8-spored, bitunicate, fissitunicate, cylindrical, short pedicellate, rounded apex with a minute ocular chamber. *Ascospores* 8–13 × 4–6 µm (\bar{x} = 10 × 5 µm, n = 40), uniseriate, slightly overlapping, ellipsoid with obtuse ends, brown, 1-septate, slightly to not constricted at the septum, with smooth wall. Asexual state: unknown.

Notes: We observed the holotype of *D. decolorans* (S, F 12021), which was examined by Aptroot (1995a) and synonymized under *D. fulvis*. *D. decolorans* grew inside the fruiting bodies of a *Leptosphaeria* or *Pleospora*-like species (Fig. 3b–c). *D. decolorans* and *D. fulvis* share similar characters in having 1-septate ascospores and trabeculate pseudoparaphyses, mainly anastomosing above the asci, but differ in the structure of the peridium (brown pseudoparenchymatous cells of *textura angularis* versus hyaline pseudoparenchymatous compressed cells of *textura intricata*), ascospore wall ornamentation (smooth versus spinulose) and the habitat (fungicolous versus saprobic), respectively. Fresh collections of *D. decolorans* and sequence data analysis are needed to confirm if this is a *Didymosphaeria* species, as *D. decolorans* is not typical of the genus because of its fungicolous nature and the structure of the peridium (composed of brown pseudoparenchymatous cells of *textura angularis*).

Material examined:—GERMANY. Oberammergau, Bayern, on dry branches of *Salix caprea*, August 1894 and 1896, leg. *Schnabl*, determined by H. Rehm (S, F 12021!, holotype).

Didymosphaeria rubi-ulmifolii Ariyawansa, Erio Camporesi & K.D. Hyde, *sp. nov.* MycoBank MB 808165 (Fig. 4a–l)

Etymology:—The specific epithet *rubi-ulmifolii* is based on the host genus from which the fungus was isolated.

Saprobic on wood. Sexual state: *Ascomata* 140–250 × 150–280 µm (\bar{x} = 210 × 230 µm, n = 10), scattered, or in small groups, immersed to slightly erumpent under clypeus, globose to sub globose, membranous, papillate. *Papilla* black, with a pore-like ostiole, ostiolar canal filled with periphyses. *Peridium* 6–12 (\bar{x} = 10 µm, n = 10) µm wide, 1-layered, composed of hyaline to light brown pseudoparenchymatous compressed cells of *textura intricata*, fusing at the outside with the host. *Hamathecium* of dense, 0.5–1 µm (\bar{x} = 0.7 µm, n = 20) broad, long, trabeculate pseudoparaphyses, anastomosing mostly above the asci, embedded in mucilage. *Asci* 60–90 × 4–8 µm (\bar{x} = 75 × 7 µm, n = 20), 8-spored, bitunicate, fissitunicate, cylindrical, pedicellate, rounded apex with and an indistinct ocular chamber. *Ascospores* 6–10 × 3–6 µm (\bar{x} = 10 × 5 µm, n = 40), uniseriate, slightly overlapping, ellipsoid with obtuse ends, brown, 1-septate, slightly to not constricted at the septum, with granulate ornamentation. Asexual state: not produced in culture.

Material examined:—ITALY. Forli-Cesena, Ravaldino, on dead branch of *Rubus ulmifolius*, 14 January 2012, Erio Camporesi (MFLU 12-2215!, holotype), ex-type living culture (MFLUCC 14-0023 and 14-0024)

Notes:—Based on morphological characteristics, *Didymosphaeria rubi-ulmifolii* fits in the generic concept of *Didymosphaeria* in having 1-septate ascospores and trabeculate pseudoparaphyses, mainly anastomosing above the asci. Morphologically *D. rubi-ulmifolii* and *D. fulvis* are similar in having immersed ascomata under clypeus with single layered peridium comprising hyaline, compressed cells of *textura intricata*, trabeculate pseudoparaphyses, anastomosing mostly above the cylindrical asci, and brown, 1-septate ascospores. Differences are in the size of the ascomata, ornamentation of the ascospores (granulate versus spinulose) and host (Table 1). Based on the original description of *D. rubi*, *D. rubi-ulmifolii* and *D. rubi* differ in width of the peridium (thin versus broad) and the ornamentation of the ascospores (granulate versus foveate). We could not observe *D. rubi* due to the unavailability of the holotype specimen in G or in any other herbarium and an isotype in W reported only as a coelomycete (Aptroot 1995a). *Didymosphaeria rubicola* Berl., differs from *D. rubiae* in having carbonaceous ascomata and comparatively large spores (20–23 × 10–12 µm versus 6–10 × 3–6 µm) and the host (*Rubus fruticosus* versus *R. ulmifolius*) (Saccardo 1891). *D. fulvis*, *D. decolorans* and *D. rubiae* are compared in Table 1.

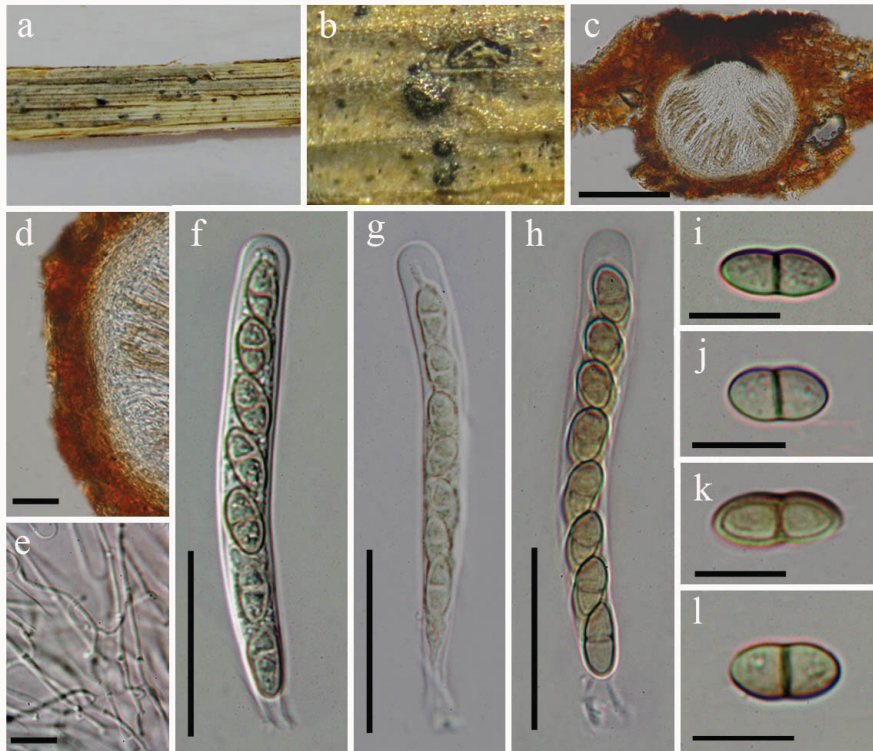


FIGURE 4. *Didymosphaeria rubi-ulmifolii* (holotype). a. Immersed ascomata on the host surface b. Close-up of the ascomata c. Section of an ascoma. d. Close-up of peridium. e. Trabeculate, anastomosing and branching pseudoparaphyses. f–h. Asci with 8 spores. i–l. Brown, 1-septate ascospores with granulate ornamentation. Scale bars: c = 100 μ m, d = 20 μ m, e = 10 μ m f–h = 30 μ m, i–l = 10 μ m.

Placement of excluded genera

Rousoellaceae J.K. Liu *et al.*, In Press MycoBank: MB 804651

Based on immersed gregarious, clypeate ascomata with trabeculate pseudoparaphyses, embedded in a gel matrix, long cylindrical bitunicate asci without obvious fissitunicate dehiscence and brown, 1–2-septate ornamented ascospores, Liu *et al.* (2014) introduced the new family Rousoellaceae in the order Pleosporales. Most of the taxa in Rousoellaceae are from bamboo, palms and grasses.

Appendispora K.D. Hyde, *Sydowia* 46(1): 29 (1994)

Generic description

Saprobic on palms. *Sexual state*: *Ascostromata* immersed beneath a stroma, axis horizontal, oblique or vertical to the host surface, with up to five clustered locules and a central ostiolar canal. *Peridium* thin, inner layer composed of hyaline elongated cells and outer layers comprising small brown cells. *Hamathecium* of dense, long, trabeculate pseudoparaphyses. *Asci* 8-spored, bitunicate, fissitunicate, cylindrical, short pedicellate, apically rounded with an ocular chamber and faint ring. *Ascospores* uniseriate to partially overlapping, fusoid, brown, 1-septate, appendaged, slightly constricted at the septum. *Asexual state*: unknown.

Type species: *Appendispora frondicola* K.D. Hyde, *Sydowia* 46(1): 30 (1994)

Notes:—*Appendispora* was introduced by Hyde (1994) and typified by *A. frondicola*. Currently two epithets (*A. australiensis* and *A. frondicola*) are listed for *Appendispora* in Index Fungorum (2014), however, molecular data is lacking. The genus was described as a saprobe of palms, and is characterized by clustered ascostromata, which are

immersed beneath a stroma, with their axis horizontal, oblique or vertical to the host surface, with minute ostioles visible through cracks or blackened dots on the host surface, trabeculate pseudoparaphyses, brown, and 1-septate, appendaged ascospores with irregular wall striations (Hyde 1994). Hyde (1994) suggested that the genus can be referred to Didymosphaeriaceae based on trabeculate pseudoparaphyses embedded within gel matrix and brown ascospores and this was followed by Barr (1987b). *Appendispora* has similarities with *Didymosphaeria*, but differs in having ascospores with reticulate wall ornamentation and appendages (Hyde 1994). Furthermore, ascomata in *Didymosphaeria* are immersed under a clypeus with their axis vertical to the host surface, whereas in *Appendispora* ascomata are clustered under stromata and their axes are horizontal, oblique or vertical (Hyde 1994). In Hyde (1994, Fig. 1) the locules appear to be arranged in a ring with a common central ostiole. One *Didymosphaeria* species has been reported from the branches of *Cytisus multiflorus*, with appendaged ascospores (*D. polytrichospora* M.T. Lucas & Sousa da Câmara) by Lucas & DaCâmara (1953). *D. polytrichospora* has brown two-celled ascospores provided with 6-7 appendages at each end, which are illustrated as seta-like (Hyde 1994). After observing the type species of *D. polytrichospora*, Aptroot (1994) concluded that this taxon has unitunicate asci with a J+ subapical ring. The taxonomic position of *D. polytrichospora* is uncertain, but it is far removed from *Appendispora*, which has bitunicate asci lacking a J+ ring. *Appendispora* shares similarities with Roussoellaceae in having trabeculate pseudoparaphyses, embedded in a gel matrix, long cylindrical asci and 1-septate, brown ascospores and furthermore, most of the taxa in Roussoellaceae have been described from bamboo (Hyde *et al.* 2013, Liu *et al.* 2014) and palms (Hyde *et al.* 2013, Liu *et al.* 2014). *Appendispora* is also recorded from palms, but differs from other genera of Roussoellaceae in the arrangement of locules in the ascostromata with a central ostiole and its appendaged ascospores (Hyde *et al.* 2013, Liu *et al.* 2014). Based on the above comparison we place *Appendispora* in Roussoellaceae.

Roussoella Sacc., in Saccardo & Paoletti, Atti Ist. Veneto Sci. lett. ed Arti, Sér. 3 6: 410 (1888)

Type species:—*Roussoella nitidula* Sacc. & Paol., Atti Ist. Veneto Sci. lett. ed Arti, Sér. 3, 6: 410 (1888)

Notes:—*Roussoëlla* was introduced by Saccardo for the single species *R. nitidula* (Saccardo & Paoletti 1888). The genus is characterized by immersed ascomata with long cylindrical asci and brown 1-septate ornamented ascospores (Hyde *et al.* 1996). Phylogeny based on multigene analysis has shown that the species of *Roussoella*, *Roussoellopsis* as well as *Arthopyrenia salicis* form a robust clade, which forms a sister group with pleosporalean families, but the generic type of *Roussoella* (*R. nitidula*) was not included in the phylogenetic study (Tanaka *et al.* 2009, Zhang *et al.* 2012). Liu *et al.* (In Press) will introduce a new family Roussoellaceae to accommodate *Roussoella*, a natural classification verified by molecular data and supported by morphology.

Montagnulaceae M.E. Barr, Mycotaxon 77:194 (2001) MycoBank: MB 82111

Barr (2001) introduced the family, Montagnulaceae with three genera including *Kalmusia*, *Montagnula* and *Didymosphaerella* in the order Pleosporales (Hyde *et al.* 2013). The family is characterized by ascomata immersed under a clypeus, a small celled pseudoparenchymatous peridium, cylindrical or oblong, fissitunicate, pedicellate asci and brown ascospores (Hyde *et al.* 2013).

Phaeodothis Syd. & P. Syd., Annls mycol. 2(2): 166 (1904)

Generic description

Saprobic or parasitic in terrestrial habitats Sexual state: *Ascostromata* subglobose to hemispherical, immersed, scattered to gregarious, dark brown to black. *Peridium* comprising several layers of hyaline compressed cells. *Hamathecium* of dense, narrow, cellular pseudoparaphyses often in a gelatinous matrix. *Asci* 8-spored, bitunicate, cylindrical, short pedicellate. *Ascospores* fusiform with rounded ends, olivaceous-brown. Asexual state: unknown.

Type species:—*Phaeodothis tricuspis* Syd. & P. Syd., Annl. mycol. 2(2): 166 (1904) (Fig. 5)

Saprobic in terrestrial habitats. Sexual state: *Ascostromata* 235–290 × 120–180 μm (\bar{x} = 254 × 137 μm, n = 10), immersed to nearly superficial under clypeus, scattered to gregarious, subglobose to hemispherical, dark brown to black, papillate, smooth. *Papilla* black, with a pore-like ostioles, ostiolar canal filled with periphyses. *Peridium* 15–28 μm (\bar{x} = 22 μm, n = 20) wide, comprising several layers of hyaline compressed cells. *Hamathecium* of dense, 2–4 μm (\bar{x} = 2 μm, n = 20), aseptate, narrow, cellular pseudoparaphyses often in a gelatinous matrix. *Asci* 55–80 × 7–12 μm (\bar{x} = 68 × 10 μm, n = 20), 8-spored, bitunicate, fissitunicate, cylindrical, short pedicellate with a minute ocular chamber. *Ascospores* 14–18 × 4–5 μm (\bar{x} = 16 × 5 μm, n = 30), biseriate and partially overlapping, fusiform with rounded ends, olivaceous-brown, 1-septate, slightly constricted at the septum. Asexual state: unknown.

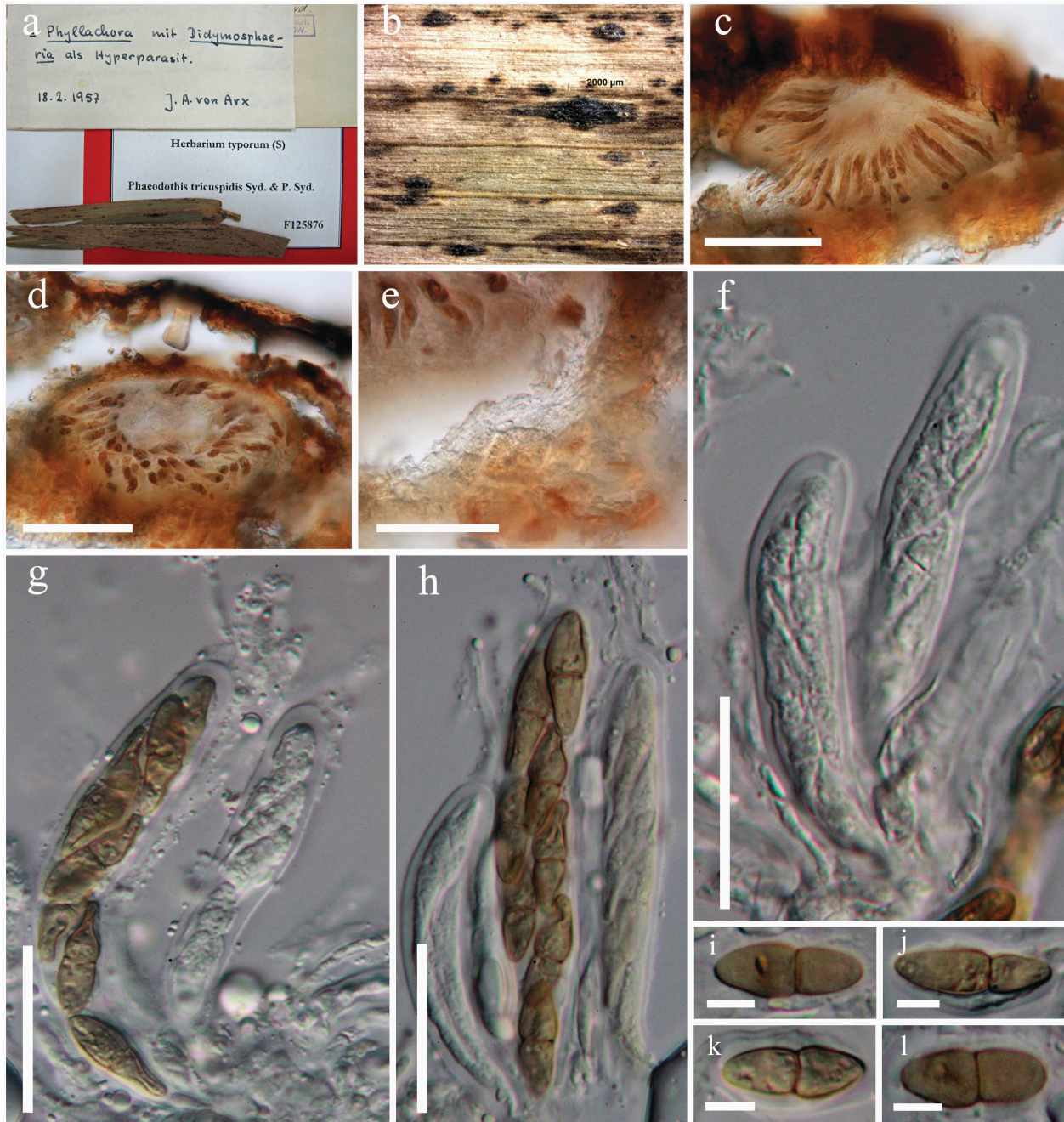


FIGURE 5. *Phaeodothis tricuspis* (holotype) a. Herbarium packet and specimen. b. Close-up of ascomata. c–d. Sections through ascoma. e. Peridium comprising hyaline compressed cells. f–h. Mature and immature asci surrounded by pseudoparaphyses. i–l. Fusiform, olivaceous-brown ascospores. Scale bars: c–d = 100 μm, e = 50 μm, f–h = 25 μm, i–l = 5 μm.

Material examined:—ARGENTINA. San José, Salta, 12 February 1873, *P.G. Lorentz* (S F125876!, holotype).

Notes:—*Phaeodothis* is characterized by its immersed to nearly superficial ascostromata, a sparse hamathecium consisting of thin pseudoparaphyses and 1-septate ascospores (Aptroot 1995). The genus was placed in *Didymosphaeria* by Niessl (1875), but Aptroot (1995) transferred the genus to Phaeosphaeriaceae. *Phaeodothis* has similarities with Montagnulaceae in having ascomata immersed under a clypeus, a pseudoparenchymatous peridium with small cells, cylindrical asci and brown ascospores. *Phaeodothis* however, differs from the other genera of Montagnulaceae in having a hamathecium consisting of thin, sparse pseudoparaphyses and 1-septate ascospores. A recent phylogenetic analysis based on multiple genes (LSU, SSU, RBP1, RBP2 and EF-1) concluded that a strain named *Phaeodothis winteri* (a synonym of *P. tricuspis* Syd. & P. Syd.) clustered within the Montagnulaceae clade (Schoch *et al.* 2009). Currently 27 epithets are listed for the genus (Index Fungorum 2014), while GenBank has seven hits for the genus including the putative strain of *Phaeodothis winteri* (CBS 182.58). Based on the above morphological characters and available molecular data, we suggest that *Phaeodothis* can be referred to Montagnulaceae.

Testudinaceae Arx, Persoonia 6: 365 (1971) MycoBank: MB 81456

The family Testudinaceae was introduced by von Arx (1971), based on ascomata with a dark peridium, which is often made up of plates, with bitunicate asci, and dark 2-celled ascospores, about 10 μm long and referred to the order *Pseudosphaeriales* (= *Pleosporales*). Currently the family contains five genera *viz* *Lepidosphaeria*, *Neotestudina*, *Testudina*, *Verruculina* and *Ulospora* (Schoch *et al.* 2009, Suetrong *et al.* 2009, Lumbsch & Huhndorf 2010, Hyde *et al.* 2013).

Verruculina Kohlm. & Volkm.-Kohlm., Mycol. Res. 94(5): 689 (1990)

Type species:—*Verruculina enalia* (Kohlm.) Kohlm. & Volkm.-Kohlm., Mycol. Res. 94(5): 689 (1990)

≡ *Didymosphaeria enalia* Kohlm., Ber. dt. bot. Ges. 79: 28 (1966)

≡ *Lojkania enalia* (Kohlm.) M.E. Barr, N. Amer. Fl., Ser. 2, 13: 56 (1990)

Notes:—*Verruculina* was introduced by Kohlmeyer & Volkmann-Kohlmeyer (1990) to accommodate an obligate marine species *Verruculina enalia* (Kohlm.) Kohlm. & Volkm.-Kohlm. The genus is characterized by a single marine species with immersed, clypeate, carbonaceous, papillate ascomata containing trabeculate pseudoparaphyses, with cylindrical asci with short pedicels and ellipsoidal, 1-septate, dark brown, verrucose or verruculose ascospores (Kohlmeyer & Volkmann-Kohlmeyer 1990). *Verruculina* is similar to *Didymosphaeria* in having partly or completely immersed clypeate ascomata, but differs in the dark peridium with thick-walled cells, forming a *textura angularis*, and stipitate asci with a clear ocular chamber (Kohlmeyer & Volkmann-Kohlmeyer 1990). *Verruculina* shows similarities with Testudinaceae in possessing septate, dark brown, verrucose or verruculose ascospores but can be differentiated based on the immersed, clypeate, carbonaceous papillate ascomata containing trabeculate pseudoparaphyses and additionally the marine habitat (Hyde *et al.* 2013, Suetrong *et al.* 2009, Schoch *et al.* 2009, Tanaka *et al.* 2009). Phylogenetic studies based on multigene analysis shows that *Verruculina enalia* clustered within Testudinaceae (Hyde *et al.* 2013, Suetrong *et al.* 2009).

Discussion

Molecular data play a pivotal role in modern mycological taxonomy, but have some constraints in application. The most significant and unsettled problem is that the phylogeny inferred from any gene may not reveal the evolutionary history of the organism (Uilenberg *et al.* 2004). It is therefore better to incorporate a polyphasic taxonomy including genotypical and phenotypical characteristics in any studies (Uilenberg *et al.* 2004). Taxonomic modifications should not only be based on small portions of the genome, but also use morphological characters (Uilenberg *et al.* 2004). The genome also needs to be evaluated (Uilenberg *et al.* 2004).

Genera with trabeculate pseudoparaphyses and 1-septate, brown ascospores viz *Bicrouania*, *Caryosporella*, *Lineolata*, *Phaeodothis*, *Roussoella*, *Verruculina*, were generally classified under Didymosphaeriaceae. Molecular studies have shown that these particular morphological characters have evolved in different families. *Verruculina enalia* clustered within Testudinaceae in order Pleosporales (Suetrong *et al.* 2009). *Lineolata rhizophorae* (Kohlm. & E. Kohlm.) Kohlm. & Volkm.-Kohlm., was originally introduced by Kohlmeyer & Kohlmeyer (1966) as a species of *Didymosphaeria* (*D. rhizophorae*). The genus is characterized by obpyriform ascomata with long trabeculate pseudoparaphyses and 1-septate, brown ascospores. Three isolates of *Lineolata rhizophorae* from varied geographic localities were analyzed by Suetrong *et al.* (2009) and formed a monophyletic group but in an unresolved clade with *Caryosporella rhizophorae* Kohlm., in a sister group (long branch length) in Dothideomycetidae and thus excluded from Pleosporomycetidae and Pleosporales. *Phaeodothis* was previously assigned to *Didymosphaeria* by Niessl (1875) but recent phylogenetic analysis showed that *Phaeodothis winteri* (a synonym of *P. tricuspis* Syd. & P. Syd.) nested within the clade of Montagnulaceae (Schoch *et al.* 2009).

There have been few molecular investigations of Didymosphaeriaceae when compared to morphological studies. Zhang *et al.* (2012) accepted only *Appendispora*, *Didymosphaeria* and *Phaeodothis* in the family. The present study concluded that the phylogenetic trees obtained from maximum likelihood and minimum evolution inference yielded trees with similar overall topology at subclass and family relationship in agreement with previous work based on maximum likelihood (Hyde *et al.* 2013, Schoch *et al.* 2009, Suetrong *et al.* 2009, Zhang *et al.* 2012). The putative strains of *Didymosphaeria futilis* (HKUCC 5834 and CMW 22186) in GenBank, clustered with Cucurbitariaceae and Didymellaceae, respectively, in the present study as well as in previous studies (Zhang *et al.* 2012). In the present study two new strains of the new species *D. rubi-ulmifolii* clustered in the family Montagnulaceae, but were separated from other genera of the family with high bootstrap support. Comparison of the generic type, *D. futilis*, and the new species, *D. rubi-ulmifolii* shows that they have similar morphology and thus represent *Didymosphaeria*. Therefore, based on the available molecular data and morphology, *Didymosphaeria* can be referred to the family Montagnulaceae and Didymosphaeriaceae can be treated as a probable synonym of Montagnulaceae.

A confusing fact is that the newly generated strains of *Didymosphaeria rubi-ulmifolii* and the putatively named strains of the genus type obtained from GenBank (HKUCC 5834 and CMW 22186) clustered in different families. This is an example of the problems of sequence data in GenBank and other public databases. The morphology and identification of the putative strains (HKUCC 5834 and CMW 22186) of *D. futilis* in GenBank as far as we can ascertain, cannot be checked, as they are not linked to any herbarium material. In this study we provide illustrations and descriptions of the new species *D. rubi-ulmifolii* and the holotype of *D. futilis* which, with the exception of ascospores ornamentation and host, are identical and should represent the same genus, *Didymosphaeria*.

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