



Article

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Taxonomy and multigene phylogenetic evaluation of novel species in *Boeremia* and *Epicoccum* with new records of *Ascochyta* and *Didymella* (Didymellaceae)

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Abstract

Four microfungi collected from Italy and Russia are reported. Morphological examination revealed that all four species can be accommodated in the family *Didymellaceae* (Pleosporales) and more precisely in the genera *Ascochyta*, *Boeremia*, *Didymella* and *Epicoccum*. ITS, LSU, RPB2 and β -TUB sequence data were analysed to investigate their phylogenetic relationships. Two new species, *Boeremia galiicola* and *Epicoccum mackenziei* are introduced, while *Ascochyta medicaginicola* and *Didymella macrostoma* are new records from Russia and Italy. A sexual stage is reported for the first time in *Ascochyta medicaginicola*. All taxa are described and illustrated with discussion on their taxonomic placement. Multigene phylogenetic analyses provide further evidence to support the introduction of these taxonomic novelties and their classification within the *Didymellaceae*.

Keywords – asexual morph, Dothideomycetes, Pleosporales, rDNA, RPB2, β -TUB

Introduction

De Gruyter et al. (2009) introduced *Didymellaceae* to accommodate the type species *Didymella exigua* (Niessl) Sacc. Subsequent researchers confirmed the placement of *Didymellaceae* in Pleosporineae, Pleosporales (Chen et al. 2015). Nineteen genera are presently assigned to the *Didymellaceae* based on morphology and phylogeny: *Allophoma*, *Ascochyta*, *Boeremia*, *Calophoma*, *Chaetasbolisia*, *Dactuliochaeta*, *Didymella*, *Didymellocamarosporium*, *Epicoccum*, *Heterophoma*, *Leptosphaerulina*, *Macroventuria*, *Neoascochyta*, *Neodidymelliopsis*, *Neomicrosphaeropsis*, *Nothophoma*, *Paraboeremia*, *Phoma*, *Phomatodes* and *Xenodidymella* (Hyde et al. 2013, Wijayawardene et al. 2014, 2016, Chen et al. 2015, Thambugala et al. 2016). *Ascochyta*, *Phoma* and *Staganosporopsis* are common pathogens and significant in quarantine decisions (Aveskamp et al. 2010, Zheng et al. 2017). Of the 19 genera, sexual morphs are known for only twelve genera, i.e. *Ascochyta*, *Boeremia*, *Didymella*, *Leptosphaerulina*, *Macroventuria*, *Neoascochyta*, *Neodidymelliopsis*, *Neomicrosphaeropsis*, *Paraboeremia*, *Phoma*, *Staganosporopsis* and *Xenodidymella* (Wijayawardene et al. 2012a, Woudenberg et al. 2009, Chen et al. 2015, Thambugala et al. 2017).

The family *Didymellaceae* is characterized by immersed, rarely superficial, separate or gregarious, globose to flattened, ostiolate, ascomata, with 2–5(–8) layers of pseudoparenchymatal cells. Asci are bitunicate, cylindrical to clavate or saccate, 8-spored and arise from a broad hymenium among pseudoparaphyses. Ascospores are mostly hyaline or brownish and 1-septate to multiseptate. *Phoma* species are common within the *Didymellaceae* including the representative strain of *P. herbarum* (CBS 615.75, Boerema et al. 2004), which is type species of the genus (Aveskamp et al. 2010). Presently *Phoma herbarum* is the type species of as *Phoma sensu stricto*, which belongs to *Didymellaceae* (Aveskamp et al. 2010). Coelomycetous or hyphomycetous asexual morphs are formed on natural substrates or culture (Woudenberg et al. 2009, Chen et al. 2015). *Epicoccum* has a hyphomycetous synanamorph, which is characterized by dark sporodochia with branched conidiophores and mono- to polyblastic, colourless conidiogenous cells that produce coloured, sometimes verruculose, dictyoconidia (Seifert et al. 2011). The coelomycetous asexual stage is characterized by the production of pycnidial conidiomata with monophialidic, doliiform to flask-shaped conidiogenous cells that producing unicellular, hyaline conidia in culture or nature (Aveskamp et al. 2010). In addition to *Ascochyta*, the genera *Calophoma*, *Didymella*, *Epicoccum*, *Heterophoma*, *Neodidymelliopsis* and *Xenodidymella* produce chlamydospores in culture (Woudenberg et al. 2009, Chen et al. 2015).

In the quest to assess fungal diversity of these bitunicate fungi from different regions and hosts, taxa collected from Italy and Russia resulted in two new species and two new records within the family *Didymellaceae*. The objectives of this study are as follows:

- 1) To identify and describe the new taxa and new records;
- 2) To link sexual and asexual morphs (Wijayawardene et al. 2012b) and
- 3) To investigate their phylogenetic relatedness to other *Didymellaceae* members based on multigene DNA sequence analyses.

Material and methods

Sample collection and specimen examination

Fresh samples were collected from Italy (Arezzo and Forli-Cesena Provinces) and from Russia (Arkhangelsk region). Slides were prepared and photographed using a Carl Zeiss stereo microscope fitted with an AxioCam ERC 5 S camera. A Motic SMZ 168 Series microscope was used to examine the microscopic characters (peridium, asci, ascospores, pycnidial wall, conidiogenous cells and conidial morphology). Hand sections of the fruiting structures (ascomata and conidiomata) were mounted in water for microscopic studies and photomicrography. All measurements were calculated using Tarosoft Image FrameWork and Adobe Photoshop CS6 Extended v.10.0 software was used for the preparation of figures (Adobe Systems, USA).

Single spore isolation was performed as outlined by Chomnunti et al. (2014). Ascospores which germinated within 24 h were transferred to malt extract agar (MEA) plates, and incubated at 18–25 °C. After one month of incubation, asexual morph and chlamydospore characters were observed from culture. Axenic cultures were kept for further examination of any asexual morphs from the chlamydospores. However, for *E. mackenziei* (MFLUCC 16-0335) we could not obtain asexual morphs in culture. Specimens were deposited in the herbarium of Mae Fah Luang University (Herb. MFLU), Herbarium of Cryptogams Kunming Institute of Botany Academia Sinica (HKAS), China Fungarium PDD, New Zealand and the living cultures were deposited in culture collection of Mae Fah Luang University (MFLUCC), Thailand and Culture Collection of Kunming Institute of Botany (KUMCC), China or BIOTEC Culture Collection (BCC), Bangkok, Thailand. Index Fungorum numbers and Facesoffungi numbers were obtained as in Index Fungorum (2016) and Jayasiri et al. (2015). Establishment of new species were based on recommendations outlined by Jeewon & Hyde (2016).

DNA extraction, PCR amplification and sequencing

Axenic cultures originating from single ascospores were grown on malt extract agar (MEA) for 14 days at 18–25 °C. Genomic DNA was extracted from the growing mycelium using the Biospin Fungus Genomic DNA Extraction Kit (BioFlux®) following the manufacturer's protocol (Hangzhou, P.R. China). DNA amplifications were performed by Polymerase Chain Reaction (PCR). The partial large subunit nuclear rDNA (LSU) was amplified by using primer pairs LROR and LR5 (Vilgalys & Hester 1990). Primer pairs ITS1 and ITS4 were used for amplification of internal transcribed spacer (ITS) regions (White et al. 1990). The RNA polymerase II second largest subunit (RPB2) gene was amplified by using primers fRPB2 and fRPB2-7cR (Liu et al. 1999, Sung et al. 2007). Beta-tubulin (β -TUB) gene was amplified by using primers Btub2fdG and Btub4fd (Woudenberg et al. 2009). Purified PCR profiles were as outlined by Chen et al. (2015) and Jeewon et al. (2002, 2003). PCR products were sequenced by Sangon Biotech (Shanghai), China using same primers as described above.

Phylogenetic analyses

Appropriate taxa for the analyses were initially selected following BLAST searches of GenBank (<http://www.ncbi.nlm.nih.gov/>). 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 (Table 1). Four different datasets were used to estimate phylogenies of the four genera (*Ascochyta*, *Boeremia*, *Didymella* and *Epicoccum*). *Ascochyta* and *Didymella* datasets were analysed to investigate phylogenetic relationships of two new records while *Boeremia* and *Epicoccum* datasets were analysed to substantiate establishment of new species. All introns and exons were aligned separately. Regions containing many leading or trailing gaps were removed from the LSU, ITS RPB2 and β -

TUB alignments prior to tree building. All sequences were obtained from GenBank. A Maximum likelihood analysis was performed at CIPRES using RAxML v.7.2.8 as part of the “RAxMLHPC2 on TG” tool (Stamatakis et al. 2008, Miller et al. 2010). The general time reversible model (GTR) using proportion of invariable sites were applied with a discrete gamma distribution and four rate classes.

Using MrModeltest 2.2, model of nucleotide substitution was performed (Nylander 2004). The Markov Chain Monte Carlo sampling (MCMC) in MrBayes v.3.0b4 were used to obtain Posterior probabilities (Rannala & Yang 1996, Huelsenbeck & Ronquist 2001, Zhaxybayeva & Gogarten 2002). Six simultaneous Markov chains were run from random trees for 2,000,000 generations and trees were sampled every 100th generation MCMC heated chain was set with a “temperature” value of 0.15. The distribution of loglikelihood scores were examined to determine stationary phase for each search and to decide if extra runs were required to achieve convergence, using the program Tracer 1.5 (Rambaut & Drummond 2007). The first 3000 trees were discarded as the burn-in phase. The remaining trees used for calculate posterior probabilities (split frequencies were reached to less than 0.01 in standard deviation). Near to each node are given Bayesian posterior probabilities equal or greater than 0.90. Phylogenetic trees were drawn using FigTree v. 1.4 (Rambaut & Drummond 2008). New strain sequences generated in this study are deposited in GenBank and accession numbers are under material examined.

Table 1. Comparison of alignment properties of genes and nucleotide substitution models used in the study.

Genes /loci	ITS	LSU	RPB2	β-TUB
Alignment strategy (MAFFT v6)	FFT-NS-2+manual	FFT-NS-2+manual	FFT-NS-2+manual	FFT-NS-2+manual
Nucleotide substitution models for Bayesian analysis (determined by MrModeltest)	GTR + I + G			

Results

Phylogeny

The combined ITS, LSU, RPB2 and β-TUB datasets were analyzed using ML and Bayesian analyses for *Ascochyta*, *Boeremia*, *Didymella* and *Epicoccum* (Figs 1, 2, 3, 4). The general time reversible model with inverse gamma rates (GTR + I + G) was selected by MrModeltest as the best for all four loci (Table 1). The single locus phylogenies of ITS, LSU and β-TUB resulted in lower resolution than the RPB2 phylogeny at the species level. The ITS, LSU, RPB2 and β-TUB single gene analyses also resulted in topologies that were congruent and therefore we analysed only the multigene DNA sequence data. All trees generated under different optimality criteria were similar in topology and did not differ significantly with respect to the position of the new taxa (data not shown). Maximum likelihood bootstrap values (MLBS) equal or greater than 70% and Bayesian posterior probabilities (BPP) greater than 0.90 are given near to each node (Figs. 1, 2, 3 & 4).

Phylogeny of *Ascochyta*

The final concatenated alignment contained 26 ingroup taxa with a total of 2, 748 characters including gaps (1327 characters for LSU, 492 for ITS, 597 for RPB2 and 332 for β -TUB). The best scoring tree for *Ascochyta* had final likelihood value of -5979.065132. The Bayesian analysis resulted in 20000 trees after 2,000,000 generations. The first 3000 trees, representing the burn-in phase of the analyses were discarded, while the remaining tree was used for calculating posterior probabilities in the majority rule consensus tree and is shown in Fig. 1. Maximum likelihood and Bayesian analysis showed that our isolate (MFLUCC 16-0599) grouped with *A. medicaginicola* (CBS 112.53, CBS 316.90, CBS 404.65) with high statistical support (Fig. 1; 100 MLBS /1 BPP). Therefore *A. medicaginicola* var. *medicaginicola*. (MFLUCC 16-0599) is a new record of *A. medicaginicola* var. *medicaginicola* from Russia.

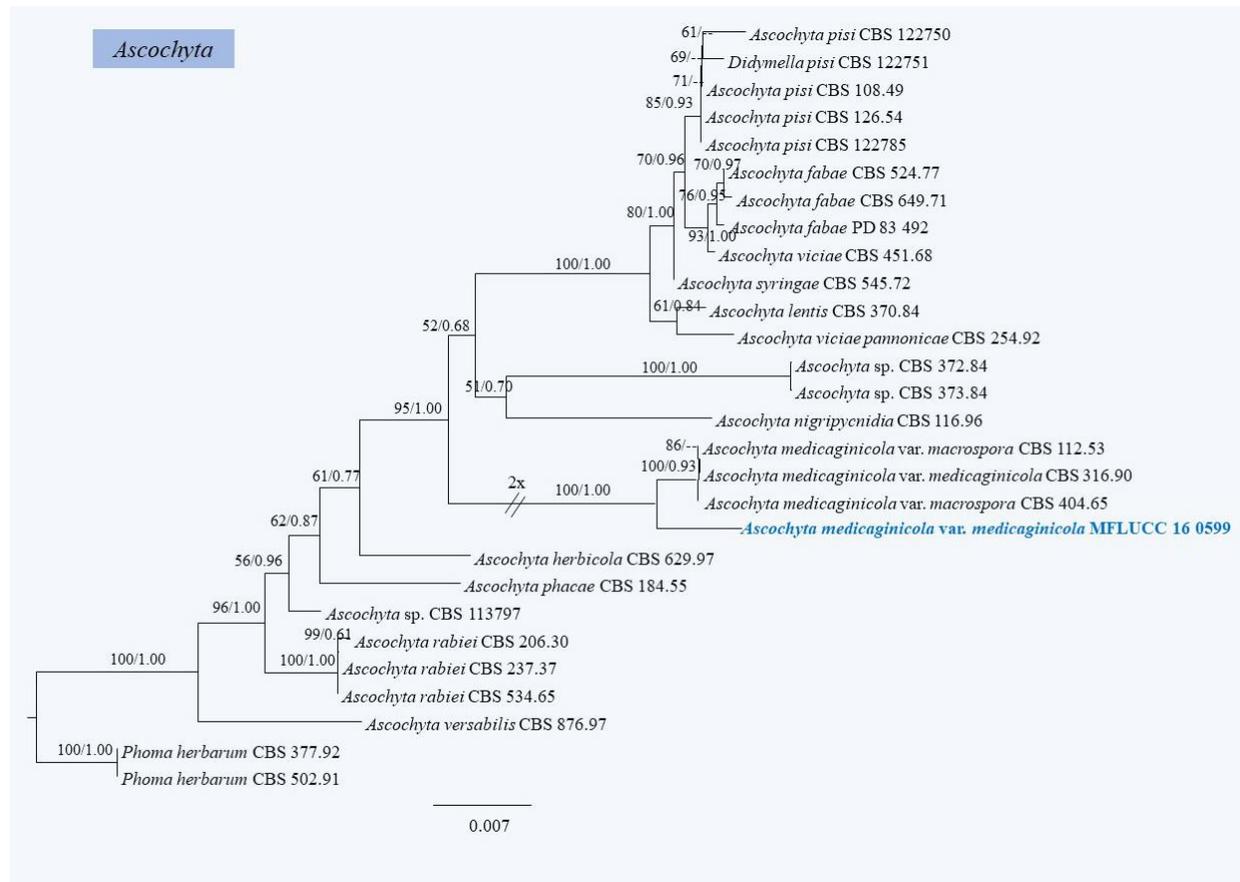


Fig. 1 – Phylogenetic tree inferred from a maximum likelihood analysis based on analyses of a concatenated alignment of LSU, RPB2, ITS and β -TUB sequence data of 26 strains representing the genus *Ascochyta*. The RAxML bootstrap support values above 70 % and Bayesian posterior probabilities above 0.90 are given above branches. One branch, indicated by two diagonal lines with the number of times it was shortened to fit the page. The tree is rooted to *Phoma herbarum* (CBS 377.92; CBS 502.91).

Phylogeny of *Boeremia*

The final concatenated alignment contained 34 ingroup taxa with a total of 2, 748 characters including gaps (1328 characters for LSU, 491 for ITS, 597 for RPB2 and 332 for β -

TUB). The best scoring tree for *Boeremia* genus had final likelihood value of -5435.934917. The Bayesian analysis resulted in 20000 trees after 2,000,000 generations. The first 3000 trees, representing the burn-in phase of the analyses were discarded, while the remaining tree was used for calculating posterior probabilities in the majority rule consensus tree and is shown in Fig. 2. Maximum likelihood and Bayesian analysis show that *Boeremia galiicola* is nested in between *B. lycopersici*, *B. foveata* and *B. hedericola* and all these taxa are basal to other *Boeremia* species (Fig. 2).

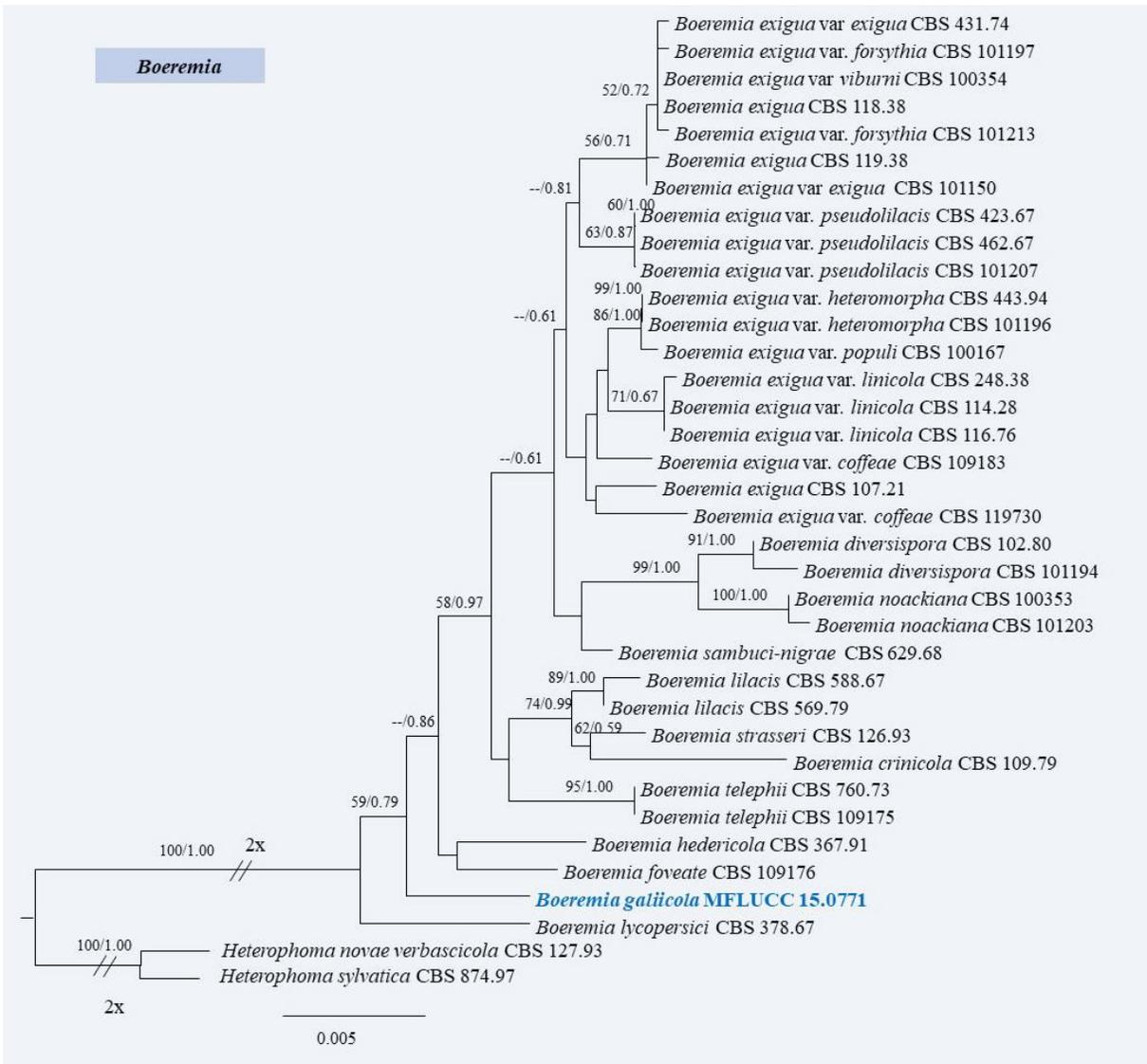


Fig. 2 – Phylogenetic tree inferred from a maximum likelihood analysis based on analyses of a concatenated alignment of LSU, RPB2, ITS and β -TUB sequence data of 34 strains representing the genus *Boeremia*. The RAxML bootstrap support values above 70 % and Bayesian posterior probabilities above 0.90 are given above branches. Two branches, indicated by two diagonal lines with the number of times a branch was shortened. The tree is rooted to *Heterophoma* sp. (CBS 127.93; CBS 874.97).

Phylogeny of *Didymella*

The final concatenated alignment contains 64 ingroup taxa with a total 2,764 characters including gaps (1327 characters for LSU, 492 for ITS, 596 for RPB2 and 349 for β -TUB). The best scoring tree for *Didymella* genus had a final likelihood value of -11996.202152. The Bayesian analysis resulted in 20000 trees after 2,000,000 generations. The first 3000 trees, representing the burn-in phase of the analyses were discarded, while the remaining tree was used for calculating posterior probabilities in the majority rule consensus tree and is shown in Fig. 3. In the maximum likelihood and Bayesian analysis, *D. macrostoma* clusters with other *D. macrostoma* strains with high support (Fig. 3).

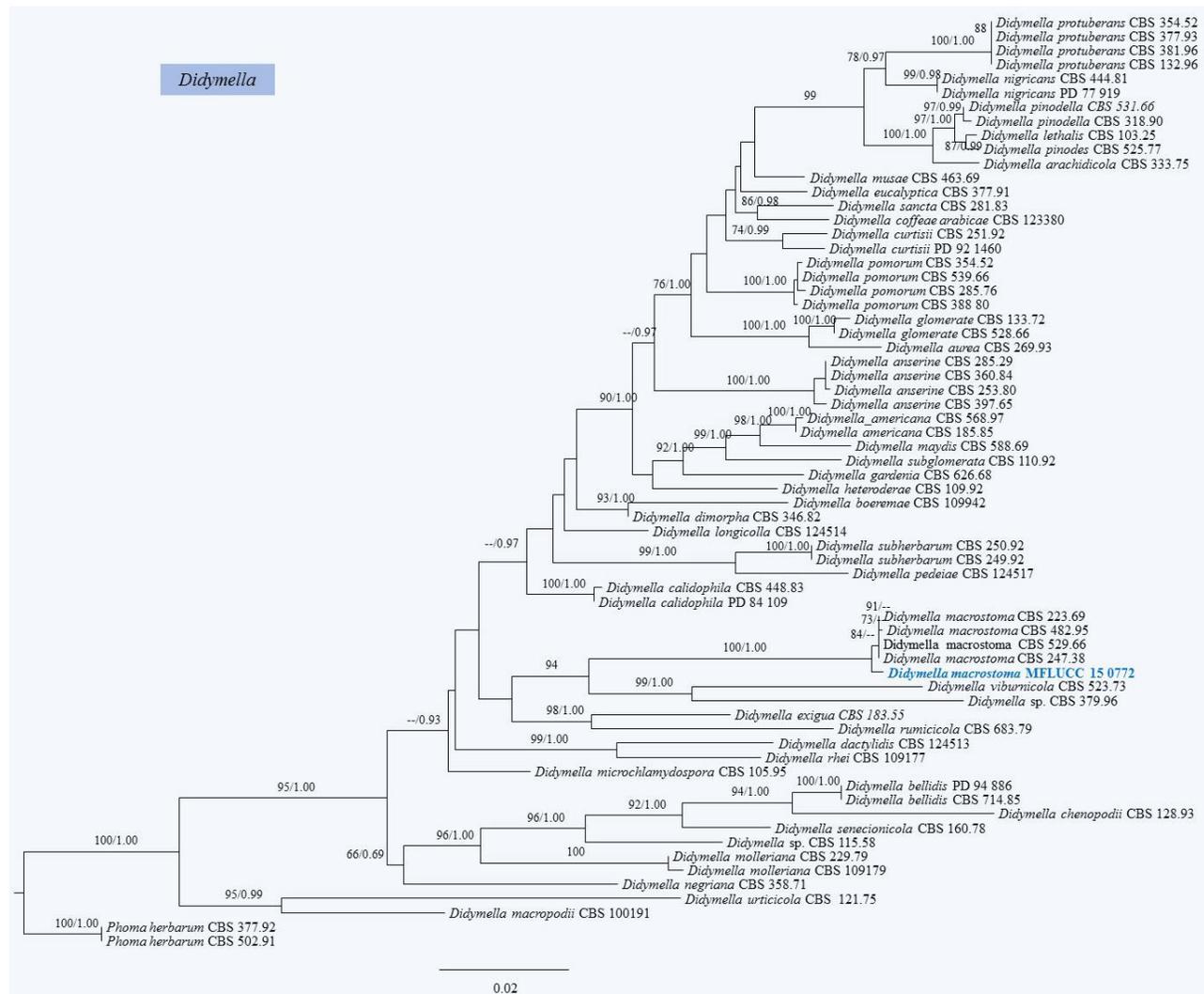


Fig. 3 – Phylogenetic tree inferred from a maximum likelihood analysis based on sequence analyses of a concatenated alignment of LSU, RPB2, ITS and β -TUB sequence data of 64 strains representing the genus *Didymella*. The RAxML bootstrap support values above 70 % and Bayesian posterior probabilities above 0.90 are given above branches. The tree is rooted to *Phoma herbarum* (CBS 377.92; CBS 502.91).

Phylogeny of *Epicoccum*

The final concatenated alignment contained 12 ingroup taxa with a total 2,763 characters including gaps (1327 characters for for LSU, 493 for ITS, 599 for RPB2, 344 and for β -TUB). The best scoring tree for *Epicoccum* genus was had a final likelihood value of 7336.036651. The Bayesian analysis resulted in 20000 trees after 2,000,000 generations. The first 3000 trees, representing the burn-in phase of the analyses were discarded, while the remaining tree was used for calculating posterior probabilities in the majority rule consensus tree and is shown in Fig. 4. Maximum likelihood and Bayesian analyses indicate that *Epicoccum mackenziei* is closely related to *E. nigrum* but constitutes an independent lineage with high statistical support (Fig. 4; 100 MLBS /1 BPP).

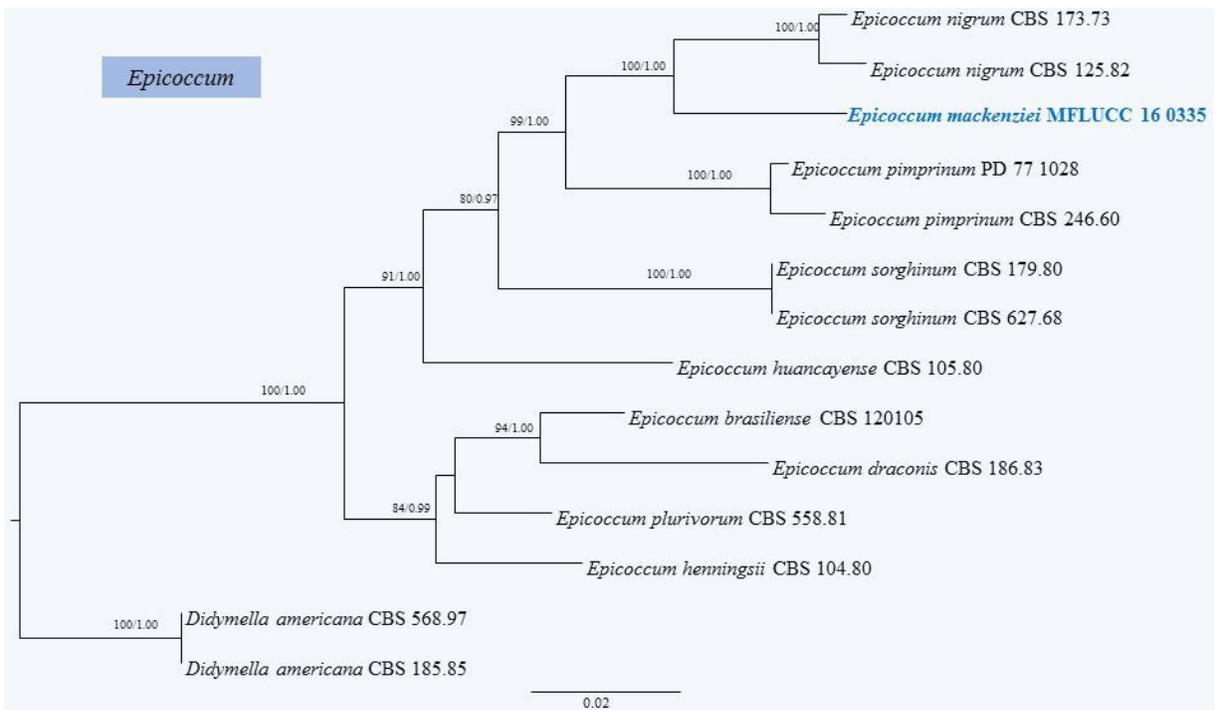


Fig. 4 – Phylogenetic tree inferred from a maximum likelihood analysis based on analyses of a concatenated alignment of LSU, RPB2, ITS and β -TUB sequence data of 12 strains representing the genus *Epicoccum*. The RAxML bootstrap support values above 70 % and Bayesian posterior probabilities above 0.90 are given above branches. The tree is rooted to *Didymella americana* (CBS 568.97; CBS 185.85).

Taxonomy

Ascochyta medicaginicola var. *medicaginicola* Q. Chen & L. Cai 2015

Figs 5–7

≡ *Phoma medicaginis* var. *medicaginis* Malbr. & Roum., Rev. Mycol. 8: 91. 1886.

Facesoffungi number: FoF 02500

Pathogenic on living branches of *Melilotus albus* noticeable as black, circular dots on the host surface. **Sexual morph:** *Ascomata* 165–190 μ m high, 170–210 μ m wide, scattered or gregarious, superficial to immerse on the substrate, black, subglobose to globose. *Ostiole* narrow, without periphyses. *Peridium* 18–41 μ m thick, 3–4-layered, equally thick at the apex and base,

with ostiole thicker than other areas, composed of dark brown cells of *textura angularis*. *Hamathecium* lacking pseudoparaphyses. *Asci* 50–84 × 8–14 μm, 8-spored, bitunicate, obclavate to cylindrical, short pedicellate, with a small ocular chamber. *Ascospores* 12–16 × 3–5 μm (\bar{x} = 14 × 4.5 μm; n = 20), obliquely to irregularly uniseriate to biseriate, unequally 2-celled, with rounded, wider apical cell tapering at apex in some, smaller and conical at base, ellipsoid to obovoid, straight, guttulate, hyaline, constricted at the septum, smooth-walled, lacking a mucilaginous sheath. **Asexual morph:** coelomycetous (Fig. 7). *Conidiomata* 200–250 μm high, 300–350 μm diam., pycnidial, solitary, scattered or gregarious, globose, semi-immersed to superficial, unilocular, dark brown, without ostiole. *Conidiomata wall* 15–30 μm wide composed of hyaline to dark brown cells of *textura angularis*, many layers, equally thickening. *Conidiophores* reduced to conidiogenous cells, forming from the inner layer of wall cells of the conidiomata. *Conidiogenous cells* 4–10 μm long × 3–5 μm wide, hyaline, phialidic, globose to bottle-shaped. *Conidia* 4–8 × 1.5–3 μm wide (\bar{x} = 5.5 × 2 μm, n = 20), ellipsoidal to cylindrical, rounded at both ends, hyaline, straight, guttulate, aseptate, thin and smooth-walled.

Culture characters – Colonies 15–20 mm diameter on MEA after one month at 25 °C, grey to brown, outer layer off white, flattened with dense, circular colony middle in yellow, filamentous, aerial, fluffy hyphae; reverse black in the middle, off white hyphae go through black part, white at the edge layer and without any diffusible pigments.

Material examined – RUSSIA, Arkhangelsk Region, Arkhangelsk City, wood waste landfill, on living branch of *Melilotus albus* Medik. (*Fabaceae*), 2 August 2015, G.V. Okatov AR 055 (MFLU 15-3195); HKAS, living cultures MFLUCC 16-0599; KUMCC 16-0068, GenBank accession numbers LSU: KX698025, ITS: KX698036, RPB2: KX698033, β-TUB: KX698029.



Fig. 5 – a *Melilotus albus*. host of *Ascochyta medicaginicola* var. *medicaginicola* b Symptoms on host branch.

Notes – This is the first record of sexual morph for *Ascochyta medicaginicola* and characters clearly indicate that it is identical to *A. medicaginicola* var. *medicaginicola* (Boerema et al. 2004, Chen et al. 2015). The multigene phylogenetic analysis shows that this collection groups in the *Ascochyta medicaginicola* clade with 100 MLBS/1.00 PP bootstrap support (Fig. 1). This collection also fits with the generic concept of *Ascochyta* in having erumpent, flattened ascomata with an ostiole, bitunicate, subcylindrical to subclavate, slightly curved asci with short pedicels, and ovoid to ellipsoidal, hyaline, asymmetric, 1-septate ascospores (Jellis & Punithalingam, 1991, Trapero-Casas & Kaiser, 1992, Kaiser et al. 1997, Chilvers et al. 2009).

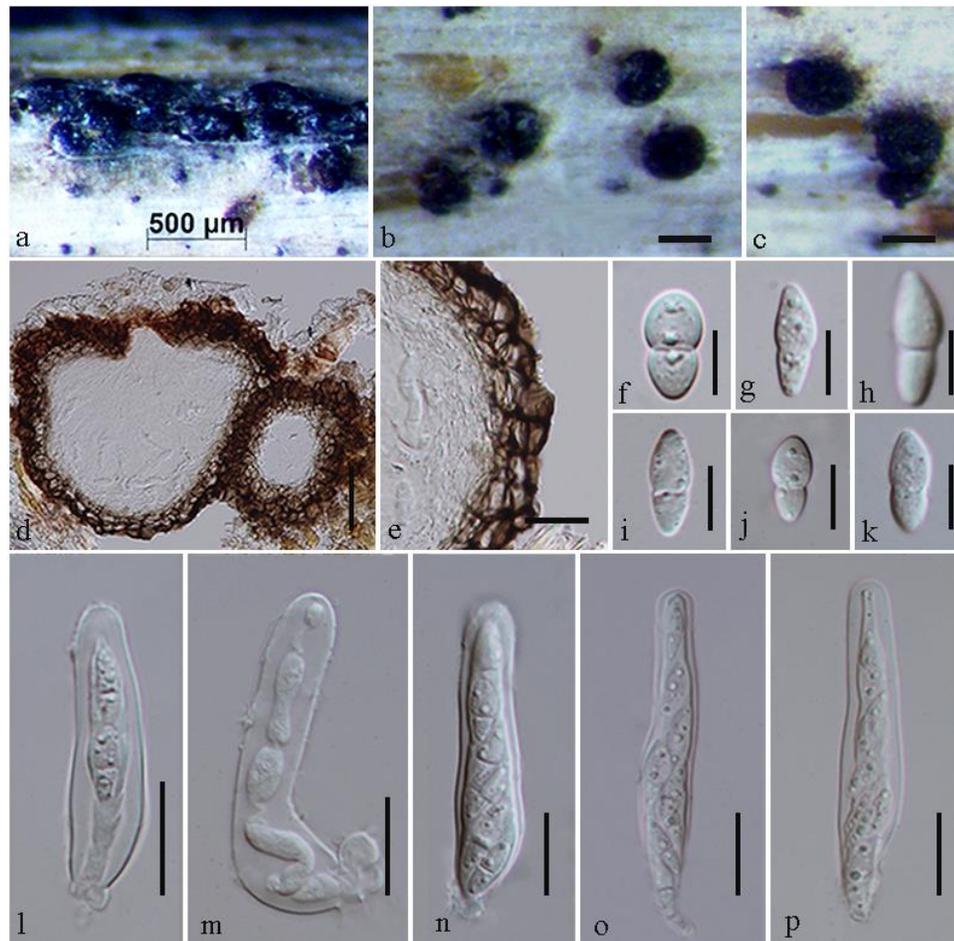


Fig. 6 – *Ascochyta medicaginicola* var. *medicaginicola*. a–c Host substrate with ascomata. d Section through ascoma. e Peridium. f–k Ascospores. l–p Asci. Scale bars: b, c = 200 μ m, d = 50 μ m, e = 30 μ m, f–k = 10 μ m, l–p = 20 μ m.

Boeremia galiicola Jayasiri, Camporesi & K.D. Hyde, *sp. nov.*

Figs 8–9

Index Fungorum number: IF552360, *Facesoffungi* number: FoF 02501.

Etymology: The specific epithet *galiicola* is based on the host genus from which the holotype was collected.

Holotype: MFLU 15-2279

Saprobic on a dead stem of *Galium* sp., forming black circular spots on the host surface. **Sexual morph**: *Ascomata* 125–200 (–270) μ m high \times 150–250 μ m wide (\bar{x} = 180 \times 200 μ m, n = 10), immersed, without subiculum covering the host, solitary, globose to subglobose, brown to dark brown. *Ostiole* central, without periphyses. *Peridium* 30–60 μ m wide, 6–8 rows of cells, with outer part comprising rows of dark brown cells, with inner 3–4 row, comprising hyaline cells of *textural angularis*. *Hamathecium* 1.5–2 μ m wide, filiform to cylindrical pseudoparaphyses. *Asci* 65–81 \times 7–11 μ m (\bar{x} = 78 \times 9 μ m, n = 20), 8-spored, bitunicate, fissionate, slightly curved, cylindrical-clavate, short-pedicellate, smooth-walled, apically rounded, with an ocular chamber. *Ascospores* 17–22 \times 2.5–5 μ m (\bar{x} = 20 \times 4 μ m, n = 20), ellipsoid to obovoid, hyaline, 1-septate, constricted at septum, 1–3-seriate, overlapping, widest at the center and tapering toward narrow ends, straight or slightly curved, guttulate, thick and smooth-walled. **Asexual morph**:

coelomycetous (Fig. 9). *Conidiomata* 70–80 μm high, 90–100 μm diam., pycnidial, globose, separate, lacking ostioles. *Conidiophores* 4–7.5 μm long \times 0.5–1 μm wide, attached together in a bunch, cylindrical, hyaline. *Conidiogenous cells* 2–3.5 μm long \times 0.5–1 μm wide, holoblastic, cylindrical, hyaline, smooth, rounded at tip. *Conidia* 2.5–3.5 μm long \times 0.5–1.5 μm wide, hyaline, cylindrical, truncate at both ends, aseptate, smooth-walled.

Culture characters – Ascospores germinated on MEA within 24 h and germ tubes produced from an end. Colonies growing on MEA, reaching 30 mm diam. after 5 days at 16–18°C, flat, margin crenate, forward white to olivaceous grey, reverse yellowish brown, fine mycelium, medium dense to dense, septate, hyaline.

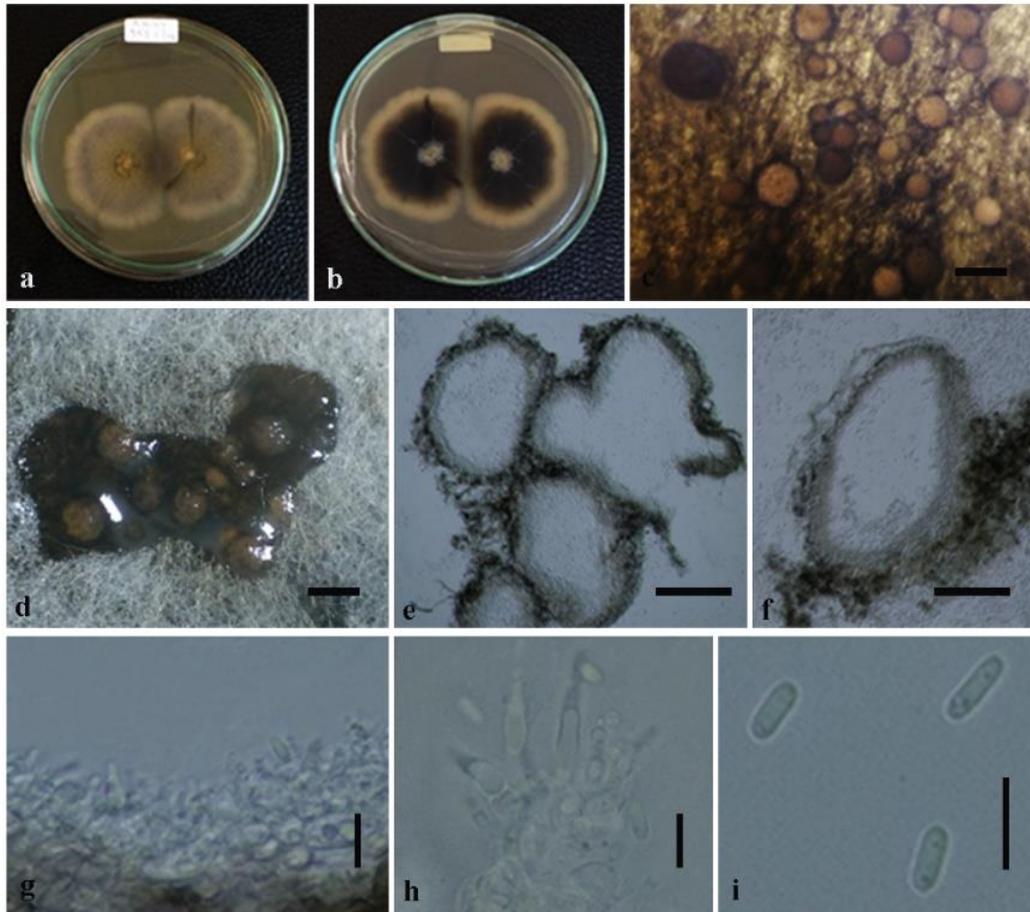


Fig. 7 – Asexual morph of *Ascochyta medicaginicola* var. *medicaginicola* (from culture). a Top view of colony on PDA. b Reverse view of colony. c, d Conidiomata in culture. e, f Section through conidiomata. g, h Conidiogenous cells. i Conidia. Scale bars: a, b = 3 cm, c, d = 200 μm , e, f = 100 μm , g = 20 μm , h, i = 10 μm .

Material examined – ITALY, Province of Arezzo [AR], Papiano Alto - Stia, on dead aerial stem of *Galium* sp. (*Rubiaceae*), 12 May 2014, E. Camporesi IT1866 (MFLU 15-2279, **holotype**); (isotype in PDD), ex-type living culture, MFLUCC 15-0771; KUMCC 16-0069; BCC, GenBank accession numbers LSU: KX698026, ITS: KX698037, β -TUB: KX698030.

Notes – *The* genus *Boeremia* represents species that are morphologically similar to what is currently known as *Phoma exigua* Desm. (Aveskamp et al. 2010), but molecular data separates

them into two distinct groups (Aveskamp et al. 2010). *Boeremia galiicola* fits into the generic concept of *Boeremia* in having subglobose pseudothecial ascomata, 8-spored, bitunicate, subcylindrical to subclavate asci and ellipsoidal, 1-septate ascospores (Aveskamp et al. 2010). Molecular data indicate that *Boeremia galiicola* is phylogenetically distinct from other *Boeremia* species. A close phylogenetic affinity is noted between *Boeremia lycopersici* (Cooke) Aveskamp et al. and *B. galiicola* (Fig. 2), but pairwise sequence alignment of LSU, ITS and β -TUB reveals a difference of 2, 3 and 12 base pair respectively, and therefore, they are not conspecific. The sexual morph is only rarely recorded for *Boeremia lycopersici* in nature, with subglobose ascomata, up to 300 μm diam., asci cylindrical or subclavate, measuring $50\text{--}95 \times 6\text{--}10 \mu\text{m}$, always 8-spored, biseriate and ascospores ellipsoid, measuring $12\text{--}18 \times 5\text{--}6 \mu\text{m}$, uniseptate (Aveskamp et al. 2010). *Boeremia galiicola* shares these characters, however, it differs from *B. lycopersici* in characters of ostiole, peridium and hamathecium. *Boeremia galiicola* is only the second sexual morph recorded for this genus.

Didymella macrostoma (Mont.) Q. Chen & L. Cai 2015

Figs 10–11

Facesoffungi number: FoF 02502

Saprobic on a dead branch of *Ailanthus altissima* (P. Mill), noticeable as minute black dots on host surface. **Sexual morph:** *Ascomata* 100–150 μm high \times 125–175 μm wide (\bar{x} = 132 \times 154 μm , n = 10), immersed or erumpent, globose to subglobose, solitary or scattered, brown to dark brown, without subiculum covering host. *Ostiole* central, opening to the outside. *Peridium* 10–23 μm wide, equally thickened, comprising dark brown cells of *textura angularis*. *Hamathecium* without pseudoparaphyses. *Asci* 82–95 \times 9–13 μm (\bar{x} = 89 \times 11 μm , n = 10), 8-spored, bitunicate, fissitunicate, cylindric-clavate, smooth-walled, slightly curved, apically rounded, short pedicellate, with an ocular chamber. *Ascospores* 18–20 \times 5–7 μm (\bar{x} = 19 \times 6 μm , n = 20), 1–2-seriate, overlapping in the ascus, ellipsoid to obovoid, hyaline, 1-septate, constricted at the septum, lower cell longer and narrower than upper cell, widest at the center and tapering towards the end, straight or slightly curved, thin and smooth-walled. **Asexual morph:** coelomycetous (Fig. 11). *Conidiomata* 150–250 μm high, 250–350 μm diam., pycnidial, globose, superficial to subperidermal, unilocular, separate, thin-walled, non-ostiolate. *Conidiomata wall* 11–21 μm wide, composed of 2–3 layers, outer dark brown and inner hyaline cells of *textura angularis*. *Conidiophores* reduced to conidiogenous cells. *Conidiogenous cells* 6–11 \times 3–5 μm , holoblastic, hyaline, smooth-walled, short, unbranched, globose, formed from the innermost layer of wall cells. *Conidia* 6–11 \times 2.5–5 μm (\bar{x} = 7.5 \times 3.3 μm ; n = 20), hyaline, oval to globose, guttulate, rounded at both ends, aseptate, smooth-walled.

Culture characters – Ascospores germinated on MEA and germ tube produced from an end within 24 h. Colonies reaching 40–50 mm diam. after 7 days at 16–18°C on MEA, no clear margin, upper dark brown to grey and covered by white mycelia, lower dark brown, when reached maximum growth reddish grey and formed asexual structures, fine mycelium, medium dense to dense, septate, hyaline.

Material examined – ITALY, Province of Forlì-Cesena [FC], near Lago Pontini - Bagno di Romagna, on dead aerial branch of *Ailanthus altissima* (Mill.) Swingle (*Simaroubaceae*), 21 May 2015, E. Camporesi IT2501 (MFLU 15-1319); HKAS, living cultures MFLUCC 15-0772; KUMCC 16-0070, GenBank accession numbers LSU: KX698027, ITS: KX698038, RPB2: KX698034, β -TUB: KX698031.



Fig. 8 – *Boeremia galiicola* (from holotype). a, b Ascomata on host surface. c, d Sections through ascomata. e Peridium. f Pseudoparaphyses. g–j Asci. k–o Ascospores. p Germinated ascospore. Scale bar: c, d = 100 μm , e, g–j, p = 20 μm , e, g = 30 μm , f, k–o = 10 μm .

Notes – The genus *Didymella* was emended to accommodate *Peyronellaea* and several other associated phoma-like species that are phylogenetically related to *D. exigua* the type species of *Didymella* (Chen et al. 2015). Our fungus was found as the sexual morph in nature but

produced the asexual morph in culture. The collection can be accommodated in *Didymella* as it is characterized by having immersed, flattened ascomata with ostioles, bitunicate, subcylindrical to subclavate, slightly curved asci with short pedicels and cymbiform to ellipsoidal, hyaline, asymmetrical aseptate ascospores. Our isolate is phylogenetically close to *D. macrostoma* (Mont.) Q. Chen & L. Cai (CBS 223.69, CBS 482.95, CBS 529.66 and CBS 247.38) with only three base pair differences in the RPB2 gene. In addition, it is similar in having globose to irregular confluent pycnidia, globose to bottle-shaped conidiogenous cells, and subglobose to ellipsoidal, hyaline ascospores with guttules. However, the non-ostiolate pycnidia are different from *D. macrostoma* (MFLUCC 15-0772). Our collection of *D. macrostoma* (MFLUCC 15-0772) and *D. sancta* (Aveskamp, Gruyter & Verkley) Q. Chen & L. Cai share the same host species, but they are phylogenetically distinct. *Didymella sancta* also differs from *D. macrostoma* (MFLUCC 15-0772) in having, macro- and micropycnidia with thick-walled, large chlamydospores and relatively short conidia (*D. sancta* 5–7 μm ; *D. macrostoma* 6–11 μm conidia).

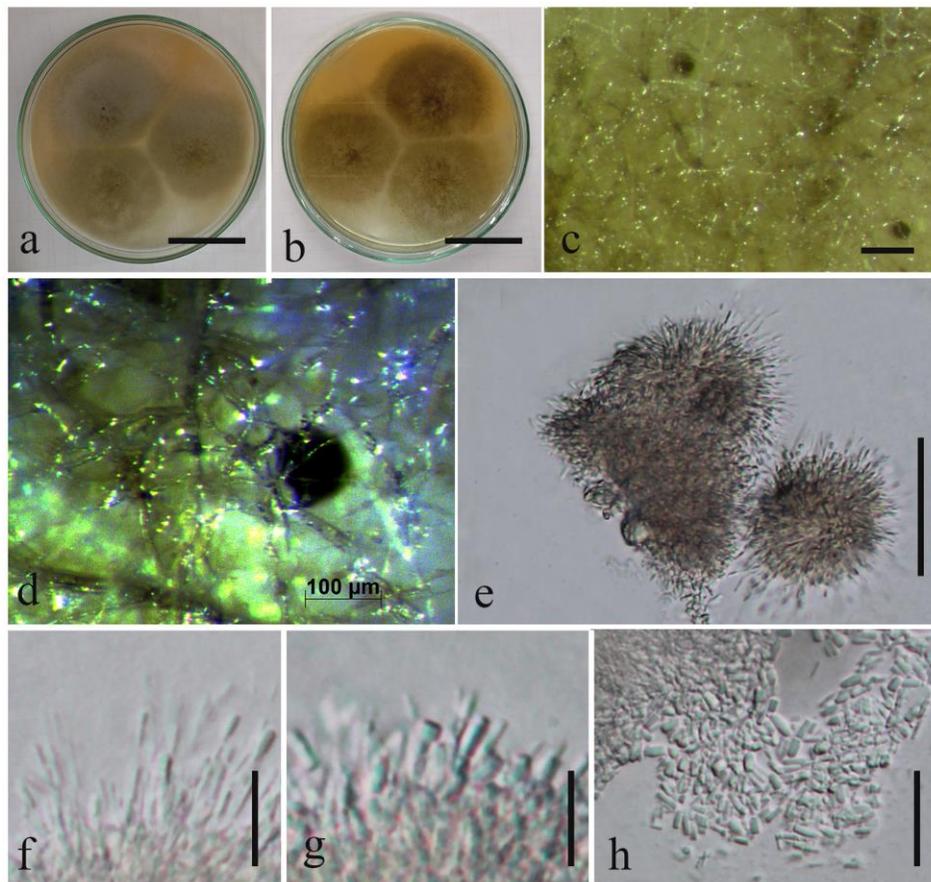


Fig. 9 – Asexual morph of *Boeremia galiicola*. a Top view of colony on PDA. b Reverse view of colony. c, d Conidiomata in culture. e Squashed conidioma. f, g Conidiogenous cells. h Conidia. Scale bars: a, b = 3 cm, c = 500 μm , e = 100 μm , f-h = 10 μm .

Epicoccum mackenziei Jayasiri, Camporesi & K.D. Hyde, *sp. nov.*

Figs 12–13

Index Fungorum number: IF552362, *Facesoffungi* number: FoF 02503

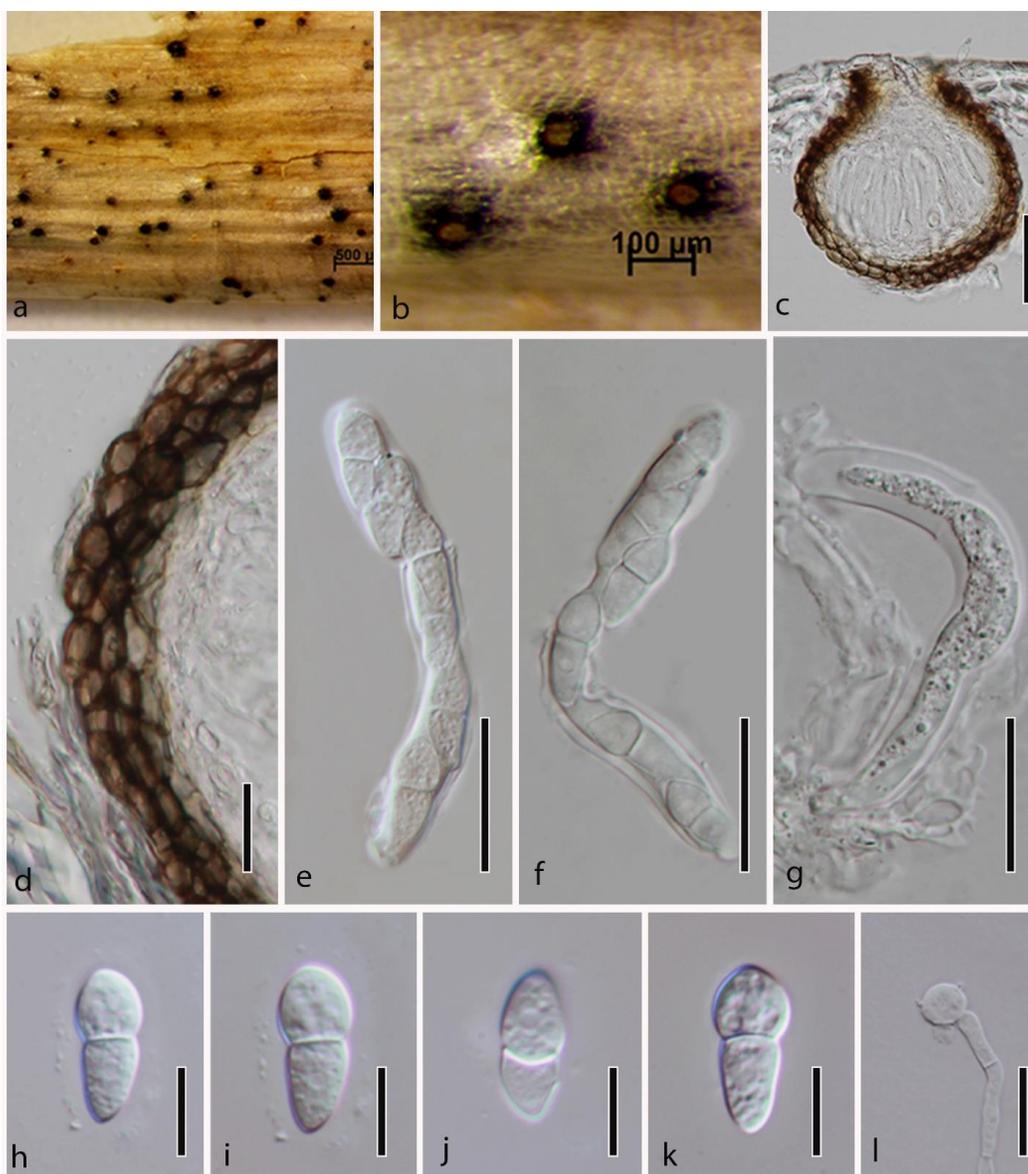


Fig. 10 – *Didymella macrostoma*. a, b Ascomata on host surface. c Section through ascoma. d Peridium. e–g Asci. h–k Ascospores. l Germinated ascospore. Scale bar: c = 50 μm, d= 20 μm, e–g = 30 μm, h–l = 10 μm.

Etymology: With reference to Dr. Eric McKenzie for his contribution to the study of microfungi

Holotype: MFLU 15-2601

Fungicolous or *saprobic* on dead stem of *Ononis spinosa*. **Sexual morph**: *Ascomata* 250–270 μm high × 205–245 μm width (\bar{x} = 260 × 230 μm, n = 10), immersed or superficial, globose, conical globose to lenticular, scattered or clustered, papillate or apapillate, ostiolate. *Peridium* 32–55 μm thick, composed of several layers of brown to hyaline cells of *textura angularis*, fusing at the outside with the host ascomata. *Hamathecium* with 1.5–2 μm wide, dense, filamentous, septate, branching and hyaline, cellular pseudoparaphyses. *Asci* 85–120 × 6.5–15 μm (\bar{x} = 99.4 × 11.3 μm, n = 20), 8-spored, bitunicate, fissitunicate, clavate to cylindrical,

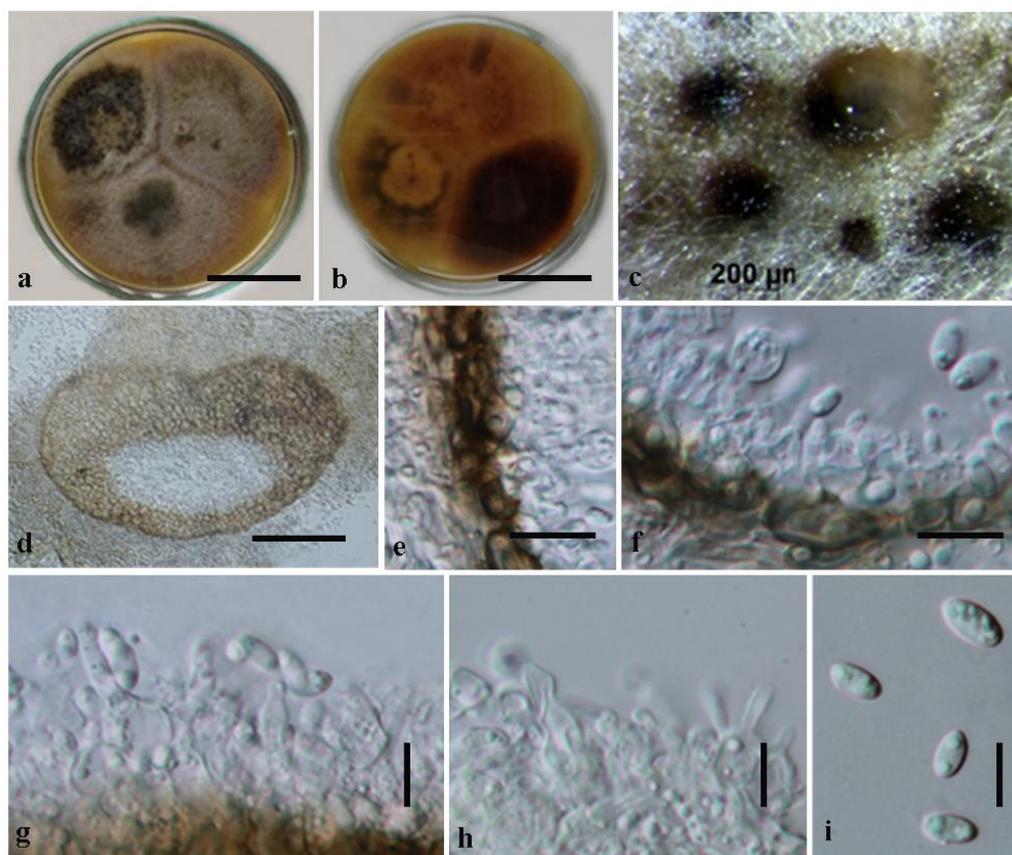


Fig. 11 – Asexual morph of *Didymella macrostoma* (from culture). a Top view of colony on PDA. b Reverse view of colony. c Conidiomata in culture. d Section through conidioma. e Cells of conidiomata wall. f–h Conidiogenous cells. i Conidia. Scale bars: a, b = 4 cm, d = 100 μm , e, f = 20 μm , g–i = 10 μm .

short-pedicellate, rounded at apex, with an ocular chamber. *Ascospores* 21–25 \times 2–6 μm (\bar{x} = 23.5 \times 4.3 μm , n = 20), overlapping 2–3-seriate, cylindrical to cylindrical-clavate, hyaline, 1–3-septate, constricted at middle septum, containing up to four refractive oil globules, irregular, hyaline, gelatinous sheath observed when mounted in Indian ink. **Asexual morph:** Not observed conidiomata in culture. *Chlamydospores* 9–16 μm \times 7–15 μm (\bar{x} = 13 \times 12 μm , n = 20), variable, irregular, unicellular or multicellular, intercalary or terminal, solitary or in chains, smooth, verruculose or incidentally tuberculate, subhyaline to dark brown, when multicellular globose or irregular-shaped (Fig. 13).

Culture characters – Ascospores germinated producing germ tubes near the septa on MEA within 24 h. Colonies reaching 20 mm diam. after 5 days at 25°C, irregular at margin, white to olivaceous grey, with white mycelial groups in the colony, middle black gummy substances (chlamydospores).

Material examined – ITALY, Province of Forlì-Cesena [FC], near Passo dei Mandrioli - Bagno di Romagna, on dead aerial stem of *Ononis spinosa* L. (*Fabaceae*), 31 August 2015, Erio Camporesi IT 2593 (MFLU 15-2601, **holotype**), (isotype in PDD), ex-type cultures, MFLUCC 16-0335; KUMCC 16-0071; BCC GenBank accession numbers LSU: KX698028, ITS: KX698039, RPB2: KX698035, β -TUB: KX698032.

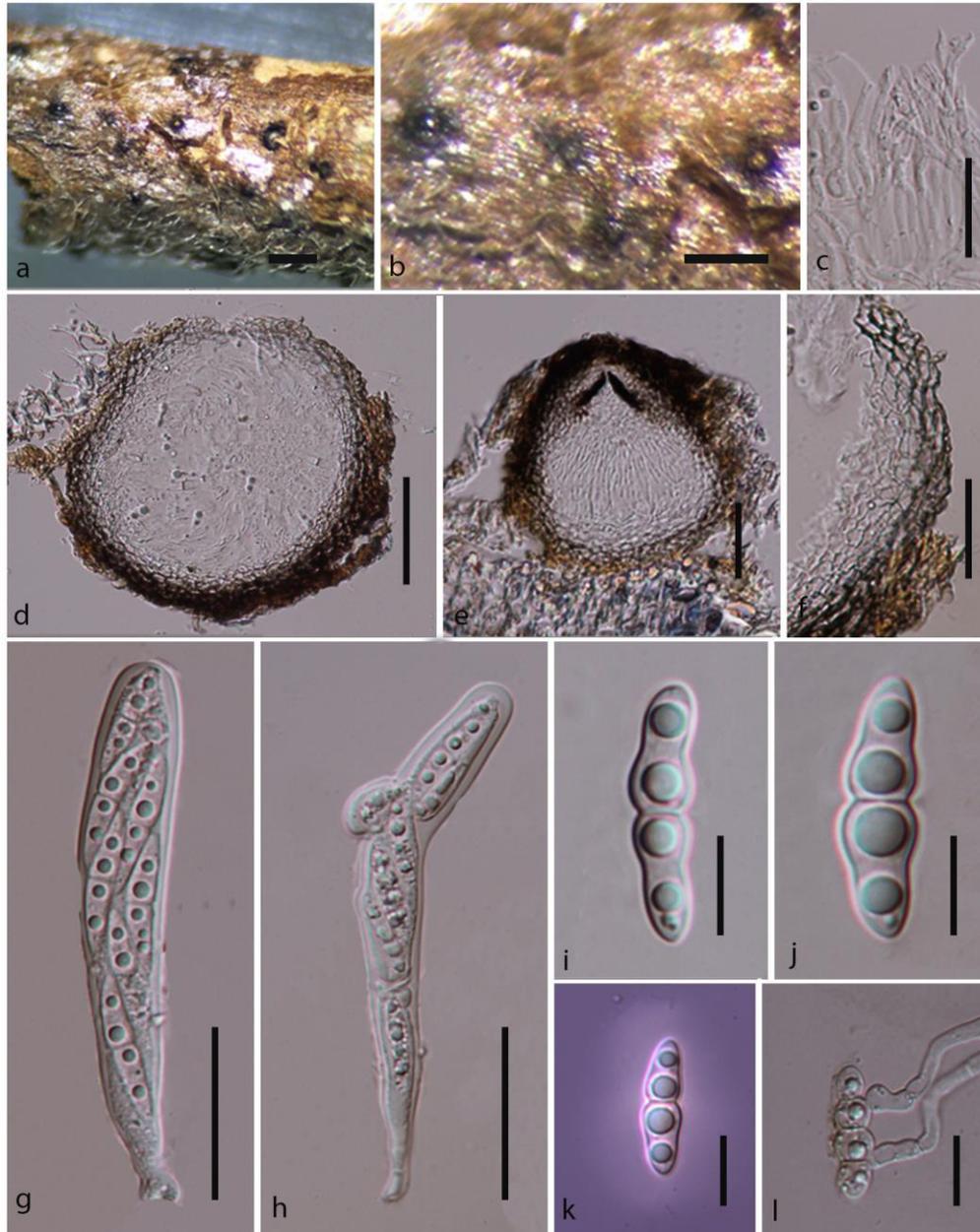


Fig. 12 – *Epicoccum mackenziei* (holotype). a, b Ascomata on host surface. c Pseudoparaphyses. d, e Section through ascomata. f Peridium. g, h Asci. i–j Ascospores. k Ascospore in Indian ink. l Germinated ascospore. Scale bars: a, b = 500 μ m, c = 10 μ m, d, e = 100 μ m, f = 50 μ m, g–h = 30 μ m, i–l = 10 μ m.

Notes – This fungus was found as the sexual morph in nature and as chlamydospores in culture. This species fits with the generic concept of *Epicoccum* in having variable and irregular, multicellular, intercalary or terminal subhyaline chlamydospores in chains (Punithalingam et al. 1972, Boerema et al. 2004, Aveskamp et al. 2010). There is no previous record regarding the sexual morph of this genus. Multigene DNA sequences indicate that *Epicoccum mackenziei* is phylogenetically distinct from other species and its distinctiveness is highly supported (100% MLBS/1.00 PP, 98% MLBS/1.00 BPP, respectively) from *E. nigrum* Link (CBS 125.82, CBS

173.73) and *E. pimprinum* (P.N. Mathur, S.K. Menon & Thirum.) Aveskamp, de Gruyter & Verkley (PD 77/1028, CBS 246.60). *Epicoccum pimprinum* and *E. nigrum* have 72 and 66 base pair differences within the RPB2 DNA sequence when compared to *E. mackenziei* respectively. Unfortunately, there are no further characters to compare these taxa as this is the first record of a sexual morph and only chlamydospores were observed in culture. This is also the first record of an *Epicoccum* species from an *Ononis* host (Fabaceae) (Chen et al. 2015, Farr et al. 2017).

Epicoccum has a hyphomycetous synanamorph (Chen et al. 2015) which is characterized by dark sporodochia with branched conidiophores and mono- to polyblastic, colourless conidiogenous cells that produce coloured, sometimes verruculose, dictyoconidia (Seifert et al. 2011). The other synanamorph known for the genus is a coelomycetous one, characterized by formation of conidia in pycnidial conidiomata (Chen et al. 2015).

Discussion

The phenotypic concept based on morphological and physiological characteristics was the classic approach for delimiting species (Inui et al. 1965, Guarro et al. 1999). The use of morphology and host specificity to recognize plant-associated fungi may have resulted in the description of an excessive number of species, with few character differences (Hibbett et al. 2007). The generic concept of *Phoma* is broadly defined, with nine sections being recognized based on morphological characters (de Gruyter et al. 2009). Species belonging to the genus *Phoma* and related coelomycetes are often encountered as serious plant pathogens (de Gruyter et al. 2009, Chen et al. 2015). Furthermore, it has been suggested that the *Phoma* classification system could be improved by adding DNA phylogenetic data, and delineating more natural groups (Grondona et al. 1997, Torres et al. 2005). Chen et al. 2015 was prompted by the question of how to delineate natural genera in the *Ascochyta-Didymella-Phoma* complex, which represents a dilemma to plant pathologists and mycologists alike (Chilvers et al. 2009, Aveskamp et al. 2010, Hyde et al. 2013). Recent studies such as those of Aveskamp et al. (2010), de Gruyter et al. (2009) and Chen et al. (2015), have revised the classification of the *Didymellaceae* by using combined multi-locus data of ITS, LSU, RPB2 and β -TUB in their phylogenetic analyses.

In this study, we provide phylogenetic trees for *Ascochyta*, *Boeremia*, *Didymella* and *Epicoccum* using as much vouchered sequence data as possible. Two new species and two new records are proposed herein with support from our analyses of ITS, LSU, RPB2 and β -TUB sequence data. *Boeremia galiicola* is phylogenetically distinct from other *Boeremia* species with close phylogenetic affinity to *Boeremia lycopersici* (2, 3 and 12 base pair differences within LSU, ITS and β -TUB regions, respectively). *Boeremia galiicola* shares same ascotal, features asci and ascospores characters with *B. lycopersici*, however, it differs in lacking the ostiole, peridium and hamathecial characters of *B. lycopersici*. Berner et al (2015) also investigated the phylogeny of *Boeremia exigua* var. *rhapontica* based on actin, β -TUB, calmodulin, elongation factor, and ITS genes. However, we also obtained the same topology using ITS, LSU, RPB2 and β -TUB genes as did Chen et al (2015). *Epicoccum mackenziei* is the first sexual morph recorded for the genus *Epicoccum* in nature and only chlamydospores were observed in the culture. Therefore, this species is introduced based on the sexual morph and phylogenetic data. *Epicoccum mackenziei* is phylogenetically distinct from *E. nigrum* and *E. pimprinum* with high statistical support. Our *Ascochyta* species (formerly *Phoma medicaginis*) is a new collection of *Ascochyta medicaginicola* (Boerema et al. 2004, Chen et al. 2015) from Russia on *Melilotus albus*. This collection further substantiates a host parasite specialization of this fungus on

Melilotus albus (Fabaceae). The multigene phylogenetic analysis shows that this species groups in the *Ascochyta medicaginicola* clade with high statistical support. In addition, there are morphological similarities in conidial and ascomatal characters that support its inclusion in *Ascochyta medicaginicola*. However, this is the first record of an *Ascochyta* species from Russia and it differs from *As. nigripynidia* by having septate conidia, country of origin and host genus (*Vicia*, Fabaceae). Our *Didymella* isolate is phylogenetically close to *D. macrostoma* with only three base pair differences in the RPB2 sequenced region. In addition, they share similar features in having globose to irregular confluent pycnidia, globose to bottle-shaped conidiogenous cells, and subglobose to ellipsoidal, hyaline ascospores with guttules. Therefore, in this study our isolate is maintained as a new record of *D. macrostoma* from Italy.

The present study indicates that the total number of *Didymellaceae* species is possibly high and warrants further investigations, and this could be considered as a step towards a systematic research of *Didymellaceae* in temperate areas such as Europe and Russia.

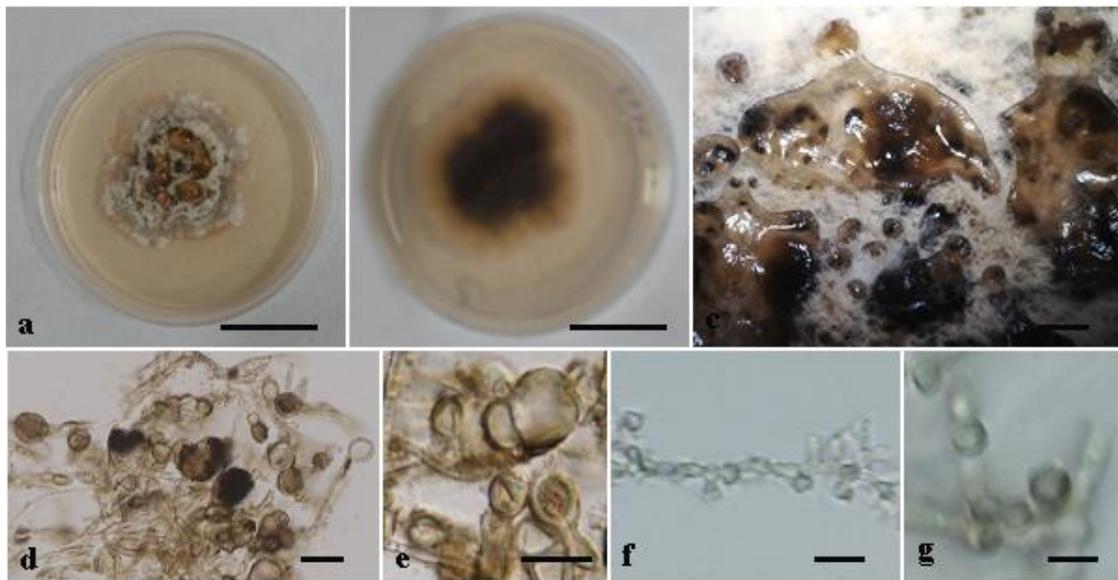


Fig. 13 – *Epicoccum mackenziei* (ex-type culture). a Top view of colony on PDA. b Reverse view of colony. c Asexual structures in culture. d–g Chlamydospores in culture. Scale bars: a, b = 2 cm, c = 500 μ m, d, e = 10 μ m, f, g = 5 μ m.

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