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Meristematic and meristematic-like fungi in *Dothideomycetes*

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Abstract: Meristematic fungi are mainly defined as having aggregates of thick-walled, melanised cells enlarging and reproducing by isodiametric division. *Dothideomycetes* black meristematic and meristematic-like fungi have been allied to *Myriangiales*, which currently has two accepted families, *Myriangiaceae* and *Elsinoaceae*, with fungi mainly regarded as pathogens, parasites, saprobes and epiphytes of different plant species. This study aimed to verify the phylogenetic position using four nuclear markers (SSU, LSU, ITS and *RPB2*) of the *incertae sedis* genera associated with *Myriangiales*, namely *Endosporium*, *Gobabebomyces*, *Lembosiniella* and *Phaeosclera*, and the new genus, *Endophytium gen. nov*. (including *E. albocacti sp. nov*. and *E. cacti sp. nov*.), established for endophytic fungi occurring in cacti in Brazil. Based on morphology, lifestyle and phylogenetic inferences, these black meristematic and meristematic-like fungi cannot be accommodated in *Myriangiales*. Combining these results, three new orders and two new families are introduced: *Endophytiales ord. nov*. (including *Endophytiaceae fam. nov*. for *Endophytium gen. nov*.), *Endosporiales ord. nov*. (including *Endosporiaceae* for *Endosporium*) and *Phaeosclerales ord. nov*. (including *Phaeoscleraceae fam. nov*. for *Phaeosclera*). *Gobabebomyces* and *Lembosiniella* remained *incertae sedis* due to their disposition in the phylogenetic tree, that moved among clades accordingly with the gene analysed. Our results show that the inclusion of endophytic fungi obtained from plants in dry forests can contribute to the discovery of new taxa, clarify the phylogenetic position of allied taxa and confer information to the estimation of national and global fungal diversity.

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INTRODUCTION

Dothideomycetes is the largest class of ascomycete fungi (Haridas *et al.* 2020, Hongsanan *et al*. 2020). Members of this class are known to have a wide range of ecological habitats and occur as plant pathogens (Fan *et al.* 2017), saprophytes (Jayasiri *et al*. 2019), endophytes (Bezerra *et al.* 2017a, b), epiphytes (Hongsanan *et al.* 2016), or are fungicolous (Trakunyingcharoen *et al*. 2014), marine (Jones *et al.* 2020), lichenised or lichenicolous fungi (Zhang *et al*. 2020). Some of them are ubiquitous rock-inhabiting fungi (Ruibal *et al*. 2009).

Dothideomycetes have been well studied in the past, and their higher-level classification has also been revisited (Pem *et al.* 2019, 2021, Hongsanan *et al.* 2020). Schoch *et al.* (2009) studied the phylogenetic position of 41 families in *Dothideomycetes*. Subsequently, Lumbsch & Huhndorf (2010), in the Outline of *Ascomycota*, included 41 families and 249 genera, and 116 remaining genera were placed as *incertae sedis* in *Dothideomycetes*. Hyde *et al.* (2013) accepted 22 orders, 105 families and estimated around 19 000 species in *Dothideomycetes*. These publications did not include some fungi known as meristematic that have been related to *Dothideomycetes*. Recently, genome-based assessments (Haridas *et al*. 2020) and phylogenies including meristematic-like taxa (Pem *et al.* 2019, 2021, Crous *et al.* 2020, Hongsanan *et al*. 2020), revealed new phylogenetic relationships and ecology of these fungi. The Outline of Fungi and fungus-like taxa (Wijayawardene *et al.* 2022) reports 49 orders and 223 families in *Dothideomycetes*, including meristematic fungi such as *Endosporium*, *Gobabebomyces*, *Phaeosclera*, and meristematic-like taxa, such as *Lembosiniella*.

Historically, meristematic fungi have been described and placed in different orders in *Dothideomycetes* due to the plasticity of morphological characters and uncertainties about

their phylogenetic position, which remains unsettled in this class (Wollenzien *et al.* 1996, Pem *et al.* 2019, 2021, Crous *et al*. 2020, Hongsanan *et al.* 2020, Wijayawardene *et al*. 2022). The term meristematic fungi was introduced to accommodate fungi that have aggregates of thick-walled, melanised cells that enlarge and reproduce by isodiametric division (de Hoog & Hermanides-Nijhof 1977, Sterflinger *et al.* 1999). Different genera, such as *Sarcinomyces* (Lindner 1898), *Phaeotheca* (Sigler *et al.* 1981), *Hyphospora* (Ramaley 1996), *Endoconidioma* (Tsuneda *et al.* 2004), *Endosporium* (Tsuneda *et al*. 2008) and the recently described *Gobabebomyces* (Crous *et al.* 2020), have been considered to be members of this unique group of fungi.

The historical classification of these genera is problematic, and several authors placed them in distinct orders or as *incertae sedis*. Ruibal *et al.* (2008) studied ITS sequences of melanised fungi (including *Coniosporium*, *Friedmanniomyces*, *Cryomyces* and *Cystocoleus*) from rock formations in Spain and placed these specimens in *Capnodiales*, *Dothideales* and *Pleosporales* (*Dothideomycetes*). Later, based on multigene phylogenetic analyses, the same authors suggested that these fungi belonged to *Capnodiales*, *Dothideales*, *Pleosporales* and *Myriangiales* with one lineage related to *Arthoniomycetes*, a sister class of *Dothideomycetes* (Ruibal *et al*. 2009). Kirk *et al.* (2008), in the *Dictionary of fungi*, included *Phaeosclera* in *Dothideomycetes* and *Sarcinomyces* in *Eurotiomycetes*. On the order hand, Hyde *et al*. (2011), on the compilation of asexual fungi in a natural classification, included *Endosporium* in the family *Elsinoaceae* (*Myriangiales*) and *Phaeosclera* and *Sarcinomyces* as *incertae sedis* in *Myriangiales*. A similar classification was adopted by Seifert *et al*. (2011) in *The Genera of Hyphomycetes*; these authors also placed *Phaeosclera* and *Endosporium* in *Myriangiales*. Recently, Pem *et al.* (2019) introduced *Endosporiaceae* as an *incertae sedis* family within *Dothideomycetes* to accommodate *Endosporium*, which is closely related to *Myriangiales*. In 2020, Crous *et al.* introduced *Gobabebomyces* as a lineage related to *Lembosiniella* and *Endosporium*, being closely related, but distinct from *Myriangiales*. Wijayawardene *et al.* (2022) placed *Endosporium* and *Gobabebomyces* in *Endosporiaceae* as an *incertae sedis* family in *Dothideomycetes*, and *Lembosiniella* as an *incertae sedis* genus in *Dothideomycetes*.

The present paper is part of a series of publications describing novel taxa occurring endophytically in the Caatinga biome in Brazil, mainly from cacti (Bezerra *et al*. 2017a, b, 2018, 2019a, b, Barbosa *et al.* 2020, Silva *et al*. 2021, Nascimento *et al*. 2021, Santiago *et al*. 2023). The Caatinga is the largest tropical forest region in South America and a biome restricted to Brazil. It is characterised by a semiarid climate with low precipitation levels and a high diversity of plants, animals and fungi, including rare and endemic species (da Silva *et al.* 2017, Rosa 2021). Among the plant families in the Caatinga forest, *Cactaceae* is one of the most diverse having about 60 species distributed in 18 genera (Flora e Funga do Brasil 2023). Our studies on fungal endophytes suggest that the Caatinga tropical dry forest represents a hotspot of endophytic fungal diversity in Brazil.

While studying endophytic fungi associated with cacti species in a tropical dry forest (Caatinga) in Brazil, isolates were obtained with meristematic growth as the main diagnostic characteristic. Multi-locus phylogenetic analyses demonstrated that these endophytes are closely related to, but distinct from, *Myriangiales*. In the present study, using morphological characteristics, ecology and phylogenetic evidence, three new orders, two new families, one new genus and two new species are proposed to accommodate black meristematic, meristematic-like fungi, and related taxa within *Dothideomycetes*.

MATERIAL AND METHODS

Isolates

Endophytic fungi were isolated from the cacti *Tacinga inamoena* and *Pilosocereus gounellei* subsp. *gounellei* as described by Bezerra *et al.* (2013). Plant material was collected in the Brazilian tropical dry forest (Caatinga), Catimbau National Park, Buíque city, Pernambuco state, Brazil (8°36'35"S, 37°14'40°W) from November to December 2013. The expeditions were authorised by the Ministério do Meio Ambiente (MMA)/Instituto Chico Mendes de Conservação da Biodiversidade (ICMBio); permission number: 40331-1/authentication code 87451826 issued on 4th November 2013. Our study was registered in the Sistema Nacional de Gestão do Patrimônio Genético (SisGen)/MMA/Conselho de Gestão do Patrimônio Genético (CGen); registration number: A7902F3. In addition, we studied the strains *Gobabebomyces vachelliae* CBS 146779, *Phaeosclera dematioides* CBS 157.81 and *Lembosiniella eucalyptorum* CBS 144603 obtained from the CBS culture collection of the Westerdijk Fungal Biodiversity Institute, Utrecht, The Netherlands.

Endophytic strains were deposited at the University Recife Mycology (URM) culture collection (Micoteca URM Profa. Maria Auxiliadora Cavalcanti) of the Universidade Federal de Pernambuco, Recife, Brazil, and in the FCCUFG working collection of the Laboratório de Micologia at the Instituto de Patologia Tropical e Saúde Pública (IPTSP) of the Universidade Federal de Goiás, Goiânia, Brazil, and at the CBS culture collection in the Westerdijk Fungal Biodiversity Institute, Utrecht, The Netherlands (under Material Transfer Agreement – MTA No. 05/2015/Micoteca URM, issued on 14 Apr. 2015). Microscope slides (holotype) with fungal reproductive structures were deposited at the Herbário UFG of the Universidade Federal de Goiás, Goiânia, Brazil.

Morphology and culture characterisation

Isolates of endophytic fungi were cultured on malt extract agar (MEA), potato dextrose agar (PDA), oatmeal agar (OA), synthetic nutrient deficient agar (SNA), potato-carrot agar (PCA), Czapek-Dox agar (CZA), and V8 juice agar (V8) (Crous *et al.* 2009), and incubated at 22 °C and 30 °C in the dark. Macro- (*e.g.*, colony diameter, texture, pigmentation, margin appearance, exudates and colours) and micromorphological (*e.g.*, hyphae, conidia, conidiogenous cells and colours) analyses were performed after 2 wk of fungal growth. Slides were prepared in clear 85 % lactic acid, and the colony colours were evaluated based on the colour chart of Rayner (1970). The growth of endophytic isolates under different temperatures was verified on MEA Petri dishes incubated in the dark for 2 wk at a temperature gradient, starting at 6 °C and reaching 40 °C in 3 °C intervals.

DNA extraction, amplification (PCR) and sequencing

Endophytic strains were cultivated on MEA, and genomic DNA extraction was performed using the Wizard® Genomic DNA Purification Kit (Promega, USA) according to the manufacturer's instructions. The primers NS1 and NS4 (White *et al.* 1990) were

used to amplify part of the nuclear ribosomal RNA small subunit gene (SSU), LR0R and LR5 (Vilgalys & Hester 1990, Vilgalys & Sun 1994) to amplify part of the nuclear ribosomal RNA large subunit gene (LSU), ITS5 and ITS4 (White *et al.* 1990) to amplify the internal transcribed spacer region (ITS) of the nrDNA operon and intervening 5.8S nRNA gene, EF1-983F and EF1-2218R (Rehner & Buckley 2005) to amplify part of the translation elongation factor 1-alpha gene (*TEF1*) and RPB2-5F2 and RPB2-7cR (Liu *et al.* 1999) to amplify part of the second largest subunit of the DNA-directed RNA polymerase II gene (*RPB2*). Amplification reactions, PCR conditions, sequencing, and sequence analyses were performed as described by Bezerra *et al.* (2017a, b).

Phylogenetic analyses

DNA consensus sequences of our endophytes were compared with other sequences deposited in the NCBI GenBank nucleotide database using the BLASTn tool in order to identify related taxa. Firstly, we constructed an LSU dataset of 97 sequences belonging to *Dothideomycetes* based on reference papers (Schoch *et al.* 2006, 2009, Tsuneda *et al.* 2008, Ruibal *et al.* 2009, Tsuneda *et al.* 2011, Jayawardena *et al*. 2014, Pem *et al*. 2019, 2021, Crous *et al.* 2020, Hongsanan *et al.* 2020). The dataset was aligned using MAFFT v. 7 (http://mafft.cbrc.jp/alignment/server/; Katoh and Standley 2013). Subsequently, the aligned matrices of all loci (ITS, LSU, SSU and *RPB2*) composed of *Dothideomycetes* species and other related taxa reported as *incertae sedis* were individually examined and thereafter combined in MEGA v. 7 (Kumar *et al.* 2016). The *TEF1* sequences were not included in the phylogenetic analyses due to a lack of reference sequences available for comparison, but are available in the GenBank nucleotide database for further verification (accessions OR762062–OR762064).

The Maximum Likelihood analysis (ML) was performed with IQ-TREE v. 1.6.12 software (Nguyen *et al.* 2015) and RAxML-HPC BlackBox v. 8.2.12 (Stamatakis 2014). The IQ-TREE ML analysis involved 5 000 replications and the ultrafast bootstrap (UFboot2) method was used to calculate branch supports (Hoang *et al.* 2018). The software ModelFinder included in IQ-TREE v. 1.6.12 (Kalyaanamoorthy *et al.* 2017) was used to determine the partitioning strategy and models based on the Akaike information criterion (AICc) for the dataset using four predefined data blocks (LSU, SSU, *RPB2*, ITS). The models were respectively: GTR+G (for LSU), and GTR+I+G (for ITS, SSU and *RPB2*) (Supplementary Table S1). The RAxML-HPC BlackBox ML analysis was performed with default system options in the CIPRES science gateway (Miller *et al*. 2010; http://www.phylo.org/), using the GTR+I+G model. Additional ML analyses on the individual markers were performed with IQ-TREE v. 1.6.12 and RAxML-HPC BlackBox v. 8.2.12 to estimate the clades disposition between the different regions.

The Bayesian inference (BI) analysis was conducted using MrBayes v. 3.2.7a in the CIPRES science gateway, using the same models and partitions as the ML analysis (IQ-TREE). Four independent Metropolis-coupled MCMC chains were run twice. Ten million generations were run with trees saved every 1 000th generation, after which the runs stopped automatically and a burn-in of 25 % of the initially sampled trees was applied. The remaining trees were used to reconstruct a 50 % majority-rule consensus tree and calculate Bayesian posterior probabilities (BPP) of the clades (Supplementary Table S1).

The IQ-TREE ML topology is presented here (Fig. 1) with the bootstrap support (BS) values from IQ-TREE UFboot2-BS and RAxML-BS analyses (both BS ≥ 70 %) and Bayesian posterior probability (BPP ≥ 0.95) values plotted at the nodes. The phylogenetic tree was visualised using FigTree v. 1.4.4 (Rambaut 2012). New DNA sequences obtained in our study were deposited in GenBank (Supplementary Table S2) and the final alignment was deposited in Figshare (doi: https://doi.org/10.6084/m9.figshare.24475114).

RESULTS

Phylogenetic analyses

The final combined alignment consisted of 97 taxa belonging to *Dothideomycetes*, and contains 3 305 characters including alignment gaps (ITS: 669 bp, LSU: 836 bp, SSU: 1 008 bp and *RPB2*: 789 bp), of which 1 574 are conserved, 1 678 variable and 1 432 parsimony-informative. The best-scoring maximum likelihood tree with a final optimisation likelihood value was *-ln* = -49901.350. For Bayesian inference, the average standard deviation of split frequencies was 0.001811 after 10 million generations.

In our combined phylogenetic analysis (Fig. 1), our endophytes (URM 8869, URM 8870 and URM 8871) formed a clade related to *Gobabebomyces* and *Lembosiniella* (UFboot2-BS = 79 %; RAxML-BS = < 70 %; BPP = 0.95), while *Endosporium* species (UFboot2-BS = 100 %; RAxML-BS = 99 %; BPP = 1) and the monotypic genus *Phaeosclera* (UFboot2-BS = 100 %; RAxML-BS = 98 %; BPP = 1) represented independent lineages. Therefore, based on morphological features, ecology and multi-locus phylogenetic analysis, we introduce a new genus, *Endophytium*, to accommodate two new species, *E. albocacti* and *E. cacti* (UFboot2-BS = 100 %; RAxML-BS = 100 %; BPP = 1), in a clade including *Gobabebomyces* and *Lembosiniella* as closest related genera. *Myriangiales*, represented by *Elsinoe*, *Myriangium*, *Mendogia* and *Anhellia* is fully supported in our phylogeny (UFboot2-BS = 100 %; RAxML-BS = 100 %; BPP = 1). The node connecting *Endophytium*, *Gobabebomyces* and *Lembosiniella* to *Myriangiales* has moderate support (UFboot2-BS = 93 %; RAxML-BS = 82 %; BPP = 0.97), while the node including *Endosporium* is poorly supported (UFboot2-BS = 83 %; RAxML-BS = < 70 %; BPP = < 0.95) and the node adding *Phaeosclera* into this assemblage is better supported (UFboot2-BS = 100 %; RAxML-BS = 98 %; BPP = 1). The order *Dothideales* was placed as an independent lineage (UFboot2-BS = 100 %; RAxML-BS = 100 %; BPP = 1) and did not share direct phylogenetic relationships with any other order-level clade. In addition, our analyses also revealed that *Mycosphaerellales* (UFboot2-BS = 98 %; RAxML-BS = 98 %; BPP = 1), *Capnodiales* (UFboot2-BS = 100 %; RAxML-BS = 100 %; BPP = 1) and *Cladosporiales* (UFboot2-BS = 99 %; RAxML-BS = 100 %; BPP = 1) are closely related lineages.

In the independent ML inference phylogenies for each gene (Supplementary Figs S1–S4), the topology of the ML trees varied in the arrangements of these groups. In the ITS ML tree (Supplementary Fig. S1), the genera *Endophytium*, *Gobabebomyces* and *Lembosiniella* were placed as a unique and moderately supported clade (UFboot2-BS = 97 %; RAxML-BS = 85 %), while the node linking *Phaeosclera* and *Endosporium* had low support (UFboot2-BS = 81 %; RAxML-BS = < 70 %); in

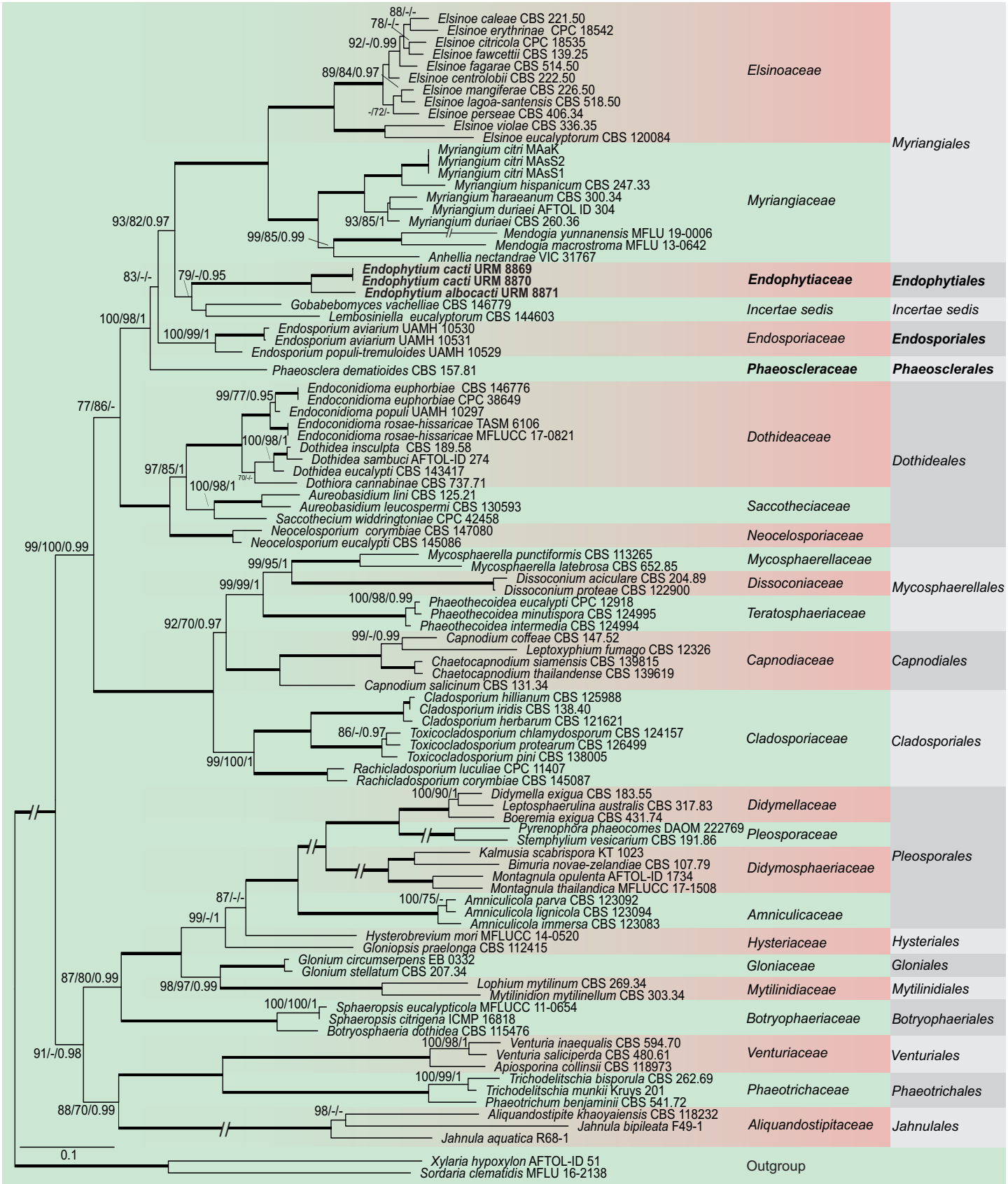

Fig. 1. Maximum Likelihood (ML) phylogenetic tree of the combined ITS, LSU, SSU and *RPB2* sequences of the new genus *Endophytium* and related meristematic and meristematic-like fungi in *Dothideomycetes*. New taxa are in **bold** face. Thickened branches indicate full statistical support in all analyses. Confidence values for ML-BS ≥ 70 % (UFboot2/RAxML) and BPP ≥ 0.95 are included near the nodes and the "-" indicates statistical support below the threshold values. The scale bar represents the expected number of changes per site. The tree was rooted to *Xylaria* (*Xylariales*, *Sordariomycetes*) and *Sordaria* (*Sordariales*, *Sordariomycetes*). Families and orders are shown in coloured blocks and indicated to the right of the tree.

the ITS tree, these clades were located on a three-pronged polytomy with *Dothideales*, distinct from *Myriangiales*. In the LSU ML tree (Supplementary Fig. S2), *Endophytium* (UFboot2-BS = 100 %; RAxML-BS = 99 %), *Endosporium* (UFboot2-BS = 99 %; RAxML-BS = 97 %) and *Phaeosclera* (UFboot2-BS = 99 %; RAxML-BS = 97 %) were placed as independent and strongly supported lineages basal to *Myriangiales* but without a well-supported backbone structure, while *Gobabebomyces* and *Lembosiniella* formed a clade without support and positioned between the clades of *Endophytium* and *Endosporium*. In the SSU ML tree (Supplementary Fig. S3), *Endophytium*, *Endosporium* and *Phaeosclera* were positioned similarly as in the LSU ML tree with a poorly supported backbone. In the *RPB2* ML tree (Supplementary Fig. S4), *Endophytium* appeared as a unique fully supported clade (UFboot2-BS = 99 %; RAxML-BS = 93 %) related to *Gobabebomyces* with low support and as basal sister to *Myriangiales*. We could not obtain any *RPB2* sequences of *Endosporium* to include in the phylogenetic analyses. To summarise, *Myriangiales* in the narrower sense (*i.e.* consisting of *Anhellia*, *Elsinoe*, *Mendogia*, and *Myriangium*) is represented by a monophyletic lineage in the ITS (UFboot2-BS = 100 %; RAxML-BS = 99 %), LSU (UFboot2-BS = 98 %; RAxML-BS = 87 %), SSU (UFboot2-BS = 100 %; RAxML-BS = 98 %) and *RPB2* (UFboot2-BS = 79 %; RAxML-BS = < 70 %) phylogenies. The inclusion of *Endophytium*, *Endosporium*, *Gobabebomyces*, *Lembosiniella* and *Phaeosclera* in *Myriangiales* in the broadest sense of the clade has support in the LSU (UFboot2-BS = 99 %; RAxML-BS = 97 %), SSU (clustered but with support values below the thresholds: UFboot2-BS = < 70 %; RAxML-BS = < 70 %) and *RPB2* (UFboot2-BS = 92 %; RAxML-BS = 77 %) phylogenies, but not in the ITS (these genera clustered with *Dothideales*, not *Myriangiales*, but with low support: UFboot2-BS = 86 %; RAxML-BS = < 70 %), this fact could be due long branch attraction or the presence of ambiguously aligned sequence regions.

Based on the analyses above, we had three main options (references to clades in the options refer to Fig. 1): 1) Expand the *Myriangiales* to span *Elsinoaceae* to *Phaeoscleraceae* (most basal node with the following support values: UFboot2-BS = 100 %; RAxML-BS = 98 %; BPP = 1); or 2) Define *Myriangiales* to only include *Elsinoaceae* and *Myriangiaceae* (connecting node fully supported in all analyses), and introducing orders for the remaining families connected to the basal node in option 1 above; or 3) Limit *Myriangiales* to only include *Myriangiaceae*, and introducing orders for the remaining families connected to the basal node in option 1 above. Phylogenetic distances between *Elsinoaceae* and *Myriangiaceae* are similar to distances observed in for example *Mycosphaerellales* and *Pleosporales* and we therefore prefer not to introduce an order for each of the two former families (option 3). Likewise, a similar distribution of branch lengths is observed if *Myriangiales* is applied broadly (option 1) compared to for example *Mycosphaerellales*/*Capnodiales*/*Cladosporiales* or *Pleosporales*/*Hysteriales*/*Gloniales*/*Mytilinidiales*. Therefore, if we choose to follow option 1, then synonymies for other orders will also be required. We choose to apply option 2 as this would be more consistent with how other families and orders are treated in the class. For now, we prefer to treat both *Gobabebomyces* and *Lembosiniella* as *incertae sedis* at the family and order level as their phylogenetic position tends to move around depending on the marker analysed (Supplementary Figs S1–S4); a loose association with *Endophytium* is often observed, but often not as a pure sister relationship and if so, not always highly supported.

Therefore, based on the phylogenetic results presented above, three new orders related to *Myriangiales* and two new families are proposed below: *Endophytiales ord. nov*. to accommodate *Endophytiaceae fam. nov*., *Endosporiales ord. nov*. to accommodate *Endosporiaceae*, and *Phaeosclerales ord. nov*. for *Phaeoscleraceae fam. nov*.; and *Gobabebomyces* and *Lembosiniella* retained as *incertae sedis*.

TAXONOMY

Endophytiales G.G. Barreto, Souza-Motta, G.A. Silva, J.Z. Groenew., Crous & J.D.P. Bezerra, *ord. nov.* MycoBank MB 850809.

Etymology: Named after the genus *Endophytium*.

Asexual morph: black meristematic, meristematic-like to yeast-like white to pale yellow, mucoid and glistening. *Hyphae* aerial, pale brown, cylindrical or torulose, septate. *Conidiophores* reduced to conidiogenous cells. *Conidia* blastic, unicellular, hyaline, ellipsoid, ovoid to obovoid. *Sexual morph*: unknown.

Type genus: *Endophytium* G.G. Barreto, Souza-Motta, G.A. Silva, J.Z. Groenew., Crous & J.D.P. Bezerra

Endophytiaceae G.G. Barreto, Souza-Motta, G.A. Silva, J.Z. Groenew., Crous & J.D.P. Bezerra, *fam. nov.* MycoBank MB 850810.

Etymology: Named after the genus *Endophytium*.

Asexual morph: black meristematic, meristematic-like to yeast-like white to pale yellow, mucoid and glistening. *Hyphae* aerial, pale brown, cylindrical or torulose, septate. *Conidiophores* reduced to conidiogenous cells. *Conidia* blastic, unicellular, hyaline, ellipsoid, ovoid to obovoid. *Sexual morph*: unknown.

Type genus: *Endophytium* G.G. Barreto, Souza-Motta, G.A. Silva, J.Z. Groenew., Crous & J.D.P. Bezerra

Included genus: *Endophytium* G.G. Barreto, Souza-Motta, G.A. Silva, J.Z. Groenew., Crous & J.D.P. Bezerra

Endophytium G.G. Barreto, Souza-Motta, G.A. Silva, J.Z. Groenew., Crous & J.D.P. Bezerra, *gen. nov.* MycoBank MB 850811.

Etymology: Named in reference to the endophytic ecological habitat from which the fungus was first isolated.

Asexual morph: *Hyphae* hyaline becoming pale to medium brown, verruculose, dividing into several globose to subglobose cells. *Conidiophores* reduced to conidiogenous cells. *Conidiogenous cells* hyaline to brown, chains, smooth to verruculose, thick-walled, oblong, ellipsoid to obovoid, obclavate, septate. *Conidia* blastic, unicellular, hyaline, ellipsoid, ovoid to obovoid, arising from cells. *Sexual morph*: unknown.

Type species: *Endophytium cacti* G.G. Barreto, Souza-Motta, G.A. Silva, J.Z. Groenew., Crous & J.D.P. Bezerra

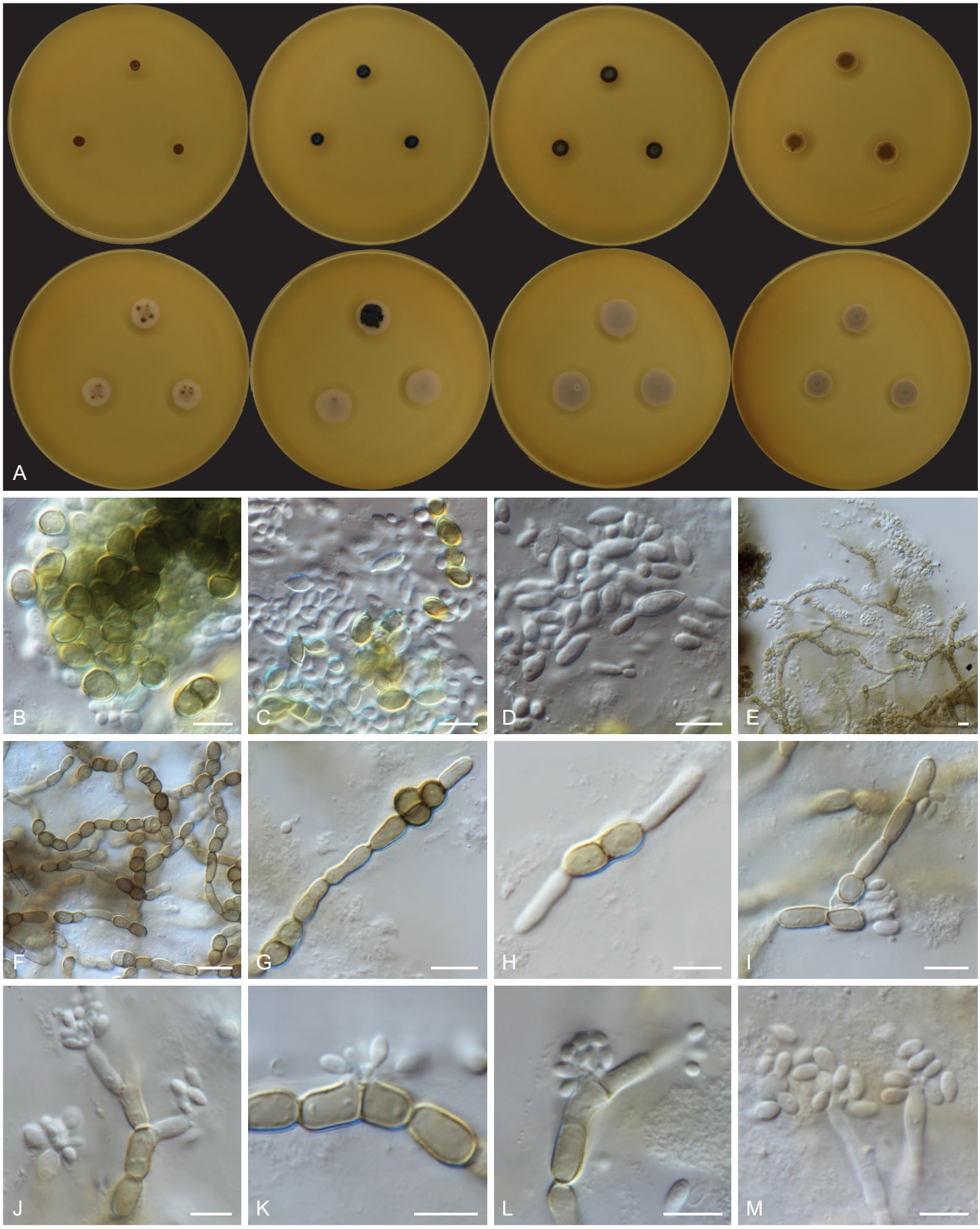

Fig. 2. *Endophytium albocacti* URM 8871. **A.** Colonies from left to right on MEA after 7 d at 9, 12, 15 and 18 °C (top row) and at 21, 24, 27 and 30 °C in the dark. **B.** Details of cells dividing into globose to subglobose cells. **C, D.** Yeast-like cells and conidia. **E–I.** Hyphae toruloid-like and conidia. **J–M.** Details of conidiogenous cells and conidia. Scale bars = 10 µm.

Endophytium albocacti G.G. Barreto, Souza-Motta, G.A. Silva, J.Z. Groenew., Crous & J.D.P. Bezerra, *sp. nov.* MycoBank MB 850812. Fig. 2.

Etymology: Named in reference to the similarity and phylogenetic relationship to *Endophytium cacti*, but differs mainly in the yeast-like growth and colony colour variation at different temperatures.

Asexual morph: *Hyphae* toruloid, sub-hyaline when young and becoming pale to dark brown with age, verruculose, branched, septate, swollen, 2–3(–4.5) µm wide; cells dividing into several globose cells, which are released upon rupture of the cell wall, yeast-like. *Conidiophores* reduced to conidiogenous cells. *Conidiogenous cells* sub-hyaline to dark brown, undifferentiated, intercalary, inconspicuous denticles, becoming brown and verruculose, thick-walled, somewhat guttulate, oblong, ellipsoid to obovoid, obclavate, 1(–2)-septate, (7.5–)8–9(–11.5) × (2.5–)3–5.5(–9) µm. *Conidia* blastic, aseptate, abundant, hyaline, guttulate, ellipsoid, ovoid to obovoid, 5–5.5(–6) × 2.5–3(–3.5) µm. *Sexual morph*: unknown.

Culture characteristics: Colonies yeast-like; on MEA, SNA, V8 and CZA similar, white to pale yellow (cream), bright; on OA, PDA and PCA olive to pale brown in the central area; growing up to 1 cm diam on MEA, OA, PDA and V8, and on SNA up to 5 mm diam at 22 °C and 30 °C in 2 wk. The optimal temperatures for growth were 24 °C and 27 °C. No growth was observed at 36 °C and 40 °C. Colonies yeast-like, becoming filamentous on MEA at 6 °C. Colonies are olive to dark brown at 6–15 °C, at 18 °C the central area of colonies is olive to dark brown, and at 33 °C the whole colony is olive to dark brown.

Typus: **Brazil**, Pernambuco, Buíque, Catimbau National Park (8°36’35’’S, 37°14’40’’W), isolated as an endophytic fungus from *Pilosocereus gounellei* subsp. *gounellei,* Sep. 2013, *J.D.P. Bezerra* (**holotype** UFG 36499; **isotype** CBS H-22683; culture ex-type URM 8871 = FCCUFG 33).

Endophytium cacti G.G. Barreto, Souza-Motta, G.A. Silva, J.Z. Groenew., Crous & J.D.P. Bezerra, *sp. nov.* MycoBank MB 850813. Fig. 3.

Etymology: Named in reference to the host, a cactus species of the genus *Tacinga*.

Asexual morph: *Hyphae* toruloid, sub-hyaline when young and becoming melanised, pale to dark brown with age, verruculose, branched, septate, swollen, 5.5–6.5(–8.5) µm wide; cells dividing into several globose to subglobose cells, which are released upon rupture of the cell wall. *Conidiophores* reduced to conidiogenous cells. *Conidiogenous cells* sub-hyaline to brown, in chains, becoming brown and verruculose, thick-walled, somewhat guttulate, globose to subglobose, (0–)1(–2)-septate, (2.5–)3.5–5(–6.5) × 2.5–4 µm. *Conidia* blastic, aseptate, abundant, hyaline, somewhat guttulate, ellipsoid, globose to subglobose, (3–)5–6.5(–7.5) × (2–)2.5–3(–4) µm. *Sexual morph*: unknown.

Culture characteristics. Colonies on MEA, PDA, SNA, PCA and V8 are similar, black, glistening, cerebriform, irregular, and elevated; on OA mucoid in the central area, and on CZA dark brown to black; aerial mycelium sometimes observed on SNA; growing up to 2 cm diam on MEA, OA, PDA and V8, and on SNA up to 7 mm diam at 22 °C and 30 °C in 2 wk. The optimal temperatures for growth were 24 °C, 27 °C and 30 °C. No growth was observed at 33 °C, 36 °C and 40 °C.

Typus: **Brazil**, Pernambuco, Buíque, Catimbau National Park (8°36’35’’S, 37°14’40’’W), as endophytic fungus from *Tacinga inamoena,* Sep. 2013, *J.D.P. Bezerra* (**holotype** UFG 36498; **isotype** CBS H-22685; culture ex-type URM 8870 = CBS 141544 = FCCUFG 31).

Additional material examined: **Brazil**, Pernambuco, Buíque, Catimbau National Park (8°36’35’’S, 37°14’40’’W), as endophytic fungus from *Tacinga inamoena,* Sep. 2013, *J.D.P. Bezerra*, culture URM 8869 = CBS 141543 = FCCUFG 32.

Notes: *Endophytium* is morphologically similar to other meristematic-like fungi, such as *Phaeotheca*, *Phaeosclera*, *Sarcinomyces* (Sigler *et al.* 1981), *Phaeothecoidea* (Crous *et al*. 2007), *Endosporium* (Tsuneda *et al.* 2008) and *Gobabebomyces* (Crous *et al*. 2020). *Endophytium* differs from *Phaeosclera* by the production of bulbil-like masses of sclerotic cells by conversion of short strands of hyphae (Sigler *et al*. 1981), from *Endosporium*, which can be differentiated by the production of aerial and determinate hyphae on the surface of colonies and conidiogenous cellular clumps (Tsuneda *et al*. 2008) and from *Gobabebomyces* by the production of pycnidial conidiomata, phialidic conidiogenous cells and aseptate conidia, ellipsoid, thick-walled with obtuse ends and medium brown (Crous *et al.* 2020). Phylogenetically, *Endophytium* is also related to *Lembosiniella*, although easily differentiated morphologically by the production of ascomata (Crous *et al.* 2019). In addition, *Endophytium* has morphological similarities to *Phaeotheca* that produce mother cells containing one to several endoconidia and no growth at 25 °C (Crous *et al.* 2007), but they are not phylogenetically related (*Capnodiales*, *Phaeothecaceae*). *Sarcinomyces* (*Eurotiomycetes*, *Chaetothyriales*) which present blastic conidia from multicellular sclerotic bodies (Sigler *et al.* 1981) and *Phaeothecoidea* (*Mycosphaerellales*, *Mychosphaerellaceae*) differs from *Endophytium* by not producing blastic conidia and mainly having brown and thick-walled endoconidia (Crous *et al.* 2007). Our phylogenetic analyses support the introduction of the new genus (*Endophytium*) with two new species (*E. albocacti* and *E. cacti*) and a new family (*Endophytiaceae*) and order (*Endophytiales*) to accommodate *Endophytium*, which is related to *Myriangiales*. The phylogenetic position of *Endophytium* is, with the exception of the ITS phylogeny, always basal to *Myriangiales* but with low to no support (Supplementary Figs S1–S4); in the ITS phylogeny, *Endophytium*, *Gobabebomyces* and *Lembosiniella* are located as a basal polytomy with *Dothideales*, *Endosporium* and *Phaeosclera*. The latter two genera always have some association with *Endopythium* and *Myriangiales* in the LSU and SSU analyses and only with *Endopythium* in the ITS analyses; no *RPB2* sequences are available for comparison (Supplementary Figs S1–S4). The association of *Endophytium*, *Gobabebomyces* and *Lembosiniella* with *Dothideales* in the ITS phylogeny could be due to artifacts such as long branch attraction or the presence of ambiguously aligned sequence regions resulting in saturation.

Endosporiales G.G. Barreto, Souza-Motta, G.A. Silva, J.Z. Groenew., Crous & J.D.P. Bezerra, *ord. nov.* MycoBank MB 850814.

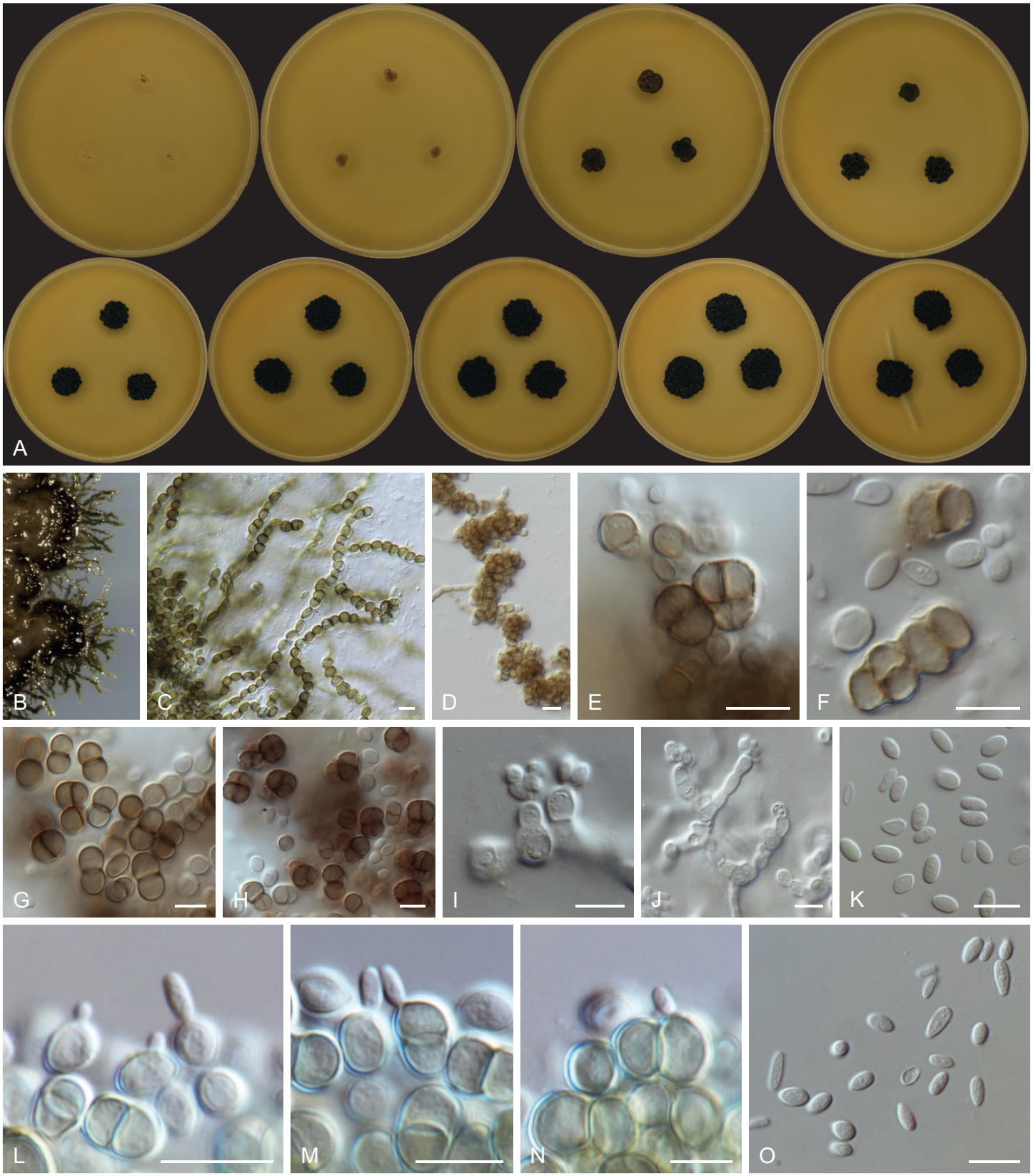

Fig. 3. *Endophytium cacti* URM 8870. **A.** Colonies from left to right on MEA after 7 d at 6, 9, 12 and 15 °C (top row) and at 18, 21, 24, 27 and 30 °C (bottom row) in the dark. **B.** Details of colony on PDA. **C, D.** Hyphae toruloid-like. **E–H.** Details of cells dividing into globose to subglobose cells. **I, J.** Conidiogenous cells and conidia. **K, O.** Conidia. **L–N.** Details of conidiogenous cell forming conidia. Scale bars = 10 µm.

Etymology: Named after the genus *Endosporium*.

Asexual morph: *Hyphae* dematiaceous, cylindrical-shaped or toruloid, light to dark brown, branched or unbranched, muriform and darkly pigmented bodies, septate. *Bulbil-like* masses, smooth, dark brown, with the production of sclerotic cells. *Endoconidia* unicellular, hyaline, ellipsoidal and subglobose to globose, schizolytic secession. *Conidia* unicellular, abundant, hyaline or light brown, cylindrical to ellipsoidal, regularly truncate at the base, sometimes globose, obovoid, fusoid, arising from cells of cellular clumps or seldom from sides of hyphae. *Sexual morph* unknown (based on Pem *et al.* 2019).

Type genus: *Endosporium* Tsuneda

Included genus: *Endosporium* Tsuneda

Notes: *Endosporiales* is introduced here in *Dothideomycetes* based on our multi-locus phylogenetic inference (Fig. 1), to accommodate *Endosporiaceae*. The genus *Endosporium* was the first black meristematic fungus producing endoconidia proposed to be related to *Myriangiales* (Tsuneda *et al.* 2008), but differing morphologically and phylogenetically from the fungi included in this order (*e.g.*, *Myriangium* and *Elsinoe*, Fan *et al*. 2017). Pem *et al.* (2019) introduced *Endosporiaceae* as an *incertae sedis* family within *Dothideomycetes*, justifying this decision based on their phylogenetic inference and morphological differences from all species in the class *Dothideomycetes*. The authors also highlighted that 'it is not clear whether *Endosporium* should be in *Myriangiales* or not' (Pem *et al*. 2019). Our phylogenetic inference including sequences from *Endophytium*, *Gobabebomyces*, *Lembosiniella* and *Phaeosclera*, showed similar proximity of *Endosporium* with *Myriangiales* as observed by Pem *et al.* (2019) and Crous *et al.* (2020), but distant from it, justifying the introduction of *Endosporiales* in *Dothideomycetes*.

Phaeosclerales G.G. Barreto, Souza-Motta, G.A. Silva, J.Z. Groenew., Crous & J.D.P. Bezerra, *ord. nov.* MycoBank MB 850815.

Etymology: Named after the genus *Phaeosclera*.

Hyphae are dematiaceous and septate. *Bulbil-like masses* are smooth, dark brown, and with variable size, occasionally covered in a slimy coating. *Sclerotic* cells are produced by the conversion of short strands of hyphae.

Type genus: *Phaeosclera* Sigler, Tsuneda & J.W. Carmich.

Included genus: *Phaeosclera* Sigler, Tsuneda & J.W. Carmich.

Phaeoscleraceae G.G. Barreto, Souza-Motta, G.A. Silva, J.Z. Groenew., Crous & J.D.P. Bezerra, *fam. nov.* MycoBank MB 850816.

Etymology: Named after the genus *Phaeosclera*.

Hyphae dematiaceous and septate, rapidly converting to thick-walled bulbil-like masses. *Bulbil-like masses* smooth, dark brown, variable in size with age, and occasionally covered in a slimy coating.

Type genus: *Phaeosclera* Sigler, Tsuneda & J.W. Carmich.

Included genus: *Phaeosclera* Sigler, Tsuneda & J.W. Carmich.

Notes: We analysed the isotype strain CBS 157.81 of *P. dematioides* and did not observe any other morphological or reproductive structures which may differ from the original description. Well-defined sexual or asexual structures are indeterminate or unknown. In addition, the cultural characteristics of the strain were similar as described in Sigler *et al.* (1981). *Phaeosclera* has been frequently placed as an *incertae sedis* genus in *Dothideomycetes* (*e.g.*, Wijayawardene *et al.* 2022). Based on our multi-locus phylogenetic analyses (Fig. 1), *P. dematioides* appears to be related to a clade having representatives of *Myriangiales* and the newly introduced orders *Endosporiales* and *Endophytiales*, but differed from it based on molecular data, morphology and ecology. Further collections are needed to better understand the morphology, lifestyle, and phylogenetic relationship of *Phaeosclera*. We introduced a new order (*Phaeosclerales*) and family (*Phaeoscleraceae*) to clarify the phylogenetic position of this genus in *Dothideomycetes*.

DISCUSSION

Dothideomycetes is the largest class of *Ascomycota* (Abdollahzadeh *et al.* 2020, Haridas *et al*. 2020, Jiang *et al.* 2020). However, several orders of *Dothideomycetes* still have numerous undescribed taxa and taxa with unresolved phylogenetic relationships (Liu *et al*. 2017, Haridas *et al.* 2020, Hogsanan *et al.* 2020, Ametrano *et al.* 2020, Wijayawardene *et al.* 2022).

Among the *Dothideomycetes*, the order *Myriangiales* comprises two families, *Myriangiaceae* and *Elsinoaceae*, with the former having 11 genera and the latter two (Fan *et al*. 2017, Wijayawardene *et al*. 2022). Members of this order are mainly known from their sexual morphs, and their species occur as saprobes, parasites or endophytes (Dissanayake *et al.* 2014, Fan *et al.* 2017, Jiang *et al.* 2020). Our phylogenetic analyses showed that the clade supporting *Myriangium* (*Myriangiaceae*) and *Elsinoe* (*Elsinoaceae*) is related, but distinct, to the clades of meristematic and meristematic-like fungi *Endophytium*, *Gobabebomyces*, *Lembosiniella*, *Endosporium* and *Phaeosclera*. In our analyses, the *Endophytiaceae* clade, represented by *Endophytium*, is phylogenetically related to *Myriangiales*. *Endosporium*, described by Tsuneda *et al.* (2008) as a meristematic fungus, is morphologically and ecologically similar to the members of *Myriangiales* and the newly introduced order *Phaeosclerales*. In addition, *Endosporium* is also morphologically similar to *Phaeotheca*, but phylogenetically unrelated, as the last belongs to *Capnodiales* (Abdollahzadeh *et al.* 2020). Recent phylogenetic inferences regarding *Endosporium* species showed that they are closely related to *Myriangiales*, although distinct within all orders in *Dothideomycetes* (Pem *et al.* 2019, Crous *et al*. 2020). A new order, *Endosporiales*, is therefore proposed to accommodate *Endosporiaceae* in *Dothideomycetes*.

Endosporium is a taxon of meristematic fungi that forms associations with trees and birds. Morphologically, this genus is similar to *Phaeotheca*, *Phaeosclera* and *Sarcinomyces* (Sigler *et al.* 1981), but the latter is phylogenetically placed in *Chaetothyriales* (*Eurotiomycetes*). However, the genera

Comminutispora (Ramaley 1996), *Endoconidioma* (Tsuneda *et al*. 2004) and *Phaeothecoidea* (Crous et al. 2007) are phylogenetically associated with *Capnodiales*, *Dothideales* and *Mycosphaerellales* within *Dothideomycetes* (Abdollahzadeh *et al.* 2020, Wijayawardene *et al.* 2022). Multi-locus phylogenetic analyses demonstrated that *Endosporium* and *Phaeosclera* are related to *Myriangiales*, but their phylogenetic affinities were still unclear (Pem *et al*. 2019, Crous *et al*. 2020, Wijayawardene *et al*. 2022).

Phaeosclera is a monospecific genus, and the type species, *P. dematioides* (holotype UAMH 4265 and isotype CBS 157.81), was observed on the pith of *Pinus contorta* in Alberta (Canada) (Sigler *et al*. 1981). Using maximum parsimony analysis of DNA sequences, Tsuneda *et al.* (2008) suggested that this genus was allied to *Myriangiales*, as a sister clade to the *Endosporium-Myriangiales* clade. Our analyses demonstrated that the genus *Phaeosclera* forms a clade closely related to *Endosporiales*, *Endophytiales*, *Gobabebomyces*, *Lembosiniella*, and *Myriangiales*. Therefore, *Phaeosclerales* and *Phaeoscleraceae* are proposed to accommodate *Phaeosclera* and solve the *incertae sedis* position of this genus in *Dothideomycetes*.

These new orders and families proposed here to accommodate the genera *Endophytium*, *Endosporium*, and *Phaeosclera* need more studies to confirm their ecological, medical and phytopathogenic importance, since members of these taxa are reported as endophytes, phytopathogens, human pathogens (causing mycosis), isolated from the skin of birds and from rock formations (Ruibal *et al.* 2008, 2009, Tsuneda *et al*. 2008, Ohm *et al.* 2012, Crous *et al.* 2019, Crous *et al*. 2020). Similarly, more studies are needed for *Gobabebomyces* and *Lembosiniella* since our analyses could not confirm their phylogenetic affinities. Furthermore, the deeply melanised cell walls, meristematic growth, and a yeast-like lifestyle (Tsuneda *et al.* 2008) may represent adaptations of these fungi to live in habitats with high ultraviolet radiation, temperature and drought, and suggest that these fungi are derived from ancestral lineages of *Dothideomycetes* (Knapp *et al*. 2015, Ametrano *et al*. 2020).

Members of the new genus *Endophytium* were isolated as endophytes from two cacti species growing in a tropical dry forest (Caatinga) in Brazil. Similarly, *Gobabebomyces* was found on leaves of *Vachellia erioloba* (*Fabaceae*) in an arid environment of the Namib Desert (Crous *et al*. 2020). The relations between plants and fungi, especially endophytes, still need to be better understood, as the lifestyle and metabolites produced by these fungi may have a specialised role and benefit a diversity of plants (Bezerra *et al.* 2012, 2013, 2017a, b, 2019, Bhunjun *et al.* 2023). The protection of these plant species in natural ecosystems can supply tools to investigate fungal diversity in different biomes, mainly in dry environments, and contribute to the national and global census of fungal diversity (*e.g.*, Flora e Funga do Brasil, 2023).

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Conflict of interest: The authors declare that there is no conflict of interest.

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Fig. S1. Maximum Likelihood (ML) phylogenetic tree of the ITS sequence alignment of the new genus *Endophytium* and related meristematic and meristematic-like fungi in *Dothideomycetes*. New taxa are in **bold** face. Thickened branches indicate full statistical support in all analyses. Confidence values for ML-BS ≥ 70 % (UFboot2/RAxML) are included near the nodes and the "-" indicates statistical support below the threshold values. The scale bar represents the expected number of changes per site. The tree was rooted to *Xylaria* (*Xylariales*, *Sordariomycetes*) and *Sordaria* (*Sordariales*, *Sordariomycetes*). Families and orders are shown in coloured blocks and indicated to the right of the tree.

Fig. S2. Maximum Likelihood (ML) phylogenetic tree of the LSU sequence alignment of the new genus *Endophytium* and related meristematic and meristematic-like fungi in *Dothideomycetes*. New taxa are in **bold** face. Thickened branches indicate full statistical support in all analyses. Confidence values for ML-BS ≥ 70 % (UFboot2/RAxML) are included near the nodes and the "-" indicates statistical support below the threshold values. The scale bar represents the expected number of changes per site. The tree was rooted to *Xylaria* (*Xylariales*, *Sordariomycetes*) and *Sordaria* (*Sordariales*, *Sordariomycetes*). Families and orders are shown in coloured blocks and indicated to the right of the tree.

Fig. S3. Maximum Likelihood (ML) phylogenetic tree of the SSU sequence alignment of the new genus *Endophytium* and related meristematic and meristematic-like fungi in *Dothideomycetes*. New taxa are in **bold** face. Thickened branches indicate full statistical support in all analyses. Confidence values for ML-BS ≥ 70 % (UFboot2/RAxML) are included near the nodes and the "-" indicates statistical support below the threshold values. The scale bar represents the expected number of changes per site. The tree was rooted to *Xylaria* (*Xylariales*, *Sordariomycetes*) and *Sordaria* (*Sordariales*, *Sordariomycetes*). Families and orders are shown in coloured blocks and indicated to the right of the tree.

Fig. S4. Maximum Likelihood (ML) phylogenetic tree of the *RPB2* sequence alignment of the new genus *Endophytium* and related meristematic and meristematic-like fungi in *Dothideomycetes*. New taxa are in **bold** face. Thickened branches indicate full statistical support in all analyses. Confidence values for ML-BS ≥ 70 % (UFboot2/RAxML) are included near the nodes and the "-" indicates statistical support below the threshold values. The scale bar represents the expected number of changes per site. The tree was rooted to *Xylaria* (*Xylariales*, *Sordariomycetes*) and *Sordaria* (*Sordariales*, *Sordariomycetes*). Families and orders are shown in coloured blocks and indicated to the right of the tree.

Table S1. Summary of phylogenetic information for the different analyses in this study.

Table S2. Species, strains/vouchers and GenBank accession numbers of sequences used in phylogenetic analyses. Newly generated sequences are in bold.