Wood staining fungi revealed taxonomic novelties in *Pezizomycotina*: New order *Superstratomycetales* and new species *Cyanodermella oleoligni*

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Abstract: A culture-based survey of staining fungi on oil-treated timber after outdoor exposure in Australia and the Netherlands uncovered new taxa in *Pezizomycotina*. Their taxonomic novelty was confirmed by phylogenetic analyses of multi-locus sequences (ITS, nrSSU, nrLSU, mitSSU, *RPB1*, *RPB2*, and *EF-1α*) using multiple reference data sets. These previously unknown taxa are recognised as part of a new order (*Superstratomycetales*) potentially closely related to *Trypetheliales* (*Dothideomycetes*), and as a new species of *Cyanodermella*, *C. oleoligni* in *Stictidaceae* (*Ostropales*) part of the mostly lichenised class *Lecanoromycetes*. Within *Superstratomycetales* a single genus named *Superstratomyces* with three putative species: *S. flavomucosus*, *S. atroviridis*, and *S. albomucosus* are formally described. Monophyly of each circumscribed *Superstratomyces* species was highly supported and the intraspecific genetic variation was substantially lower than interspecific differences detected among species based on the ITS, nrLSU, and *EF-1α* loci. Ribosomal loci for all members of *Superstratomyces* were noticeably different from all fungal sequences available in GenBank. All strains from this genus grow slowly in culture, have darkly pigmented mycelia and produce pycnidia. The strains of *C. oleoligni* form green colonies with slimy masses and develop green pycnidia on oatmeal agar. These new taxa could not be classified reliably at the class and lower taxonomic ranks by sequencing from the substrate directly or based solely on culture-dependent morphological investigations. Coupling phenotypic observations with multi-locus sequencing of fungi isolated in culture enabled these taxonomic discoveries. Outdoor situated timber provides a great potential for culturable undescribed fungal taxa, including higher rank lineages as revealed by this study, and therefore, should be further explored.

Key words: Classification of *Pezizomycotina*, *Cyanodermella oleoligni*, *Dothideomycetes*, Fungal cultures, Multi-locus phylogeny, Oil-treated wood, *Ostropales*, *Superstratomycetales*.

Taxonomic novelties: New order: *Superstratomycetales* van Nieuwenhuijzen, Miadlikowska, Lutzoni & Samson, **New family:** *Superstratomycetaceae* van Nieuwenhuijzen, Miadlikowska, Lutzoni & Samson, **New genus:** *Superstratomyces* van Nieuwenhuijzen, Miadlikowska & Samson, **New species:** *Cyanodermella oleoligni* van Nieuwenhuijzen & Samson, *Superstratomyces albomucosus* van Nieuwenhuijzen & Samson, *S. atroviridis* van Nieuwenhuijzen & Samson, *S. flavomucosus* van Nieuwenhuijzen & Samson.

INTRODUCTION

Microbial community composition can be an informative characteristic of an organism, substrate, or habitat. A distinction is made between desirable balanced microbial communities and unbalanced or disturbed communities, for example, in intestines (Roeselers *et al.* 2011, Gouba *et al.* 2013) and on skin (Findley *et al.* 2013, Lloyd-Price *et al.* 2016), as well as in tap water (Roeselers *et al.* 2015, Babič *et al.* 2016) soils (Barot *et al.* 2007, Creamer *et al.* 2016) or on wood (Sailer *et al.* 2010, Purahong *et al.* 2016). In order to broaden the understanding, and improve applications, of beneficial microbial communities, it is essential that all taxa in these communities are identified.

As part of a study on natural fungal-based wood finishes (biofinishes), fungal compositions on outdoor exposed wood samples were studied using a culture-based method. Fungi were sampled from untreated and oil-treated wood that contained dark fungal stains due to outdoor exposure. Several oil-treated wood samples had dense stained surfaces that met the desirable biofinish criteria of surface coverage and pigmentation (van Nieuwenhuijzen *et al.* 2015). DNA sequencing of the resulting fungal cultures showed that these biofinishes were composed of multiple genera, always containing the common wood staining

fungus *Aureobasidium* (van Nieuwenhuijzen *et al.* 2016, unpubl. data). However, not all cultures, even if characterised with molecular data, could be identified taxonomically.

Among the predominant cultured fungal colonies obtained from fungal stained wood surfaces, two types of coelomycetes remained unclassified (van Nieuwenhuijzen *et al.* unpubl. data). One type involved isolates with darkly coloured pycnidia (referred here as the "dark" group) obtained from several oil-treated and untreated wood samples situated at an outdoor test site in the Netherlands. Isolates of the other type had green coloured pycnidia (referred here as the "green" group) and were obtained from a single oil-treated wood sample exposed to outdoor conditions at a selected site in Australia. Neither morphological nor molecular data for these two fungal groups matched currently known described species.

The aim of this study was to further investigate the phylogenetic affiliations and taxonomic identities of 26 pycnidia-producing fungi isolated from oil-treated wood exposed to outdoor conditions. We sequenced four ribosomal and three protein-coding loci of representative fungal strains and inferred their phylogenetic relationships using multiple data sets including kingdom-, subphylum-, class- and order-wide taxonomic contexts. Furthermore, we conducted a detailed morphological

investigation of these fungal isolates. As a result, a new mono-generic order *Superstratomycetales* (*Dothideomycetes*) containing three newly proposed species, and a new species *Cyanodermella oleoligni* (*Ostropales*, *Lecanoromycetes*) are formally described here.

MATERIALS AND METHODS

Fungal-stained substrates

The substrates from which fungal isolates were obtained for this study consists of thirteen oil-treated wood samples that contained dark fungal stains due to outdoor exposure. The samples were prepared, exposed and handled as described in van Nieuwenhuijzen *et al.* (2015). Wood samples were impregnated with olive oil (Carbonel, iodine value 82 and 0,34 % free fatty acids), raw linseed oil (Vereenigde Oliefabrieken, iodine value 183 and 0,81 % free fatty acids), or stand linseed oil (Vliegenthart, viscosity P45). Fungi were sampled from the following oil-treated types of wood: pine (*Pinus sylvestris* L.) sapwood (sw), pine that mainly contained heartwood (hw), spruce (*Picea abies*) and ilomba (*Pycnanthus angolensis*). Information about the substrate (wood species and oil type used) and the geographical location of the outdoor exposure sites for each fungal isolate is provided in Table 1.

Fungal isolation

Wood staining fungi were collected using the swab sampling method as described in van Nieuwenhuijzen *et al.* (2015). After incubation on agar plates, the total number of colonies and the number of phenotypically predominant colonies were counted (van Nieuwenhuijzen *et al.* unpubl. data). Twenty-two colonies representing dark pycnidia producing fungi that originated from wood samples in the Netherlands ("dark" group) and four colonies representing green pycnidia producing fungi that originated from Australia ("green" group) were transferred to fresh malt extract agar (MEA) plates. These isolates were deposited in the CBS-KNAW Culture Collection and/or in the working collection of the Applied and Industrial Department (DTO) housed at the CBS-KNAW Fungal Biodiversity Centre, The Netherlands (Table 1).

Molecular data acquisition

Isolates were grown on MEA plates, prepared according to Samson *et al.* (2010), for at least one week prior to DNA extraction. Genomic DNA was extracted from cultures using the Ultraclean Microbial DNA isolation kit (MoBio Laboratories, Carlsbad, CA, USA) according to the manufacturer's instructions. The first DNA amplifications and sequencing were done on the following two loci: the two nuclear internal transcribed spacers and 5.8S rRNA gene (ITS) for each of the 26 fungal wood-stain isolates, and the nuclear ribosomal large subunit (nrLSU) for 23 isolates. All ITS and nrLSU sequences of both types of pycnidia producing fungi ("dark" and "green" groups) were subjected to BLAST searches (Wheeler *et al.* 2007) using the NCBI database to confirm fungal origin of each sequence fragment and to reveal their putative taxonomy. Top hits based on both 'maximum identity' and 'maximum query cover' were recorded. Subsequently, ITS and nrLSU sequences were screened

against the CBS-KNAW database. Based on this screening, strain CBS 353.84 was added to the 22 fungal isolates of the "dark" group, subjected to morphological examination, and DNA was extracted from a fresh culture (Table 1). Subsequently, the translation elongation factor 1 alpha (*EF*-1α) was obtained for all strains of the "dark" group. The nuclear ribosomal small subunit (nrSSU), the mitochondrial ribosomal small subunit (mitSSU), and two additional protein-coding genes, namely the largest and second largest subunits of RNA polymerase II (*RPB1* and *RPB2*, respectively), were obtained for five strains (Table 1). Each of the selected strains represented either a group of isolates with identical ITS sequences ("green" group) or one of the resulting clades of the ITS single-locus phylogenetic analysis ("dark" group).

Primers and PCR conditions used for the amplification and the resulting amplicon lengths are provided in Table S1. PCR reactions were performed as described in van Nieuwenhuijzen *et al.* (2015), including 0.50 μL of each (forward/reverse) primer (10 μM) and 0.1 μL Taq polymerase per 25 μL reaction mixture. PCR-products were sequenced with the BigDye Terminator v. 3.1 Cycle Sequencing Kit (Applied Biosystems, USA) and the same primer sets as used for PCR amplification. Sequence products were analysed on an ABI PRISM 3730XL genetic analyser (Applied Biosystems, USA) and Seqman Pro v. 9.0.4 (DNAstar Inc.) was used to assemble bio-directional traces into sequence contigs.

Reference data sets and multi-locus phylogenetic analyses

To determine the phylogenetic affiliation and taxonomic identities of the two unknown fungal groups based on multi-locus sequences, we assembled seven different data sets. Phylogenies were inferred based on Maximum Likelihood (ML) using RAxML-HPC2 version 7.2.8 (Stamatakis 2006, Stamatakis *et al.* 2008) as implemented on the CIPRES portal (Miller *et al.* 2010). Optimal tree and bootstrap searches were conducted with the rapid hill-climbing algorithm for 1 000 replicates with GTRGAMMA substitution model (Rodríguez *et al.* 1990). Five data sets were used to infer the phylogenetic placement of the "dark" group and three data sets were used for the "green" group (Table 2). Sequences of the unknown fungi were added to each reference data set and aligned manually with MacClade 4.08 (Maddison & Maddison 2005) using the "Nucleotide with AA color" option for guiding all alignments for protein-coding genes. Ambiguously aligned regions (Lutzoni *et al.* 2000) of the alignments were re-adjusted manually and excluded from subsequent phylogenetic analyses. Partition subsets for the RAxML searches and rooting of the resulting phylogenies were in accordance to the original studies that generated the reference data matrices (James *et al.* 2006 – Analysis 1; Carbone *et al.* unpubl. data – Analysis 2; Nelsen *et al.* 2014 and Ertz *et al.* 2015 – Analysis 5; Miadlikowska *et al.* 2014 – Analysis 6; Resl *et al.* 2015 – Analysis 7), except for Analyses 3 and 4, which were based on a newly assembled data set by restricting the *Pezizomycotina* (Carbone *et al.* unpubl. data) taxon sampling to *Arthoniomycetes* + *Dothideomycetes* only. For these two analyses, two members of *Geoglossomycetes* were used to root the trees. The data set for Analysis 3 was partitioned into seven subsets corresponding to each non-protein coding locus and each coding position, whereas for Analysis 4 the data set was partitioned into nine subsets (5.8S+nrLSU; mitSSU; nrSSU; 1st

Table 1. Fungal isolates included in this study with their CBS collection numbers, substrate types, geographic origins, and GenBank accession numbers for the sequenced loci.

CBS no.	DTO no.	Phenotype	Substrate	Locality	GenBank accession numbers						
					ITS	nrLSU	*EF-1α*	nrSSU	mitSSU	*RPB1*	*RPB2*
353.84	305-C3	"dark" group	Leaf of *Hakea multilinearis*	Australia, Perth	KX950411	KX950438	KX950470	KX950466	KX950462	KX950493	KX950497
140270	277-D2	"dark" group	Ilomba treated with olive oil	The Netherlands	KX950412	KX950439	KX950471	KX950467	KX950463	KX950494	KX950498
140271	277-I3	"dark" group	Pine sw treated with raw linseed oil	The Netherlands	KX950413	KX950440	KX950472	KX950468	KX950464	KX950495	KX950499
140272	305-E1	"dark" group	Pine sw treated with raw linseed oil	The Netherlands	KX950414	KX950441	KX950473	KX950469	KX950465	KX950496	KX950500
140273	277-C8	"dark" group	Pine sw treated with stand linseed oil	The Netherlands	KX950415	KX950442	KX950474	–	–	–	–
140274	277-C9	"dark" group	Pine sw treated with stand linseed oil	The Netherlands	KX950416	KX950443	KX950475	–	–	–	–
140275	277-D3	"dark" group	Ilomba treated with olive oil	The Netherlands	KX950417	KX950444	KX950476	–	–	–	–
140276	277-D4	"dark" group	Ilomba treated with olive oil	The Netherlands	KX950418	KX950445	KX950477	–	–	–	–
140277	277-H6	"dark" group	Pine sw treated with raw linseed oil	The Netherlands	KX950419	KX950446	KX950478	–	–	–	–
140278	277-H7	"dark" group	Pine sw treated with raw linseed oil	The Netherlands	KX950420	KX950447	KX950479	–	–	–	–
140279	277-H8	"dark" group	Pine sw treated with stand linseed oil	The Netherlands	KX950421	KX950448	KX950480	–	–	–	–
140280	277-I2	"dark" group	Spruce treated with olive oil	The Netherlands	KX950422	KX950449	KX950481	–	–	–	–
140281	277-I4	"dark" group	Pine sw treated with stand linseed oil	The Netherlands	KX950423	KX950450	KX950482	–	–	–	–
140282	277-I5	"dark" group	Ilomba treated with raw linseed oil	The Netherlands	KX950424	KX950451	KX950483	–	–	–	–
140283	277-I6	"dark" group	Spruce treated with olive oil	The Netherlands	KX950425	KX950452	KX950484	–	–	–	–
140284	277-I7	"dark" group	Spruce treated with olive oil	The Netherlands	KX950426	KX950453	KX950485	–	–	–	–
140285	277-I8	"dark" group	Pine sw treated with stand linseed oil	The Netherlands	KX950427	KX950454	KX950486	–	–	–	–
140286	277-I9	"dark" group	Spruce treated with olive oil	The Netherlands	KX950428	KX950455	KX950487	–	–	–	–
140343	278-A2	"dark" group	Ilomba treated with raw linseed oil	The Netherlands	KX950429	KX950456	KX950488	–	–	–	–
140287	278-A3	"dark" group	Spruce treated with olive oil	The Netherlands	KX950430	KX950457	KX950489	–	–	–	–
140288	305-D9	"dark" group	Pine sw treated with raw linseed oil	The Netherlands	KX950431	KX950458	KX950490	–	–	–	–
140289	305-E2	"dark" group	Pine sw treated with raw linseed oil	The Netherlands	KX950432	KX950459	KX950491	–	–	–	–
140344	305-E3	"dark" group	Pine sw treated with raw linseed oil	The Netherlands	KX950433	KX950460	KX950492	–	–	–	–
140290	301-G1	"green" group	Pine hw treated with raw linseed oil	Australia	KX950434	KX950461	–	KX999145	KX999144	KX999146	KX999147
–	301-G2	"green" group	Pine hw treated with raw linseed oil	Australia	KX950435	–	–	–	–	–	–
–	301-G3	"green" group	Pine hw treated with raw linseed oil	Australia	KX950436	–	–	–	–	–	–
–	301-G4	"green" group	Pine hw treated with raw linseed oil	Australia	KX950437	–	–	–	–	–	–

Analyses	Data set	Source of data set	No. of taxa	No. of loci	No. of char.	Phylogenetic placement
"Dark" group						
Analysis 1 (Fig. S1)	*Fungi*	James *et al.* (2006)	219	6	8 482	*Arthoniomycetes* + *Dothideomycetes* clade (BS = 80 %); *Dothideomycetes* (BS = 64 %); sister to *Trypethelium* (BS = 39 %)
Analysis 2 (Fig. S2)	*Pezizomycotina*	Carbone *et al.* (unpubl. data)	986	6	7 269	*Arthoniomycetes* + *Dothideomycetes* clade (BS = 58 %); *Dothideomycetes* (BS = 36 %); sister to *Trypetheliales* (BS = 37 %)
Analysis 2A	*Pezizomycotina*	Carbone *et al.* (unpubl. data)	983	6[1]	7 269	*Dothideomycetes* (BS = 30 %); *Botryosphaeriaceae* (BS = 56 %)
Analysis 2B	*Pezizomycotina*	Carbone *et al.* (unpubl. data)	986	6[2]	7 269	*Dothideomycetes* (BS = 40 %); related to *Kirschsteiniotheliaceae* and part of *Phaeotrichaceae* (BS = 32 %)
Analysis 3 (Fig. 1, Table S3)	*Dothideomycetes* + *Arthoniomycetes*	Carbone *et al.* (unpubl. data) and Schoch & Grube (2015)	236	7	7 072	*Dothideomycetes* (BS = 68 %); sister to *Trypetheliales* (BS = 86 %)
Analysis 4 (Fig. S3, Table S3)	*Dothideomycetes* + *Arthoniomycetes* + additional *Trypetheliales*	Carbone *et al.* (unpubl. data) and Nelsen *et al.* (2009, 2011)	260	7	7 072	*Dothideomycetes* (BS = 75 %); sister to *Trypetheliales* (BS = 49 %)
Analysis 5 (Fig. S4)	*Trypetheliales*	Nelsen *et al.* (2014) and Ertz *et al.* (2015)	95	2	1 011	Outside of *Trypetheliales*
"Green" group:						
Analysis 1 (Fig. S1)	*Fungi*	James *et al.* (2006)	219	6	8 482	*Lecanoromycetes*, *Ostropomycetidae*, *Ostropales* (BS = 80 %)
Analysis 6 (Fig. S5)	*Lecanoromycetes*	Miadlikowska *et al.* (2014)	1 319	5	7 433	*Ostropales* (BS = 21 %); sister to *Cyanodermella viridula* (BS = 100 %)
Analysis 7 (Fig. 3)	*Ostropomycetidae*	Resl *et al.* (2015)	207	8	6 665	*Ostropales* (BS = 76 %); *Stictidaceae* (BS = 43 %); sister to *Cyanodermella viridula* (BS = 100 %)

[1] "dark" group is represented by the ribosomal RNA loci only (ITS, nrLSU, and nrSSU).
[2] "dark" group is represented by the protein-coding loci only (*RPB1*, *RPB2* and *EF-1α*).

position *RPB1*, *RPB2*; 2nd position *RPB1*, *RPB2*; 3rd position *RPB1*, *RPB2*; 1st position *EF-1α*; 2nd position *EF-1α*; and 3rd position *EF-1α*). Data partitions were estimated with Partition-Finder (greedy algorithm to explore all the nucleotide substitution models under the BIC selection criterion; Lanfear *et al.* 2012).

To infer the general phylogenetic placement (at the class level) of the two unknown pycnidia producing groups within the fungal kingdom, and to delineate the phylogenetic context for the next round of phylogenetic analyses, we added multi-locus sequences of five unknown strains to a data set which has been used to infer the phylogeny of the kingdom *Fungi* (Analysis 1). Based on these results, the subsequent data sets were restricted to *Pezizomycotina* (Analysis 2), "*Dothideomyceta*": *Dothideomycetes* and *Arthoniomycetes* (Analyses 3), "*Dothideomyceta*" with additional *Trypetheliales* data (Analysis 4) and *Trypetheliales* (Analysis 5) for the phylogenetic placement of the "dark" group, and to *Lecanoromycetes* (Analyses 6) and *Ostropomycetidae* (Analysis 7) for the placement of the "green" group.

Analysis 1: The multi-locus sequences of four strains from the "dark" group (CBS 353.84, CBS 140270, CBS 140271 and CBS 140272) and one strain from the "green" group (CBS 140290) were added to the James *et al.* (2006) data set containing six gene regions (three nuclear ribosomal loci: nrLSU, nrSSU, and 5.8S, and three protein-coding genes: *EF-1α*, *RPB1* and *RPB2*). The maximum likelihood analyses were performed on 8 482 characters for 219 taxa (Table 2). **Analysis 2**: Sequences of the four representatives from the "dark" group were

added to the six-locus supermatrix (5.8S, nrLSU, nrSSU, mitSSU, *RPB1*, *RPB2*) used to generate the most comprehensive phylogeny for *Pezizomycotina* (986 taxa) as part of the online T-BAS tool (http://tbas.hpc.ncsu.edu; Carbone *et al.* unpubl. data). We also completed analyses on the same data set but our fungal strains were represented by the ribosomal (Analysis 2A; 5.8S, nrLSU, and nrSSU) or protein-coding loci (Analysis 2B; *RPB1*, *RPB2*, and *EF-1α*) only. **Analysis 3**: Based on the placement of the unknown isolates resulting from Analyses 1 and 2 (inside of *Dothideomycetes*, sister to *Trypetheliales*), we restricted this data set to *Dothideomycetes* + *Arthoniomycetes* using the T-BAS function for downloading alignments of selected clades (http://tbas.hpc.ncsu.edu; Carbone *et al.* unpubl. data). We supplemented the six-locus supermatrix by sequences of another protein-coding locus, *EF-1α* available in GenBank (70 sequences; Schoch & Grube 2015) and completed a maximum likelihood analysis on 7 072 unambiguously aligned characters for 236 taxa. **Analysis 4**: For this set of analyses, we extended the seven-locus data set (5.8S, nrLSU, nrSSU, mitSSU, *RPB1*, *RPB2*, *EF-1α*) used in Analysis 3 by adding more members of *Trypetheliales* (24 taxa; Nelsen *et al.* 2009, 2011) for a total of 260 taxa (7 072 characters) to better infer the placement of the four fungal strains from the "dark" group within the *Dothideomycetes*. **Analysis 5**: To infer, more specifically, the phylogenetic placement of the unknown "dark" group fungi with regard to *Trypetheliales*, we used the two-locus (nrLSU and mitSSU) data set for the family

Trypetheliaceae from Nelsen *et al.* (2014) supplemented by nrLSU sequences of six representatives from the recently described family *Polycoccaceae* (Ertz *et al.* 2015), for a total of 95 taxa and 1 011 characters. **Analysis 6**: Based on the placement of the single representative from the "green" group resulting from Analysis 1, we used the *Lecanoromycetes* data set generated by Miadlikowska *et al.* (2014) and added the same representative for a total of 1 319 taxa (7 433 characters). **Analysis 7**: Phylogenetic analyses for the "green" group was performed with the most recent data set (ITS, nrLSU, nrSSU, mitSSU, *MCM7*, *RPB1*, *RPB2*, *EF-1α*) available for *Ostropomycetidae* (Resl *et al.* 2015) and the addition of *Cyanodermella viridula* for a total of 207 taxa and 6 665 included characters (Table 2). Selected multi-locus matrices and the resulted RAxML trees are available in TreeBASE (http://purl.org/phylo/treebase/phylows/study/TB2:S20205).

Single-locus phylogenetic analyses

To delimit species (and to identify clades for which we obtained additional genes for selected representatives; Table 2) within the "dark" group, we followed the phylogenetic species concept relying on the monophyly criterion and the pattern of congruence among single-locus phylogenies. We assembled and completed ML analyses (as described above) on single-locus data sets of 23 strains: for the ITS (702 sites, one indel excluded), nrLSU (759 sites), and *EF-1α* (960 sites). Strain CBS 353.84 was used to root the trees based on the phylogenetic relationships among strains inferred with Analyses 1–4 (Table 2). Pairwise distances among 23 strains representing the "dark" pycnidia-containing fungal clade were calculated with PAUP 4.0a147 (Swofford 2003).

Morphological data

To characterise phenotypically the newly delimited taxa, the isolates of the "green" and the "dark" group were subjected to morphological observations. Strains were inoculated in a three point positions onto MEA, potato dextrose agar (PDA), oatmeal agar (OA) and synthetic nutrient-poor agar (SNA) plates. In addition, the strains of the "dark" group were inoculated on a sterilised nettle stem embedded in OA. All agar media were prepared as described by Samson *et al.* (2010). Strains were grown in dual sets and incubated at 25 °C. Isolates representing the "green" group were studied after seven days of incubation and the "dark" group strains were studied after two and five weeks of incubation. Culture images were captured with a Canon 400D digital camera and a Nikon SMZ25 stereomicroscope. Colony diameters, colony colours and other macroscopic features were recorded. Microscopic characteristics of cultures on OA were studied with an Axio Imager A2 light microscope.

RESULTS

Taxonomic identity of fungal isolates based on BLAST

The results of BLAST searches against GenBank data using ITS and nrLSU sequences were highly inconclusive and varied considerably even at the class level. The top matches, depending on the locus, length of the entry sequence and sorting option of the GenBank hits, indicated that the newly sequenced strains represent two taxonomically different groups of fungi: the top hits for the "dark" group were mostly *Dothideomycetes* or *Sordariomycetes* (based on 22 isolates), whereas for the "green" group top hits represented *Lecanoromycetes* or *Leotiomycetes* (based on four isolates) (Table S2). Overall, the top BLAST matches, regardless of the locus, had relatively low maximum identity scores ($\leq$85 % and 92 % for the "dark" and "green" groups, respectively) and most query coverages were low as well ($\leq$91 % and 94 % for each group), except for the largest fragment of the nrLSU for the "green" isolate (100 %). The sequences of the "dark" fungi could represent *Dothideomycetes* based on top matches ranked by maximal identity score of ITS (top hit: *Valsaria neotropica*), nrLSU long fragment (LR0R-LR7 sequence top hits: *Verminconia calcicola* and *Preussia minima*), and nrLSU short fragment (top hit LR0R-LR5: *Cystocoleus ebeneus*). However, some top BLAST matches with the higher query cover showed affiliation with other fungal classes, *Leotiomycetes* and *Sordariomycetes* (Table S2). The "green" isolates matched mostly the class Lecanoromycetes, subclass *Ostropomycetidae* when using ITS sequences (top hit *Stictis radiata*) or *Umbilicariomycetidae* when using nrLSU sequences (top hit *Umbilicaria torrefacta*). Only the top hit of the ITS based on maximum identity showed an affiliation with another class, *Leotiomycetes* (*Leptodontidium elatius*, Table S2).

After screening the ITS and nrLSU sequences from the CBS database, one strain (CBS 353.84) had a high LSU sequence similarity (more than 97 %) to isolates from the "dark" group. This strain was isolated from a leaf of *Hakea multilinearis* in Australia by W. Gams in 1984, however, it lacked a proper taxonomic name. The strain was included in the subsequent phylogenetic analyses and morphological examinations of the "dark" group (Table 1).

Because all four ITS sequences from the "green" group were identical, a single strain (CBS 140290) was selected to represent this group in all multi-locus phylogenies. The ITS sequences from the "dark" group were more variable. They contained ten different sequences in total. The ML phylogeny based on ITS showed at least four clades (Fig. 2) and strains CBS 353.84, CBS 140272, CBS 140271, and CBS 140270 were selected to represent these clades in the multi-locus phylogenies.

Phylogenetic placement of the "dark" group of isolates within *Dothideomycetes*

The four strains representing the "dark" group formed monophyletic clades in all reconstructed multi-locus phylogenies (bootstrap support [BS] of 100 %; Figs 1, S1–S4). Based on the phylogenetic analysis of the James *et al.* (2006) data set that covered the fungal kingdom (Analysis 1; Fig. S1, Table 2), the "dark" group lineage was found sister to *Trypethelium* sp., a member of the family *Trypetheliaceae* in the order *Trypetheliales* (taxa from the family *Polycoccaceae* were not included in the multi-locus analyses 1–4 because only the nrLSU sequences were available in GenBank; Ertz *et al.* 2015). Although, this relationship, as well as the monophyly of the entire class *Dothideomycetes* where *Trypetheliales* are currently classified (e.g., Spatafora *et al.* 2006, Nelsen *et al.* 2009, 2011, Schoch *et al.* 2009a), received a low bootstrap support (of 39 % and 64 %, respectively), "*Dothideomyceta*" (a clade encompassing

Arthoniomycetes and *Dothideomycetes*; Schoch *et al.* 2009b, Nelsen *et al.* 2009) was highly supported (80 %) in this phylogeny. Overall, the phylogenetic relationships and their stability in this tree are congruent with the phylogeny from James *et al.* (2006).

For the subsequent phylogenetic analysis, we restricted the reference taxa to *Pezizomycotina* utilizing the most taxon-, and locus-comprehensive data set available (Analysis 2, 986 taxa; Carbone *et al.* unpubl. data). Based on this multi-locus analysis, the sister relationship of the "dark" group lineage with *Trypetheliaceae* (represented by five genera) was also recovered, but similar to the results from Analysis 1, with very low support (BS = 37 %). Both relationships, the monophyly of *Dothideomycetes* and its close affiliation with *Arthoniomycetes*, were poorly supported as well (Fig. S2, Table 2).

Because the ribosomal genes from the "dark" group were very different from fungal sequences available in GenBank, we performed additional analyses on the *Pezizomycotina* data set where isolates from the "dark" group lineage were represented exclusively by the ribosomal (Analysis 2A) versus the protein coding loci (Analysis 2B). Only the latter analysis showed an increased support (BS 40 %) compared to analysis 2, but still remained below 70 %. In all three analyses, the phylogenetic relationships of known taxa and their stability within *Pezizomycotina* were generally in agreement with published phylogenies (e.g., Schoch *et al.* 2009b, Carbone *et al.* unpubl. data).

Because the subphylum-wide context (*Pezizomycotina*) of this data set resulted in numerous and often large ambiguously aligned regions that had to be excluded from the phylogenetic analyses, we limited the taxon sampling to "*Dothideomyceta*" in order to increase the phylogenetic resolution and tree robustness for Analyses 3 and 4 (Tables 2 and S3). The phylogeny resulting from Analysis 3 confirmed for the first time the sister relationship of the "dark" fungal lineage to *Trypetheliaceae* with high confidence (BS = 86 %; Fig. 1, Table 2). Because in Analyses 2 and 3, the order *Trypetheliales* was represented by a few taxa only (five and four, respectively; Figs S2 and 1), in Analysis 4 we increased the number of individuals to 28 from twelve genera (Fig. S3, Tables 2, S3). Although the sister relationship of the unknown fungal lineage with *Trypetheliaceae* was recovered, bootstrap support went down from 86 % (Fig. 1) to 49 % (Fig. S3, Table 2). Clades representing *Dothideomycetes* received bootstrap support above 70 %, and the relationships among orders and families within both classes were in agreement with previous phylogenies (e.g., Schoch *et al.* 2009b). While the phylogenetic placement of *Trypetheliales* within *Dothideomycetes* remains unsettled (e.g., Schoch *et al.* 2009a, Nelsen *et al.* 2009, and this study), the order *Trypetheliales* was often recovered as one of the earliest evolutionary splits in the class *Dothideomycetes*, but always with low support. To better infer the phylogenetic placement of the "dark" fungal lineage in relation to *Trypetheliales* (i.e., outside versus nested within the order) we used a two-locus (nrLSU and mitSSU) data set (Nelsen *et al.* 2014) encompassing 79 members from *Trypetheliaceae* and six from *Polycoccaceae* (Analysis 5). The resulting phylogeny demonstrated that the unknown fungal lineage was placed outside of the order (its monophyly as well as the monophyly of each family was highly supported; >70 %), and therefore recognized as sister to *Trypetheliales* (Fig. S4, Table 2). Delimitations of the putative genera and informal groups, and their relationships in *Trypetheliaceae* and *Polycoccaceae* are in agreement with Ertz *et al.* (2015) and Nelsen *et al.* (2014), however, bootstrap support for

many internodes went down compared to their phylogenies, most likely because we excluded broader ambiguously aligned regions.

Species delimitation within the unknown lineage of *Dothideomycetes*

All multi-locus phylogenies suggested the presence of multiple species in the "dark" group of fungi, which were phylogenetically placed in *Dothideomycetes*. Within this unknown lineage of *Dothideomycetes*, the sister relationship between strains CBS 140271 and CBS 140270, and their close affiliation with strain CBS 140272 was recovered in all (Figs 1, S1–S3) but one phylogeny (Fig. S4 shows the alternative arrangement involving sister relationship of the latter strain with CBS 353.84 based on two ribosomal loci, nrLSU and mitSSU with BS = 75 %). Using the relationships among strains in the multi-locus phylogenies, we chose CBS 353.84 (the only non-wood isolate) to root single-locus (ITS, nrLSU, and *EF-1α*) phylogenies inferred for 22 strains for which these three markers were sequenced (Fig. 2, Table 1). In all phylogenies the wood isolates were clustered in two highly supported (BS = 100 %) groups. We considered strain CBS 353.84 as putative species 1, while the two main clades were recognised as putative species 2 and 3. The ITS and *EF-1α* provided more phylogenetic structure than nrLSU within species 3, which was also represented by the greatest number of isolates (19 strains versus three in species 2, and one in species 1) and may include additional putative taxa. The recognition of three potential species, as shown in Fig. 2, is corroborated by the higher genetic distance observed among them in comparison to genetic variation found within each species (Table S4). The *EF-1α* sequence for CBS 140344 was very different from the remaining individuals in species 3 (differ by 15 nucleotides) and might represent another taxon. However, multiple copies of *EF-1α* in fungi have incidentally been reported (James *et al.* 2006, Henk & Fisher 2012, Ekanayake *et al.* 2013) since the first evaluation of this gene as a phylogenetic marker in eukaryotes (Roger *et al.* 1999).

Phylogenetic placement of the "green" group of isolates within *Ostropales*

Based on the phylogeny spanning the fungal kingdom (James *et al.* 2006), the selected representative strain of the "green" group was placed within the order *Ostropales* (BS = 80 %), subclass *Ostropomycetidae* in the mostly lichenised class *Lecanoromycetes* (Fig. S1, Table 2). Analysis 6 on the data set restricted to *Lecanoromycetes* (Miadlikowska *et al.* 2014) confirmed the affiliation of these fungi with *Ostropales*, although with low bootstrap support (Fig. S5). More specifically, it showed a sister relationship with *Cyanodermella viridula* (BS = 100 %; Fig. S5), a member of the family *Stictidaceae* (Eriksson 1981). The large scale *Ostropomycetidae* data set for eight loci from the study by Resl *et al.* (2015) used in Analysis 7, enabled us to confirm the placement of the "green" group within *Ostropales* (BS = 76 %) (Fig. 3). However, the family *Stictidaceae*, including the highly supported clade containing *Cyanodermella viridula* and the unknown fungal lineage, was poorly supported (below 50 %). Ambiguously aligned regions in our reassembled alignments were more broadly delimited, and therefore more characters were excluded, compared to Resl *et al.* (2015), especially
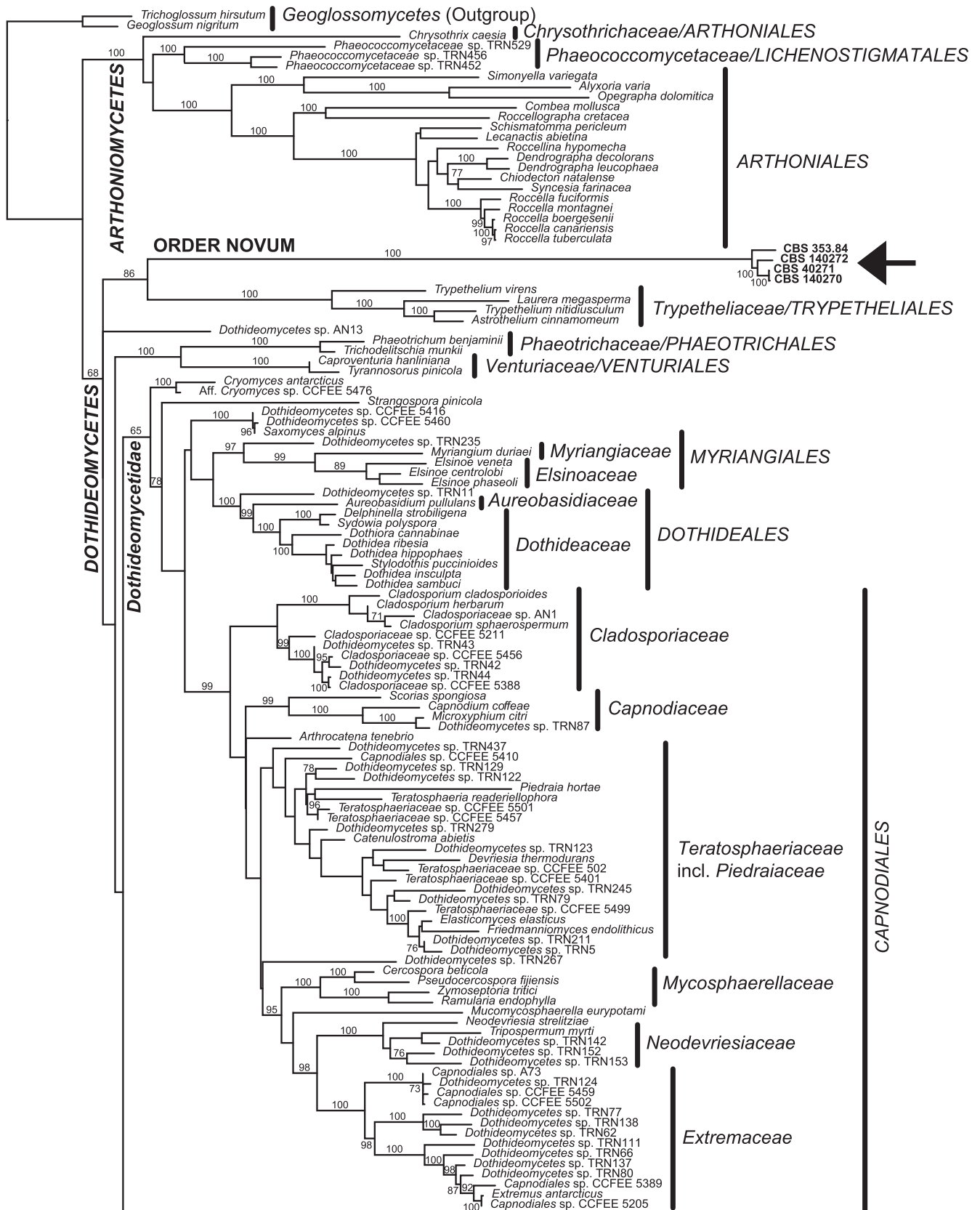
Fig. 1. ML phylogeny based on a seven-locus data set (5.8S, nrSSU, nrLSU, mitSSU, *RPB1*, *RPB2*, and *EF-1α*) for 236 taxa representing "*Dothideomyceta*" (Analysis 3, Table 2): *Arthoniomycetes* (21 taxa) and *Dothideomycetes* (209 taxa) including four strains from the "dark" group, and two representatives of *Geoglossomycetes* (used to root the tree). Families and higher taxa in *Arthoniomycetes* were delimited following Ertz *et al.* (2014) and in *Dothideomycetes* according to Wijayawardene *et al.* (2014). Because of incomplete taxon sampling for *Pleosporales*, many families are underrepresented and not monophyletic and, consequently, are not annotated. Bootstrap support values above 70 % (with the exception for *Dothideomycetes* and *Dothideomycetidae*; BS = 68 % and 65 %, respectively) are shown for each internode. Taxon selection and alignments (except for *EF-1α* locus; Schoch & Grube 2015) were assembled using T-BAS (Carbone *et al.* unpubl. data). Arrow indicates the placement of "dark" group strains as a separate monophyletic lineage (BS = 100 %; a putative new order) sister to *Trypetheliales* (BS = 86 %).
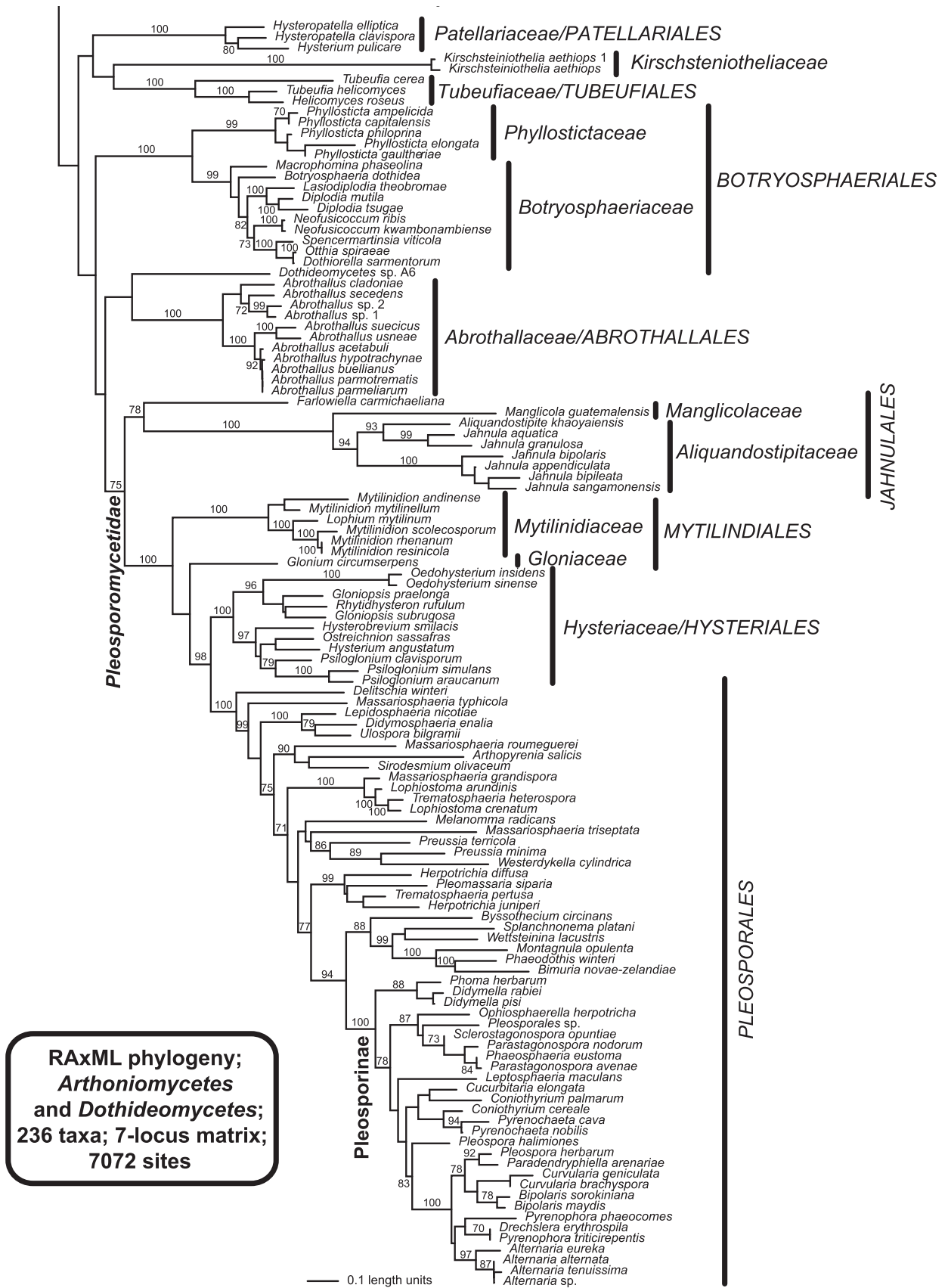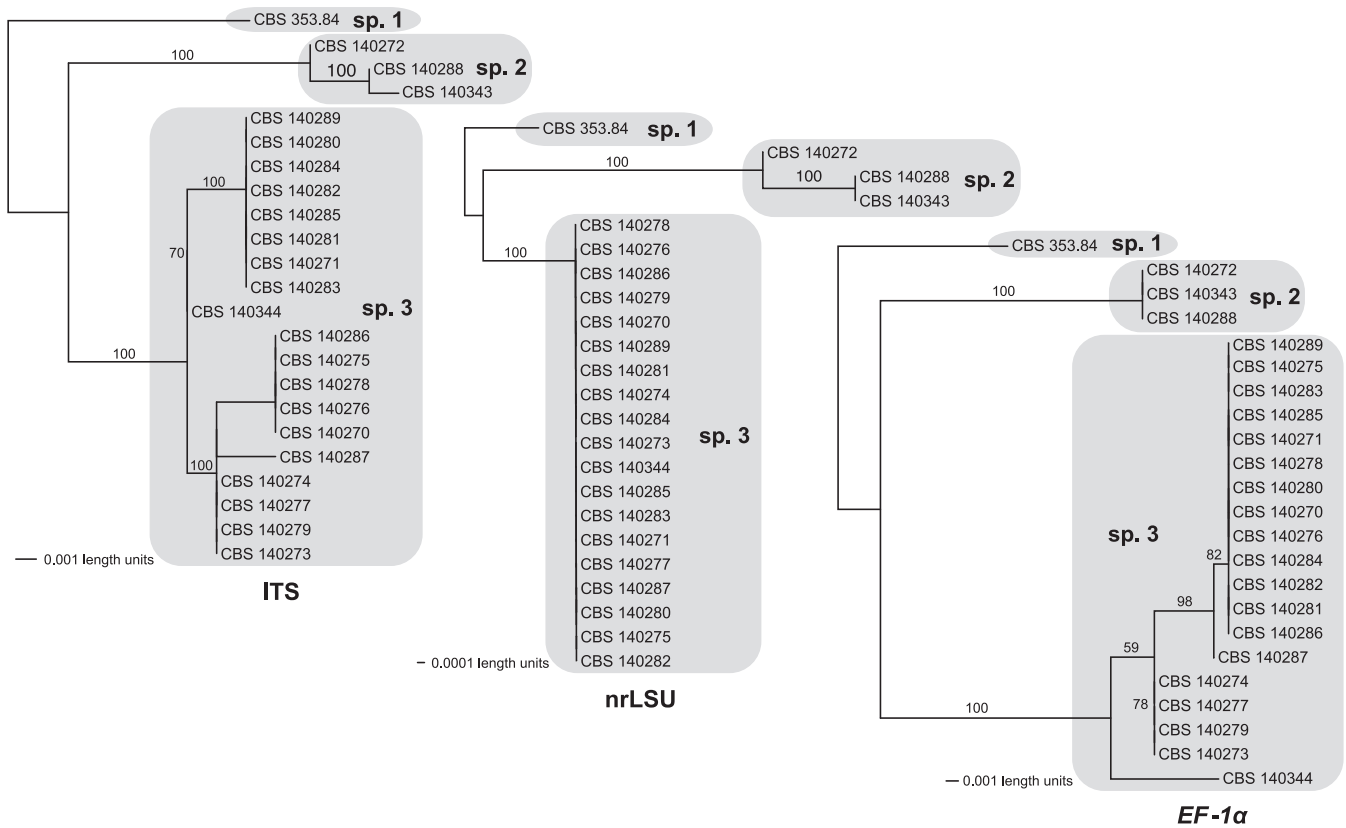
Fig. 1. (Continued)

Fig. 2. Single-locus ML phylogenies based on the ITS (701 characters), nrLSU (759 characters), and *EF-1α* (960 characters) for 23 dark pycnidia-forming fungal strains (indicated by CBS culture numbers) strongly supporting the monophyletic delimitation of clades recognized as putative species (sp. 2 and sp. 3) within the newly discovered order of *Dothideomycetes* (Fig. 1). The third putative species (sp. 1) represented by strain CBS 353.84 was used to root each tree based on the multi-locus phylogenies (Figs. 1, S2 and S3). Bootstrap support values ≥70 % are shown above internodes. The branch length units represent the number of substitutions per site.

from the ITS1 and ITS2 regions (6 665 sites were included in our phylogenetic study versus 8 978 sites in Resl *et al.* 2015). Nevertheless, the overall delimitation of taxa and their relationships (with a few exceptions above the order level, but not well supported), as well as branch robustness, remained at a similar level (37 internodes supported above 70 %). We observed a decrease in bootstrap values (below 70 %) for eleven nodes, but also an increase in support for two internodes in *Ostropales* (Fig. 3) in comparison to the phylogeny from Resl *et al.* (2015).

Taxonomy of the newly discovered taxa

Superstratomycetales van Nieuwenhuijzen, Miadlikowska, Lutzoni & Samson, **ord. nov.** MycoBank MB819160.

Etymology: From Latin "super" = above/on the top, and "stratum" = layer of material and from Greek "myces" = organisms, referring to fungal stains forming a top layer or covering the surface of a material.

Diagnosis: Phylogenetically placed in the class *Dothideomycetes* and potentially affiliated with *Trypetheliales*, but outside all currently recognised orders of fungi (James *et al.* 2006, Carbone *et al.* unpubl. data; Schoch & Grube 2015).

Type genus: *Superstratomyces* van Nieuwenhuijzen, Miadlikowska & Samson

Includes a single family *Superstratomycetaceae* and a single genus *Superstratomyces*.

Superstratomycetaceae van Nieuwenhuijzen, Miadlikowska, Lutzoni & Samson, **fam. nov.** MycoBank MB819161.

Type genus: *Superstratomyces* van Nieuwenhuijzen, Miadlikowska & Samson

Diagnosis: Same as the order Superstratomycetales (see above).

Superstratomyces van Nieuwenhuijzen, Miadlikowska & Samson, **gen. nov.** MycoBank MB819162

Diagnosis: Same as the order Superstratomycetales (see above).

Type species: *Superstratomyces albomucosus* van Nieuwenhuijzen & Samson
Includes three species.

Superstratomyces albomucosus van Nieuwenhuijzen & Samson, **sp. nov.** MycoBank MB819163. Fig. 4.

Etymology: From Latin "albus" = white, "mucosus" = slimy; refers to conidia in white slimy masses.

Diagnosis: Slow growing olive to light grey green colonies with thin white edge (MEA and OA), pycnidia forming aggregated masses (MEA and OA) and white coloured slimy masses (OA). Differs phylogenetically (based on the ITS, nrLSU and *EF-1α* loci) from the remaining two species in the genus.
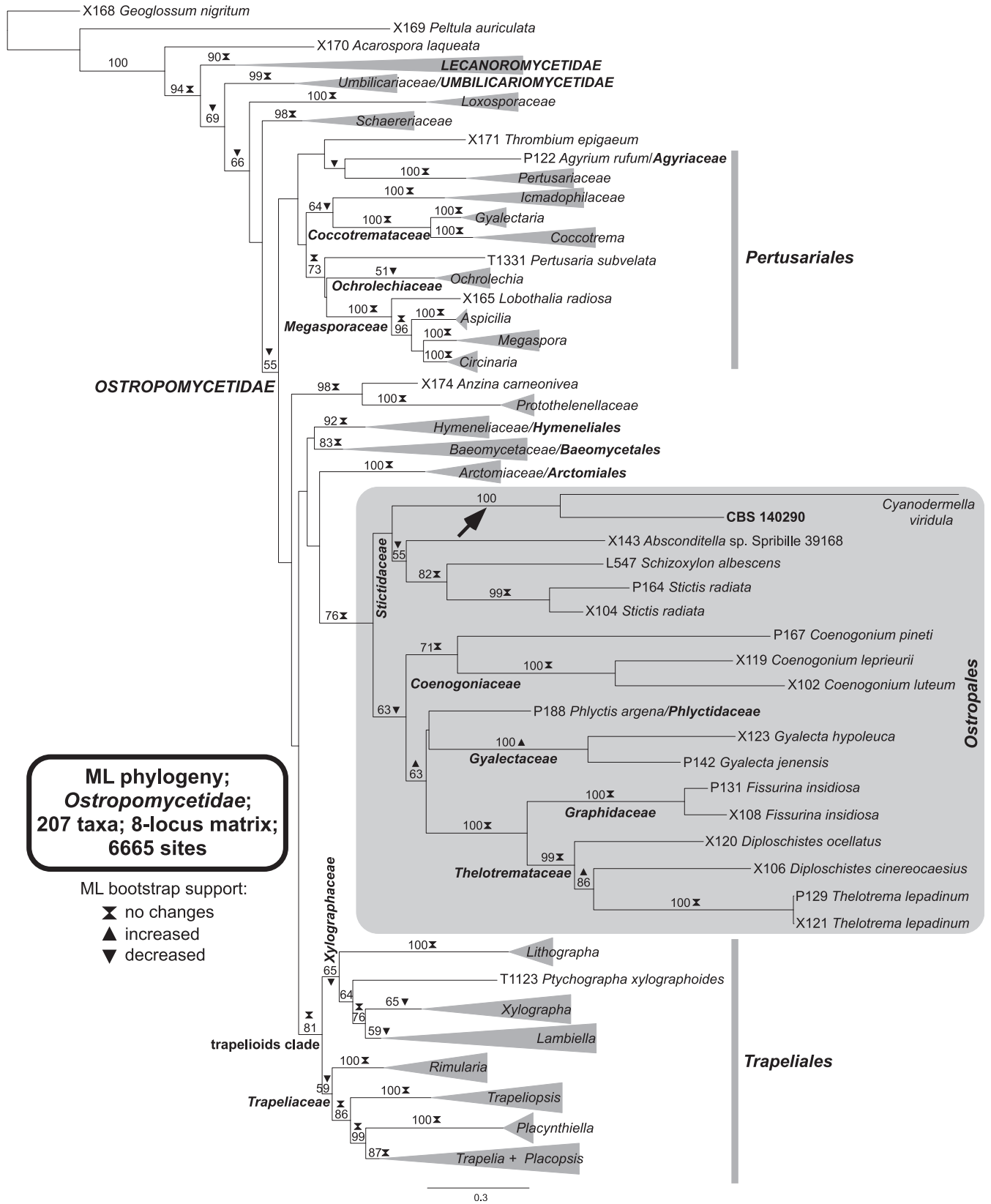
Fig. 3. ML phylogeny (Analysis 7, Table 2) based on an eight-locus data set (ITS, nrLSU, nrSSU, mitSSU, *MCM7*, *RPB1*, *RPB2*, *EF-1α*) for 207 taxa representing *Ostropomycetidae*, the outgroup species from *Lecanoromycetes* and *Lichinomycetes*, and one representative of *Geoglossomycetes* (used to root the tree). Sequences of the fungal strain CBS 140290 and *Cyanodermella viridula* were added to the concatenated matrix of the 205 reference taxa derived from Resl *et al.* (2015). Branches outside *Ostropales* represent genera and families (collapsed when monophyletic and represented as grey triangles). Families and higher taxa were delimited following Miadlikowska *et al.* (2014) and Resl *et al.* (2015). Boostrap support values above 50 % and its comparison to ML bootstrap support values obtained by Resl *et al.* (2015) are provided above each internode (see figure legend for symbol explanations).
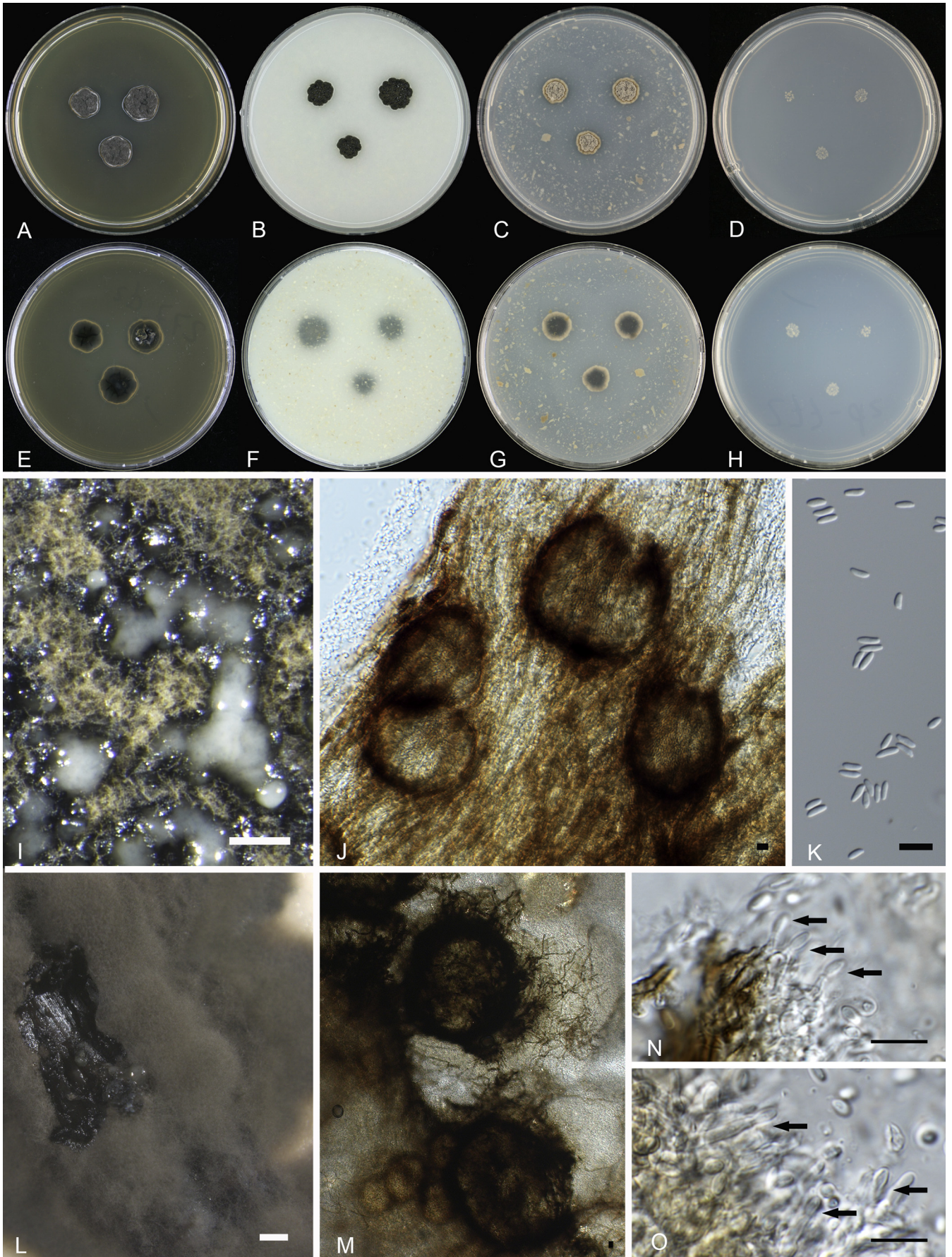
Fig. 4. *Superstratomyces albomucosus.* **A–H.** Colonies 2 wk old 25 °C; **A.** MEA obverse; **B.** OA obverse; **C.** PDA obverse; **D.** SNA obverse; **E.** MEA reverse; **F.** OA reverse; **G.** PDA reverse; **H.** SNA reverse; **I.** Macroscopic structure of colony on OA showing aerial hyphae and white coloured slimy masses; **J.** Pycnidia, hyphae and released conidia; **K.** Conidia; **L.** Macroscopic structure of a partly scratched colony on a nettle stem embedded in OA; **M.** Pycnidia and released conidia; **N–O.** Conidiogenous cells. Scale bars: I, L = 1 000 μm; J, K, M–O = 10 μm.

Type: **The Netherlands**, Utrecht, outdoor exposed *Pycnanthus angolensis* impregnated with olive oil, collector E.J. van Nieuwenhuijzen, 09 Sept 2013 (**holotype** H-22668; culture ex type CBS 140270 = DTO 277-D2).

Colony characteristics: colony diameters, 2 wk, in mm, 25 °C: MEA 2–13; OA 4–13; PDA 4–12; SNA: 1–5, poor growth. MEA colony: obverse olive to grey-green or light grey-green with thin white edge; reverse dark green or dark green with thin white edge; knotted cone-shaped; slimy masses inconspicuous. OA colony: obverse olive to grey-green or light grey-green with thin white edge; reverse dark green; colony edge slightly elevated; white slimy masses containing abundant amounts of conidia. PDA colony: obverse light grey with thin white edge; reverse dark green or dark green with thin white edge or light brown with thin white edge, tufted colony with centre elevated; slimy masses absent.

Micromorphology OA, 25 °C, 2–5 wk: pycnidia forming brown to black aggregated masses, individual fruiting bodies not present; dark pigmented hyphae; conidia smooth walled, oval typically with blunt ends, length 2.8–6.0 μm, width 1.5–3.0 μm; aerial mycelium hyaline. Micromorphology nettle stem OA, 25 °C, 4–5 wk: pycnidia brown/black, spherical to subspherical, diameter 80–300 μm; conidiogenous cells phialidic.

Ecology and distribution: All strains were isolated from wood impregnated with olive or linseed oil that was exposed to the outdoors in Utrecht, The Netherlands.

Additional cultures examined: **The Netherlands**, Utrecht, outdoor situated log of timber impregnated with oil (wood species and oil types specified in Table 1) 09 Sept 2013 (CBS 140273 = DTO 277-C8, CBS 140274 = DTO 277-C9, CBS 140275 = DTO 277-D3, CBS 140276 = DTO 277-D4), 13 Sept 2013 (CBS 140271 = DTO 277-I3, CBS 140277 = DTO 277-H6, CBS 140278 = DTO 277-H7, CBS 140279 = DTO 277-H8, CBS 140280 = DTO 277-I2, CBS 140281 = DTO 277-I4, CBS 140282 = DTO 277-I5, CBS 140283 = DTO 277-I6, CBS 140284 = DTO 277-I7, CBS 140285 = DTO 277-I8, CBS 140286 = DTO 277-I9, CBS 140287 = DTO 278-A3), 1 May 2014 (CBS 140289 = DTO 305-E2 and CBS 140344 = DTO 305-E3), E.J. van Nieuwenhuijzen.

Superstratomyces atroviridis van Nieuwenhuijzen & Samson, **sp. nov.** MycoBank MB819165. Fig. 5.

Etymology: From Latin "ater" = dark, black, "viride" = green; referring to colonies coloured dark green/ black on agar plates.

Diagnosis: Slow growing grey/olive to dark green colonies, with pycnidia forming aggregated masses (MEA and OA) and little or no slimy masses up to five weeks (OA). Differs genetically (based on the ITS, nrLSU and *EF-1α* loci) from the remaining two species in the genus.

Type: Utrecht, The Netherlands, outdoor exposed *Pinus sylvestris* impregnated with raw linseed oil, collector E.J. van Nieuwenhuijzen, 01 May 2014 (**holotype** = H-22669; culture ex type = CBS 140272).

Colony characteristics: colony diameters, 2 wk, in mm, 25 °C: MEA 4–11; OA 7–13; PDA 6–9; SNA: 3–5, poor growth. MEA colony: obverse dark olive to grey green; reverse dark green; tufted colonies with centre elevated; slimy masses inconspicuous. OA colony: obverse dark green; reverse dark green; tufted colonies with centre elevated; incidentally small spots of

superficial transparent slimy masses. PDA colony: obverse dark green or grey brown centre and dark green edge; reverse dark green; tufted colonies with centre elevated; slimy masses absent.

Micromorphology OA, 25 °C, 2–5 wk: aggregated mass of dark brown to black pycnidia, individual fruiting bodies not visible; dark pigmented hyphae; conidia smooth walled, oval typically with blunt ends, length (2 wk) 2.6–6.5 μm, width 1.6–2.8 μm; aerial mycelium hyaline. Micromorphology nettle stem OA, 25 °C, 4–5 wk: individual fruiting bodies inconspicuous; conidiogenous cells phialidic.

Ecology and distribution: All three strains were isolated from wood impregnated with linseed oil that was exposed to the outdoors in Utrecht, The Netherlands.

Additional cultures examined: **The Netherlands**, Utrecht, outdoor situated log of *Pinus sylvestris* sapwood impregnated with raw linseed oil, 01 May 2014 (CBS 140288 = DTO 305-D9) and *Pycnanthus angolensis* impregnated with raw linseed oil, 13 Sept 2013 (CBS 140343 = DTO 278-A2), E.J. van Nieuwenhuijzen.

Superstratomyces flavomucosus van Nieuwenhuijzen & Samson, **sp. nov.** MycoBank MB819164. Fig. 5.

Etymology: From Latin "flavus" = yellow, "mucosus" = slimy; refers to conidia in yellow slimy masses.

Diagnosis: Slow growing olive to grey green colonies with pycnidia forming aggregated masses and yellow coloured slimy masses (MEA and OA). Differs genetically (based on the ITS, nrLSU and *EF-1α* loci) from the remaining two species in the genus.

Type: **Australia**, Perth, leaf of *Hakea multilinearis*, collector W. Gams, 01 Aug 1983 (**holotype** H-22667; culture ex type CBS 353.84).

Colony characteristics: colony diameters, 2 wk, in mm, 25 °C: MEA 8–16; OA 13–16; PDA 10–13; SNA: 4–5, poor growth. MEA colony: obverse olive to grey green; reverse dark green; knotted cone-shaped; yellow slimy masses containing abundant amounts of conidia. OA colony: obverse olive to grey green; reverse dark green; colony edge slightly tufted; yellow slimy masses containing abundant amounts of conidia. PDA colony: light grey with dark green edge; reverse dark green; colony centre clearly tufted; slimy masses absent.

Micromorphology OA, 25 °C, 2 wk: pycnidia forming yellow to dark brown aggregated masses, individual fruiting bodies not visible; dark pigmented hyphae; conidia smooth walled and oval; conidia length 3.4–6.1 μm, width 2.0–3.4 μm; aerial mycelium hyaline. Nettle stem OA, 25 °C, 4–5 wk: pycnidia brown/yellow, spherical to subspherical, diameter 80–170 μm; conidiogenous cells phialidic.

Ecology and distribution: A single strain representing this species was isolated from a leaf of *Hakea multilinearis*, Perth, Australia.

Cyanodermella oleoligni van Nieuwenhuijzen & Samson, **sp. nov.** MycoBank MB819166. Fig. 6

Etymology: From Latin "lignum" = wood, which is the main element of the substrate and "oleum" = oil, which is describing
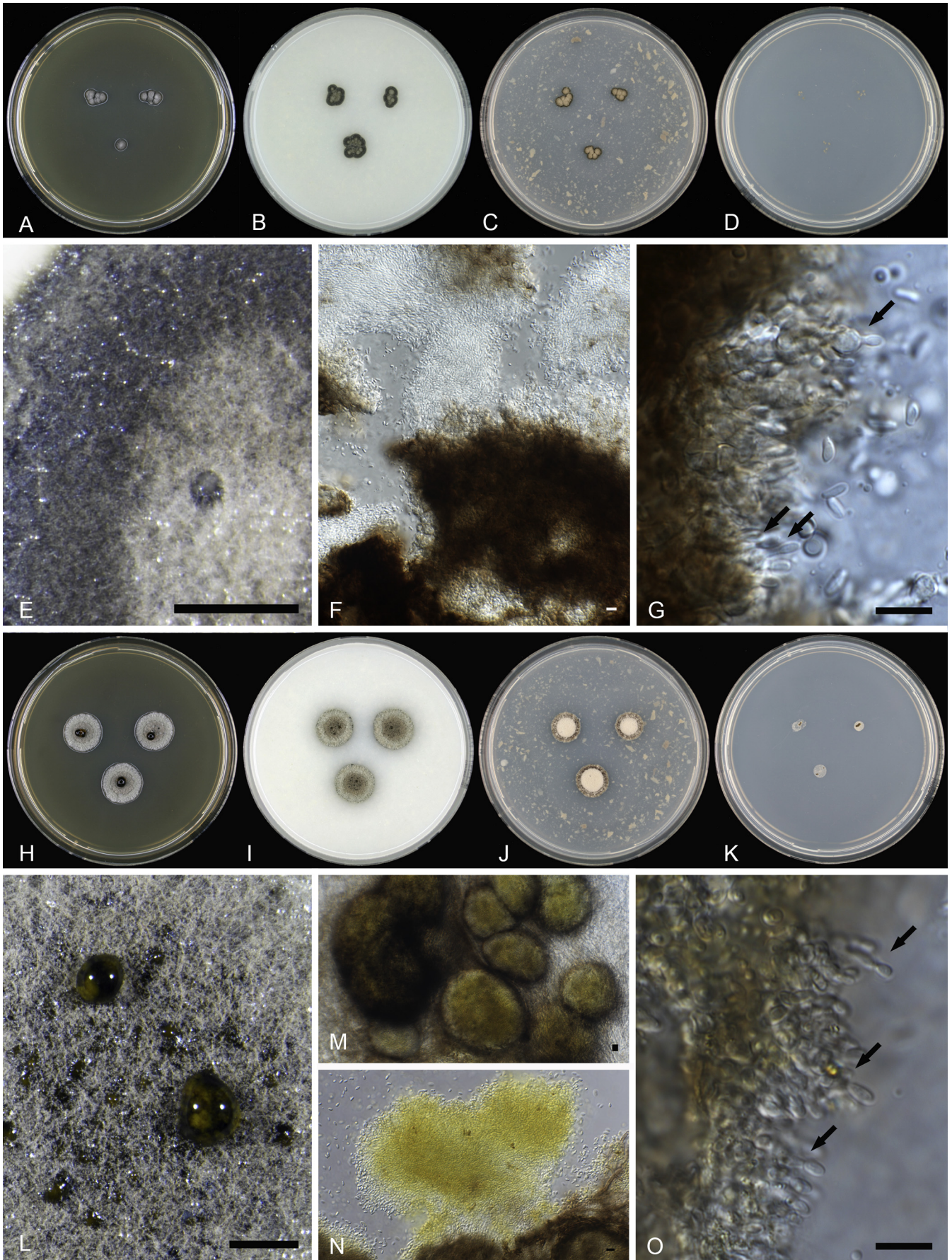
Fig. 5. *Superstratomyces atroviridis.* **A–D.** Colonies 2 wk old 25 °C; **A.** MEA obverse; **B.** OA obverse; **C.** PDA obverse; **D.** SNA obverse; **E.** Macroscopic structure of colony on OA showing aerial hyphae and one spot of a white coloured slimy mass; **F.** Clusters of hyphae and conidia; **G.** Conidiogenous cells. *Superstratomyces flavomucosus.* **H–K.** Colonies 2 wk old 25 °C; **H.** MEA obverse; **I.** OA obverse; **J.** PDA obverse; **K.** SNA obverse; **L.** Macroscopic structure of colony on OA showing aerial hyphae and yellow coloured slimy masses; **M.** Pycnidia; **N.** Clusters of hyphae and conidia; **O.** Conidiogenous cells. Scale bars: E, L = 1 000 μm; F, G, M, N, O = 10 μm.

the special type of wood. The genitive of the noun is selected to refer to the isolation (of the fungus) from the substrate.

Diagnosis: Medium to slow growing grey green (MEA) and green colonies (OA) with transparent to white coloured slimy masses (MEA) or green spherical pycnidia, which are mostly solitary, but occasionally aggregated (OA). Phylogenetically placed in the order *Ostropales* (*Ostropomycetidae*, *Lecanoromycetes*), sister to *Cyanodermella viridula*.

Type: **Australia**, Adelaide, *Pinus sylvestris* impregnated with raw linseed oil, collector E.J. van Nieuwenhuijzen, 05 Feb 2014 (**holotype** H-22666; ex type = CBS 140290).

Colony characteristics: colony diameters, 1 wk, in mm, 25 °C: MEA 7–10; OA 6–10; PDA 6–10; SNA: 3–4, poor growth. MEA colony: obverse wrinkled and greenish white with white edge; reverse dark green with white edge; transparent to white coloured slimy masses. OA colony: obverse pattern of green to dark green pycnidia and a white edge without green pycnidia; reverse dark green with white edge; transparent slimy masses. PDA colony: obverse wrinkled and white to olive green with white edge; reverse white to olive green with white edge; slimy masses absent.

Micromorphology OA, 25 °C, 1 wk: pycnidia green, spherical, diameter 25–150 μm, typically solitary, occasionally aggregated. Separated or single layered hyphae and conidia hyaline, dense or double layered biomass dark green. Conidiogenous cells phialidic. Conidia smooth walled, oval typically with blunt ends, length 4.9–8.1 μm, width 1.9–3.0 μm. Aerial mycelium and teleomorphic structures absent.

Ecology and distribution: Four strains were isolated from the surface of a piece of wood (*Pinus sylvestris*) treated with raw linseed oil, located outdoors in Dover gardens, Adelaide, Australia.

Additional cultures examined: **Australia**, Adelaide, outdoor situated log of *Pinus sylvestris* impregnated with raw linseed oil (DTO 301-G2, DTO 301-G3, DTO 301-G4; deposited in culture collection of the Applied and Industrial Department [DTO] housed at the CBS-KNAW Fungal Biodiversity Centre), 05 Feb 2014, E.J. van Nieuwenhuijzen.

DISCUSSION

All reconstructed phylogenies (Figs 1, S1–S4) were in agreement (although mostly with low support, except for analyses 3 and 4; see Tables 2, S3 and Fig. 1) for the placement of the fungal "dark" group as sister to *Trypetheliales* in *Dothideomycetes*. Although the order *Trypetheliales*, recently extended with two lichenicolous genera (*Clypeococcum* and *Polycoccum*; Ertz *et al.* 2015), also includes non-lichenised taxa, the fungal "dark" group is morphologically and phylogenetically distinct. We propose to recognise this new lineage at the order level – *Superstratomycetales*, with a single family *Superstratomycetaceae* and a single genus *Superstratomyces* containing three species (*S. albomucosus*, *S. flavomucosus*, and *S. atroviridis*), each representing a distinct monophyletic lineage based on multiple multi- and single-locus phylogenies (Figs 1, 2, S2–S4). All three species are well defined by the barcoding gap approach based

on three loci, including ITS (the official DNA barcode marker for fungi; Schoch *et al.* 2012). Morphological characteristics, mainly of the slimy masses produced on agar plates, support the classification of these three species within the genus *Superstratomyces*.

Molecular data are necessary for the reliable identification of species in the genus *Superstratomyces* and for distinguishing them from other pycnidia-producing coelomycetous fungi. To our knowledge, members of the newly described genus *Superstratomyces* lack unique characteristics and, therefore, cannot be identified based solely on their phenotypes. For example, the observed shapes of the conidiophores and conidia were also observed in pycnidia of species belonging to other genera. DNA sequencing facilitated numerous systematic revisions, and resulted in the addition of new genera to the long-standing coelomycetes such as *Septoria*, *Mycosphaerella*, *Phoma*, *Coniothyrium* and *Phomopsis* (*Diaporthe*) (Crous *et al.* 2001, de Gruyter *et al.* 2013, Gomes *et al.* 2013, Verkley *et al.* 2013, Verkley *et al.* 2014). Therefore, a comprehensive study was needed to place them with high confidence in the context of a phylogenetically-based classification. The ITS and nrLSU loci, which are the markers commonly used in molecular systematic studies of fungi, were particularly different for *Superstratomyces* compared to all available sequences in GenBank, when we conducted this study.

The 23 examined strains of the fungal "dark" group have enlightened the existence of a new order with at least three species. Nevertheless, it is likely that one can find other *Superstratomyces* taxa if a larger set of isolates is examined, especially when strains are included from other substrates or locations. For example, *Superstratomyces* was also abundant among the culturable fungi of untreated wood samples in Utrecht, The Netherlands (van Nieuwenhuijzen *et al.* unpubl. data), and might be present on other substrates at the same test site.

Based on the close phylogenetic relationship of the "green" group to *Cyanodermella viridula* (Figs 3 and S5), a species classified in *Stictidaceae* (*Ostropales*, *Lecanoromycetes*) (Lumbsch & Huhndorf 2010) we propose to recognise the green pycnidia-producing fungal strains as a new species within the genus *Cyanodermella* and provided a formal description for *C. oleoligni*. Our phylogenies (Figs 3 and S5) show a strong support for the placement of the genus *Cyanodermella* in *Ostropales*, however, its affiliation with the family *Stictidaceae* received a low bootstrap support (Table 2). Most phylogenetic studies on *Stictidaceae* did not include *Cyanodermella* (Wedin *et al.* 2005, Fernández-Brime *et al.* 2011, Baloch *et al.* 2013), however, the two phylogenies, which contain this taxon, showed similar results to ours (Winka 2000, Baloch *et al.* 2010). The placement of *Cyanodermella* within *Ostropales* seems to be well established, but its affiliation at the family level remains to be confirmed with higher confidence. Currently, the genus *Cyanodermella* contains two species: *C. viridula* and *C. candida*. Both species were initially classified based on their teleomorph structure by Berkeley & Curtis in 1919 and Setchell in 1924 (Eriksson 1967), and were later renamed by Eriksson (Eriksson 1967, 1981). Molecular data and herbarium samples (including old leaves of *Leymus arenarius*) are available for *C. viridula* only. Although descriptions of cultured isolates of *C. viridula* and *C. candida* are missing, their general characteristics include the presence of ascomata with a characteristic blue-green colour. Because the pycnidia of *C. oleoligni* clearly showed a blue-green colour, the classification of this species within *Cyanodermella*
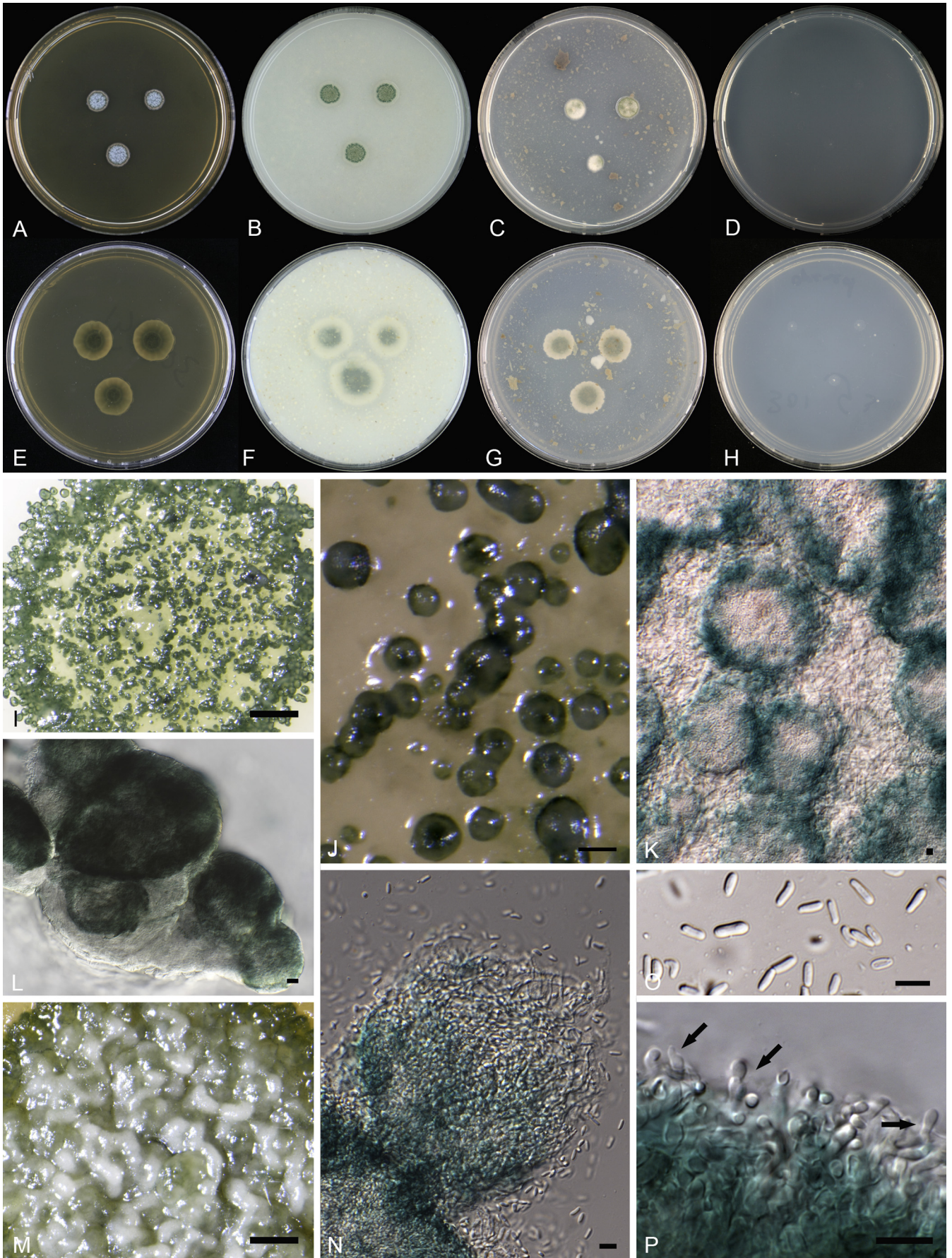
Fig. 6. *Cyanodermella oleoligni*. **A–D.** Colonies 1 wk old 25 °C; **A.** MEA obverse; **B.** OA obverse; **C.** PDA obverse; **D.** SNA obverse; **E–H.** Colonies 2 wk old 25 °C; **E.** MEA reverse; **F.** OA reverse; **G.** PDA reverse; **H.** SNA reverse; **I.** Macroscopic structure of colony on OA; **J.** Macroscopic structure of pycnidia on OA; **K.** Pycnidia, hyphae and conidia; **L.** Pycnidia; **M.** Macroscopic structure of colony on MEA; **N.** Cluster of hyphae and conidia; **O.** Conidia; **P.** Conidiogenous cells. Scale bars: I, M = 1 000 µm; J = 100 µm; L, N, K, O, P = 10 µm.

based on its phylogenetic relationship, is corroborated by this phenotypic trait. The order *Ostropales* includes mostly crustose taxa with high species richness in the tropics. This order is well known, especially *Stictidaceae* species, for their non-lichenised members in addition to mostly lichen-forming taxa (Lutzoni *et al.* 2001, Wedin *et al.* 2006, Cannon & Kirk 2007; Schoch *et al.* 2009b, Baloch *et al.* 2010, 2013, Spribille *et al.* 2014). The inclusion of another, most likely, saprotrophic wood-inhabiting species of *Cyanodermella* (*C. oleoligni*) in the order *Ostropales* fits well within the broad biological spectrum of life strategies of this order (and *Ostropomycetidae*) where multiple shifts from symbiotrophy (lichen-forming fungi) to saprotrophy (wood-inhabiting fungi), i.e., losses of lichenisation, have been reported (Lutzoni *et al.* 2001, Spribille *et al.* 2014).

A culture-based approach coupled with multi-locus phylogenetics is confirmed here as a powerful methodology to classify fungi that generate highly inconclusive BLAST searches. This strategy was implemented by Gazis *et al.* (2012) and Chen *et al.* (2015) and led to the discovery of a new class of Fungi – *Xylonomycetes*, and a new order – *Phaeomoniellales* – within *Eurotiomycetes*. ITS or nrLSU BLAST results from GenBank can be used to identify the taxonomic placement of fungal strains, but our study showed that for fungi with top hits below 92 % identity and low coverage (Table S2) BLAST results can be misleading. For example, the top hits based on maximum identity scores turned out to be accurate in predicting the class to which the unknown "dark" group belong (*Dothideomycetes*), while most of the hits based on highest query coverage were contradictory (Table S2). For the "green" group, the accuracy of the class assignment was the other way around, i.e., where the top hits based on the highest query coverage were correct (*Lecanoromycetes*), whereas the maximum identity score also suggested an affinity with *Leotiomycetes* (Table S2). The BLAST results obtained for lower taxonomic levels, such as family and genus, were even more scattered across various fungal taxa and inconsistent. In some cases, the contradictory results may be due to misidentifications or co-sequenced fungi submitted to GenBank.

A comprehensive understanding of microbial communities in time and space, relies on large-scale and robust phylogenies. Fungi, are among the most species-rich clades of the tree of life (Gazis *et al.* 2012). Estimations of their species number range from 1,5 million (Hawksworth 2001) to 5,1 million (O'Brien *et al.* 2005, Blackwell 2011). Only a small fraction of the fungal biodiversity has been taxonomically described (Blackwell 2011, Bass & Richards 2011), whereas the remaining diversity is known only from molecular data or remains to be discovered (e.g., Arnold *et al.* 2009, U'Ren *et al.* 2016). The number of newly described higher-level taxa, especially in the class *Dothideomycetes*, has been increasing substantially. For example, since the recognition of the *Dothideomycetes* as a supraordinal taxa of *Ascomycota* (Eriksson & Winka 1997), sixteen new orders have been added to this class and half of them in the last four years (according to the current information from GenBank). The increase of accessible DNA sequences for fungi across the fungal kingdom in the last ten years coupled with the application of multi-locus phylogenetic analyses facilitates the recognition of new families and orders. A recent phylogenetic treatment of the class *Dothideomycetes* based on a four-gene combined analysis provided support for 75 of 110 currently recognized families and 23 orders, including seven newly described taxa (Wijayawardene *et al.* 2014).

Exploration of unique habitats, such as sapwood in remote forests (Gazis *et al.* 2012, 2014), cacti in tropical dry forest (Bezerra *et al.* 2013, 2016), marine borderline lichens (Pérez-Ortega *et al.* 2016), deep sea water (Jebaraj *et al.* 2010), and of the Antarctic (Stchigel *et al.* 2001), revealed new deep lineages in the fungal tree of life. This study, as part of a research project on fungal-based wood finishes, showed that mundane substrates modified by humans, are also a valuable potential source of hidden fungal taxonomic novelties.

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APPENDIX A. SUPPLEMENTARY DATA

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.simyco.2016.11.008.

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