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

E.J. van Nieuwenhuijzen1, J.M. Miadlikowska2*, J.A.M.P. Houbraken3*, O.C.G. Adan3, F.M. Lutzoni2, and R.A. Samson1

1CBS-KNAW Fungal Biodiversity Centre, Uppsalalaan 8, 3584 CT Utrecht, The Netherlands; 2Department of Biology, Duke University, Durham, NC 27708, USA; 3Department of Applied Physics, Eindhoven University of Technology, P.O. Box 513, 5600 MB Eindhoven, The Netherlands

*Correspondence: J.M. Miadlikowska, jolantam@duke.edu; J.A.M.P. Houbraken, j.houbraken@cbs.knaw.nl

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, mtlSSU, 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*.


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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, Babic 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 (biofinish), 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 affinities and taxonomic identities of 26 pycnidia-producing fungi isolated from oil-treated wood exposed to outdoor conditions. We sequenced four ribosomal and three proteincoding 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 monogenic order Superstratomycetales (Dothideomycetes) containing three newly proposed species, and a new species Cyanodermella oleicligni (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 (Caronel, 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 (DNAsar 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 (Rodriguez 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 searchings 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; Carbene et al. unpubl. data – Analysis 2; Nielsen 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; 18S; RPB1+RPB2; EF-1α; RPB1; EF-1α; RPB2).
<table>
<thead>
<tr>
<th>CBS no.</th>
<th>DTO no.</th>
<th>Phenotype</th>
<th>Substrate</th>
<th>Locality</th>
<th>GenBank accession numbers</th>
</tr>
</thead>
<tbody>
<tr>
<td>353.84</td>
<td>305-C3</td>
<td>“dark” group</td>
<td>Leaf of <em>Hakea multilinearis</em></td>
<td>Australia, Perth</td>
<td>KX950411 KX950438 KX950470 KX950467 KX950462 KX950493 KX950497</td>
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<tr>
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<td>277-D2</td>
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<td>Ilomba treated with olive oil</td>
<td>The Netherlands</td>
<td>KX950412 KX950439 KX950471 KX950467 KX950463 KX950494 KX950498</td>
</tr>
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<td>277-I3</td>
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<td>Pine sw treated with raw linseed oil</td>
<td>The Netherlands</td>
<td>KX950413 KX950440 KX950472 KX950468 KX950464 KX950495 KX950499</td>
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<td>KX950414 KX950441 KX950473 KX950469 KX950465 KX950496 KX950500</td>
</tr>
<tr>
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<td>277-C8</td>
<td>“dark” group</td>
<td>Pine sw treated with stand linseed oil</td>
<td>The Netherlands</td>
<td>KX950415 KX950442 KX950470 KX950467 KX950463 KX950494 KX950497</td>
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<td>301-G1</td>
<td>“green” group</td>
<td>Pine hw treated with raw linseed oil</td>
<td>Australia</td>
<td>KX950434 KX950461 – KX999145 KX999144 KX999146 KX999147</td>
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<tr>
<td>–</td>
<td>301-G2</td>
<td>“green” group</td>
<td>Pine hw treated with raw linseed oil</td>
<td>Australia</td>
<td>KX950435 – – – – – –</td>
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<td>–</td>
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<td>“green” group</td>
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<td>Australia</td>
<td>KX950436 – – – – – –</td>
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<tr>
<td>–</td>
<td>301-G4</td>
<td>“green” group</td>
<td>Pine hw treated with raw linseed oil</td>
<td>Australia</td>
<td>KX950437 – – – – – –</td>
</tr>
</tbody>
</table>
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 PartitionFinder (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 four strains from Ostropomycetidae (Analysis 1). Based on these results, the subsequent data sets were restricted to Pezizomycotina (Analyses 2a and 3), and to Lecanoromycetes (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

### Table 2. Number of taxa, loci, and characters included in each data set. Sources of reference sequences for each analysis are cited. ML phylogenetic placements of the two unknown groups of pycnidia-producing fungi are reported with bootstrap support (BS).

<table>
<thead>
<tr>
<th>Analyses</th>
<th>Data set</th>
<th>Source of data set</th>
<th>No. of taxa</th>
<th>No. of loci</th>
<th>No. of char.</th>
<th>Phylogenetic placement</th>
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<tr>
<td>“Dark” group</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Analysis 1 (Fig. S1)</td>
<td>Fungi</td>
<td>James et al. (2006)</td>
<td>219</td>
<td>6</td>
<td>8 482</td>
<td>Dothideomycetes + Arthoniomycetes clade (BS = 80%); Dothideomycetes (BS = 64%); sister to Trypethelium (BS = 39%)</td>
</tr>
<tr>
<td>Analysis 2 (Fig. S2)</td>
<td>Pezizomycotina</td>
<td>Carbone et al. (unpubl. data)</td>
<td>986</td>
<td>6</td>
<td>7 269</td>
<td>Arthoniomycetes + Dothideomycetes clade (BS = 58%); Dothideomycetes (BS = 36%); sister to Trypetheliales (BS = 37%)</td>
</tr>
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<td>Pezizomycotina</td>
<td>Carbone et al. (unpubl. data)</td>
<td>983</td>
<td>61</td>
<td>7 269</td>
<td>Dothideomycetes (BS = 30%); Botryosphaeriaceae (BS = 56%)</td>
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<tr>
<td>Analysis 2B</td>
<td>Pezizomycotina</td>
<td>Carbone et al. (unpubl. data)</td>
<td>986</td>
<td>62</td>
<td>7 269</td>
<td>Dothideomycetes (BS = 40%); related to Kirschsteiniotheliaceae and part of Phaeotrichiaceae (BS = 32%)</td>
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<tr>
<td>Analysis 3 (Fig. 1, Table S3)</td>
<td>Dothideomycetes + Arthoniomycetes</td>
<td>Carbone et al. (unpubl. data) and Schoch &amp; Grube (2015)</td>
<td>236</td>
<td>7</td>
<td>7 072</td>
<td>Dothideomycetes (BS = 68%); sister to Trypethelium (BS = 86%)</td>
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<tr>
<td>Analysis 4 (Fig. S3, Table S3)</td>
<td>Dothideomycetes + Arthoniomycetes + additional Trypetheliales</td>
<td>Carbone et al. (unpubl. data) and Nelsen et al. (2009, 2011)</td>
<td>260</td>
<td>7</td>
<td>7 072</td>
<td>Dothideomycetes (BS = 75%); sister to Trypetheliales (BS = 49%)</td>
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<tr>
<td>Analysis 5 (Fig. S4)</td>
<td>Trypetheliales</td>
<td>Nelsen et al. (2014) and Ertz et al. (2015)</td>
<td>95</td>
<td>2</td>
<td>1 011</td>
<td>Outside of Trypetheliales</td>
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<td>“Green” group:</td>
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<td>Analysis 1 (Fig. S1)</td>
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<td>James et al. (2006)</td>
<td>219</td>
<td>6</td>
<td>8 482</td>
<td>Lecanoromycetes, Ostropomycetidae, Ostragales (BS = 80%)</td>
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<td>Analysis 6 (Fig. S5)</td>
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<td>Miadlikowska et al. (2014)</td>
<td>1319</td>
<td>5</td>
<td>7 433</td>
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<tr>
<td>Analysis 7 (Fig. 3)</td>
<td>Ostropomycetidae</td>
<td>Reist et al. (2015)</td>
<td>207</td>
<td>8</td>
<td>6 665</td>
<td>Ostragales (BS = 76%); Stictidaeae (BS = 43%); sister to Cyanodermella viridula (BS = 100%)</td>
</tr>
</tbody>
</table>

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).
*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, *MCMT*, *RPB1*, *RPB2*, *EF-1α*) available for *Ostromycectidae* (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 (Swoford 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 (<85 % and 92 % for the “dark” and “green” groups, respectively) and most query coverages were low as well (<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: *Valsaia neotropica*), nrLSU long fragment (LROR-LR7 sequence top hits: *Vermicinia calcicola* and *Preussia minima*), and nrLSU short fragment (top hit LROR-LR5: *Cystococcus ebeneus*). However, some top BLAST matches with the higher query covered showed affiliation with other fungal classes, *Leotiomycetes* and *Sordariomycetes* (Table S2). The “green” isolates matched mostly the class Lecanoromycetes, subclass *Ostromyctetidae* when using ITS sequences (top hit *Sticis radiata*) or *Umbilicariomycetidae* when using nrLSU sequences (top hit *Umbilicaria forresta*). 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 multilinaria* 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 *Trypetheliaceae* 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 “Dothideomycetae” 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 mtSSU) data set (Nelsen et al. 2014) encompassing 79 members from Trypetheliales 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 mtSSU 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 (Rogier 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 (Mladilokowska 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 Cyanoderma 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 Cyanoderma 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 "Dothideomycetae" (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)

RAXML phylogeny; Arthoniomycetes and Dothideomycetes; 236 taxa; 7-locus matrix; 7072 sites
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 Trypetheliaceae, 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.*

**Superstratomyces** 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.

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**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.
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). Bootstrap 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).

Colonies: 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:** 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).

**Colonies:** 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 80–300 μm; conidiogenous cells phialidic.

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

*Cyanoderma 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 = 1000 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, Lecanoromyces), 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.


**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 Polyccicum; 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 — Superstratomyces, with a single family Superstratomyctaceae 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 (Diapothor) (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 rLSU 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, Lecanoromyces) (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. candia**. 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. candia** 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
The inclusion of another, most likely, saprotrophic wood-
inhabiting species of Cyanoderma (C. oleoidigni) 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-loci phyloge-
netics 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 – Phaeomoniliellales – within
Eurotiomycetes. ITS or nLSU 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 (Lecano-
romycetes), 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). Estimates 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, ÚRen 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 in-
crease of accessible DNA sequences for fungi across the fungal
kingdom in the last ten years coupled with the application of
multi-loci 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
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