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Phylogenetic analyses of eurotiomycetous endophytes reveal their close affinities to Chaetothyriales, Eurotiales, and a new order – Phaeomoniellales

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ABSTRACT

Symbiotic fungi living in plants as endophytes, and in lichens as endolichenic fungi, cause no apparent symptoms to their hosts. They are ubiquitous, ecologically important, hyperdiverse, and represent a rich source of secondary compounds for new pharmaceutical and biocontrol products. Due in part to the lack of visible reproductive structures and other distinctive phenotypic traits for many species, the diversity and phylogenetic affiliations of these cryptic fungi are often poorly known. The goal of this study was to determine the phylogenetic placement of representative endophytes within the Eurotiomycetes (Pezizomycotina, Ascomycota), one of the most diverse and evolutionarily dynamic fungal classes, and to use that information to infer processes of macroevolution in trophic modes. Sequences of a single locus marker spanning the nuclear ribosomal internal transcribed spacer region (nrITS) and 600 base pairs at the 5′ end of the nuclear ribosomal large subunit (nrLSU) were obtained from previous studies of >6000 endophytic and endolichenic fungi from diverse biogeographic locations and hosts. We conducted phylum-wide phylogenetic searches using this marker to determine which fungal strains belonged to Eurotiomycetes and the results were used as the basis for a class-wide, seven-locus phylogenetic study focusing on endophytic and endolichenic Eurotiomycetes. Our cumulative supermatrix-based analyses revealed that representative endophytes within Eurotiomycetes are distributed in three main clades: Eurotiales, Chaetothyriales and Phaeomoniellales *ord. nov.*, a clade that had not yet been described formally. This new order, described herein, is sister to the clade including Verrucariales and Chaetothyriales. It appears to consist mainly of endophytes and plant pathogens. Morphological characters of endophytic Phaeomoniellales resemble those of the pathogenic genus *Phaeomoniella*. This study highlights the capacity of endophytic and endolichenic fungi to expand our understanding of the ecological modes associated with particular clades, and provides a first estimation of their phylogenetic relationships in the Eurotiomycetes.

1. Introduction

All plant species sampled to date harbor endophytic fungi, which are fungal symbionts inhabiting living tissues such as roots, leaves and stems without causing obvious symptoms (Rodriguez et al., 2009; Saikkonen et al., 1998). Many studies have reported the extremely high biodiversity of fungal endophytes in aboveground tissues of plants (e.g., Arnold and Lutzoni, 2007; Lodge et al., 1996; Zimmerman and Vitousek, 2012). Endophyte species richness is predominately found within the subphylum Pezizomycotina (Ascomycota) encompassing the majority of the filamentous ascomycetes (see Arnold et al., 2009; U'Ren et al., 2010, 2012). In addition to important roles in plant physiology and ecology (Ernst et al., 2003; Hubbard et al., 2014; Rodriguez et al., 2008), the potential of endophytes as a resource for biological control (Arnold et al., 2003; Backman and Sikora, 2008) and pharmaceuti-

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cal products (e.g., Stierle et al., 1993) is also widely acknowledged. Despite their ubiquity and potential importance, studies of endophytes have focused primarily on diversity at the species level (e.g., Gazis et al., 2011; Ovaskainen et al., 2010; but see U'Ren et al., 2012), with relatively little information available so far regarding their phylogenetic relationships or broader contributions to the fungal tree of life (but see Arnold et al., 2009; Qadri et al., 2014; Spatafora et al., 2007).

Much like endophytes in plants, endolichenic fungi are endophyte-like symbionts that live inside apparently healthy lichen thalli, primarily in association with algal and/or cyanobacterial cells (Arnold et al., 2009). Endolichenic fungi are distinct from mycobionts, which make up the lichen thallus, and from lichenicolous fungi, the reproductive structures of which (sexual and/or asexual) can be often observed on living lichens (Arnold et al., 2009). They also are largely distinct at the species level from foliar endophytic fungal communities in vascular plants, but are frequently shared with co-occurring bryophytes (even when not growing in close physical proximity; see U'Ren et al., 2010, 2012). U'Ren et al. (2010, 2012, 2014) also reported that endolichenic fungal communities are abundant and diverse.

Relationships of endophytic and endolichenic fungi (hereafter, collectively referred to as fungal endophytes or endophytes) have been explored only once in a phylum-wide phylogenetic framework (Arnold et al., 2009). Since that time many studies have documented additional endophyte taxa (e.g., Del Olmo-Ruiz and Arnold, 2014; Gazis and Chaverri, 2010; Larkin et al., 2012; U'Ren et al., 2012, 2014; Zimmerman and Vitousek, 2012), and knowledge of the Pezizomycotina tree of life has advanced substantively (e.g., Gazis et al., 2012; Prieto et al., 2013; Schoch et al., 2009), prompting new exploration of the phylogenetic relationships of endophytes in a phylogenetically broad and robust context.

The kingdom Fungi is one of the most diverse groups of eukaryotes on earth (Blackwell, 2011; Hibbett and Taylor, 2013). Although the total species richness of fungi is estimated to be up to 5.1 million (Blackwell, 2011), only about 100,000 have been described (Blackwell, 2011; Kirk et al., 2008). Many fungal species are believed to be living cryptically, in symbiosis with other organisms such as plants and insects (Blackwell, 2011). Although culture-independent methods and advancements in high-throughput sequencing technology have greatly hastened the discovery of fungal biodiversity, the phylogenetic placement and taxonomy of most fungal endophytes have not been explored. This is in part because (1) some endophytes are not culturable (e.g., Arnold et al., 2007; Impullitti and Malvick, 2013; Pancher et al., 2012); (2) even when culturable, many fungal endophytes do not form *in vitro* the morphological structures that are traditionally used in fungal taxonomy (Petrini and Petrini, 1985); and (3) most culture-independent studies of fungi rely on the nuclear ribosomal internal transcribed spacer region (nrITS), which is not amenable to broad-scale phylogenetic analysis, or use short reads that provide limited resolving power (Lindner and Banik, 2011; Porter and Golding, 2011). Moreover, (4) the great majority of endophyte species have not been described; thus, even if many sequences of endophytes have been deposited in GenBank, they usually provide limited taxonomic information (e.g., see Gazis et al., 2012; Nilsson et al., 2014; U'Ren et al., 2009). These issues lead to uncertainty with regard to how best to delimit species and other taxonomic groups for endophytes and related fungi, limiting ecological inferences and diminishing our ability to address evolutionary questions.

The class Eurotiomycetes (Pezizomycotina, Ascomycota) includes species with highly varied metabolic abilities, many of which are important in human health and sustainability (e.g., Geiser et al., 2006). The order Eurotiales includes mainly saprotrophic genera like *Aspergillus* and *Penicillium*, but also animal-associated genera such as *Trichophyton* and *Onygena*. The orders Pyrenulales and Verrucariales include some lichen mycobionts. Extreme environments, such as xeric rock surfaces, are colonized by some Chaetothyriales, and by members of the subclass Chaetothyriomycetidae (containing Chaetothyriales, Pyrenulales and Verrucariales) in general (Geiser et al., 2006; Gueidan et al., 2008). Several groups also contain opportunistic human pathogens that can switch from saprotrophic to pathogenic lifestyles (e.g., Barker et al., 2007; Hohl and Feldmesser, 2007).

In addition to interacting with dead plant tissue and living animals, many Eurotiomycetes associate closely with living plants and lichens. For example, *Phaeomoniella chlamydospora* is a causal agent of Petri disease of grapevine (Crous and Gams, 2000) and *Elaphomyces* forms ectomycorrhizal associations with trees (Castellano et al., 2012). Some species occur as lichenicolous fungi (e.g., Diederich et al., 2013; Réblová et al., 2013), and others – especially *Aspergillus* and *Penicillium* – can be isolated as endophytes (see Arnold et al., 2009; Naik et al., 2009; Peterson et al., 2005; Sandberg et al., 2014; U'Ren et al., 2012; Vega et al., 2010).

Here, we resolve the phylogenetic and taxonomic affinities of representative endophytes within Eurotiomycetes, and explore for the first time the origin of endophytism and endolichenism within this diverse class. Previous culture-based work, which characterized endophytic and endolichenic fungi from diverse biomes across North America using sequence data from the nrITS and a portion of the adjacent nuclear ribosomal large subunit (nrLSU) (i.e., Arnold et al., 2009; U'Ren et al., 2010, 2012), suggested the placement of a number of strains within Eurotiomycetes based on BLAST. We used these data as the basis for a seven-locus phylogenetic approach to address the following questions: (1) What is the evolutionary history of endophytes within Eurotiomycetes? (2) How do endophytes fit within the current classification of Eurotiomycetes? (3) What are the geographical, ecological and, phenotypic features of these endophytic taxa? Further, (4) we tested the reliability of using the nr5.8S + LSU from our target nrITS-LSU locus to infer relationships of unknown endophytes within the broad scope of the Pezizomycotina, as a complementary approach to BLAST analysis with nrITS (the fungal DNA barcode; Schoch et al., 2012). Our results reveal an order (Phaeomoniellales) that was observed but not described formally in previous work within Eurotiomycetes (Gueidan et al., 2014; Rossman et al., 2010). Phaeomoniellales appears to be composed mainly of endophytic fungi and plant pathogens. We use data from the nrLSU to infer the most comprehensive phylogenetic tree to date for this new order of fungi, and draw from metadata provided by ecological studies to evaluate major trends in their geographic- and host affiliations.

2. Materials and methods

2.1. Endophyte isolation, DNA extraction, and nrITS-LSU sequence acquisition

We used a collection of 6521 fungal cultures isolated through studies examining the abundance, diversity, ecology, and evolution of fungal endophytes (i.e., Arnold and Lutzoni, 2007; Arnold et al., 2009; Arnold, unpubl. data; Higgins et al., 2007; Hoffman and Arnold, 2008; U'Ren et al., 2010, 2012). These endophytes were collected in 2003–2009 from six major lineages of land plants (bryophytes, lycophytes, monilophytes, gymnosperms, monocots, and eudicots; Fig. 1A) and three functional groups of lichens (epiphytic, saxicolous, and terricolous/muscicolous; Fig. 1B). These samples represent diverse geographic and biogeographic provinces, as described in full by Arnold and Lutzoni (2007), Arnold et al. (2009), Higgins et al. (2007), Hoffman and Arnold (2008) and

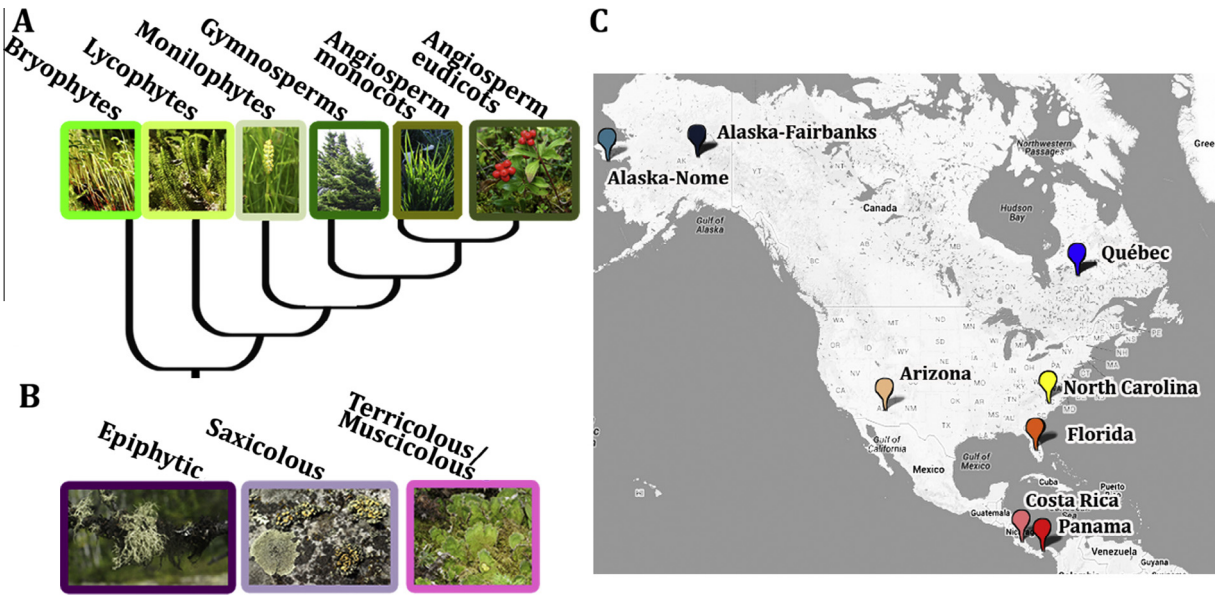

Fig. 1. Hosts and locations sampled to generate the endophyte database evaluated for this study (for collection information, see Arnold and Lutzoni, 2007; Arnold et al., 2009; U'Ren et al., 2010, 2012). (A) Simplified schematic representation of evolutionary relationships of plant hosts from which fungal endophytes were isolated. Different green outlines indicate distinct plant lineages. These colors are used in Fig. 3 to represent the major host plant groups. (B) Lichens sampled for endolichenic fungi. The purple tones correspond to primary substrates of focal lichens. These colors are used in Fig. 3 to represent these three main ecological lichen groups. (C) Fungal endophytes were isolated from eight geographic areas across North and Central America (references listed above). The same colors are used in Fig. 3 to represent the geographic origin of each fungal strain.

U'Ren et al. (2010, 2012): coniferous montane forest in the southwestern USA (Arizona); the boreal treeline in Alaska, USA (near Fairbanks and Nome); mountain and Piedmont areas of the southeastern USA (North Carolina); subtropical scrub forest (Florida); boreal forest in Québec, Canada; and lowland tropical forests and highland sites (Costa Rica and Panama) (Fig. 1C). The fungi were collected from asymptomatic photosynthetic tissues; non-living tissues of a limited number of plants were also sampled in Arizona, Alaska-Nome, Alaska-Fairbanks, North Carolina, and Florida (see U'Ren, 2011; U'Ren et al., 2010). For the overall workflow see Supplementary Fig. 1.

2.2. OTU determination and selection of endophytes belonging to Eurotiomycetes using nrITS-LSU similarity assembly and Ascomycota nr5.8S + LSU phylogeny

Information on DNA extraction, amplification and bidirectional sequencing of the nrITS-LSU locus (ITS1F-LR3 primers, ≈1200 bp; see Supplementary Fig. 1B), and data assembly are included in the original studies (Arnold and Lutzoni, 2007; Arnold et al., 2009; Arnold, unpubl. data; Higgins et al., 2007; Hoffman and Arnold, 2008; U'Ren et al., 2010, 2012). Operational taxonomic units (OTUs) were defined by ⩾95% similarity among strains, as determined by Sequencher v4.5 (Gene Codes Corporation, Ann Arbor, MI) (see Arnold et al., 2007; U'Ren et al., 2009).

In preparation for the present work, all OTUs were verified as monophyletic groups by phylogenetic analysis of a concatenated nr5.8S + LSU dataset containing 2492 terminal taxa (397 reference taxa selected across Ascomycota, obtained from GenBank, and 2095 non-redundant sequences of fungal endophytes obtained from the aforementioned samples and selected strains from other localities; see Arnold et al., 2009; U'Ren et al., 2010, 2012). The nr5.8S + LSU dataset was aligned using MacClade v4.08 (Maddison and Maddison, 2003) and finalized with Mesquite v2.75 (Maddison and Maddison, 2011). The nuclear ribosomal large subunit was aligned according to secondary structure as described in Miadlikowska et al. (2006). Ambiguously aligned regions (*sensu* Lutzoni et al., 2000) and introns were delimited manually and excluded from subsequent analyses.

The resulting dataset was analyzed using RAxML v7.2.8 (Stamatakis, 2006; Stamatakis et al., 2008) with the nrLSU and nr5.8S considered as two distinct partitions. The search for the most likely tree and bootstrap analysis (each with the GTRGAMMA substitution model; 1000 bootstrap replicates) were performed using a backbone constraint tree (see Arnold et al., 2007) containing 397 reference taxa, which was built based on well established and strongly supported multi-locus phylogenetic studies (e.g., Geiser et al., 2006; James et al., 2006; Lutzoni et al., 2004; Schoch et al., 2009; Spatafora et al., 2006). Two *Neolecta* isolates (Taphrinomycotina) were used to root the tree (Spatafora et al., 2006). Strains placed within Eurotiomycetes were selected for further study, as described below.

2.3. Multi-locus data acquisition

From each OTU group that was (1) placed in the Eurotiomycetes and (2) verified as monophyletic in the nr5.8S + LSU phylogeny described above, one representative isolate was selected for seven-locus sequencing (Supplementary Table 1). Seven loci were targeted, including three nuclear ribosomal RNA-coding genes: the nuclear ribosomal small subunit gene (nrSSU), nuclear ribosomal large subunit gene (nrLSU) and nuclear ribosomal 5.8S gene (nr5.8S); one mitochondrial ribosomal RNA-coding gene (mitSSU); and three protein-coding genes: the RNA polymerase II largest subunit gene (*RPB1*), the RNA polymerase II second largest subunit gene (*RPB2*), and minichromosome maintenance complex component 7 gene (*MCM7*). In some cases, nrLSU and nr5.8S data were obtained *de novo* in this study; in other cases these data were obtained from previous work (see Supplementary Table 1 and references listed therein).

Primers, PCR protocols and PCR amplification conditions for each locus are shown in Supplementary Tables 2–4 and are described in previous studies (Arnold et al., 2009; Gargas and Taylor, 1992; Hofstetter et al., 2007; Kauff and Lutzoni, 2002; Liu et al., 1999;

Miadlikowska and Lutzoni, 2000; Reeb et al., 2004; Rehner and Samuels, 1994; Schmitt et al., 2009; Stiller and Hall, 1997; Vilgalys and Hester, 1990; White et al., 1990; Zoller et al., 1999). PCR products were cleaned using ExoSAP-IT following the manufacturer's instructions (USB Corporation, Cleveland, OH, USA). Amplicons consisting of multiple bands were cloned using the TOPO TA Cloning Kit (Invitrogen™, Life Technologies, Carlsbad, CA).

Amplicons were sequenced in both directions at the Duke Genome Sequencing & Analysis Core Facility or the University of Arizona Genomics Core using Applied Biosystems BigDye® chemistry with an ABI 3730xl DNA Analyzer (PE Applied Biosystems, Foster City, CA). Each 10 μl sequencing reaction included 1 μl of primer, 0.75 μl BigDye® (BigDye® Terminator Cycle sequencing kit, ABI PRISM version 3.1; Perkin-Elmer, Applied Biosystems, Foster City, CA, USA), and 3.25 μl of Big Dye buffer; the amount of PCR product and double distilled water depended on the concentration of the PCR product visualized by gel electrophoresis. Endophyte strains and sequences used in this study, as well as their host and locality information, are shown in Supplementary Table 1.

Reference taxa included representative species from all known classes of the Leotiomyceta. Two recently circumscribed classes, Xylonomycetes (Gazis et al., 2012) and Coniocybomycetes (Prieto et al., 2013), were included in the seven-locus phylogeny but not the nr5.8S + LSU Ascomycota phylogeny described above.

2.4. Sequence alignments

Sequence fragments obtained for this study were assembled and edited using Sequencher v4.5. Datasets consisting of nrSSU and nrLSU were manually aligned with Mesquite v2.75 (Maddison and Maddison, 2011). Alignments were improved according to the secondary structure of these molecules from *Saccharomyces cerevisiae* (Kjer, 1995). The nr5.8S and mitSSU sequences were aligned using MAFFT (Katoh and Standley, 2013), and the alignments were adjusted manually in Mesquite. *RPB1*, *RPB2*, and *MCM7* were aligned manually using the amino acid visualization tool in Mesquite. All ambiguously aligned regions and introns were excluded from phylogenetic analyses (Table 1). Alignments were submitted to TreeBASE (http://purl.org/phylo/treebase/phylows/study/TB2:S17149).

2.5. Seven-locus datasets and phylogenetic analyses

Four datasets were assembled and analyzed using a cumulative supermatrix approach (see Gaya et al., 2012; Miadlikowska et al., 2006, 2014). Because our main supermatrix includes reference taxa and OTUs with 1–7 available gene sequences, certain sequences are missing for particular taxa. The other three datasets, which include fungal strains with a minimum of three genes (i.e., 3 + 4 + 5 + 6 + 7 genes), five genes (5 + 6 + 7), and 6 genes (6 + 7), are missing fewer genes, but also include fewer OTUs (Table 2). The analyses were not performed on datasets containing 2–7 genes and 4–7 genes because the taxa included in the 2–7 gene dataset are the same as those in the 1–7 gene dataset, the 4–7 gene dataset and the 5–7 gene dataset differ by only 30 taxa. Reference sequences were selected according to Geiser et al. (2006) and Gueidan et al. (2008) to represent all major known lineages of Eurotiomycetes. Several taxa with uncertain taxonomic and phylogenetic placements also were included (Diederich et al., 2013; Rossman et al., 2010). Outgroup selection consisted of one to three species of every known class within Leotiomyceta (see Gazis et al., 2012; Prieto et al., 2013). Sequence information pertaining to reference taxa is shown in Supplementary Table 5.

A Maximum Likelihood (ML) bootstrap analysis (1000 replicates) was conducted with RAxML (Stamatakis, 2006) on each gene separately. Majority rule bootstrap trees were compared to detect topological conflicts among genes (Mason-Gamer and Kellogg, 1996). The sequences responsible for the conflicts were removed if conflicting relationships were highly supported (i.e., bootstrap support [BS] $\geqslant$70%). An ML search for the optimal tree along with 1000 bootstrap replicates were performed on all genes combined, as well as on all taxa for which sequences for at least six, five, and three of the seven genes were present in the supermatrix (Table 2). The following 13 data partitions were predefined: nrLSU, nrSSU, nr5.8S, and mitSSU represented four separate partitions; and the three codon positions of the three protein coding genes were recognized as nine separate partitions (*RPB1*/1st, 2nd, 3rd; *RPB2*/1st, 2nd, 3rd; *MCM7*/1st, 2nd, 3rd). PartitionFinder v1.1.1 (Lanfear et al., 2012) was run separately on all datasets (1–7, 3–7, 5–7, 6–7 genes) to determine optimal partitions and substitution models (Table 2) using a Bayesian Information Criterion (BIC) and limiting searches to "RAxML", "Greedy" (Lanfear et al., 2014).

2.6. Cumulative supermatrix approach to assess the effect of missing data on phylogenetic confidence

Majority-rule consensus trees (50%) were built with PAUP* v4.0 (Swofford, 2003) using the 1000 bootstrap trees generated with RAxML for the four (1–7, 3–7, 5–7, 6–7 genes) datasets. The module "Hypha" (Oliver et al., 2013; see also Miadlikowska et al., 2014) in Mesquite was used to report support values and conflicts derived from all four bootstrap consensus trees onto each internode of the best tree derived from the 1–7 genes dataset.

2.7. Identification of eurotiomycetous endophytes: similarity-based BLAST search of the nrITS "DNA barcode" and phylogeny of nr5.8S + LSU

The results of two additional approaches to identifying strains were compared with the seven-locus phylogeny of Eurotiomycetes. First, we queried representative nrITS sequences of the 20 endophyte OTUs belonging to Eurotiomycetes (verified by the seven-locus phylogeny described above) against the GenBank database using the BLASTn algorithm. BLASTn hits full sequence coverage (i.e., 100%) and high similarity (i.e., 97–100%) were examined for taxonomic information.

Second, an additional set of phylogenetic analyses was conducted with the nr5.8S + LSU alone using the same set of taxa as in the seven-locus dataset of Eurotiomycetes (Supplementary Tables 1 and 5). The resolving power of the nr5.8S + LSU was compared with the resolution and phylogenetic support achieved using the seven-locus dataset. Only the nr5.8S (158 bp) of the nrITS region and partial nrLSU (518 bp) was alignable across Eurotiomycetes. The nr5.8S was treated as a distinct partition in ML phylogeny recon-

Table 1
Summary of the seven alignments used for this study.

Gene name	Number of taxa (total: 157)	Number of endophytic taxa (total: 20)	Alignment length (bp)	Number of included sites (bp)
nrLSU[a]	148	19	4066	1223
nrSSU	135	15	10,007	1540
mitSSU	115	16	2829	545
$RPB1_{A\text{-}G}$	111	9	5589	2736
$RPB2_{5\text{-}11}$	62	5	3923	1878
MCM7	28	13	2562	603
5.8S[a]	126	19	158	158

Total number of included sites: 8683 bp.
[a] Generated by Arnold et al. (2009), Higgins et al. (2007), Hoffman and Arnold (2008), U'Ren (2011) and U'Ren et al. (2010, 2012).

Table 2
Descriptive summary for the four multi-gene datasets.

Datasets	6 + 7 (taxa with 6–7 genes)	5 + 6 + 7 (taxa with 5–7 genes)	3 + 4 + 5 + 6 + 7 (taxa with 3–7 genes)	1 + 2 + 3 + 4 + 5 + 6 + 7 (taxa with 1–7 genes)
Number of taxa	41	91	122	157
Missing percentage of loci	11	21	26	34
Number of partitions	6	7	7	7
Model	GTRGAMMAI	GTRGAMMAI	GTRGAMMAI	GTRGAMMAI

struction in RAxML. The GTRGAMMAI model was specified for the analysis.

2.8. nrLSU tree for taxa within Phaeomoniellales ord. nov.

nrLSU sequences in GenBank that were most similar to the nrLSU sequences from members of Phaeomoniellales (see below) were selected using BLAST and a literature search (Booth and Ting, 1964; Crous et al., 2008, 2009; Crous and Groenewald, 2011; Damm et al., 2010; Groenewald et al., 2001; Gueidan et al., 2014; Langenfeld et al., 2013; Larkin et al., 2012; Lee et al., 2006; Norden et al., 2005; Peršoh and Rambold, 2012; Supplementary Table 6). Bayesian and ML analyses were conducted with BEAST v1.6.1 (Drummond and Rambaut, 2007) and RAxML, respectively, on the resulting nrLSU dataset (1293 characters included). The substitution model GTRGAMMAI used for the RAxML and BEAST analyses was selected based on the BIC results of jModelTest (Posada, 2008). Phylogenetic uncertainty for the ML search was assessed with 1000 bootstrap replicates. For the Bayesian analysis, a Yule speciation model and uncorrelated lognormal-distributed relaxed clock model were employed. Four independent runs were conducted in BEAST with 50,000,000 iterations. Trees were sampled every 5000 iterations, resulting in 10,000 trees. The log files of four independent runs were examined manually with Tracer v1.5 (Rambaut and Drummond, 2009) to assess convergence. The first 1000 trees were discarded and the remaining 9000 trees were pooled to generate a majority consensus tree and posterior probabilities using TreeAnnotator v1.6.1 (Rambaut and Drummond, 2010).

2.9. Morphological data

Our analyses detected a distinctive clade within Eurotiomycetes that was not formally described before. We examined morphological traits for members of this novel lineage, focusing on 15 isolates representing five of the six OTUs from the apparently new order (subsequently identified as Phaeomoniellales, below; 9419, AZ0857, AZ0871, AZ0887, AZ0952, AZ0963, AZ0988, AZ0989, AZ0993 [OTU AZ0963]; 9352, FL0080 [OTU FL0086]; FL0854 [OTU FL0854]; FL1432 [OTU FL1432], NC1419, and NC1564 [OTU 4466]).

For our morphological study, focal isolates were grown on Difco Malt Extract Agar (MEA, 2%) for up to three months at room temperature with natural light. Microscopic observations were made using a Zeiss Axioplan 2 imaging system. Colony morphology was observed using a Leica MZ12.5 stereomicroscope, and pictures were taken with a Canon EOS Rebel XSi camera. Attempts to obtain sexual and asexual states were made by growing additional cultures on Synthetic Nutrient Agar (Damm et al., 2010) with autoclaved leaves of oak, grape, juniper, loblolly pine and grass, as well as lichen thalli (*Usnea* species).

3. Results

3.1. Phylogenetic affiliations of endophytes within Eurotiomycetes

Eighty of 6521 fungal isolates considered for this study were resolved within Eurotiomycetes based on the reconstructed nr5.8S + LSU Ascomycota-wide phylogeny (results not shown). These 80 isolates represent 20 OTUs (i.e., 95% nrITS-LSU similarity groups that were resolved as monophyletic in nr5.8S + LSU analyses; Supplementary Table 1). According to our seven-locus phylogenetic tree, three OTUs belong to Chaetothyriales, 11 belong to Eurotiales, and six are grouped into a well-supported lineage that has not been formally described before, Phaeomoniellales *ord. nov.* (Figs. 2 and 3; Supplementary Table 7).

Within Eurotiomycetes, the order Eurotiales is the most rich in fungal endophytes given the taxon sampling available for this study (54 isolates, 11 OTUs). Of the 11 OTUs in Eurotiales, seven were represented by more than one isolate. Among those seven OTUs, four were isolated from both plants and lichens; two were found only in plants; and one OTU (represented by NC0339) was found only in lichen thalli. Most of these OTUs are closely related to well-known soil- or fruit-borne saprotrophs. However, FQ10867A is most closely related to *Trichocoma paradoxa*, a wood-inhabiting saprotroph (Fig. 3).

Within Chaetothyriales, strain 422 was most closely related to two animal-associated fungi (*Exophiala oligosperma* and *E. jeanselmei*). AK1130 and AK0094 are recovered as long terminal branches, with no close relatives based on current availability of DNA sequences from this order (at similarity >97%) (Fig. 3; Supplementary Table 7). Although known Chaetothyriales are rarely associated with plants, all three isolates were cultured from the interior of surface sterilized plant leaves (Supplementary Table 1).

Phaeomoniellales *ord. nov.* represents a clade discovered by Rossman et al. (2010), and provisionally named Celotheliales *ad int.* by Gueidan et al. (2014). This clade includes 23 of 80 isolates identified as Eurotiomycetes in our analyses. These 23 isolates represent six OTUs (Fig. 3). Four of these OTUs have uncertain affiliations due to low bootstrap support. However, FL0086 is closely related to *Phaeomoniella effusa*, a fungus associated with *Prunus* tree necrosis (Damm et al., 2010). AZ0963 is sister to *Phaeomoniella zymoides*, which has been isolated from *Prunus* tree necrosis and as an epiphyte in pine (Damm et al., 2010; Lee et al., 2006) (Supplementary Table 6). All four OTUs represented by more than one isolate were isolated from plants.

The monophyly of Eurotiomycetes is well supported (i.e., BS values $\geqslant$70%) in analyses of three of the four datasets (i.e., the 1–7, 3–7, 5–7 gene datasets; Fig. 2). The eight currently recognized orders within Eurotiomycetes are well-resolved with high phylogenetic support (Fig. 2). The *Dactylospora* clade represents an apparently undescribed lineage of mycoparasitic and saprotrophic fungi. The rock-inhabiting fungus "TRN_242" is sister to Chaetothyriales but with poor support. However, the placements of these two lineages are in agreement with three previous studies (Diederich et al., 2013; Gueidan et al., 2008, 2014).

3.2. Molecular-based identification of endophytes: nrITS BLAST and nr5.8S + LSU phylogeny

Of the 20 OTU within Eurotiomycetes (shown in Supplementary Table 1), six BLAST to known fungi with 100% coverage and 100% identity (Supplementary Table 7; nrITS BLAST, primer sets: ITS1F-ITS4). One of these is a member of the Chaetothyriales, whereas the other five are most similar to members of the Euro-

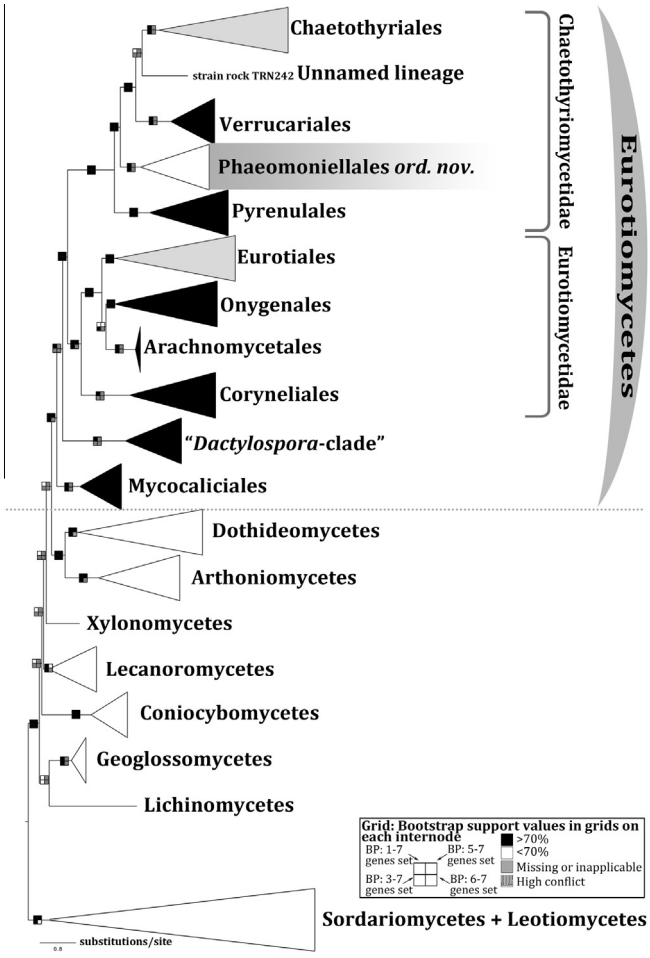

Fig. 2. Summary of phylogenetic analyses designed to confirm the affiliations of endophyte strains within the Eurotiomycetes. The clades highlighted in gray represent orders within the Eurotiomycetes containing fungal endophytes. One of these clades is named here for the first time as the order Phaeomoniellales. The combined 7-locus dataset (nrLSU, nrSSU, nr5.8S, mitSSU, *RPB1*, *RPB2* and *MCM7*) was analyzed using a cumulative supermatrix approach (e.g., Miadlikowska et al., 2014). Clades within orders were collapsed within the class Eurotiomycetes. Clades within classes were collapsed in the outgroup (i.e., below the dotted line). The 4-box grids on each internode show bootstrap (BS) values from different combined datasets (see legend). A black box indicates a highly supported (BS ⩾70%) internode; a white box indicates medium to low support (BS <70%); a gray box indicates that an internode did not exist due to missing taxa or was not recovered by the bootstrap analysis; a box with black stripes indicates conflicts (with bootstrap values ⩾70%) among different datasets.

tiales. When using 100% coverage as a cutoff, five of the endophytes in Eurotiales BLAST to more than one known taxon with >97% similarity, including strain "DC568" and "AK0184" which had BLAST hits to two *Penicillium* species and two *Aspergillus* species, respectively, with 100% identity. None of the endophytes nested in Phaeomoniellales (see below, Figs. 3 and 4) had BLAST hits to known fungi with similarities higher than 97% (Supplementary Table 7).

Overall, the BLASTn option in GenBank using nrITS was a reliable method to determine if unknown endophytes belonged to the class Eurotiomycetes when sequences of closely related known fungi were available. For example, BLAST searches are accurate in identifying a query endophyte to Eurotiomycetes regardless of its order-level placement. At the ordinal level, BLAST searches were only accurate if an unknown isolate belongs to the order Eurotiales rather than Chaetothyriales or Phaeomoniellales. These results reflect the fact that members of Eurotiales are more extensively studied and sequenced compared to Chaetothyriales and Phaeomoniellales. At finer scales (i.e., at the genus and species ranks), BLASTn provided limited information regarding the taxonomic identity of unknown fungi. However, in Eurotiales, it is difficult to assign species names with confidence to unknown endophytes. The difficulty is due in part to BLAST searches usually hitting multiple species with high similarity in Eurotiales (Supplementary Table 7).

We also evaluated information regarding endophyte taxonomy provided by a single-amplicon (nrITS-LSU) phylogeny. We found that the nr5.8S + LSU part of this locus placed endophytes within the same orders as when using our concatenated seven-locus cumulative supermatrix (Fig. 3; Supplementary Fig. 2), but in general with low support (e.g., BS = 21% for Eurotiales, BS = 51% for Chaetothyriales, BS = 61% for Phaeomoniellales). We also noted that three reference taxa—*Dactylospora mangrovei*, *D. haliotrepha* and *Sclerococcum sphaerale*, which form an undescribed clade—were misplaced in Phaeomoniellales using nr5.8S + LSU data alone.

3.3. Phylogenetic relationships and host association within Phaeomoniellales

Topologies resulting from the ML and Bayesian analyses of the nrLSU dataset for Phaeomoniellales were similar, with no conflicts detected. Phaeomoniellales was well supported as a monophyletic group in both analyses, with a bootstrap value of 98% and posterior probability of 0.985 (Fig. 4). The first split within Phaeomoniellales, although without high support, leads to a clade formed by the lichen-forming genus *Celothelium* (for which the degree of lichenization can be minimal to absent; see Aptroot, 2009) and the endolichenic strain FL0854. The remaining members of the order Phaeomoniellales occur as endophytic, saprotrophic, and pathogenic fungi in a variety of gymnosperms and angiosperms (Fig. 4).

Although most of the deep nodes within Phaeomoniellales have low bootstrap support or have only high Bayesian probabilities, several clades were well-supported by both analyses, allowing us to examine the fungus–host association patterns observed within this order (Fig. 4). The *Phaeomoniella prunicola–Phaeomoniella dura* clade includes pathogenic fungi isolated from tree trunks of *Prunus*. An OTU isolated from living leaves of *Aristida stricta* (Poaceae) (OTU FL1432; U'Ren et al., 2012) and saprotrophic fungi (*Moristroma* spp.) isolated from dead branches and trunks of *Quercus* are grouped together. The "AZ1057-Fungal sp BG79" clade consists of endophytes of gymnosperm leaves. In contrast, the "*Phaeomoniella niveniae–Phaeomoniella zymoides*–Fungal sp BG47" clade includes strains that are endophytic in gymnosperms and pathogenic on a broad array of angiosperms. Similarly, the "FL0086-*Phaeomoniella effusa*" clade contains endophytes and plant pathogens isolated from gymnosperms and angiosperms, respectively (Supplementary Table 6).

3.4. Morphology of endophytes of the order Phaeomoniellales

Among the 23 isolates placed within the monophyletic Phaeomoniellales, only one (FL0854) was isolated from a lichen thallus (i.e., is recognized as an endolichenic fungus). The remaining isolates were isolated from plant leaves as endophytic fungi, and share common morphological features with the genus *Phaeomoniella* (Fig. 5).

Endophytic Phaeomoniellales have white, pale yellow to pale pink, flat colonies on 2% MEA. Some form aerial hyphae and velvety hyphae with mycelium folded toward the center (Fig. 5B and C). Others are moist to mucoid, showing wrinkled growth and forming yeast-like colonies (Fig. 5A). Sporulation was sometimes observed directly from the colony surface or embedded in the mycelium (Fig. 5C), whereas some strains produced pycnidia (Fig. 5D).

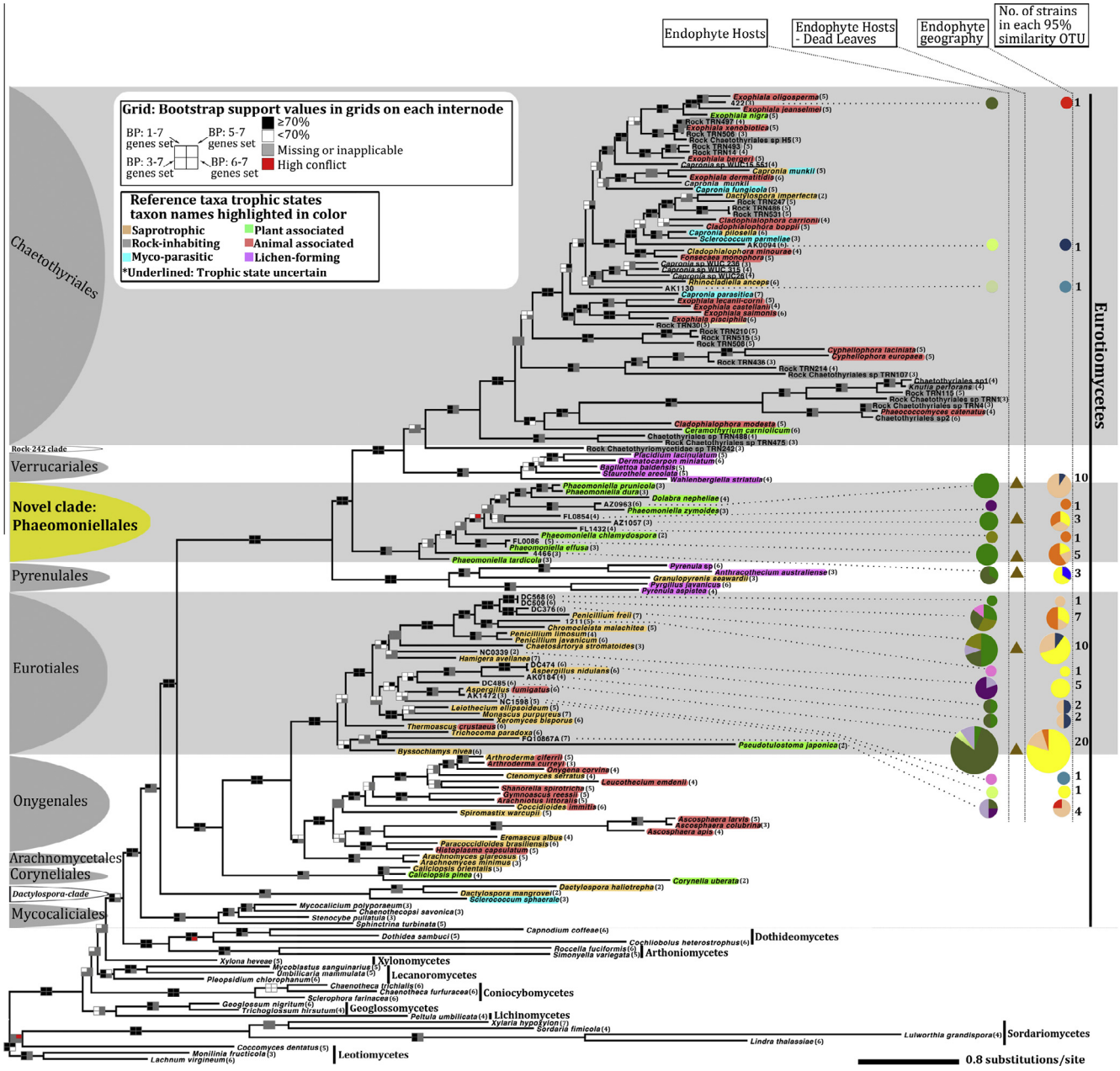

Fig. 3. Phylogenetic placement of fungal endophytes in Eurotiomycetes based on seven genes (nrLSU, nrSSU, mitSSU, *RPB1*, *RPB2*, *MCM7*). Clades highlighted in gray represent lineages containing fungal endophytes. Representative fungal endophytes are annotated with host information (first column, pie charts color-coded according to Fig. 1A and B). Brown triangles in the second column indicate that at least one strain from the OTU was isolated from senescent leaves collected in the canopy or from host-associated leaf litter (U'Ren et al., 2010; U'Ren, 2011), which suggest that these OTUs are found as both endophytes and saprotrophs. The third column reports geographical information (pie charts color-coded according to Fig. 1C) The number of strains, corresponding to the size of the pie charts, is shown in the fourth column. Reference taxon names are highlighted in color representing their primary/known trophic states (see legend); names with multiple colors indicate more than one trophic state is known for those fungi. Numbers in parentheses after species names and isolate numbers indicate the number of genes for which DNA sequences were available for this phylogenetic analysis. The 4-box grids on each internode show bootstrap (BS) values from different combined datasets (see legend). A black box indicates a highly supported (BS ⩾70%) internode; a white box indicates medium to low support (BS <70%); a gray box indicates that an internode did not exist due to missing taxa in one of the analyses of the 3–7, 5–7, or 6–7 gene data sets, or was not recovered by the bootstrap analysis; a red box indicates conflicts (with bootstrap values ⩾70%) among different datasets in the cumulative supermatrix approach.

Although attempts were made to grow these fungi on different substrates (see Section 2), sporulating structures were only observed on inoculated needles of loblolly pine (Fig. 5E). Typical conidia are straight and hyaline (Fig. 5F). Conidia-like structures (e.g., microconidia) with uncertain functions also were observed in several isolates (Fig. 5G).

The single endolichenic isolate (FL0854) is phenotypically very distinct from endophytic isolates: its colonies have a raised, melanized center (Fig. 5H). In addition, sporulating structures are pycnidia-like (Fig. 5I). Small uniform conidia were produced along with large, deformed, conidia-like structures, potentially macroconidia (Fig. 5J).

We observed morphological variation among nine strains that belong to OTU AZ0963 (95% similarity group). For example, some strains (9419, AZ0952, AZ0857, AZ0871, AZ0887, AZ0963) had slimy, yeast-like colonies that produced abundant pycnidia, but other strains (AZ0993, AZ0989, AZ0988) had velvety hyphae with few pycnidia observed.

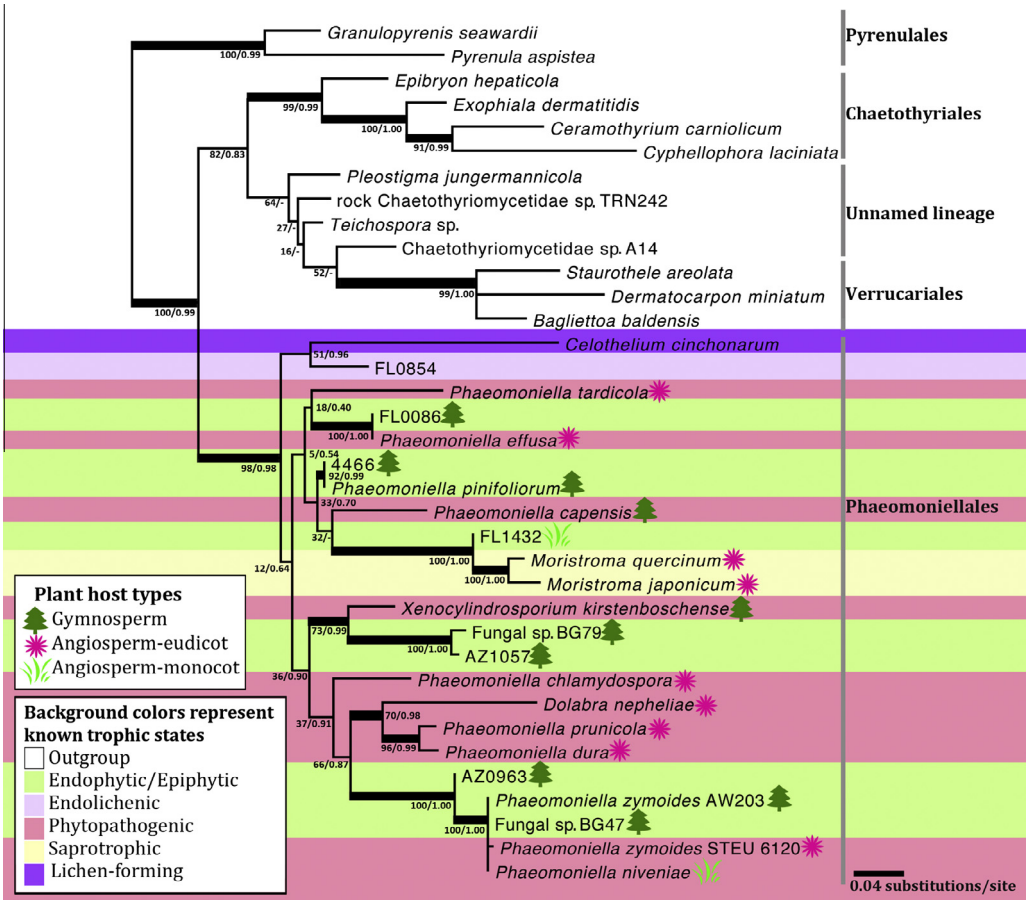

Fig. 4. ML tree for the order Phaeomoniellales based on nrLSU. Thickened branches represent bootstrap values ⩾70% and posterior probabilities ⩾95%. Values associated with internodes represent bootstrap values/posterior probabilities; "dash" indicates that the internode was not present in the Bayesian phylogeny. Symbols following taxon names are indicative of their plant host. OTUs are highlighted in color (background color) according to their trophic states.

4. Discussion

4.1. Eurotiomycetous endophytes: abundance and function

Endophytes appear to be ubiquitous symbionts of plants and lichens, and are especially species-rich among the Pezizomycotina (Ascomycota) (see Arnold et al., 2009). Most studies using culture-based approaches have highlighted the predominance of endophytes from classes such as the Sordariomycetes, Dothideomycetes, Leotiomycetes, and Pezizomycetes in photosynthetic tissues and lichen thalli (e.g., Arnold and Lutzoni, 2007; Arnold et al., 2009; Petrini and Petrini, 1985; Petrini et al., 1990; Shipunov et al., 2008; Stone et al., 2004; Bacon and White, 2000). When surveys of endophytes were conducted using malt extract agar as the isolation medium (i.e., Arnold et al., 2009; U'Ren, 2011; U'Ren et al., 2010, 2012), and resulting cultures were evaluated using the phylogenetic approach described above, a low proportion (0.015% of isolates) represented Eurotiomycetes. This result agrees with several culture-dependent (e.g., Higgins et al., 2007; Lodge et al., 1996; Larkin et al., 2012; Shipunov et al., 2008) and culture-independent studies (e.g., U'Ren et al., 2014; Zimmerman and Vitousek, 2012), but see Vega et al. (2010) and Sandberg et al. (2014). Notably, U'Ren et al. (2014) showed that the frequency with which eurotiomycetous endophytes are observed can differ as a function of how plant/lichen tissues are treated prior to DNA extraction in culture-free studies.

Of the three clades that contain fungal endophytes within the class Eurotiomycetes, the order Eurotiales includes most of the isolates examined here, followed by the Phaeomoniellales.

Of the eurotiomycetous endophytes, isolates of *Penicillium* and *Aspergillus* in the order Eurotiales have been studied most extensively. Studies on *Penicillium* and *Aspergillus* endophytes have revealed a wide array of bioactive compounds (Rai et al., 2014; Suryanarayanan et al., 2009). In addition, some of these endophytes can produce plant hormones such as gibberellins and indoleacetic acid, which can promote plant growth and alter plant responses to abiotic stress (e.g., Khan et al., 2011; Waqas et al., 2012). Exploration for such compounds in endophytes within the order Chaetothyriales and Phaeomoniellales is likely to uncover new bioprospecting materials and will provide insights into the evolution of endophytes and Eurotiomycetes in general.

Overall, eurotiomyceteous endophytes are more commonly found in plants than in lichens (see U'Ren et al., 2012), and they were isolated more frequently from southern temperate to subtropical forests relative to boreal sites in Alaska and Québec (see U'Ren et al., 2012; Figs. 1 and 3). The three endophytic isolates belonging to the Chaetothyriales came from various biomes, but their abundance was insufficient to detect any biogeographical trend.

4.2. Evolution of host affiliation and trophic states in Eurotiomycetes

Spatafora et al. (2007) revealed that grass endophytes from the Clavicipitaceae (Hypocreales, Sordariomycetes) arose from animal-pathogenic ancestors. None of the eurotiomycetous endophytes in this study were resolved within Onygenales, which is especially rich in animal pathogens (Geiser et al., 2006). A culture-free study

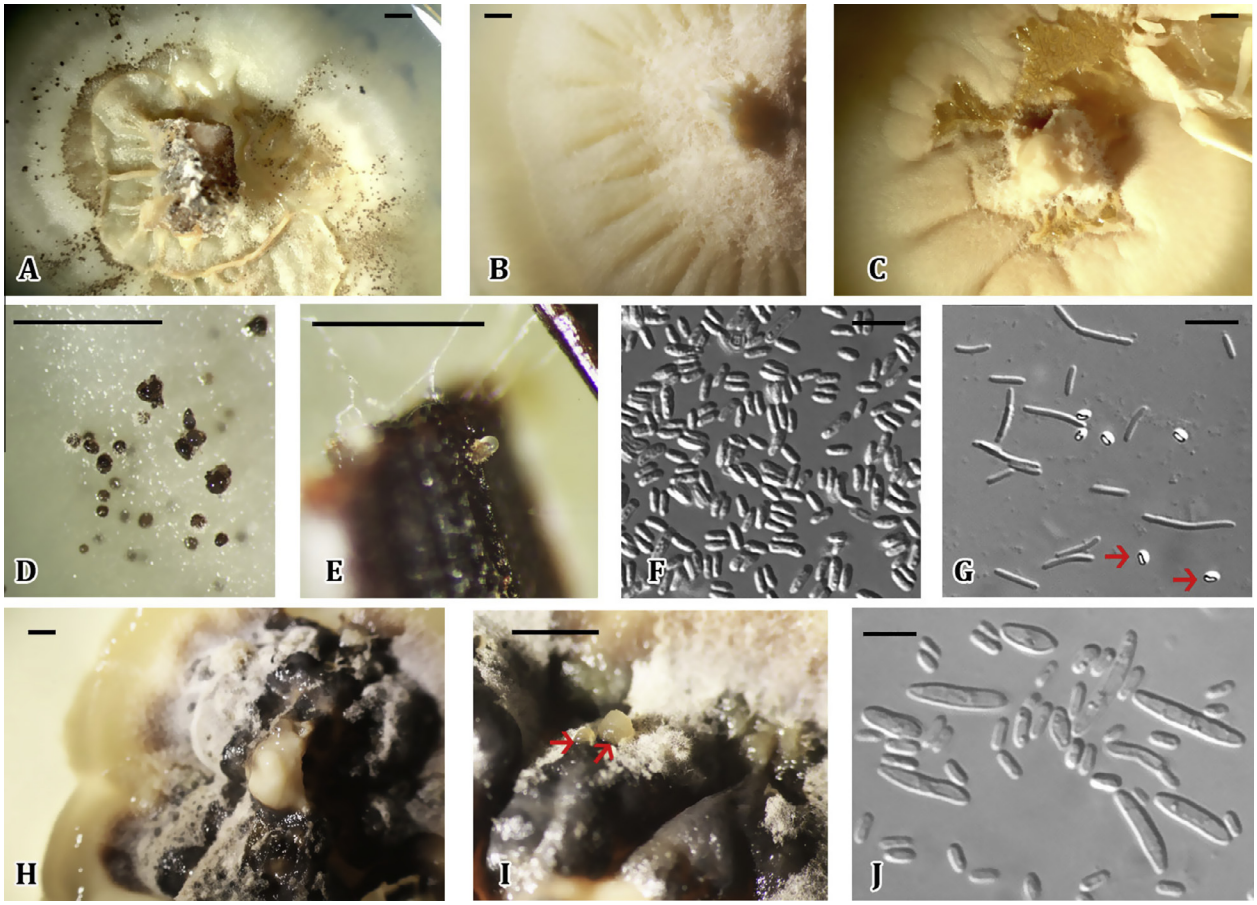

Fig. 5. Morphological and anatomical features of endophytic and endolichenic strains of Phaeomoniellales. (A) AZ0857 (OTU AZ0963; Fig. 4; Supplementary Table 1) – colony morphology on 2% MEA after 90 days. Colony pale yellow, moist to mucoid, yeast-like; with black dots representing growing pycnidia (see Fig. 5D). (B) AZ0993 (OTU AZ0963) – colony morphology on MEA after 90 days. Colony white to pale yellow, velvet hyphae folded toward the center. (C) NC1564 (OTU 4466) – colony morphology on MEA after 90 days. Colony pale yellow, folded irregularly, with buff region representing exposed sporulating areas. (D) AZ0857 (OTU AZ0963) – black pycnidia. (E) 9352 (OTU FL0086) – pycnidia and conidial masses on pine needle. (F) AZ0871 (OTU AZ0963) – conidia. (G) AZ0952 (OTU AZ0963) – conidia-like structures (red arrows). (H) FL0854 (OTU FL0854) – colony morphology (pale yellow, blackened from the center, raised, moist but with velvet regions) on MEA after 90 days. (I) FL0854 (OTU FL0854) – pycnidia-like structure (red arrows) on the surface. (J) FL0854 (OTU FL0854) – conidia-like structures (the biggest and deformed cells) and conidia (small, uniform, bacilliform cells). Scale bars: A, B, C, D, H, I = 1 mm; E = 0.5 mm; F, G, J = 5 μm.

from similar hosts also found no evidence of endophytic and endolichenic Onygenales (U'Ren et al., 2014). Endophytes within the order Eurotiales are intermingled with opportunistic pathogens of animals (e.g., *Aspergillus fumigatus* [Schoch et al., 2009]) in our multi-locus phylogeny. Chaverri and Samuels (2013) examined the ancestral states of endophytes affiliated with *Trichoderma* (Hypocreaceae, Hypocreales, Sordariomycetes), providing evidence of multiple inter-kingdom host jumps (e.g., from fungal hosts to plant hosts and vice versa). Chaetothyriales include fungi that grow on diverse hosts such as animals and other fungi (Geiser et al., 2006), but they were too rare in our study to permit conjecture regarding their evolution. Most fungi within the Phaeomoniellales clade are plant-inhabiting, with the exception of one endolichenic fungus and one lichen-forming species representing *Celothelium*.

We found that endophytes within Eurotiales were closely related to many saprotrophic taxa. All but one of the plant-associated OTUs in Phaeomoniellales include at least one isolate from the interior of senescent leaves in the canopy or host-associated leaf litter (see U'Ren et al., 2010; U'Ren, 2011), suggesting a potential saprotrophic stage in their life cycle (Fig. 3). Such evolutionary switches between endophytism and saprotrophy are common in Sordariomycetes (e.g., *Trichoderma* [Chaverri and Samuels, 2013]; *Colletotrichum* and *Fusarium* [Promputtha et al., 2007]), in Leotiomycetes (e.g., *Lophodermium* [Lantz et al., 2011]); and in Dothideomycetes (Pleosporales [Zhang et al., 2009]). On the other hand, many endophytes are closely related to pathogenic fungi on plants (e.g., *Colletotrichum* [Hyde et al., 2009], *Fusarium* [Aimé et al., 2013]; diverse Botryosphaeriales [Slippers et al., 2013] and Pleosporales [Zhang et al., 2009]), as we observed in the Phaeomoniellales. Our results suggest a general pattern in the Phaeomoniellales in which fungal strains are endophytic on gymnosperms but pathogenic on angiosperms (Fig. 4). Some fungi are known to switch trophic states (i.e., to shift from endophytism to pathogenicity or saprotrophy) at different life stages or under different conditions (e.g., Arnold et al., 2009; Delaye et al., 2013; Eaton et al., 2011; Kuo et al., 2014; Osono and Hirose, 2011). U'Ren et al. (2010) revealed a 25% overlap in communities found simultaneously within living and senescent leaves of the same hosts. Kuo et al. (2014) provided experimental evidence that *Neurospora crassa* shifts among endophytic, saprotrophic, and pathogenic states. Other studies have used data from fungal genomes and transcriptomes to explain trophic plasticity of endophytes (O'Connell et al., 2012; Zuccaro et al., 2011). Phaeomoniellales represent an opportunity to extend our knowledge of fungal evolution. Although the Chaetothyriales includes many lichenicolous fungi (Schoch et al., 2009; Geiser et al., 2006), none of our isolates in that clade were found in lichens. The scarcity of endolichenic fungi in Chaetothyriales agrees with Arnold et al. (2009) in that licheni-

colous fungi and endolichenic fungi are often phylogenetically distinct.

4.3. An evaluation of the identification power of nrITS (DNA barcode) BLAST vs. nr5.8S + LSU phylogeny

The nrITS BLAST approach successfully identified unknown sequences as Eurotiomycetes. However, affiliations of endophytes to members of Phaeomoniellales could not be detected readily using BLAST because there are no known fungi of similarity higher than 97% to these query endophytes. In general this speaks to the limitations of BLAST as a function of taxa represented in the database (e.g., Matsen et al., 2010).

We examined whether the nrITS-LSU amplicon (primer pair ITS1F-LR3) used systematically in previous studies (e.g., Arnold and Lutzoni, 2007; Arnold et al., 2009; U'Ren et al., 2010, 2012), can provide phylogenetic signal to accurately resolve, as a first approximation, the phylogenetic placements of fungal endophytes within the Eurotiomycetes. Because this marker can be amplified as a single amplicon, it can be widely applied in ecological and environmental sampling to estimate phylogenetic placement. Our results showed that nr5.8S + LSU was able to place endophytes into the same order as revealed by the seven-locus phylogeny. However, the deep nodes have very low support in the nr5.8S + LSU tree, such that the results of that analysis alone would need to be interpreted with caution.

The ITS1 and ITS2 of the nrITS region cannot be aligned for broad large-scale phylogenetic analyses (Porter and Golding, 2011; U'Ren et al., 2009). For this reason, albeit with caveats (U'Ren et al., 2009), they are useful in estimating OTU, but not necessarily for inferring the relationships of OTU to one another. Using them for identification, especially if close relatives have not yet been sequenced, is thus of limited power: matches via BLAST and similar tools are only good as the availability of highly similar, high-quality, and well-identified reference sequences in databases. Recently, efforts have been made to establish more accurate and comprehensive reference databases for Fungi (Kõljalg et al., 2013; Schoch et al., 2014). However, most fungal biodiversity is unknown (e.g., Blackwell, 2011). Under such circumstances, a phylogenetically-based approach is an alternative to classify unknown fungi in an incremental and tractable way (Matsen et al., 2010), provided that sufficient taxon sampling is available. Sanger sequencing can be used to amplify the larger nrITS-LSU amplicon rather than nrITS alone (e.g., Arnold and Lutzoni, 2007), which enables the implementation of both similarity- and phylogenetically-based approaches. Phylogeny-based methods originally developed for classifying short reads generated by next generation sequencing can also be applied here (e.g., SAP [Munch et al., 2008], pplacer [Matsen et al., 2010], PaPaRa [Berger and Stamatakis, 2011], PhyloSift [Darling et al., 2014]).

4.4. The origin of Phaeomoniellales

Previous studies have reported the broad spectrum of the ecological and morphological features of Eurotiomycetes within a phylogenetic framework (e.g., Arnold et al., 2009; Geiser et al., 2006; Gueidan et al., 2008, 2014; Schoch et al., 2009). Our results show that the mostly plant-associated order Phaeomoniellales diverged after the lichen-forming Pyrenulales and before the split of the orders Chaetothyriales (which includes many animal pathogens) and Verrucariales (mostly lichen-forming fungi growing on rocks). According to Gueidan et al. (2011), the divergence of the Pyrenulales is estimated at about 280 MYA, and the Chaetothyriales–Verrucariales split took place ca. 229 MYA. Therefore, the origin of the Phaeomoniellales may have occurred sometime between 230 and 280 MYA, which is shortly after the angiosperm–gymnosperm split ca. 330 MYA (Magallón et al., 2013; Smith et al., 2010). Although further analyses are required to confirm and refine these estimations, this time period (230–280 MYA) overlaps with the early diversification of extant gymnosperms, i.e., before the diversification of extant angiosperms (Magallón et al., 2013). Most endophytic Phaeomoniellales obtained for this study were derived from gymnosperm leaves despite efforts to sample diverse lineage of plants at each sampling site (e.g., see U'Ren et al., 2010, 2012; Figs. 1A and 4; Supplementary Table 1). Globally, the Phaeomoniellales encompasses endophytic, pathogenic, and saprotrophic fungi on plants across the Spermatophyta, including cycads, Pinaceae, monocots, and eudicots, as well as endolichenic and lichen-forming fungi (Fig. 4 and Supplementary Tables 1 and 6). The common ancestor of Verrucariales and Chaetothyriales was inferred to be non-lichenized and rock-inhabiting by Gueidan et al. (2008), non-lichenized by Schoch et al. (2009), but as lichen-forming in James et al. (2006) and Lutzoni et al. (2001). Available divergence time estimates suggest that the diversification and ecological success of the gymnosperms, including the establishment of inland forests (Magallón et al., 2013), may have contributed to the origin of the Phaeomoniellales and its ecological shift from rock-inhabiting/lichen-forming to being mostly plant-associated.

4.5. Ecological traits of Phaeomoniellales

Gueidan et al. (2014) discovered that the lichenized genus *Celothelium* is most closely related to members of the Phaeomoniellales. According to Aptroot (2009), individuals of this species are "usually lichenized", i.e., form thalli that are occasionally delimited by a black hypothallus or, rarely, lack the thallus entirely (no apparent association with a photobiont). Our data does not allow us to infer with confidence the evolutionary history of endophytism/lichenization within Phaeomoniellales, as most of the deep nodes, including the lineage leading to *Celothelium*, are not well-supported (Fig. 4). Future studies using ancestral state reconstruction methods and time divergence estimations that are based on a more comprehensive multi-locus phylogenetic analysis of the Phaeomoniellales (including more samples of *Celothelium*), as well as re-synthesis experiments, are needed.

Although most Phaeomoniellales inhabit plants, their trophic states are diverse (Crous et al., 2008; Crous and Groenewald, 2011; Damm et al., 2010). Several genera of Phaeomoniellales are known for their association with plant diseases (e.g., *Phaeomoniella*, *Dolabra*, *Xenocylindrosporium*). However, whether these fungi are strict plant pathogens or are endophytes, at least in part of their life cycle, awaits further investigation. For example, *Phaeomoniella chlamydospora*, which can cause Petri disease by blocking plant host vessels (Landi et al., 2012), has also been isolated from healthy (Halleen et al., 2003) and dead plant tissues (Hofstetter et al., 2012). Therefore, *Phaeomoniella chlamydospora* was suggested to be an endophyte or saprotroph (Antonielli et al., 2014; Halleen et al., 2003; Hofstetter et al., 2012) that induces plant disease during part of its life cycle. Its recently sequenced genome revealed a reduced numbers of pathogenic genes (e.g., polyketide synthetases [PKS], nonribosomal peptide synthetases [NRPS]) compared to other plant pathogens, perhaps consistent with an endophyte-like lifestyle (Antonielli et al., 2014). *Moristroma*, a saprotrophic fungus, is also nested within the Phaeomoniellales (Damm et al., 2010). Lee et al. (2006) described acid tolerance of epiphytic *Phaeomoniella* strains. Many studies focusing on endophytes (Arnold et al., 2007; Hoffman and Arnold, 2008, 2010; Larkin et al., 2012; Langenfeld et al., 2013), especially those focusing on gymnosperms, have uncovered fungal isolates closely related to members of *Phaeomoniella*. We found only one endolichenic strain that is part of the Phaeomoniellales; however, Peršoh and Rambold

(2012) discovered several *Phaeomoniella*-related strains in their study of lichen-inhabiting fungi.

4.6. Classification within the order Phaeomoniellales

The five named genera in the Phaeomoniellales clade exhibit high morphological and ecological diversity (Damm et al., 2010; Gueidan et al., 2014). *Dolabra* and *Moristroma* are holomorphic (Rossman et al., 2010; Norden et al., 2005). *Celothelium* has been observed only in a sexual state (Gueidan et al., 2014), whereas *Phaeomoniella* and *Xenocylindrosporium* are known only from an asexual state (Crous and Gams, 2000; Crous et al., 2009; Damm et al., 2010). According to our seven-locus phylogeny, *Dolabra nepheliae* is nested within the genus *Phaeomoniella*, a genus that spans almost the entire order in its current delimitation (Fig. 4). Nevertheless, *D. nepheliae* is very different morphologically from available *Phaeomoniella* strains. The deepest nodes of our nrLSU tree are not well supported (Fig. 4); thus relationships among these five genera remain uncertain.

Another taxonomic question lies within *Phaeomoniella* itself: this genus was erected to accommodate a hyphomycete, *Phaeoacremonium chlamydospora*. However, a *Phoma*-like asexual state was reported in several *Phaeomoniella* species (Crous et al., 2008; Damm et al., 2010; Lee et al., 2006), thus complicating genus-level delimitation. The two strains of *Phaeomoniella zymoides* sampled independently in Korea and South Africa are closely related, and highly similar (based on nrLSU) to *Phaeomoniella niveniae* and the unidentified fungal strain BG47. Overall, relationships of fungi within Phaeomoniellales were poorly supported in both our seven-locus and nrLSU trees. Sequencing faster-evolving markers for a broad range of strains is needed to resolve the taxonomic challenge presented by this order.

5. Taxonomy of a new main lineage within the class Eurotiomycetes

5.1. Phaeomoniellales ord. nov.

Based on their distinct phylogenetic placement, we propose the order Phaeomoniellales in the class Eurotiomycetes, subclass Chaetothyriomycetidae, to accommodate the genera *Celothelium*, *Dolabra*, *Moristroma*, *Phaeomoniella*, and *Xenocylindrosporium* as well as various fungal endophytes isolated mostly from gymnosperms from the southern part of the United States (Figs. 1, 3 and 4).

5.2. Order: Phaeomoniellales K.-H. Chen, A.E. Arnold, Gueidan & Lutzoni ord. nov.

MycoBank no.: MB 810711.

Typus: *Phaeomoniella* Crous et W. Gams, Phytopathol. Mediterr. 2000.

Diagnosis: Closely related to the clade formed by the Verrucariales and Chaetothryriales within the subclass Chaetothyriomycetidae of the class Eurotiomycetes. This order contains five described genera: *Celothelium*, *Dolabra*, *Phaeomoniella*, *Moristroma* and *Xenocylindrosporium*. These genera comprise plant pathogens, epiphytic fungi, saprotrophic fungi and lichenized fungi. Endophytic and endolichenic fungi are reported herein. Sexual states have been observed for *Celothelium*, *Dolabra* and *Moristroma*. When present, sexual states with ascoma perithecial, blackened; in *Celothelium* usually with black hypothallus; ascus bitunicate, ascospore cylindrical, ellipsoid to filiform, hyaline. Asexual states have been observed for *Dolabra*, *Phaeomoniella*, *Moristroma* and *Xenocylindrosporium*. When present, asexual states with conidia produced in pycnidia or in mycelia. Conidia long fusiform or cylindrical; light brown, light green to hyaline. Conidiogenesis enteroblastic or holoblastic; yeast-like budding behavior has been reported in some species. Colony surface green, white, orange or pale yellow; many have slimy, yeast-like colonies on solid media.

Name justification: Five previously *incertae sedis* genera (*Celothelium*, *Dolabra*, *Moristroma*, *Phaeomoniella*, *Xenocylindrosporium*) in Chaetothyriomycetidae are now part of the monophyletic Phaeomoniellales (see Fig. 4). Although *Celothelium* (Massalongo, 1860) appears to be the oldest genus name within this clade, and a temporary order Celotheliales *ad int.* was proposed by Gueidan et al. (2014), the genus *Phaeomoniella* currently encompasses most of the known biodiversity for this clade rich in plant-inhabiting fungi. An additional concern associated with the name Celotheliales is that there are very few molecular data available for *Celothelium* (only two nrLSU and three mitSSU sequences). *Dolabra* (Booth and Ting, 1964) and *Moristroma* (Romero and Samuels, 1991) were also described earlier than *Phaeomoniella*. However, *Dolabra* is a monotypic genus restricted to tropical areas (Rossman et al., 2010). *Moristroma* only has two nrITS-LSU sequences available in GenBank. For those genera with few sequences available, more molecular data are necessary to confirm their placement within this order. Therefore, the name Phaeomoniellales is chosen here for long-term nomenclatural stability.

6. Conclusion

By coupling previous culture-based studies with morphological analysis and seven-locus sequencing, we infer the phylogenetic placement and phenotypic traits of previously unknown fungal endophytes within the evolutionarily dynamic Eurotiomycetes. As a result, we describe a well-supported, endophyte-rich lineage that has not been officially described before, Phaeomoniellales.

Although previous studies have shown that fungal endophytes are distributed across at least five classes of Ascomycota (Arnold et al., 2009; Rodriguez et al., 2009; Gazis et al., 2012), this study is the first example focusing on detailed phylogenetic placements of fungal endophytes within a focal class. Our results suggest that the evolution of fungal endophytism might be concentrated in three orders instead of occurring widely in every lineage of the class Eurotiomycetes, but this has to be tested by additional sampling, ideally including culture-free methods. The phylogenetic distribution of fungal endophytes in Eurotiomycetes supports the reciprocal exclusivity between lichenization and endophytism reported by Arnold et al. (2009), where endophytes seem to evolve only in non-lichenized lineages and vice versa (endophytes were not recovered from the lichenized orders Verrucariales and Pyrenulales in Eurotiomycetes). To understand the broader evolutionary picture of fungal endophytism, a comprehensive phylogenetic study based on multiple and preferably protein-coding loci of the Pezizomycotina is needed.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ympev.2015.01.008.

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